CHAPTER SIXTEEN

Antimycobacterial Agents

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1 INTRODUCTION

Some species of mycobacteria are pathogenic for several animal species and are responsible for two important human chronic diseases, tuberculosis and leprosy, as well as for other less widespread but severe infections, traditionally called atypical mycobacterioses. *Mycobacterium leprae* (identifiedby Hansen in 1871) and M. tuberculosis (identified by Koch in 1882) were among the first bacteria recognized as causative agents, respectively, of leprosy and tuberculosis. The dramatic importance of these two still-present illnesses is well known; over the past 200 years, tuberculosis was responsible for the death of 1000 million people (1), and leprosy has been one of the most terrifying diseases since antiquity, and its stigma persists in virtually all cultures in some form (2).

More recently, diseases caused by other mycobacteria, such as M. *avium* complex in immunodepressed patients, have become of increasing importance (3). Despite the early discovery of the etiological agents of the infections, only in the past 5 decades have drugs that are highly effective in the treatment of mycobacterial diseases been discovered (Table 16.1).

Tuberculosis probably appeared in humans about 8000 years ago. Evidence of human tuberculosis, such as bone deformities suggesting Pott's disease, has been found in mummies from pre-dynastic Egypt and pre-Columbian Peru. Paintings, drawings, and, in successive eras, written descriptions witness that a disease similar to what we now define as tuberculosis was well known among ancient peoples. For a long time the popular opinion considered tuberculosis a hereditary disease, although Aristotle (384–322 B.C.), Galen (A.D. 131–201), and Avicenna (980–1037) supported the theory of a contagious etiology.

The first clinical description of phthisis is attributed to Hyppocrates (460–370 B.C.), who, besides the clinical signs, considered the inspection of sputum of great value for diagnostic and prognostic purposes. This view was shared by Aretaeus (4, 5).

For several centuries, knowledge of the disease did not progress, and treatment was based on superstitious practices (bloodletting, administration of wolfs liver boiled in wine, or boiled crocodile, "the king's touch"), as well as old Hindu remedies, also quoted in the Old Testament (milk from a lactating woman, many meats and vegetables, avoidance of fatigue).

When it became possible to perform autopsies, in the late 1400s, the morbid anatomy allowed for better insight in the pathology of disease as well as allowing correlation with clinical symptoms. Vesalius (1514–1564) was the initiator of modern anatomy, whereas about the same period, Fracastorus (1478–1553) laid the foundation of the epidemiology of infectious diseases with his book "De contagione," where he described three ways of infection: direct contact, fomites, and air. He postulated the existence of "seminaria," imperceptible particles that could exist outside the body for several years and still infect. Among other clever intuitions, we can quote that for phthisis, he postulated that "seminaria" could come not only from outside, but could arise within the body from "putrefaction of the humors." Moreover, he observed that infection is selective in affecting some organs and sparing some others, and he suggested that sterilization at the initial stage of the disease could be efficacious, eradicating the infecting organisms ("germs").

The concept of the contagious nature of phthisis was widely accepted in the following centuries, particularly in Southern Europe, while supporters of the hereditary or constitutional nature persisted in Northern Europe. The evidence of the contagious spreading of the disease induced some governments to take measures of disinfections, decontamination of houses, goods, clothes, personal objects, and some attempts of compulsory report-keeping of affected patients. The Republic of Lucca (Italy) was the first government to take these measures. Its example was later followed by Florence, Naples, and Spain. However, financial considerations and the fear that these laws could give rise to prejudices against tuberculous patients led to their demise by the end of 18th century. However, in the 19th century and the early 20th century, the number of cases and deaths for phthisis showed a continuous increase, particularly in overcrowded cities, where poverty, malnutrition, and poor

Table 16.1 Antimycobacterial Drugs

Generic Name	Trade Name	Name of Chemical Class	Who Marketed the Non-Generic Substance	Efficacy/Potency[a]	Route of Administration	Dose[b]
Synthetic products						
p-aminosalicylic acid	Paser	Aminosalicylates	Jacobus (USA)	1 μg/mL	Oral	10–12 g/day
Clofazimine	Lamprene	Riminophenazines	Geigy (USA)	0.1–1 μg/g[e]	Oral	300 mg/once a month + 50 mg/day
Dapsone	Alvosulfon	Sulfones	Wyeth Ayerst (Canada)	10 ng/mL for *M. leprae*[f]	Oral	100 mglday
Ethambutol	Myambutol	Alkylenediamines	Lederle (USA)	0.5–8 μg/mL	Oral	15–25 mg/kg/day (max. 2.5 g)
Ethionamide	Trecator	Nicotinamide	Wyeth-Ayerst (USA)	0.6–10 μg/mL	Oral	15 mg/kg/day (max. 1 g)
Isoniazid	Lamiazid and Nydrazid	Isonicotinic acids	Lannet (USA)	0.02–0.2 μg/mL	Oral	5 mg/kg/day (max. 300 mg/day)
Prothionamide[c]	Trevintix[c]	Nicotinamides	CSL (Australia)	1–10 μg/mL	Oral	15 mg/kg/day (max. 1 g)
Pyrazinamide	Tebrazid and Zinamide	Nicotinamides	ICN (Canada) Merck Sharp and Dohme (UK)	5–20 μg/mL (at pH 5.5)	Oral	20–35 mg/kg/day (max. 3 g)
Thiacetazone[c]		Thiosemicarbazones		1 μg/mL	Oral	150 mglday (or 2.5 mg/kg/day)
Thiocarlide	Isoxyl	Thioureas	Inibsa (Spain)	0.1–5 μg/mL	Oral	3–6 g/day
Antibiotics						
Amikacin[d]	Amikin	Aminoglycosides	Apothecon (USA) Bristol-Myers Squibb (UK)	4 μg/mL	IM or IV infusion	15 mg/kg/day (max. 1.5 g)

Capreomycin	Capstat	Polypeptides complex from Streptomyces capreolus	Lilly (USA)	10 μg/mL	IM	1 g/day
Cycloserine	Seromycin	Produced by Streptomyces orchidaceus or S. *garophalus*	Lilly (USA)	5–20 μg/mL	Oral	0.5–1 glday
Kanamycin[d]	Kantrex	Arninoglycosides	Aphothecon (USA)	0.5–10 μg/mL	IM	15 mg/kg/day (max. 1.5 g)
Rifabutin	Mycobutin	Rifamycins or ansamycins	Adria (USA) Pharmacia and Upjohn (Italy)	0.1–1 μg/mL	Oral	150–450 mglday
Rifamycin SV[d]	Rifocin	Rifamycins or ansamycins	Lepetit (Italy) Chepharin (Aust.)	0.1 μg/mL	IM or IV infusion	0.5–0.75 glday
Rifampicin or Rifampin[d]	Rifadin and Rimactane	Rifamycins or ansamycins	Marion-Merrel Dow (USA) Ciba (USA)	0.1–2 μg/mL	Oral (IV infusion)	600 mglday
Rifapentine[g]		Rifamycins or ansamycins		0.1–2 μg/mL	Oral	
Streptomycin[d]	Streptomycin injection	Aminoglycosides	Official preparation (USP23)	1–8 μg/mL	IM	15–20 mg/kg/day (max. 1 g/day)
Viomycin[c]		Polypeptides from Streptomyces *pumiceus* or S. floridae		2–16 μg/mL	IM	1 g/day

[a]MIC, minimum inhibitory concentration in *vitro* (μg/mL) for M. tuberculosis.
[b]Dosage is merely indicative. It depends on the type of therapeutic regimen in which it is included
"Not yet released.
[d]Drug active also against other bacterial species.
'(Foot-pad technique) for M. leprae.
[f](*In* vitro mouse tissue culture) for *M. leprae.*
[g]At present not commercially available.

work conditions in the era of initial industrialization favored contagion and spreading of the disease.

At the same time, some progresses in the clinical diagnosis could be registered: the introduction of percussion of the thorax by Auenbrugger and of auscultation by means of the stethoscope by Laennec (1819) allowed for a better diagnosis as well as a correlation of the clinical with postmortem findings. We owe to Laennec also the concept of the unity of tuberculosis, namely that tubercles present in various organs in different stages of evolution were an expression of the same disease. He reached this conclusion on the basis of the description of the pathologic lesions found at the autopsies of tuberculous patients performed by Bayle, who described miliary nodules, cavitary, and extrapulmonary lesions, but was unable to differentiate them from bronchiectasis or lung abscess. Laennec's concept of the etiological unity of the different tuberculous lesions was not uniformly accepted by the scientific community and was credited more than one century after, when the microbiologic demonstration of tubercle bacilli in lesions was possible. According to Scholnlein (1839), tubercle was to be considered the fundamental lesion of "tuberculosis," so that this word should substitute the old definitions of phthisis or consumption. The empiricism and the disputes on the etiology of tuberculosis ended in 1882 with the discovery and isolation of tubercle bacillus by Robert Koch, who could also reproduce the disease in animals.

A considerable progress in the diagnosis of the disease and its clinical stages was represented by the discovery of X-rays by Roentgen in 1885. It took some years to realize how valuable X-rays were in the clinical knowledge of the course of tuberculosis.

From the middle of 19th century, while scientific research progressed, new attitudes toward treatment emerged. The superstitious practices of the past were discarded, and the observation that rest, healthy climate, such as a long stay on the mountains, at the seashore, and spas with mineral waters and proper nutrition exerted a favorable effect on the course of the disease led to the opening of numerous sanatoria. They were based on completely different concepts on which the previous institutions were founded, that is with charitable purposes for poor people and with the aim of stopping the spread of infection in crowded towns. Sanatoria greatly contributed to ameliorate the outcome of tuberculosis and also to examine closely its clinical course, its stages, and its different manifestations. The first sanatoria were set up in Europe, particularly in Germany, Switzerland, and Italy, but quite soon they proliferated in the United States and in the rest of the world. Some controversies arose about the benefits of the sanatorium system: according to somebody's opinion, it could represent a kind of imprisonment, possibly lasting some months, some years, or the whole lifetime, with a negative influence on the patient's personality. For others, it represented the possibility to escape a situation of isolation or even rejection from community life. On the whole, considering the lack of specific therapies of the tuberculosis at that time, the experience of the sanatorium system could be considered positive.

At the same time, the awareness of the contagious character of the disease as well as of the efficacy of preventive measures led numerous national organizations to promote a campaign to educate people about the communicability of tuberculosis and about the use of hygienic measures to prevent its dissemination. A further objective was to improve the epidemiological situation of tuberculosis through legislation, research, better patient care, and establishment of hospitals specializing in tuberculosis patients. The initiative gained great success and was adopted by many countries throughout the world. In 1902, an International Central Bureau for the campaign against tuberculosis was established with an office in Berlin and then in Geneva. Since then, the campaign has reached significant achievements under the auspices of the International Union against Tuberculosis and Lung Disease and World Health Organization as well as other national institutions.

After the discovery of tubercle bacillus and the observation that guinea pigs inoculated with living or dead tubercle bacilli had a different reaction from the latter inoculation that allowed animals to survive (Koch phenomenon), Koch inferred that a treatment with a sterile filtrate of cultured organisms

(Old Tuberculin or OT) could act as a remedy for tuberculosis in humans. Unfortunately, the assumption was not correct, and most of the patients treated with this preparation died. Later, tuberculin was revealed to be one of the major methods to diagnose tuberculosis.

Before the introduction of the specific chemotherapy of tuberculosis, the main therapies were collapsotherapy and surgical resection. Collapsotherapy was based on the idea that allowing the lung to rest would hasten the healing of tuberculous lesions. The lung rest could be obtained by introducing air in the pleural space. Carlo Forlanini in Pavia (1894) performed the first successful pneumothorax, a practice that was widely adopted everywhere.

The presence of pleural adhesion prevented the effective collapse of the lung. The invention of the thoracoscope by Jacobs allowed the adhesions to be cut by introducing the apparatus through the thoracic wall into the pleural space. Also, surgery was used to obtain the collapse of the lung. It consisted in removing ribs (thoracoplasty) to bring the chest wall down to the lung; it was first applied by de Carenville in 1885. Results were quite favorable, significantly prolonging the life of patients. Resection of the diseased lung was also used. Lobectomy and pneumonectomy achieved some successes, but the incidence of complications and mortality was high. At present, surgery is uncommon; it is only applied in selected patients where the disease was caused by multiresistant mycobacteria.

Another approach against the disease was the search for avaccine. In 1921, Calmette and Guerin of the Pasteur Institute of Paris succeeded in preparing a vaccine from an attenuated strain of Mycobacterium bovis (BCG) that proved to be effective in preventing tuberculosis and was widely adopted some years later.

A first step in specific chemotherapy of tuberculosis was represented by Domagk's discovery of sulfones, which showed a certain activity on tuberculosis; however, they were toxic.

The real beginning of the modern era of antituberculous chemotherapy was the discovery of streptomycin (Waksman, 1944), which was extremely active against tubercle bacillus in vitro and in *vivo*, both in animal infection and in human disease. Unfortunately, after a brief period of treatment, mycobacteria became resistant to the adopted drug, and therapy was no longer effective. The discovery of other antituberculous agents showed that the resistance problems could be bypassed by the use of multidrug associations. The discovery of isoniazid (1952), and later of rifampicin (1966), the two most powerful antimycobacterial agents with high bactericidal activity, *allowed* the adoption of multidrug regimens, together with other agents (ethambutol, pyrazinamide, ethionamide, cycloserine, p-aminosalycilic acid, and thioacetazone) capable of eradicating M. tuberculosis from sputum in 1–2 months and curing the disease in 6–12 months (6, 7).

Since then the course of tuberculosis has been profoundly changed, and the old measures and remedies have been discarded. In industrialized countries, only a few sanatoria are now open for people whose social conditions do not insure a proper compliance to therapy at home. Collapse procedures are no longer applied, and surgical resections are limited to few selected indications.

Unexpectedly, in 1985, the number of cases of tuberculosis in western countries. which were always in constant decline, particularly after the introduction of chemotherapy,. showed a sharp increase. This alarming phenomenon prompted a series of studies and surveys to find the cause. Since 1990, this trend now seems to be stable or in decline. In the United States, even if the overall number of cases has declined, the relative proportion of cases occurring among foreign-born individuals increased from 27% to 42% in 1998 (8, 9).

The resurgence of tuberculosis has been attributed mostly to the spread of HIV infection and increasing waves of immigration. Moreover, multidrug resistant (MDR) strains of M. tuberculosis are emerging, rendering older therapies largely ineffective. New strategies have been developed or are under study to withstand this situation.

2 THE MYCOBACTERIA

The genus Mycobacterium belongs to the order Actinomycetales and the family Mycobac-

teriaceae; it is characterized by nonmotile, nonsporulating rods that resist decolorization with acidified organic solvents and alcohol (10). For this reason, they are also called acid-fast bacteria. Some mycobacterial species are pathogenic for humans: the so-called *Mycobacterium* complex includes M. tuberculosis, M. bovis, and M. africanum. M. *microti* is pathogenic for voles, field mice, and other rodents, but it was considered nonpathogenic in humans. However, in recent years, it has been demonstrated that it can cause disease in immunosuppressed, and particularly, in HIV^+ patients (11). M. leprae, pathogenic for man, was considered of uncertain taxonomy for some time, but now is definitely classified among mycobacteria. The study of its genome has contributed to clarify its position in this genus (12).

Other mycobacterial species that resemble M. tuberculosis in some morphologic aspects and cultural requirements, but show a characteristic reaction pattern in a battery of biochemical tests and little or no pathogenic effect in humans (at least in absence of some underlying conditions), were improperly defined in the past as "atypical mycobacteria." They were classified by Runyon (13) into four groups according to their growth rates and pigment production. This classification, however, is inadequate to define the different species in a clear-cut way. The development of recombinant DNA technology has allowed us to define better the species belonging to these groups, but the general definition still does not seem satisfactory. A common definition in the recent years has been "mycobacteria other than tuberculosis" (MOTT), but now "Non-Tuberculous Mycobacteria" (NTM) is preferred. The most recent and appropriate grouping of these organisms, proposed by the American Thoracic Society, is based on the type of clinical disease they produce: pulmonary disease, lymphadenitis, cutaneous disease, and disseminated disease (Table 16.2). The term mycobacteriosis is proposed for the diseases caused by these organisms (14, 15).

M. tuberculosis is a nonmotile bacillus 1–2 μm long and 0.3–0.6 μm wide. It can be demonstrated in pathologic specimens by means of specific staining procedures, the most widely used being the carbol fuchsin or Zichl-Neelsen stain or fluorescent acid-fast staining methods, using auramine as primary fluorochrome. In vitro culture of tubercle bacilli is slow on solid media and sometimes difficult. The nutritional requirements are not particularly complex, but the content of the medium greatly influences the composition of the mycobacterial cell. The most common media used for the isolation of M. tuberculosis from pathological specimens and for its maintenance are solid media, with egg yolk as a base (Petragnani, Lowenstein-Jensen, and IUTM media) or agar (Middlebrook 7H10, 7H11, and 7H12). In these media, the culture begins to appear 12–15 days after inoculation, but full growth is obtained after 30–40 days. When inoculation is made with pathological material from patients, observation must be prolonged. The most widely used maintenance liquid media are synthetic media containing albumin (Dubos, Youmans, and 7HT media). They usually allow rapid growth (8–10 days), and addition of Tween 80 makes possible to obtain uniformly dispersed growth (16, 17).

In addition to culture in solid and liquid media, recent laboratory diagnostic methods for mycobacterial infections and for susceptibility patterns to antimicrobial drugs include radiometric methods that measure the release of $^{14}CO_2$ from a C-labeled substrate (BACTEC), antigen demonstration by enzyme-linked immunosorbent assays (ELISA), DNA probes, nucleic acid amplification methods, and restriction fragment length polymorphism (RFLP) analysis of genomic DNA (18).

In uiuo pathogenic activity of mycobacteria was demonstrated in guinea pigs. The rabbit, which is susceptible to M. bovis infection but scarcely or not at all to M. tuberculosis, was used to differentiate between the two infections. M. leprae, or Hansen bacillus, is resistant to acid and alcohol and requires the Ziehl-Neelsen method of staining to be recognized. It is found in lepromatous lesions, where it is arranged mostly in clumps. It is 1–8 μm long, nonmotile, and nonsporeforming, with a doubling time of 14 days. The most difficult problem in accumulating information about the biology, the susceptibility to antibiotics, and the epidemiology of the disease was the impossibility of cultivating this mycobacterium in vitro. Tests in animals have been improved in

Table 16.2 Classification of the Non-Tuberculous Mycobacteria (NTM) Recovered From Humans

Clinical Disease	*Common Etiologic Species*	*Geographical Distribution*	*Species Rarely Causing Disease in Humans*
Pulmonary disease	• *M. avium complex*	*Worldwide*	• *M. simiae*
	• *M. kansasii*	*USA, coal mining regions Europe*	• *M. szulgai*
	M. abscessus	*Worldwide, mostly in USA*	*M. fortuitum*
	• *M. xenopi*	*Europe Canada*	*M. celatum*
	• *M. malmoense*	*UK, Northern Europe*	*M. shimiodie*
			M. haemophilum
			M. smegmatis
			M. microti
Lymphadenitis	• *M. avium complex*	*Worldwide*	*M. fortuitum*
	• *M. scrofulaceum*	*Worldwide*	*M. chelonae*
	• *M. malmoense*	*UK, Northern Europe (especially Scandinavia)*	*M. abscessus*
			• *M. kansasii*
			M. nonchromogenicum
			M. smegmatis
			• *M. haemophlum*
Cutaneous disease	• *M. marinum*	*Worldwide*	• *M. avium complex*
	M. fortuitum	*Worldwide, mostly USA*	• *M. kansasii*
	M. chelonae	*Australia, tropics, Africa, SE Asia*	*M. nonchromogenicum*
	M. abscessus		*M. haemophilum*
	• *M. ulcerans*		
Disseminated disease	• *M. avium complex*	*Worldwide*	*M. abscessus*
	• *M. kansasii*	*USA*	*M. xenopi*
	M. chelonae	*USA*	• *M. malmoense*
	M. haemophilum	*USA, Australia*	*M. genavense*
			• *M. simiae*
			M. conspicum
			• *M. marinum*
			M. fortuitum

• Slowly growing species.

Adapted from American Thoracic Society (ATS), Diagnosis and treatment of disease caused by nontuberculous Mycobacteria, *Am. J. Resp. Crit. Care Med. 156,* 51 (1997).

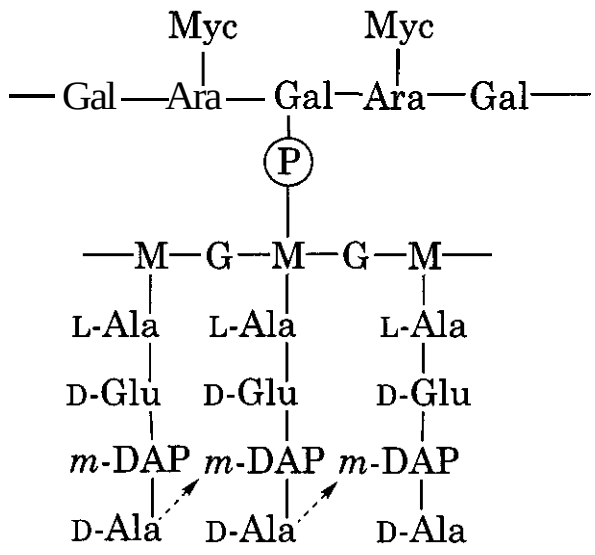
recent years with introduction of the infection of foot-pads in mice and an infection model in the armadillo, but they are complex and can be performed only in the specialized laboratories (19).

The biochemical constitution of mycobacteria is complex and an enormous amount of work has been done in this field. Novel chemical structures have been discovered, but the relationship between these and the pathogenic and biological activities of mycobacteria have not yet been satisfactorily elucidated (19–24). Information about metabolism of mycobacteria is extremely voluminous, but the overall picture of the mycobacterial metabolism is far from complete.

Some interesting differences exist in the metabolic properties of tubercle bacilli grown in *vivo* and in vitro, which must be considered when the practical value of antimycobacterial agents designed and tested in laboratory is assessed in practice. Populations of M. tuberculosis H37 Rv (a virulent strain) grown in the lungs of mice and populations grown in vitro show two different phenotypes, Phe I and Phe II, with marked differences in the metabolism of certain energy sources, in the production of detectable sulfolipids, and in immunogenicity (Phe II is a better immunogen than Phe I) (25–27). Because the shift from Phe I to Phe II is readily reversible, it can be deduced that the genome of H37 Rv remains the same (28); probably, a modification in its surface occurs.

A great deal of effort has been focused on research of the constituents of the mycobacte-

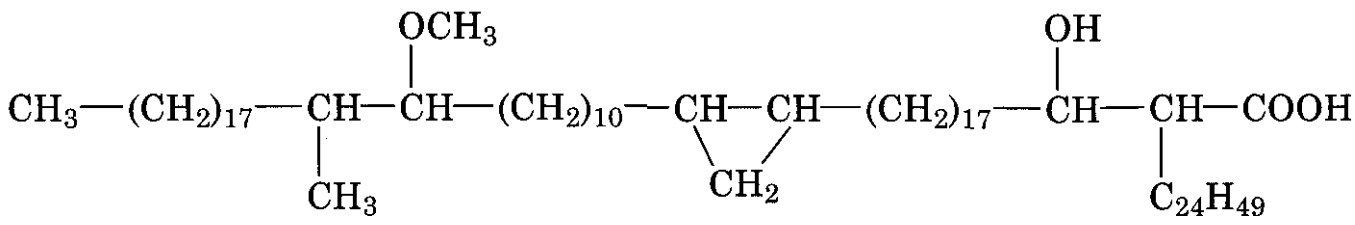
rial cell wall, because they are responsible for many of the pathogenic effects of tubercle bacilli. The core of the mycobacterial cell wall is the mycolylarabinogalactanpeptidoglycan constituted by three covalently attached macromolecules (peptidoglycan, arabinogalactan, and mycolic acid). A schematic structure, simplified from (29), is outlined in (1). The peptidoglycan (or murein) consists of a repeating disaccharide unit, in which N-acetyl-D-glucosamine (G) is linked in a 1–4 linkage to *N*-glycolyl-D-muramic acid (M) attached to L-alanine-D-glutamic acid-NH_2-meso-diaminopimelic acid-NH_2-D-alanine. This unit is linked to a polysaccharide unit, the arabinogalactan, through a disaccharide phosphate (rhamnose-galactose phosphate). The arabinogalactan is connected to a glycolipidic region constituted of esters of mycolic acids.



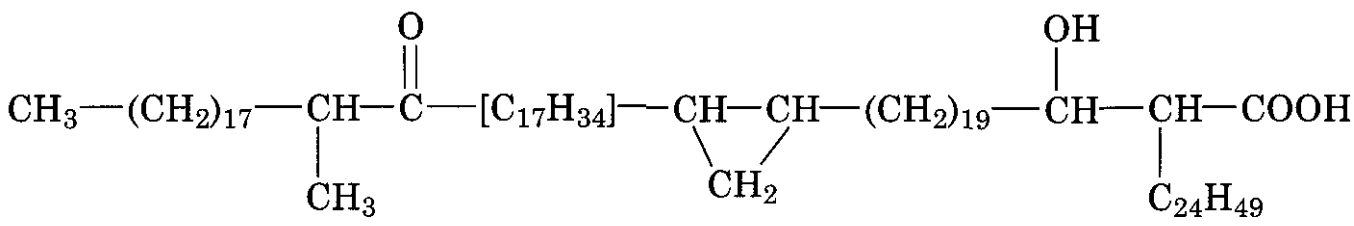
(1)

Mycolic acids are a-branched, β-hydroxylated long-chain fatty acids, of which three principal groups are known: the corynomycolic acids, the nocardic acids, and the mycobacterial mycolic acids (30, 31). Mycolic acids can also be detected in the skin lesions of patients suffering from lepromatous leprosy, indicating that the agent of leprosy is a mycobacterium containing it (32). The ,chemical structures of methoxylated mycolic acid and β-mycolic acid extracted from M. *tuberculosis* sp. *hominis* are shown in structures (2) and (3) (33). The mycolic acids are linked through their carboxy groups to the end terminal OH groups of the D-arabinofuranose (Araf) molecules, branches of the arabinogalactan of the cell wall (34–36). Mycolic acids are known to be acid-fast, because they bind fuchsin and the binding is acid-fast. Thus, it seems that the acid fastness of mycobacteria depends on two mechanisms: the capacity of the mycobacterial cell to take fuchsin into its interior and the capacity of mycolic acids to form a complex with the dye (37–39).

In addition to the lipid murein part of the rigid structure, there is a series of soluble lipid compounds that seem to be located in or on the outer part of the cell wall: lipoarabinomannan, waxes D, cord factor, mycosides, sulfolipids, and phospholipids (20–22). Lipoarabinomannan (LAM) is an essential part of the cell envelope, being a component of the plasma membrane, which lacks covalent association with the cell core. In this macromolecule, arabinan chains are attached to a mannan back-



(2)



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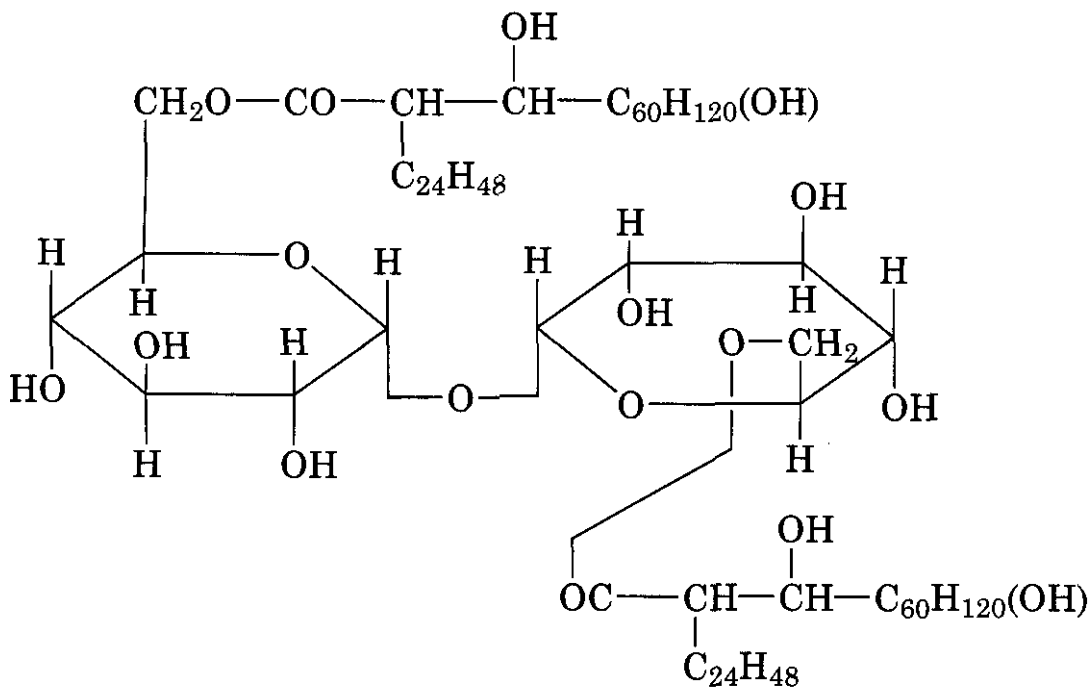


(4)

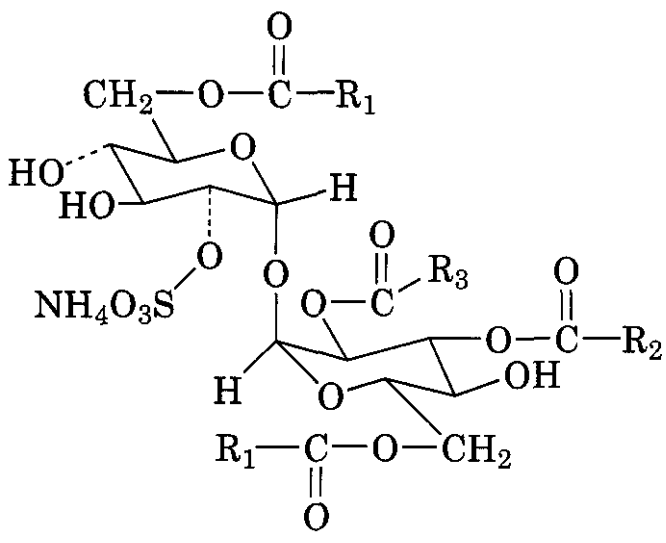
bone, which is, in turn, attached to a phosphatidylinositol esterified with fatty acids like palmitate and 10-methyloctadecanoate (tuberculostearate).

The so-called waxes D are ether-soluble, acetone-insoluble, and chloroform-extractable peptidoglycolipid components of the mycobacterial cell, probably autolysis products of the cell wall (40). Because they are esters of mycolic acids with arabinogalactan linked to a mucopeptide containing *N*-acetylglucosamine, N-glycolylmuramic-acid, L- and D-alanine, meso-diaminopimelic acid, and D-glutamic acid, it has been suggested that they are materials synthesized in excess of those needed for insertion into the cell wall (22, 39–41). The constitution of wax D differs in different varieties and strains of mycobacteria.

Cord-factor is a toxic glycolipid (6, 6′-dimycolate of trehalose) structure (4), which has been deemed to be responsible for the phenomenon of cording (42, 43) (i.e., the capacity of *M. tuberculosis* to grow in serpentine cords), a property that is correlated with its capacity to kill guinea pigs (44, 45). The detergent properties of cord factor and its location on the outer cell wall have led to the suggestion that it may play a role in facilitating the penetration of certain molecules necessary for growth of mycobacteria (39).

Mycosides are glycolipids and peptidoglycolipids type-specific of mycobacteria (46) that often have in common terminal saccharide moieties containing rhamnoses *O*-methylated in various positions (47). They can be divided into two main categories (31): phenolicglycolipids with branched-chain fatty acids and peptidoglycolipids consisting of a sugar moiety, a short peptide, and a fatty acid. The biological activity of mycosides is still obscure. They probably have a role in cellular permeability (48). Glycolipids or peptidoglycolipids are responsible for the ropelike appearance that is evident in one of the outer layers of mycobacteria when they are visualized by the technique of negative staining (39).

The sulfolipids (2, 3, 6, 6′-tetraesters of trehalose; 5) (22–28, 49), to which are attributed the cytochemical neutral-red fixing activity of viable, cordforming tubercle bacilli, seem to



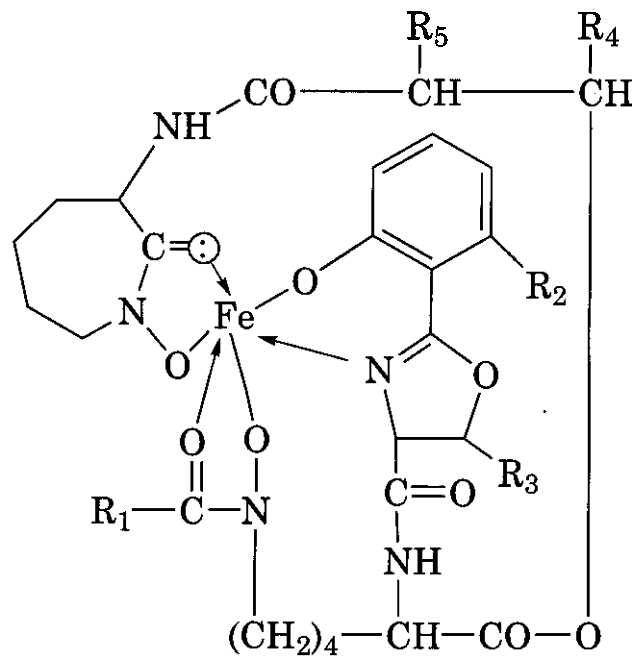
(5)

play a role in conferring virulence to tubercle bacilli and influencing their pathogenicity (49), acting synergically with the cord factor (50).

The phospholipids (cardiolipin, phosphatidylethanolamine-glycosyl diglyceride, and phosphatidylinositol-monomannosides and -oligomannosides) were considered to be antigenic substances elaborated by M. tuberculosis, but the most purified preparations have been shown to behave only as haptens (22, 30, 51).

Even though some suggestions have been made about the biological activities of the lipids of the tubercle bacillus, a clear structure-function relationship has not yet been delineated. Nor has it been determined which structural features can produce favorable or detrimental effects. Also, the biosynthetic pathways leading to the formation and assembly of the cell wall have not been clarified, and the enzymes involved have not been purified.

Other interesting substances isolated from mycobacteria are the mycobactins, which are a group of bacterial growth factors that occur only in the genus Mycobacterium (52). The isolation of the mycobactins was followed by the identification of growth factors in other microorganisms, the sideramines, which differ from mycobactins but share with them some common properties, the most relevant being strong chelating capacity for ferric ions. At least nine mycobactin groups have been isolated from different mycobacteria. They have the same basic constitution, with some variations in details of structure. They consist of an octahedral iron-binding site (containing two secondary hydroxamate groups, an oxazoline ring, and a phenolic hydroxyl group) and a hydrophobic chain (containing up to 20 carbon atoms). Mycobactin P (6), isolated in 1946, was the first example of a natural product with an exceptional iron-chelating activity, and its structure was the first to be determined. Mycobactin S (7) is the most active of these factors, showing growth stimulation at concentrations as low as 0.3 ng/mL. Mycobactin M (8) is a representative of the structure of M-type factors. The most biochemically unusual product in the structure of mycobactins is N^6-hydroxylysine, which is present in the molecule in both acyclic and cyclic forms. All the



(6) $R_1 = C_{17}H_{34}$, $R_2 = CH_3$, $R_3 = H$, $R_4 = C_2H_5$, $R_5 = CH_3$
(7) $R_1 = C_{17}H_{34}$, $R_2 = H$, $R_3 = H$, $R_4 = CH_3$, $R_5 = H$
(8) $R_1 = CH_3$, $R_2 = H$, $R_3 = CH_3$, $R_4 = C_{17}H_{34}$, $R_5 = CH_3$

known mycobactins contain either a salicylic acid or a 6-methylsalicylic acid moiety. The oxazoline rings derive from 3-hydroxy amino acids, either serine or threonine. The mycobactins are powerful iron chelators with an association constant of $>10^{30}$ and are essential growth factors for the mycobacteria. Therefore, it seems likely that they are. involved in mycobacterial iron metabolism (53). Mycobacteria respond to iron deficiency by producing salicylic or 6-methylsalicylic acid, together with mycobactins that have a great affinity for ferric iron. It has been suggested that in the mycobacteria, salicylic or methylsalicylic acid mobilizes the iron in the environment and that mycobactins are involved in the active transport of iron into the cell.

Mycobacterial proteins have been submitted to extensive research since the days of the discovery of Robert Koch and the preparation of the old tuberculin (OT), a heat inactivated concentrate of proteins released to the growth medium from stationary culture of M. tuberculosis. This product termed "tuberculin" elicits a reaction in the skin, that is an expression of delayed-type hypersensitivity (DHT), and has been used for the diagnosis of tuberculosis. At present, an ammonium sulfate precipitable protein fraction of the culture filtrate, termed "tuberculin purified protein

derivative" (PPD) is currently used to determine the "tuberculin reaction" (54).

Numerous proteins with different locations have been identified in M. tuberculosis. They can be cytoplasmic, cell-membrane, and cell-wall associated or secreted to the surrounding medium. They have been sequenced, but in most cases, their function has not been identified.

Cytoplasmic proteins are involved in amino acid and protein biosynthesis, and sometimes they can show a different behavior in virulent and avirulent strains. For instance, the virulent strains (H37 Rv) possess one type of asparaginase, and the avirulent one (H37 Ra) possesses two asparaginases. The avirulent strain has an aspartotransferase that transfers the aspartyl moiety of asparagine to hydroxylamine, whereas the avirulent strain does not (55–56).

Other proteins show a significant homology with the so called "heat shock proteins" or "stress proteins." They have many functions, particularly in assisting the microrganism in adaption to environmental changes (57). Proteins synthesized in the cytoplasm may be destined to function outside the cytoplasm either in association with the cytoplasmic membrane or cell wall or can be exported to the surrounding medium. Proteins secreted by M. tuberculosis are very potent in generating a cellular immune response. The vaccine of Calmette-Guerin (BCG), an attenuated strains of M. *bovis*, is in position to confer protection against disease only if it is alive. It has been suggested that only in this situation, where the secretion of proteins is continuous, will the reaction of T-cells responsible for recognition of the infected macrofage be elicited and lead to the control of infection at an early stage. Proteins associated with the cell membrane or the cell wall are lipoproteins, which besides fulfilling functional activities such as binding some nutrients, could be immunogenic. Proteins exported to the exterior have some distinct functions. As example we can quote superoxide dismutase (SOD), an enzyme that can paralise the host defense mechanism by inactivating the toxic oxide radicals generated by the activated macrophage (58).

The functions of mycobacterial proteins that have been found in the past years could now be confirmed, elucidated, and completed with those of numerous other proteins and heterogeneous compounds constituting the mycobacterial cells through the informations obtained by the complete sequencing of M. tuberculosis genome (virulent strain H37Rv).

This achievement, reported in 1998, represents a milestone in knowledge of biology of *M.* tuberculosis. The genome results of a composite sequence of 4.411.529 base pairs, with a guanine + cytosine (G + C) content of 65.6%, which is relatively constant throughout the genome. It contains 3924 proteins encoding genes. It is the second largest bacterial genome after E. coli. By the analysis of the proteins encoded in genes, a precise function could be attributed to a 40% of them and some information was obtained for another 44%. The remaining 16% do not resemble any known proteins and may account for specific mycobacterial functions (59, *60).*

From the composition of the genome sequence, it can be inferred that M. tuberculosis has the capacity to synthetize all essential amino acids, vitamins, and enzymes, necessary co-factors for major metabolic pathways present in the cell (lipid metabolism glycolysis, pentosephosphate pathways, tricarboxylic acid, glyoxylate cycles) as well as for a number of others.

Two major important findings emerged by the genome sequence: the identification and characterization of repeat elements as well as of regulatory genes. The presence of repeat elements, that vary from strain to strain, allows the strain subtyping by molecular techniques. The expression of regulatory genes is governed by 13 putative sigma factors, which promote the recognition of the subunit of mycobacterial RNA polymerase. More than 100 regulatory proteins are predicted, and other transcription regulators and regulatory protein pairs are present that may transmit signals from outside of the bacterial cell into the cytoplasm.

Two new families of acidic glycine-rich proteins have been recognized, the PE and PPE families, which represent about 10% of the coding capacity of the genome. Their genes are clustered. Their function is unknown except for a lipase. These proteins include one of the major antigens, the serine-rich antigen,

present in leprosy patients. Even if at present there is no proof, some immunological function could be possibly attributed to them and, according to some authors, also some role as virulence factors (61).

The complex lipidic constitution of M. tuberculosis cells led to the presumption of the presence of numerous genes encoding for lipolitic and lipogenic activity. In fact, over 250 genes for biosynthesis of various lipid classes were found in M. tuberculosis in contrast with the approximately 50 genes of the same class in E. coli. For fatty acid degradation, a process that leads to release lipids within mammalian cells and tubercles, there is a series of enzymes that are involved such as 36 acylCoA-synthases and a family of 36 related enzymes that could catalyze the first step in fatty acid degradation. The successive degradation steps are catalyzed by the enoyl CoA hydratase/isomerase superfamily of enzymes, enzymes converting 3-hydroxy fatty acid into 3-keto fatty acid, the acetyl-CoA, C-acetyltransferases, and the Fad A/Fad B β-oxidation complex. For fatty acid synthesis, two distinct families of enzymes are involved: fatty acid synthase I and II (FAS I on FAS II), which contain seven enzyme activities. Two subunits of FAS II system, the enoylreductase synthase corresponding to the enzyme *Inh*A and β-ketoacylsynthase proteins *Kat*A are target for isoniazid. Numerous other genes are contributed to the fatty acid biosynthesis (carboxylase system, Fab A–like β-hydroxyacyl-ACP-dehydrase, S-adenosyl-L-methionine-dependent enzymes, etc.).

It was found that mycobacteria also synthetize polyketides by several different mechanisms, similar to those involved in erythromycin biosynthesis. There are four classes of polyketide synthases. Their function in mycobacteria is unknown, but it is of potential interest because some polyketides such as rapamycin show antibacterial activities or immunological properties.

From genome sequencing, useful information is expected to clarify some aspects of virulence, pathogenicity, and immunology of M. tuberculosis. At present little information has been obtained about virulence. Before the completion of the genome sequence three virulence factors were known: catalase-peroxidase, with a protective activity against reactive oxygen species produced by the phagocyte; mce that encodes macrophage colonizing factor; and a sigma factor gene, *sig*A, mutations, which can lead to virulence attenuations. After genome sequence a series of phospholipases C, lipases, and esterases, which attack cellular or vacuolar membranes, could be individuated as further virulence factors.

To identify factors of virulence, the genomic analysis of M. tuberculosis H37Rv and of the closely related attenuated strains H37Ra has allowed to individuate some genetic differences that is two polimorphisms: a 480-kb fragment in M. tuberculosis H37Rv was replaced by two fragments of 220 and 260 kb in M. tuberculosis H37Ra, while there was a fragment of restriction endonuclease *Dra*I in M. tuberculosis H37Ra that had no counterpart in M. tuberculosis H37Rv (62). It has been suggested that these polymorphisms were the results of transposition of an insertion sequence (IS6110), which may have inactivated virulence genes. However complementation of M. tuberculosis H37Ra with the restriction fragment from M. tuberculosis H37Rv that encompasses the IS6110 did not restore the virulence as it could be demonstrated by infection in mice. Therefore, the role played by the genomic variation demonstrated in the two strains has not been elucidated.

A further approach to identify genes for putative virulence has been to perform comparative genomics of the members of M. tuberculosis complex, an approach facilitated by the paucity of genetic differences among them at the nucleotide level (63). The genome sequence of M. bovis is almost completed (64). A comparative study of M. bovis and M. bovis BCG (a strain of attenuated virulence used for vaccination) has shown that three regions, designated as RD1, RD2, and RD3, are deleted in M. bovis BCG relative to M. bovis, but it is not clear if these deletion regions play a role in the attenuation of M. bovis BCG. Other deletion regions were found from BCG relative to M. tuberculosis. Loss of RD5 locus removed the genes for three phospholipase C enzymes, other deletions concerned Esat6 protein, (a potent T-cell antigen), the genes involved in lipopolysaccharide biosynthesis. Although de-

finitive comparison of PE and PPE is not possible as the genome sequence of BCG is not completed, analysis so far performed revealed remarkable differences in the sequence of PE and PPE proteins, which could have a role in virulence. Therefore, it seems that comparative genomics may offer some indications to understand the attenuation of M. bovis BCG.

The genome of M. leprae, which has recently been fully sequenced, is smaller that that of M. tuberculosis, containing 3.268.182 base pairs. Its GC content circa 58% and the number of genes is also inferior (about 1800 vs. 3966 of M. tuberculosis H37Rv) (12). Assuming that the genome of M. leprae was once equivalent and similar in size to other mycobacteria, it can be inferred that extensive downsizing and rearrangement may have occurred during evolution. The leprosy bacillus may have lost more than 2000 genes. Comparison with the genome of M. tuberculosis revealed a mosaic arrangement, where areas (about 65 segments) showing synteny are flanked by unrelated genomic segments, Many sequences are noncoding and demonstrated to be pseudogenes, namely inactivated version of genes that are still functional in M. tuberculosis. The present arrangement denotes that extensive recombination events between dispersed repetitive sequences occurred. Gene deletion and decay led to the elimination or decrease of many important metabolic activities such as siderophore production, oxidative microaerophilic, and anaerobic respiratory chains as well as of many catabolic systems. On the other hand, among the outer lipids of the leprosy bacillus there is a phenolic glycolipid 1, a component not found in the envelope of *M.* tuberculosis. Several enzymes don't have any counterpart in M. tuberculosis, such as uridine-phosphorilase and adenylate cyclase, and two transport systems, one for sugars the other for divalent metal ion uptake. The composition of genome of M. leprae that shows an extended loss of functions in comparison to *M.* tuberculosis may account for some peculiar biological properties of this organism: extremely long doubling time, non-cultivability in vitro, obligate intracellular location, and pronounced tropism for the Schwann cells of the peripheral nervous system in humans.

Further insight in gene functions and comparison with genomes of other mycobacteria hopefully will reveal the mechanisms of pathogenicity and virulence and provide the basis for the design of new chemotherapeutic agents and vaccines.

3 PATHOGENESIS AND EPIDEMIOLOGY

3.1 Tuberculosis

Tuberculosis is sometimes an acute but more frequently a chronic communicable disease that derives its character from several properties of the tubercle bacillus, which in contrast with many common bacterial pathogens, multiplies very slowly, does not produce exotoxins, and does not stimulate an early reaction from the host. The tubercle bacillus is also an intracellular parasite, living and multiplying inside macrophages.

The structure and evolution of lesions caused by tubercle bacilli are determined by host aspecific defense system, immunologic response, and genetic factors. Most commonly mycobacteria reach the lung alveoli by inhalation. If the number of bacilli is low, the strain is of moderate virulence and the intrisic microbicidal activity (genetically determined) of resident alveolar macrophages is of good level,. the tubercle bacilli ingested are rapidly killed and no further evolution of infection follows. However mycobacteria may evoke a variable immunological response leading the host organism either to a state of resistance or to a state of disease, which depends on the number of infecting organisms and the already quoted host defense capacity and genetic factors.

To schematize the events that follow the penetration of tubercle bacilli in the organism, four stages have been described in the pathogenesis of tuberculosis, mainly based on the researches of Lurie (65) and Dannenberg (66) in rabbits. The results of animal research can be only partially applied to human disease. However they have contributed to a better understanding of the main aspects of its course (67).

The first stage has been described above, and because of the rapid destruction of the infecting organism, it can be self-limiting. However if the number of bacilli is high or if

the macrophage microbicidal activity is inadequate to kill them, bacilli multiply inside the cell, causing its burst. This event, through the production of chemotactic factors from the released bacilli attracts monocytes from the circulation and gives origin to the second stage.

Blood-born monocytes are not yet activated, so they cannot destroy the bacilli, which grow within the cell at a logarithmic rate. At this time, the immunological response is triggered; mycobacterial antigens processed by macrophages elicit the subset lymphocytes (CD+ T-cells) primarily involved in cell-mediated immunity, the main mechanism of protective immunity in tuberculosis. These cells secrete a number of lymphokines (particularly interferon-γ) capable of inducing macrophages to kill intracellular mycobacteria (68, 69). At this stage the key event in the immunological response to mycobacterial antigens, i.e., the formation of granuloma (tubercle), is initiated.

Stage 3 starts when the logarithmic phase of bacillary growth stops. It is characterized by the emergence of an other subset of lymphocytes, CD8+ T-cells, that probably play some role also in the late phases of stage 2 and through their action caseous necrosis is produced. CD8+ T-cells are endowed with cytotoxic activity and mediate the delayed-type hypersensitivity, which leads to caseous necrosis and to interruption of logarithmic bacillary growth. CD8+ T-cells destroy the nonactivated macrophages by eliminating the intracellular environment favorable to bacillary growth. The released bacilli will be then phagocytized by activated macrophages (whose activation is accelerated by previously sensitized lymphocytes). It is still debated how CD8+ T-cells contribute to the production of caseous necrosis; among different ideas, it has been suggested that CD8+ T-cells induce local sensitivity to tumor necrosis factor (TNF) (69–71).

Further research has been dedicated to investigate the role of apoptosis (programmed cell death) of T-lymphocytes in the pathogenesis of TB. Conflicting results have been obtained. A recent hystopathologic finding in biopsy material of TB patients seems to throw some light on this subject (72, 73). Large numbers of apoptotic T-cells and macrophages were seen in areas surrounding caseous foci. Macrophages are negative for anti-apoptotic bcl-2 protein, but positive the pro-apoptotic bax protein, whereas the associated T-cells express interferon (IFN-γ) and FasL, data that indicate that caseation is associated with apoptosis of macrophages and T lymphocytes. If these findings are confirmed, apoptosis should represent a mechanism for tissue destruction and consequently for TB transmission (74, 75).

In its typical elementary structure, granuloma shows a solid caseous center surrounded by immature macrophages (allowing intracellular bacillary growth) and activated macrophages (that can differentiate in epithelioid cells and Langhans cells, giant polynuclear cells), which kill the bacilli. The development or the arrestment of the disease depends on the balance between the two types of macrophages. Caseous material is not favorable to the bacillary growth because of acidic pH, low oxygen tension, and presence of inhibitory fatty acids; mycobacteria do not multiply but can survive for years in this environment (76–77).

The disease proceeds when stage 4 is reached, in which liquefaction of the caseous center occurs, often followed by formation of cavities where bacilli multiply extracellularly reaching very high density. They can be discharged into the airways, diffusing in other parts of the lung and in the environment. Liquefaction and cavitation as markers of disease progression occur in human disease, but not in infection model in rabbits (67). However, the schematized patterns of infection in animals find some counterpart in the course of human infection and disease. In fact, the first contact of the human organism with tubercle bacilli, which usually takes place in infancy or adolescence, normally does not produce any clinical manifestation. The anatomic lesion induced by proliferation of mycobacteria and the reactive regional adenitis are called "primary complex." At that moment the subject shows a positive tuberculin test [a cutaneous reaction obtained by injection or percutaneous application of culture filtrates of mycobacteria or of their purified protein content (PPD)], which indicates a state of hypersensitivity to the tubercle bacillus, not necessarily a state of im-

munity. Usually the primary complex remains clinically silent, but it can also progress and evolve to a state of disease.

According to the definition of the American Thoracic Society (ATS, 1981), tuberculosis infection does not mean disease (78). The state of disease is defined by the appearance of clinically, radiologically, and bacteriologically documentable signs and symptoms of infection.

Chronic pulmonary tuberculosis in adults may be caused by reactivation of the primary infection or to exogenous reinfection. Some aspects of the state of latency of M. tuberculosis in lung tissue, cause of possible reactivation of the disease, have been recently elucidated that could have some impact in clinical settings. It has been demonstrated that some mutants are not capable to maintain the state of latency; this property is associated to inactivation of cyclopropane synthetase (79) or isocytrate lyase (80), which is required for the metabolism of fatty acids. Moreover a mutant has been discovered that cannot proliferate in the lungs, although it proliferates normally in other tissues. This strain is unable to synthetize or transport the cell wall associated phtiocerol, dimycocerosate, a lipid found only in pathogenic mycobacteria (81).

A typical characteristic of tuberculosis is the formation in the infected tissue of tubercles, nodular formations, which can have different sizes and different modes of diffusion, giving rise to various clinical forms called miliary, infiltrate, lobar tuberculosis, and so on. The disease progresses by means of ulceration, caseation, and cavitation, with bronchogenic spread of infectious material. Healing may occur at any stage of the disease by processes of resolution, fibrosis, and calcification.

Control of the disease has been achieved in part through mass vaccination with BCG (the bacillus of Calmette and Guèrin, an attenuated strain of *M. tuberculosis bovis*), but above all through correct application of active chemotherapeutic agents. Chemotherapeutic treatment now available enables to stop the propagation of the disease in a high percentage of cases, by killing the pathogenic bacilli, thus permitting the organism to repair or to confine the pathological alterations. Another important consequence of chemotherapeutic treatment is the prevention of dissemination of virulent bacilli to other persons.

Despite the efficacious drugs now available for the treatment of tuberculosis, this illness is present all over the world. According to recent estimates by the World Health Organization (WHO), 8.4 million new cases occurred in 1999, up from 8.0 million in 1997. The rise affected mostly the African countries, which had a 20% increase in TB incidence attributed to the continuous spread of HIV/AIDS. The total number of smear positive cases was 1.4 million both in 1998 and 1999 (82). In developing countries, the majority of deaths occurs in young adults, between 15 and 40 years and represents 98% of all deaths caused by tuberculosis (83). Twenty-two are in the highest burden countries and more than one-half the cases occurred in India, China, Indonesia, Bangladesh, and Pakistan. The highest incidence rates pro capita were in sub-Saharan Africa (82, 84).

In developed countries after the First World War, the trend to a decrease in tuberculosis morbidity has been constant, probably related to better general hygienic conditions, preventive measures, and in the last decades, to the introduction of effective chemotherapy. However, in 1985, the reports of WHO indicated a resurgence of tuberculosis, even in advanced countries of Europe and North America. In the United States, where the incidence of tuberculosis has been declining at an average rate of 6% each year until 1985, the case rate has risen from 9.3 for 100,000 in 1985 to 10.3 in 1990 (85–88).

In April 1993, the WHO took the extraordinary step of declaring tuberculosis a "global emergency" (88). However, after this increase, case rates started to become stable or declining. The most recent data, issued in 1998, indicated that the incidence of tuberculosis in the United States was 6.8 per 100,000 per year, a decrease of 31% since 1992. However the relative proportion of cases occurring among foreign-born individuals increased from 27% in 1992 to 42% in 1998 (84). It is well known that this phenomenon occurs in poor areas of major cities in industrialized countries. A dramatic increase in the number of tuberculosis cases was notified since the early 1990s in some Eastern European countries,

particularly Georgia, Kazakhstan, Kyrgystan, Latvia, Lithuania, Romania, and the Russian Federation. In Russia, the number of cases more than doubled in the 5 years between 1992 and 1997 (89).

Bolivia, Haiti, and Peru have notification rates greater that 100 per 100,000. In Peru, where the notification rates reached 225 per 100,000 in 1994, the intensified control and the prevention and therapy measures led to a decrease in notification rates of 3–4% per year, while the treatment success rates now exceed 90%. China and Vietnam reported a remarkable increase in number of TB cases during the 1990s. Now the two countries have implemented a control program that seems to give good results. In the United States, the highest percentage of TB cases are registered among blacks and Hispanics between the ages of 25 and 44 years (89–90). In Western Europe, the Gulf States, and Australia, the majority of cases are among foreign-born immigrants. In 1997, immigrants accounted for more than one-half of tuberculosis cases in these areas.

Why the highest rate of tuberculosis is in immigrants is not fully understood, even if many factors are known to contribute to this behavior. Poor living conditions, poor socioeconomic status, and malnutrition surely play an important role.

Moreover it must be noted that the spread of HIV infection/AIDS has coincided with the recrudescence of TB (91). AIDS is the highest risk factor that favors the progression of latent infection to active tuberculosis. In HIV-infected subjects, cell-mediated immunity is depressed, because the major targets of this virus are CD4+ T lymphocytes, whose number reduction and functional impairment prevents them to mount the essential immunological response to M. tuberculosis challenge. At present it is estimated that the risk of developing tuberculosis in HIV infected adults is 3–8% annually throughout the world, with rates varying widely among geographical areas within the same country. The majority of cases are concentrated in sub-Saharan African countries where the AIDS epidemic has caused about an 100% increase in reported tuberculosis cases (92).

HIV-infected subjects may develop tuberculosis by direct progression of exogenous infection to disease or by recrudescence of previously acquired latent infection. They are at least 30-fold more likely to develop reactivation tuberculosis as HIV infection progresses. Severe outbreaks of tuberculosis have been reported when groups of HIV-infected persons have been exposed to a person with infectious tuberculosis (93–95).

Tuberculosis tends to occur in the early stages of HIV infection. Sometimes it is the first manifestation of the disease caused by HIV. The clinical course of tuberculosis is determined by the degree of immunosuppression. Very often it is a severe disease, frequently with extrapulmonary localization (92).

A further consequence of spreading HIV infection has been an increase of infections caused by mycobacteria other than tuberculous (MOTT) or non-tuberculous mycobacteria (NTM), previously defined as atypical mycobacteria. They only partially resemble the tubercle bacillus and cause diseases less frequently than it. In some geographical areas, their incidence is rather high, such as M. *kansasii* in central United States and M. ulcerans in Australia and South Africa. Unfortunately the drugs developed for the treatment of tuberculosis have a poor efficacy for these infections. Among NTM, the M. *avium intracellulare* complex (MAC), which in the past was very seldomly recognized as cause of infection, showed to be a frequent cause of severe disseminated infection in AIDS patients occurring most frequently in the late stages of the disease. However, at present, a growing number of patients have MAC infection as the initial manifestation of AIDS. In the last years, MAC has been isolated from 20% to 80% of patients fully followed from initial diagnosis of AIDS to death (96–98).

The resurgence of tuberculosis has been accompanied by another phenomenon of importance, that is the emergence of multiple drug-resistant (MDR) strains of M. tuberculosis in the etiology of tuberculosis. MDR organisms demonstrate in vitro resistance to at least two major antituberculosis drugs, usually isoniazid and rifampicin.

Cases caused by these strains are difficult to treat. Multidrug-resistant TB has emerged particularly in the states of Eastern Europe

and Russia, probably as a consequence of the collapse of public health services, associated with growing poverty, malnutrition, and war. Percentages of 9.4% for primary MDR-TB were reported from Russia in 1998, whereas percentages as high as 55% and 23% were reported from patients in Russian prisons (90, 99).

3.2 Leprosy

Leprosy is a chronic disease caused by M. leprae, a mycobacterium that multiplies even more slowly than the tubercle bacilli, having a doubling time of about 15 days. It is an obligate intracellular organism that can not be cultured in *vitro*. Mice foot-pad infection and a model of infection in armadillo are the means to grow this mycobacterium (100).

For a long time, humans were considered the only natural reservoir of M. leprae, but actually it has been demonstrated that about 15% of wild armadillos in Louisiana and Texas and some primates can be infected by M. leprae (19, 101) The mode of transmission of leprosy is not fully elucidated. Household contacts with patients with borderline leprosy and lepromatous leprosy whose nasal mucosa is heavily infected are at risk of acquiring the infection because of dissemination of infected secretions in the air, whereas dissemination through skin lesions and contaminated soil seems less important (102–104). M. leprae causes granulomatous lesions that differ according to the immune status of the patient. Granulomatous lesions with epithelioid cells, many lymphocytes, and few Mycobacterium leprae are characteristic of tuberculoid leprosy, the form of disease occurring in subjects with a good immunologic reactivity. In lepromatous leprosy, the form of disease occurring in subjects with impaired cell-mediated immunity, granuloma shows a massive infiltration of tissues with large macrophages filled with mycobacteria. Lymph nodes have hyperplastic germinal centers and in the paracortical areas there are very few lymphocytes.

The clinical manifestations of tuberculoid leprosy consist of skin macules with clear tenter that are insensitive to pain stimuli because of the involvement of peripheral nerves. The skin lesions of lepromatous leprosy consist of numerous, symmetric, small, hypopigmented, or erythematous papules in the initial phase, whereas nodules develop later in the course of the disease. Then the lesions diffuse to the eyes, upper respiratory tract, and many other organs and tissues. The damage to peripheral nerves lead to loss of sensation, deformity, and mutilation. The borderline forms of leprosy show lesions that have characteristics intermediate between the two main forms of the disease.

The majority of people exposed to leprosy will not develop it. Those who develop it, after an incubation period that can range from 1 to ≥40 years (usually 5–7 years), may have only single lesion (indeterminate leprosy) that is often self-healing or may progress to the paucibacillary or multibacillary stages. The progression and evolution of the infection is influenced by many factors: socioeconomic status, immune status of the host, genetic factors, and concomitant infections. For instance, a previous infection with M. tuberculosis may boost the immune system, diminishing the chances of developing leprosy (105). BCG vaccination seems to provide protection against leprosy, but the figures proving it are quite different, ranging from 20% to 80% (106). Conflicting results are reported also about the influence of concomitant HIV infection on the development of leprosy. So far there is no indication that the HIV epidemic is causing an increase in the number of leprosy patients. It is possible that the incubation period for leprosy is too long to discover the relationship between the two diseases (102).

The influence of genetics is documented by familial clustering (107) by the link between NRAMP1 gene (108) and susceptibility to leprosy, as well between HLA genes and the type of leprosy (109, 110).

The Ridley and Jopling classification (111) correlates the clinical manifestations of leprosy with cell-mediated immune response. The types of disease defined as indeterminate (I) and tuberculoid (TT) occur in patients who exhibit a relatively good cell-mediated immunity against M. leprae, borderline (BT), mid-borderline (BB), and borderline lepromatous (BL) in patients with less effective cell-mediated immunity, and lepromatous (LL) in patients anergic to M. leprae. In the last classes of patients, the disease is widespread and in-

volves the skin and upper respiratory tract, the anterior chamber of the eye, the testes, the lymph nodes, the periosteum, and the superficial sensory and motor nerves. The WHO classification is simpler. Leprosy is subdivided in two types: paucibacillary corresponding to I, TT, and BT and multibacillary corresponding to BB, BL, and LL (112–114). Paucibacillary leprosy is defined by five or fewer skin lesions with no bacilli on skin smears. The bacterial index can range from 0 (none found in 100 oil-immersion fields) to 6+ (over 1000 bacilli per field). Multibacillary cases have six or more skin lesions and may be skin-smear positive (100).

The use of polymerase chain reaction (PCR) for M. *leprae* DNA in skin biopsies to increase sensitivity and specificity of diagnosis has proved to be of limited use. Sensitivity is high in multibacillary cases but low in paucibacillary cases. The number of false positives is also high (115–117). At present, it represents an interesting research tool for some particular investigations.

Leprosy was an epidemic disease in Europe in medieval times but now is confined to some tropical areas, especially India, the Philippines, South America, and tropical Africa. Social and economic poverty is the main reason for the prevalence of this disease, which has the greatest distribution in underdeveloped countries. Chemotherapy offers a great possibility for eradication of the disease and multidrug therapy gave very favorable results.

The success of the multidrug therapeutic regimens recommended by WHO in 1981 led the WHO Assembly in 1991 to set a goal of elimination of leprosy as a public health problem by the year 2000 (118). Elimination was defined as a prevalence of ≤ 1 case for 10,000 population, with a case being defined as a patient receiving or requiring chemotherapy.

This goal has not been reached and it has been pushed back to 2005, but a significant decline in incidence has occurred in some countries such as China and Mexico.

The estimates of total cases of leprosy have fallen from 10–12 million to about 5.2 million in 1991. In 1999 about 800,000 cases were registered for treatment worldwide. About the same number of cases was detected in 1998. About 75% of registered patients live in South-East Asia, particularly in India, where the rate of cases per 100,000 population is 5.3 and the absolute number of patients is >500,000. However there are still 32 countries that have a leprosy prevalence of >1 case per 10,000 population (104, 112–114).

4 SCREENING AND EVALUATION OF ANTIMYCOBACTERIAL AGENTS

In the search for new antimycobacterial agents, demonstration of in vitro activity against the virulent strain of M. tuberculosis H37 Rv is one of the simplest preliminary tests. Although much more predictive than other in vitro models using avirulent or fast-growing mycobacteria (*M.* smegmatis, M. phlei), the in vitro test with M. tuberculosis gives a large number of false positives, and unfortunately, also some false negative results. Despite these limitations, the in vitro test, with various modifications of the inoculum size, the culture media, and the observation time, is still used in many laboratories for the blind primary screening of a large number of compounds. It is also used in antibiotic screening, where thousands of fermentation broths must be tested, and a primary screen using in vivo models is a practical impossibility.

Rapid susceptibility testing of M. tuberculosis can be performed using the radiometric assay of $^{14}CO_2$ produced by the action of the microorganism on ^{14}C-palmitic acid substrate. Results from this method, which are useful for screening new chemical compounds, are available after 4–10 days of incubation instead of the 20–30 days required by conventional culture media (119, 120).

To avoid the use of radioactive culture medium, the firefly bioluminescence is used to detect ATP during mycobacterial metabolism, comparing the ATP production in drug-containing broths with that of the control broths (121).

Another test, devised to shorten the time required for sensitivity testing, uses mycobacteria infected with a specific reporter phage expressing the firefly luciferase gene. The photoreaction produced by the reaction of luciferin with ATP allows the measurement of bac-

terial growth or inhibition by drugs. The assays requires small amounts of test compounds and is designed to screen a large number of antimycobacterial products (122).

The in vitro tests give only an indication of activity; quantitative evaluation of the potential usefulness of the new products must be obtained through in vivo tests. These are performed, generally speaking, by inoculating virulent mycobacteria strains into laboratory animals, administering the product to a group of them, and comparing the course of infection in treated and untreated animals. There are a variety of procedures for performing these tests, differing with respect to animal species (mouse, guinea pig, rabbit, etc.), mycobacterial strain and size of inoculum, route of product administration, and evaluation of the results. The most current procedure for the evaluation of antituberculous drugs uses mice infected with the human virulent mycobacterial strain, evaluating the results in terms of ED_{99}, survival time, the pathology of the lung, and bacterial count. The products active in the mice are then evaluated in other in vivo tests using more sophisticated techniques.

The best species for extrapolation of the results to humans is the rhesus monkey, *Macaca mulata* (123, 124). Experimental tuberculosis in this species closey parallels the human disease, and despite the difficulties in terms of time, space, and cost of the test, it is advisable to perform it, especially when doubtful results have been obtained from other species. In any case, extrapolation to the human disease of the results obtained in animals requires comparison of the kinetics and metabolism of the product in the different animal species and in humans. Differences in activity are sometimes clearly related to differences in the metabolic behaviors.

In the case of leprosy, for a long time no screening or evaluation models were available using the pathogenic agent M. leprae, which could not be cultivated in vitro or transmitted to animals. Therefore, the experimental infection of rodents with M. lepraemurium was used for evaluating the effect of potential drugs, although this model shows a low predictivity for activity in humans. For example, dapsone is inactive and isoniazid very active in this test, whereas the opposite is true for human leprosy. Only in 1960 was local infection in the mouse footpad with M. leprae set up (125). This model has been successfully used, with various procedural modifications, for screening and evaluation of drugs (126, 127). Thymectomy and body irradiation of mice inoculated with M. leprae provokes dissemination of bacilli, and this may be a model for a generalized infection (128). Another model of experimental leprosy was set up using the armadillo, which develops a severe disseminated lepromatoid disease several months after inoculation with a suspension of human leprosy bacilli (129). Limitations in the use of this model for chemotherapeutic evaluation are derived not only from its cost, but also from the importance to reserve the infected animals as a source of bacillary material for the development of a vaccine.

All the available animal models for the evaluation of antileprotic agents are too time-consuming for the screening of a large number of compounds. The search for short-term models is necessary. More recently it was reported that an in vitro culture system for M. leprae in cells harvested from livers of nine banded armadillos, inoculated earlier with human-derived microorganism had been created. The system seems to be adequate for initial screening of the drugs (129).

Activity of products against NTM is generally tested ad *hoc*, in vitro, or in vivo, and the compounds selected for this purpose are initially those showing antitubercular action, although their activity is quite variable against the various mycobacterial species. In recent years there has been increasing interest in identifying reliable in vitro susceptibility test methods against M. avium complex because of the severity of the infections caused by this microorganism and the increase of their frequency (130).

In addition to the standard in vitro assays, some in vitro studies have been carried out in presence of macrophages to determine the activity of drugs against M. auium complex and other NTM when they are located intracellularly (131–133). A model of chronic disseminated M. avium complex infection, developed in beige mice, revealed to be a useful tool in studying the in vivo activity of antimycobacterial drugs (134).

The inclusion of a number of NTM in the large antibacterial screening programs of new compounds could provide leads for finding new, more active chemotherapeutic agents for the diseases caused by these microorganisms. On the other hand, there is certainly need for more knowledge on the biochemistry and physiology of NTM and on their pathogenic behavior in laboratory animals.

Using the laboratory models available for testing and evaluating products, there are two possible approaches in the search for antimycobacterial drugs. The first approach is the blind screening of a large number of compounds, which permits the detection of a certain number of structures endowed with antimycobacterial activity. Chemical modification of these "lead" structures, accompanied by careful studies of structure-activity relationships (SARs), can yield the optimal derivative for therapeutic use. The second approach, more challenging from the scientific point of view, is based on designing drugs to act selectively on biochemical targets specific for the particular microorganism.

The development of new antimycobacterial drugs is made difficult by a number of factors (135). First, large scale screening systems for the detection of new antimycobacterial agents are particularly time-consuming and entail some problems related to the handling of the pathogens. Second, the development of an antimycobacterial drug takes more time and human resources than the development of other antimicrobial agents. Third, and probably most important, tuberculosis and leprosy are predominant in developing countries with low economic resources, and industrial laboratories are reluctant to invest in research for new products to be used in those geographic areas, where an additional drawback is the lack of patent protection. However, the actual situation makes the search for new and more effective antimycobacterial drugs necessary.

Although screening for antimycobacterial agents will continue to be a possible way to discover useful new drugs, the increasing knowledge of the biochemistry of the mycobacteria makes possible a more rational approach to the problem. In particular, the studies of the biosynthesis of the unique constituents of the mycobacterial cell will indicate the targets for inhibitors of the biosynthetic pathways present specifically in the microorganisms of the mycobacterium species.

The complete sequencing of M. tuberculosis genoma opens new opportunities to find the basic targets of the activity of antimycobacterial agents and possibly the way to design drugs specifically directed to inhibit essential enzymes. However precise functions have been attributed to about 40% of the encoded proteins, whereas there is only some information for 44%. Therefore, the horizon for screening unexploited antimicrobial targets is broad. The discovery of selective inhibitors will allow to have products ineffective on the eukariotic cells of the host, therefore, potentially with little toxicity and no activity on the normal microbial flora. At present it has been demonstrated that the fatty acid synthase (FAS II) may be the target of isoniazid (136).

A novel approach to identify new antimicrobial agents has been the application of combinatorial chemistry, which creates molecules starting from a rational building block. It represents a powerful tool for a rapid and automated screening of active components and for the creation of libraries including enormous series of derivatives.

Although some important clues have appeared in the field of specific biochemical pathways inside the mycobacteria and of the mechanism of action of antimycobacterial drugs, little progress has been made till now in overcoming the problem of drug resistance in mycobacteria. M. tuberculosis mutates spontaneously and randomly to resistance to isoniazid, streptomycin, ethambutol, and rifampicin (137), and that is in a genotype form.

The knowledge of the mechanism of mycobacterial resistance to a drug could indicate the direction in which to search for new products specifically overcoming this mechanism. In the case of aminoglycoside antibiotics, the mechanism of resistance in *Enterobacteriaceae* and some Pseudomonas strains has been extensively studied, and the inactivating enzymes have been identified. This information has allowed a rational design of chemical modifications of aminoglycoside antibiotics at the site of attack of inactivating enzymes giving new products that were active against resistant strains (138).

Unfortunately, strides toward understanding the molecular basis of mycobacterial resistance to the various drugs have been made only rather recently. The status of knowledge of the resistance mechanisms is not sufficient to conceive a rational design of molecules acting on the mutants resistant to the specific drugs. Also the knowledge of the genetic mechanisms and genotypical systems governing resistance in mycobacteria is limited because of the difficulty of using mycobacteria in classical genetic techniques (slow growth, lipid-rich cell walls impermeable to DNA uptake). Only during the last decade, application of recombinant DNA technologies to the mycobacteria has opened new doors to obtain information on some basic mechanisms of resistance to drugs. However, at present, the delay or prevention of the evolution toward resistance of mycobacterial flora is accomplished only through combination therapy, based on the complementary action of the constituents. It is well known that in this case the probability of a concomitant mutation toward resistant strains is much lower than the probability of mutation to resistance to a single drug.

5 CURRENT DRUGS ON THE MARKET

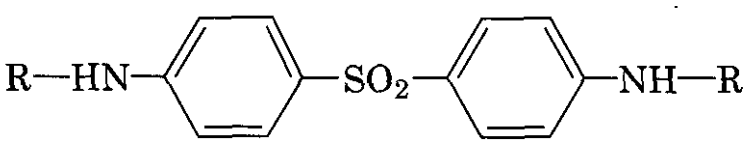
Among the several thousand compounds screened for antimycobacterial activity, only a few have had therapeutic indices sufficient to warrant introducing them into clinical use (Table 16.1). These drugs are described in some detail, together with general information about the chemical analogs, mechanism of action, pharmacokinetics and metabolism, clinical use, and effects. The information, in summary form, is intended to cover the aspects that are useful for understanding the role of each product in current therapy, the limitations of use, and the need for improvement or for further studies.

The drugs have been subdivided arbitrarily into synthetic products and antibiotics, and within these two categories they are listed in a quasi-chronological order, with products having structural similarities grouped with the representative first introduced into therapy.

5.1 Synthetic Products

5.1.1 Sulfones

5.1.1.1 History. The sulfones were synthesized by analogy to sulfonamides, which had no antimycobacterial activity. The first sulfones prepared, 4, 4'-diaminodiphenylsulfone (dapsone) (9) and its glucose bisulfite derivative, glucosulfone sodium (**10**), were found to be active in suppressing experimental tubercle infections (139–140). The usefulness in the chemotherapy of human tuberculosis was limited, but the discovery of some effect of compound (10) in leprosy experimentally induced in rats (141) opened the way to their successful introduction into the treatment of human leprosy.



(9) R = H

(10) R = $CH(CHOH)_4CH_2OH$ with SO_3Na

(11) R = CH_2SO_2Na

(12) R = $CH(SO_3Na)-CH_2-CH(SO_3Na)-C_6H_5$

(13) R = $COCH_3$

5.1.1.2 SARs. Because it was thought that the activity of compound (10) was caused by its metabolic conversion into dapsone, intensive studies have been carried out that vary the structure of the latter to find optimal activity and to improve its low solubility. Various substitutions on the phenyl ring yielded products less active than dapsone. The only product of this type that has some clinical use is acetosulfone sodium, which contains one $SO_2N(Na)—COCH_3$ group in the ortho position to the sulfone group. Its antibacterial effect seems to be caused by the unchanged drug. Substitutions in both the amino groups gave rise to products that are in general active only if they are converted metabolically into the parent dapsone.

Substitution on the amino groups to improve solubility yielded products such as the above-mentioned glucosulfone, the methanesulfonic acid derivative, sulfoxone sodium, aldenosulfone (**11**), and the cinnamaldehyde-sodium bisulfite addition product, sulfetrone sodium, solasulfone (**12**), which have limited use in leprosy treatment. They act through their metabolic conversion to dapsone in the body. Several methanesulfonic acid derivatives of 4, 4'-diamino-diphenyl sulfone have been tested for their ability to be metabolized to dapsone (142).

The 4,4′-diacetyldiaminodiphenylsulfone, acedapsone (**13**), has low activity in vitro but is used as an injectable depot sulfone, which releases dapsone at a steady rate over several weeks.

Although a large number of sulfones have been synthesized and tested as potential antileprotic agents, dapsone continues to be the basic therapeutic agent for M. leprae infections, often as a component of multidrug programs.

5.1.1.3 Activity and Mechanism of Action. Dapsone is weakly bactericidal against M. leprae at concentration estimated to be of the order of 0.003 μg/mL (143), when the microorganism has been isolated from untreated patients. M. leprae becomes resistant to dapsone and its congeners after chronic administration.

It is assumed that dapsone interferes with incorporation of p-aminobenzoic acid into dihydrofolate, in analogy with the action of sulfonamides in other bacterial systems This mechanism of action of dapsone has been proved for E. coli (144), and the finding of cross-resistance to sulfones and sulfonamides in Mycobacterium species 607 indirectly confirms their similarity of action (145). Unfortunately, the inability to cultivate M. leprae in vitro makes it difficult ultimately to verify that dapsone acts through the proposed mechanism on this organism.

5.1.1.4 Pharmacokinetics. Dapsone is usually administered orally at a daily dose of 100 mg (118). It is nearly completely absorbed from the gastrointestinal (GI) tract, well distributed into all tissues, and excreted in high percentage in the urine as mono-N-sulfamate and other unidentified metabolites (143). It is monoacetylated in humans. The characteristics of dapsone acetylation parallel those of isoniazid and sulfametazine, thereby establishing genetic polymorphism for the acetylation of dapsone in humans (146). Two metabolic factors, greater acetylation and greater clearance of dapsone from the circulation, may contribute to emergence of dapsone-resistant M. leprae (147).

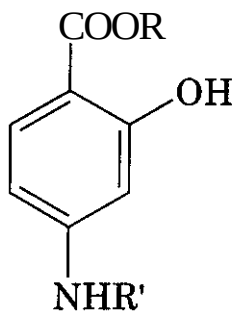
Acedapsone is administered intramuscularly in oily suspension at a dose of 225 mg every 7 weeks, The product is slowly released from the site of injection, and the plasma contains mainly dapsone and its monoacetyl derivative; the ratio of these two products depends on whether the patient is a slow or rapid acetylator, as in the case of dapsone. The advantage of this type of depot usage was also considered for the prophylaxis of people exposed to risk (148).

5.1.1.5 Adverse Effects. Dapsone and its derivatives may be administered for years with certain precautions: gradual increase of doses, rest periods to prevent cumulative effects, and laboratory and clinical supervision. Among the most common side effects are gastrointestinal and central nervous system disturbances. Also, the most common effect is hemolysis in varying degrees. There are indications that individuals with a glucose-6-phosphate dehydrogenase deficiency are more susceptible to hemolysis during sulfone therapy, although this is controversial (149).

5.1.2 Para-amino-salicylic Acid

5.1.2.1 History. The observation that benzoate and salicylates have a stimulatory effect on the respiration of mycobacteria (150) suggested that analogs of benzoic acid might interfere with the oxidative metabolism of the bacilli. When various compounds structurally related to benzoic acid were tested, it was found that some of them had limited antituberculous activity; *p*-aminosalicylic acid (PAS) (14) was the most active (151). The discovery of PAS cannot be quoted as an example of biochemically oriented chemotherapeutic research, because in fact, its mechanism of action is not by way on the respiration of mycobacteria.

5.1.2.2 SARs. The in vitro and in *vivo* antimycobacterial activity of a simple molecule



	R	R'
(14)	H	H
(15)	C_6H_5	H
(16)	H	COC_6H_5
(17)	H	$COCH_3$
(18)	H	$COCH_2NH_2$

such as PAS stimulated the synthesis and testing of many derivatives. This extensive research failed to give rise to better drugs but did provide knowledge of the structural requirements for activity in the series.

Modification of the position of the hydroxy and amino groups with respect to the carboxy group resulted in a sharp decrease of activity. The amino group confers a distinct pharmacodynamic property to the molecule, eliminating the antipyretic and analgesic activities of salicylic acid and giving the specific tuberculostatic activity. Further nuclear substitution and replacement of the amino, hydroxy, or carboxy groups with other groups yielded inactive or poorly active products. Also, functional derivatives on the amino, hydroxy, and carboxy groups of PAS are generally inactive, unless they are converted in vivo into the active molecule. Among the latter, the phenylester (15)and benzamidosalicylicacid (16) must be mentioned.

5.1.2.3 Activity and Mechanism of Action. PAS has bacteriostatic activity in vitro on M. tuberculosis at a concentration of the order of 1 μg/mL. It is active only against growing tubercle bacilli, being inactive against intracellular organisms. Other microorganisms are not affected by the compound. Most of the other mycobacteria are insensitive to the drug. Although active against M. leprae in the mouse footpad test (152), is not used in the current treatment of leprosy.

The mechanism of action of PAS is not clear. It was demonstrated earlier that *p*-aminobenzoic acid antagonizes the antibacterial activity of PAS in vitro and in vivo, and therefore, it could act by competitively blocking the synthesis of dihydrofolic acid. On the other hand the formation of mycobactin, an ionophore for iron transport, is strongly inhibited by PAS, and the bacteriostatic activity of PAS might be caused by the inhibition of the metabolic pathway for iron uptake (153–155).

5.1.2.4 Pharmacokinetics. PAS is readily absorbed by the gastrointestinal tract and well distributed throughout the body, but it does not penetrate into cerebrospinal fluid (CSF)of patients with uninflamed meninges. Peak serum levels of 7–8 μg/mL are reached 1–2 h after oral administration of 60 mg/kg of PAS. The half-life time of serum levels is very short (0.75 h) It is quickly eliminated through the urine in form of inactive metabolites, the N-acetyl (17) and the N-glycyl (18)derivatives. The common daily dose for adults is around 10–20 g, generally divided in two or four doses administered after meals, according to patient tolerance. In children the usual daily dose is 0.2–0.5 g/kg. Probenecid interferes with renal tubular secretion of PAS and increases the serum levels of the drug.

Although PAS easily undergoes decarboxylation in vitro, it seems quite stable in the body. The in vivo acetylation of PAS competes with acetylation of isoniazid, resulting in higher plasma concentration of free isoniazid than when isoniazid is given alone. This fact has no proven clinical relevance.

5.1.2.5 Adverse Effects. PAS has been used for many years in combination with streptomycin and isoniazid for the treatment of all forms of tuberculosis.

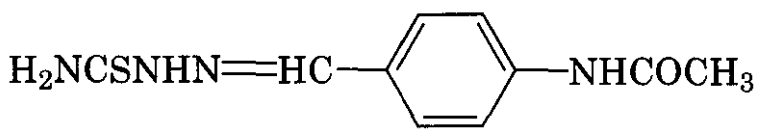
Gastrointestinal irritation is a very common side effect, with manifestations of various degrees of intensity and severity, which may lead to discontinuation of treatment. Hypersensitivity reactions occur in about 5–10% of patients, usually with manifestations like rash, fever, and pruritus, rarely followed by exfoliative dermatitis or hepatitis of allergic nature. PAS should be used with caution in patients with renal diseases, because it is largely excreted in the urine.

The therapeutic use of PAS is declining because of the high incidence of side effects and poor patient acceptance and the introduction of more potent and safer antituberculous

drugs. However it is still used in developing countries because it is inexpensive.

5.1.3 Thioacetazone

5.1.3.1 History. The synthesis of a series of thiosemicarbazones as intermediates in the preparation of analogs of sulfathiadiazole (156–157), which has weak antituberculous activity (158), led to the discovery of the thiosemicarbazone of p-acetamidobenzaldehyde (thioacetazone, amithiozone, 19), the most active substance in *vitro* and in *vivo* of the series (159–161).



(19)

5.1.3.2 SARs. In the search for better products, many modifications of the thioacetazone molecule have been made. The studies clearly indicate that the activity resides in the thiosemicarbazone structure of the aromatic aldehydes. In fact, several products with modified aromatic moieties, including some heterocyclic nuclei, have been found to be active. Some of them have been tested clinically with positive results, but thioacetazone is the only thiosemicarbazone still in clinical use.

Several thioureas that are structurally related to thiosemicarbazones have been found to be active in *vitro* and in *vivo* against M. tuberculosis, the most active being the diphenylthioureas with p-alkoxy groups in one or both the aromatic nuclei. Of these, thiocarlide (20) and thiambutosine (21) have been studied clinically with disappointing results. Thiarnbutosine was recommended for leprosy patients who do not tolerate dapsone (162), but the last WHO report on chemotherapy of leprosy does not mention thiambutosine among the currently available antileprosy drugs (118).

5.1.3.3 Activity and Mechanism of Action. Thioacetazone is active against M. tuberculosis at concentration of 1 μg/mL. Its activity in tuberculous animal infections is comparable with that of streptomycin. Its action is bacteriostatic, and the development and frequency of resistant strains is rather high. Some of the thioacetazone-resistant strains are also resistant to ethionamide (163). The mechanism of action of thioacetazone is not known. Its tuberculostatic activity is not counteracted by p-aminobenzoic acid (164), and there is a partial cross-resistance to antituberculous thioureas.

Its good activity in *vitro* and in *vivo*, and the lack of cross-resistance to isoniazid and streptomycin, indicated the use of thioacetazone as a drug to combine with these drugs to delay bacterial resistance.

5.1.3.4 Pharmacokinetics. Thioacetazone is well absorbed from the gastrointestinal tract (165). Oral administration of 150 mg of thioacetazone gives serum levels of 1.6 μg/mL at peak and of 0.2 μg/mL after 2 days. Large amounts of thioacetazone are excreted in the urine, but there is not enough information on its metabolism.

5.1.3.5 Adverse Effects. Despite relatively low toxicity in laboratory animals, thioacetazone has limitations in clinical use because of serious side effects (such as gastrointestinal disorders, liver damage, and anemia) when administered to humans at the initial proposed daily dose of 300 mg. A review of the side ef-

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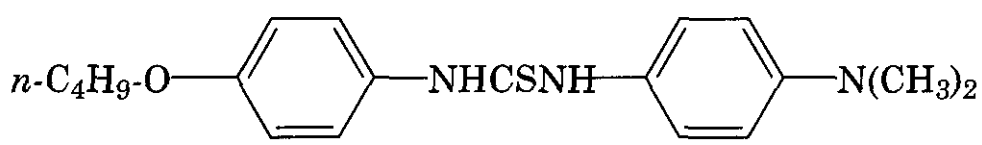

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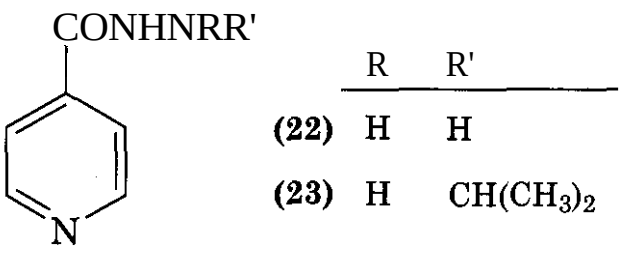
fects and efficacy of thioacetazone in relation to the dosage indicates that the drug has an activity comparable with that of PAS and an acceptable toxicity when administered at lower dosage (166).

A dose of 300 mg of isoniazid plus 150 mg of thioacetazone is an inexpensive and acceptable combination for long-term therapeutic treatment after the initial treatment with three drugs. This schedule is used in developing countries (167), although there are considerable differences in side effects among patients from different geographic areas (168). The reported effectiveness in leprosy (169) notwithstanding, thioacetazone is of limited usefulness in the treatment of this disease because of its side effects and the emergence of resistance of M. leprae to the drug.

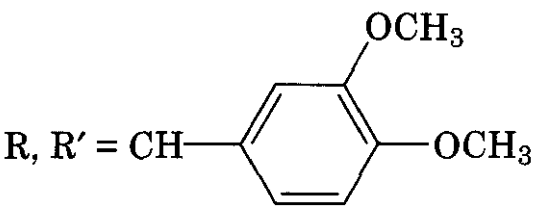
5.1.4 Isoniazid

5.1.4.1 History. After the early report that nicotinamide possesses tuberculostatic activity (170–171), several compounds related to it were examined. Attention was aimed at the isonicotinic acid derivatives, and in view of the already established antituberculous activity of thiosemicarbazones, the thiosemicarbazone of isonicotinyl aldehyde was prepared. When isonicotinyl hydrazide (isoniazid, **22**), described in the chemical literature in 1912 (172), was prepared as an intermediate in the synthesis of the aldehyde and tested, it proved to be a potent antitubercular agent in vitro and in *vivo* (173–175).

5.1.4.2 SARs. The outstanding antituberculous activity of isoniazid in experimental infections, confirmed by the clinical trials, stimulated the study of chemical modifications of this simple molecule. At least 100 analogs were prepared, but structural changes caused a reduction in or loss of activity. Among the modified forms that retained appreciable activity, the N2-alkyl derivatives should be mentioned. In particular the N2-isopropyl derivative (iproniazid, **23**), was found to be active in *vivo*. Extensive clinical trials proved the therapeutic effectiveness of iproniazid and revealed its psychomotor stimulant effect, caused by the inhibition of monoamine oxidase (176). Use of iproniazid in the treatment of tuberculosis or of psychotic and neurotic depression was discontinued because of the



	R	R'
(22)	H	H
(23)	H	$CH(CH_3)_2$



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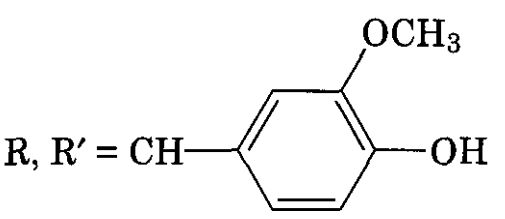

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hepatic toxicity of the drug. Although acetyl isoniazid is inactive, its hydrazones constitute a group of isoniazid congeners that have activity of the same order of the parent compound. The activity of these compounds generally is related to the rate of their hydrolysis to the parent compound. Some hydrazones have been introduced into therapeutic use, such as the 3,4-dimethoxybenzilidene (verazide, 24) and the 3-methoxy-4-hydroxy-benzilidene (phthivazid, 25) derivatives; however, their use is questionable, and products of this kind now have limited or no application.

5.1.4.3 Activity and Mechanism of Action. Isoniazid has bacteriostatic and bactericidal activity in vitro against M. tuberculosis and also against strains resistant to other antimycobacterial drugs. The minimal inhibitory concentration for the human strain is about 0.05 μg/mL. It acts on growing cells and not on resting cells and is also effective against intracellular bacilli. Its effect on the non-tuberculous mycobacteria is marginal or non-existent. Isoniazid is active in various models of experimental tuberculosis in animals. It shows limited activity against M. leprae in the mouse footpad test (177), and it is essentially inactive in human leprosy (178).

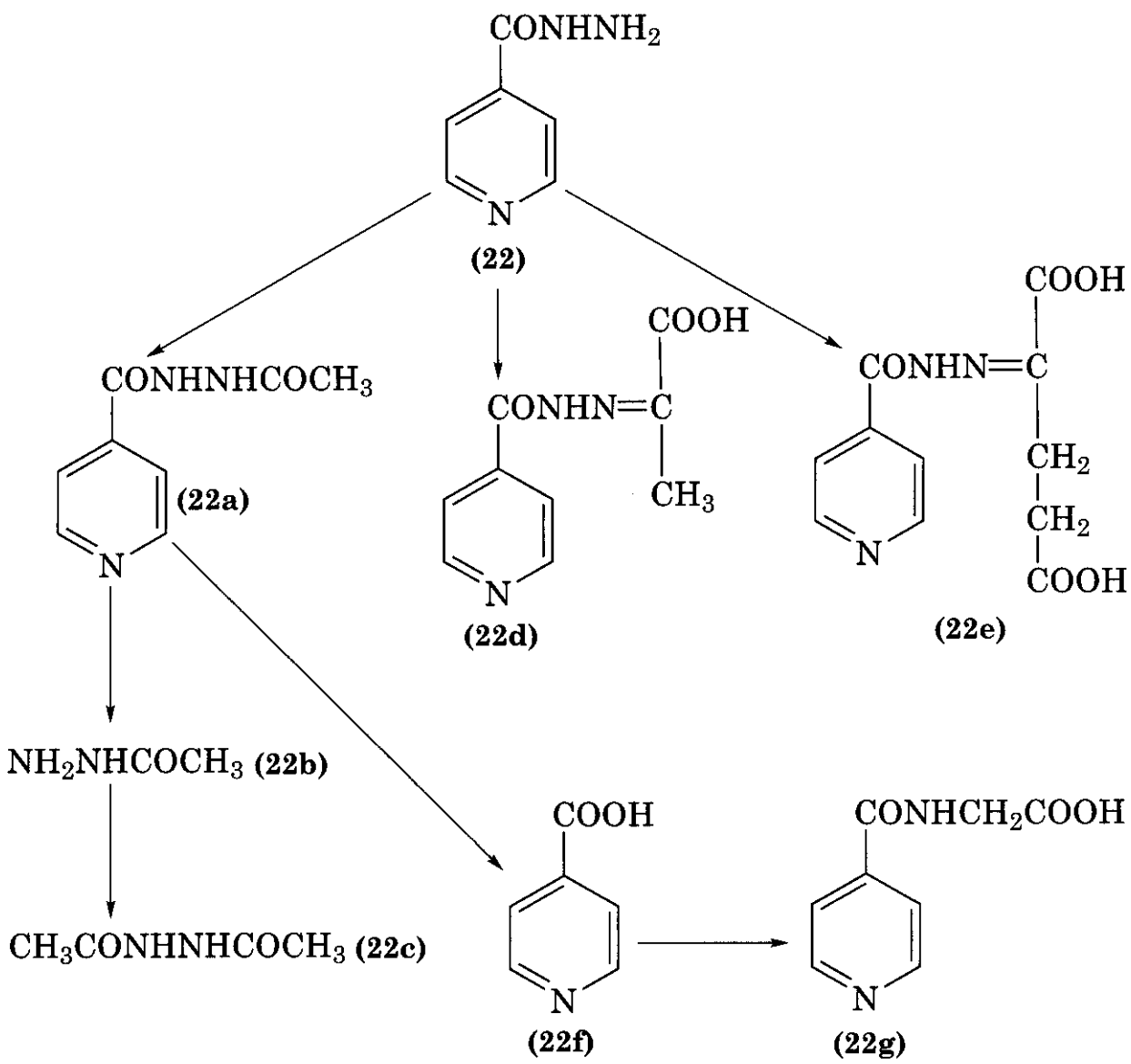
Resistance of M. tuberculosis to isoniazid develops rapidly if it is used alone in the treat-

ment of clinical infection and can be prevented or delayed by combination with other antimycobacterial agents. Resistance does not seem to be a problem when isoniazid is used alone in prophylaxis, probably because the bacillary load is low. It is recommended as a single drug in tuberculosis chemoprophylaxis, especially in household contacts and close associates of patients with isoniazid-sensitive tuberculosis.

Several hypotheses have been put forward concerning the mechanism of action of isoniazid, taking into account that the activity of this drug is very specific against mycobacteria at low concentrations. Investigations in this direction have indicated that the action of isoniazid is on the biosynthetic pathway to the mycolic acids (179–180). Apparently, isoniazid blocks the synthesis of fatty acids longer than C26 in chain length (181).

Another hypothesis suggests that it is the isonicotinic acid the responsible for the inhibitory effect of isoniazid on mycobacteria (182–184). Isoniazid is said to penetrate the cell, where it is hydrolyzed enzymatically to isonicotinic acid, which at the intracellular pH is nearly completely ionized and cannot return across the membrane and accumulates inside the cell. Isonicotinic acid is then quaternized and competes with nicotinic acid through the formation of an analog of the nicotinamide-adenine dinucleotide, which does not have the activity of the natural coenzyme. Alteration of the metabolic functions of the cell follows, particularly with respect to lipid metabolism.

More recently it has been discovered that the activity of isoniazid results from a peroxidative reaction catalysed by a catalase-peroxidase, which is encoded by the *kat* G gene (185). In fact, clinical isolates of M. *tuberculosis* highly resistant to isoniazid lack *kat* G (186). The most common and potent mechanism of resistance to INH involves loss of *Kat* G activity to prevent activation of the drug. The *kat* G–mediated oxidation of INH to a potent elec-



trophile proceeds at the expense of hydrogen peroxide, which acts as an electron sink for the reaction (187). The activated INH electrophile is then free to interact with any number of cellular nucleophiles poisoning the nicotinamide biosynthetic pool. The discovery of a novel gene, *inh A*, may complete the understanding of the mechanism of action of isoniazid. The product of *inh A* has a certain analogy with the enterobacterial *env* M enzyme, which is associated with the biosynthesis of fatty acids, phospholipids, and lipopolysaccharides (188). The inh A protein could be the primary target of action of isoniazid, but there is also the possibility that *inh A* requires NAD(H) as a coenzyme and that its activity can be affected by the incorporation of iso-NAD produced by action of catalase-peroxidase on isoniazid, leading to a block of the synthesis of mycolic acid and loss of acid fastness. It is still to be clarified the fact that *inh A* and *kat* G are also present in the non-tubercular mycobacteria naturally resistant to isoniazid. In M. *tuberculosis*, the two genes are altered in isoniazid resistant isolates (189).

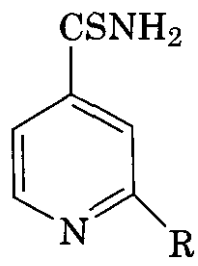
5.1.4.4 Pharmacokinetics. Isoniazid is readily absorbed from the gastrointestinal tract in humans. Peak serum levels of the order of 5 μg/mL are obtained 1–2 h after administration of a 5-mg/kg dose. After absorption, isoniazid is well distributed in body fluids and tissues, including the cerebrospinal fluid, and penetrates into the macrophages. Isoniazid is excreted mainly in the urine in unchanged form, together with various inactive metabolites: *N*-acetyl isoniazid (**22a**), monoacetyl hydrazine (**22b**), diacetyl hydrazine (**22c**), isoniazid hydrazones of pyruvic acid (**22d**), and a-ketoglutaric acid (**22e**), isonicotinic acid (**22f**), and isonicotinylglycine (**22g**).

The primary metabolic route that determines the rate at which isoniazid is eliminated from the body is acetylation in the liver to acetyl isoniazid. There are large differences among individuals in the rate at which isoniazid is acetylated. The acetylation rate of isoniazid seems to be under genetic control (190–191), and individuals can be slow or rapid acetylators of the drug. The serum half-lives of isoniazid in a large number of subjects show a bimodal distribution; the isoniazid half-lives of rapid metabolizers range from 45 to 110 min and those of slow metabolizers from 2 to 4.5 h (192). The rate of acetylation seems to be conditioned by ethnic background. The proportion of slow acetylators varies from 10% among the Japanese and Eskimos to 60% among blacks and Caucasians. The isoniazid acetylator status of tuberculosis patients treated with isoniazid-containing regimens seems to be relevant only for once-weekly treatments with the drug (193).

5.1.4.5 Adverse Effects. Isoniazid is usually well tolerated for a long period of treatment. Hepatic side effects consist of frequent subclinic asymptomatic enzyme abnormalities (increase of SGOT and bilirubin), which occasionally cause severe clinical hepatitis, especially in patients with previous hepatobiliary diseases and in alcoholics. The risk of hepatitis is age-related, being very rare in children or young people. Neurological side effects, such as peripheral neuritis, are rare at the daily dose of 5 mg/kg, but more frequent at doses of 10 mg/kg. Administration of pyridoxine to patients receiving high doses of isoniazid generally prevents neurological disturbances. Other rare effects are at the GI level and moderate hypersensitivity reactions.

5.1.5 Ethionamide

5.1.5.1 History and SARs. As mentioned before, the discovery of isoniazid was the consequence of research based on the weak antitubercular activity of nicotinamide. This lead was pursued in various directions, and among the earliest modifications, thioisonicotinamide (26) (194–196) seemed to have the in-



(26) R = H

(27) R = C_2H_5

(28) R = *n*-C_3H_7

triguing property of an *in vivo* efficacy superior to that expected from the *in vitro* activity. The hypothesis that some metabolic product

of the drug was responsible for the activity *in vivo* stimulated the synthesis as well the testing of a series of potential thioisonicotinamide metabolites and various other derivatives. Among the latter, increased activity was observed for the 2-alkyl derivatives (197). 2-ethyl thioisonicotinamide (ethionamide, 27) and the 2-n-propylanalog (prothionamide,28) were selected for clinical use. Of these two drugs, ethionamide has been more extensively studied, although prothionamide seems to possess biological properties similar to it.

5.1.5.2 Activity and Mechanism of Action. At concentrations around 0.6–2.5 μg/mL, ethionamide is active *in vitro* against *M. tuberculosis* strains, either sensitive or resistant to isoniazid, streptomycin, and p-aminosalicylic acid. It also shows activity against other mycobacteria, especially M. *kansasii*. Administered orally, ethionamide is effective in the treatment of experimental tuberculosis in animals. Although activity against M. *leprae* in animal infections has been reported, ethionamide is rarely used in the therapeutic treatment of leprosy. Bacterial resistance develops quickly when ethionamide is given alone; therefore, it is used in combination with other antimycobacterial drugs.

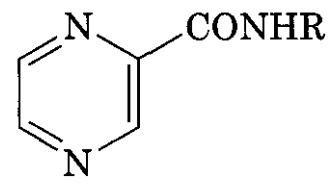
The antibacterial action of ethionamide seems to be caused by an inhibitory effect on mycolic acid synthesis, with a concomitant effect on nonmycolic acid-bound lipids (198). This pattern is like that shown by isoniazid. However, other studies indicate that ethionamide disturbs the synthesis of mycolic acid in both resistant and susceptible mycobacteria, whereas isoniazid inhibits the synthesis of all kinds of mycolic acid in the same way in all susceptible strains and has no effect on mycolic acid synthesis in resistant strains (199). On the other hand, a missense mutation within the mycobacterial *inh A* gene was shown to confer resistance to both isoniazid and ethionamide in M. *smegmatis* and *M. bovis* (188).

5.1.5.3 Pharmacokinetics. In case of tuberculosis, ethionamide is given orally at doses varying from 125 mg to a maximum of 1 g daily. It is rapidly absorbed and widely distributed in the body tissues and fluids, including the CSF. It has a short half-life and is rapidly excreted in the urine, but only a minor percentage is in the form of unaltered product. A series of metabolites has been found in the urine: the active sulfoxide, the 2-ethyl isonicotinic acid and amide, and the corresponding dihydropyridine derivatives (200).

5.1.5.4 Adverse Effects. GI side effects, sometimes very severe, are common and constitute the major cause for discontinuation of the treatment with ethionamide. Neurotoxicity with manifestations of mental disturbances is also relatively frequent during the treatment with ethionamide.

5.1.6 Pyrazinamide

5.1.6.1 History. In studies of chemical modifications of the nicotinamide structure, other heterocyclic nuclei have been investigated, and the 2-carboxamidopyrazine (pyrazinamide, 29) was synthesized and tested for

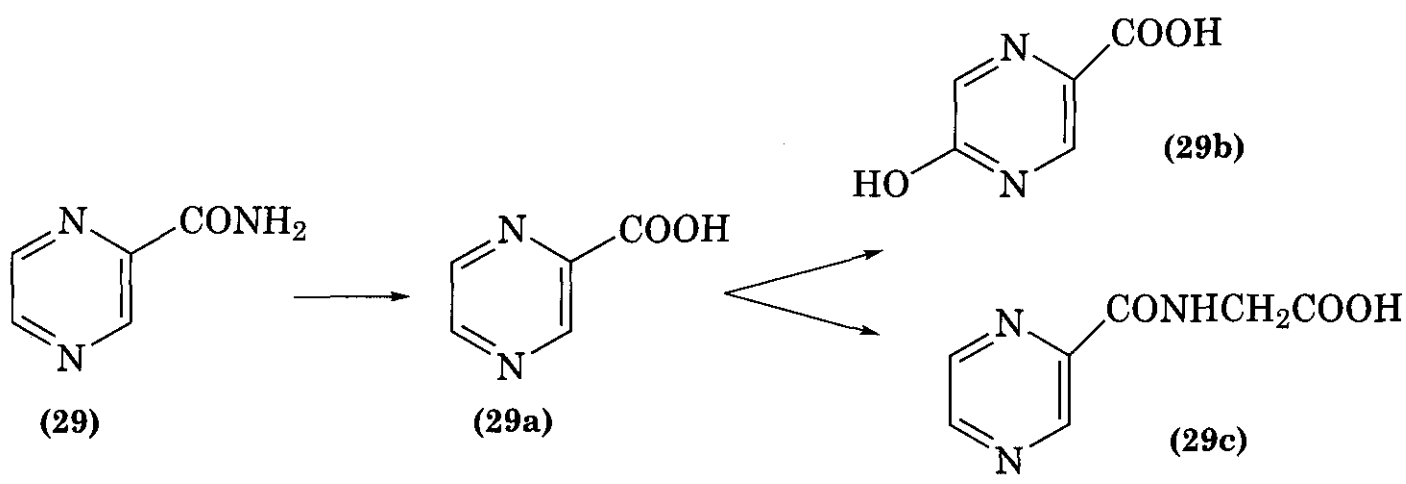


(29) R = H

(30) R = CH_2-N O

antituberculous activity (201–203). The *in vitro* activity of pyrazinamide against M. *tuberculosis* was found to be negligible at neutral pH and of the order of 5–20 μg/mL at pH 5.5. The best activity of pyrazinamide is against intracellular mycobacteria in monocytes, probably because of the low intracellular pH, which favors its activity.

5.1.6.2 SARs. Compounds with other substitutions on the pyrazine nucleus or other carboxamido heterocycles were found to be inactive or less active than pyrazinamide. The only active analog developed because of its potential superiority over pyrazinamide was morphazinamide (N-morpholinomethylamide of pyrazinoic acid, **30)** (204). Interest in this drug ceased when it was ascertained that the activity and toxicity parallel those of pyrazinamide, to which morphazinamide is converted *in vivo* (205). Some pyrazinoic acid esters (206) and some N-pyrazinylthyoureas (207) were found to be more active *in vitro* against



(29) (29a) (29b) (29c)

(29)

M. tuberculosis than pyrazinamide, but no data in *vivo* have been reported.

5.1.6.3 Activity and Mechanism of Action. The activity of pyrazinamide against intracellular mycobacteria parallels the fact that pyrazinamide is inactive in guinea pig tuberculosis, predominantly extracellular, and very active in murine tuberculosis, which has an important intracellular component. Apart from these observations, the mechanism of action of pyrazinamide remains unknown. According to some authors, the activity of pyrazinamide is caused by its intracellular conversion into pyrazinoic acid (208), but the mechanism of action of the latter is unknown. Recently, the gene *pncA*, encoding the pyrazinamidase (PZase) of M. tuberculosis has been identified (209); mutations in *pncA* have been shown to be associated with pyrazinamide resistance. Recent studies suggest that the mutations found in *pncA* occur preferentially in conserved regions of *pncA* that might be very important for the binding and processing of PZA (210). Recent work has revealed that the target of pyrazinamide is the eukaryotic-like fatty acid sinthetase 1 (FAS-1) of *M.* tuberculosis (211).

Pyrazinamide was introduced in therapy in 1952 for the treatment of tuberculosis. After a period of declining use, a renewed interest has been shown on this drug for its potential role in the short-course chemotherapy regimens. Because of its bactericidal effect on the intracellular mycobacteria, pyrazinamide is recommended especially in the first 2 months of treatment of tuberculosis in combination with rifampicin and isoniazid. The usual daily dosages are 20–35 mg/kg given orally in three or four equally spaced doses.

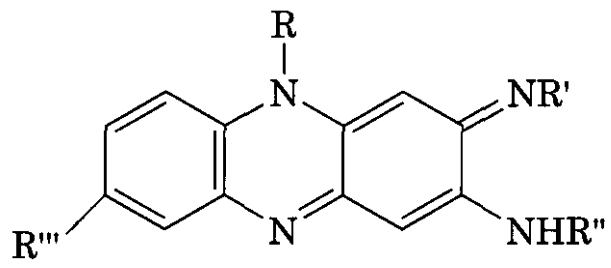
5.1.6.4 Pharmacokinetics. Pyrazinamide (**29a**) is well absorbed from the gastrointestinal tract. Peak serum concentrations occur about 2 h after a dose by mouth and have been reported to be about 35 μg/mL after 1.5 g, and 66 μg/mL after 3 g. Pyrazinamide is widely distributed in body fluids and tissues and diffuses into the CSF. The half-life time has been reported to be about 9–10 h. It is metabolized primarily in the liver by hydrolysis to the major active metabolite pyrazinoic acid (**29a**) which is subsequently converted into the major excretory product 5-hydroxypyrazinoic acid (**29b**) and pyrazinuric acid (**29c**). It is excreted through the kidney mainly by glomerular filtration. About 70% of a dose appears in the urine within 24 h, mainly as metabolites and 4–14% as unchanged drug.

5.1.6.5 Adverse Effects. Hepatotoxicity is the most common and serious side effect of pyrazinamide and is related with the dose and length of treatment. Liver functions should be checked before the administration of the drug and at frequent intervals during the therapy. Another side effect observed with pyrazinamide is arthralgia, caused by elevation of plasma uric acid levels.

5.1.7 Clofazimine

5.1.7.1 History. Clofazimine belongs to a peculiar class of phenazines called riminophenazines. Studies on these compounds derived from the original observation that treating a solution of 2-aminodiphenylamine with ferric chloride produced a red crystalline pre-

cipitate that completely inhibited the growth of tubercle bacilli H37Rv strain in vitro and was not inactivated by human serum (212–214). The in vivo activity was moderate and the toxicity low. Such a compound, named B-283 (31), was the leading structure for a se-



	R	R'	R''	R'''
(31)	H	H	H	H
(32)	*p*-ClC_6H_4	$CH(CH_3)_2$	*p*-ClC_6H_4	H
(33)	C_6H_5	CH_2-CH_2 / CH / CH_2 / CH_2-CH_2	C_6H_5	Cl

ries of riminophenazines, which are alkyl or arylimino derivatives. Among the first compounds synthesized, B-663, later named clofazimine (32), was the most active (215,216).

5.1.7.2 Activity and Mechanism of Action. Clofazimine has an in vitro inhibitory activity against M. tuberculosis at concentrations of 0.1–0.5 μg/mL. Strains resistant to isoniazid, and/or streptomycin, PAS, and thioacetazone, are susceptible to the drug. Some MOTT are also susceptible to this compound. Minimal inhibitory concentrations for M. avium complex (MAC) are 0.125–1 μg/mL (217). This activity has rendered the drug eligible for the treatment of infections caused by this organism.

Clofazimine is not only bacteriostatic, but also bactericidal (but the latter action is rather slow) and only on multiplying mycobacteria. The mechanism of action of riminophenazines has not been elucidated, mainly because of the low solubility of these compounds in aqueous media. The activity seems to be correlated with thep-quinoid system; in fact, when this is removed by reductive acylation, activity disappears. The mycobacteria under anaerobic conditions reduce the quinoid system. It has been shown that 20% of the respiratory hydrogen can be transferred from a respiratory enzyme to clofazimine (214). Its action has been also related to iron chelation, with resulting production of nascent oxygen radicals intracellularly (218).

In the treatment of murine tuberculosis. clofazimine was found to be very active, but much higher doses were necessary to achieve a therapeutic effect in guinea pigs and monkeys. Trials in chronic human pulmonary tuberculosis indicated that clofazimine had no significant effect on the disease at doses up to 10 mg/kg. In experimental infections with mycobacteria, clofazimine was found to be much more active than isoniazid against M. kansasii (219). Other studies have shown that clofazimine is also active in experimental infections with M. johnei (220), M. ulcerans (2211, M. lepraemurium (222,2231,and M. leprae (224–231). In particular, M. leprae seems to be about 10 times more susceptible to clofazimine than M. tuberculosis (177). The marked activity against leprosy was confirmed in clinical trials (224–230). Generally speaking, the activity of clofazimine is similar to that of dapsone. Dapsone-resistant mutants are susceptible to clofazimine. During treatment of lepromatous leprosy with clofazimine, the characteristic inflammatory reaction erythema nodosum leprosum (ENL) seldom develops; if clofazimine is combined with dapsone, the latter agent no longer causes ENL. This makes the concurrent use of corticosteroids unnecessary. It was suggested that clofazimine would have a corticosteroids like anti-inflammatory action (231). In a dye-hyaluronidase spreading test, clofazimine had a hyaluronidase inhibitory effect, after a single oral administration of 100–200 mg in humans (232). In agreement with these results, it was found that clofazimine (50–100 mg/kg per day) inhibited rat adjuvant arthritis and the inflammatory paw swelling following an adjuvant injection (233). It did not inhibit the primary antibody response to sheep erythrocytes or the tuberculin skin response. Thus clofazimine seems to have anti-inflammatory but not immunosuppressive activity.

5.1.7.3 Pharmacokinetics. Clofazimine is an effective alternative drug in the therapy of leprosy. It is most active when administered twice weekly or daily, but can be administered

at monthly intervals as well, permitting monitoring of the treatment. The daily dose should not exceed 100 mg. Clofazimine has a peculiar pharmacokinetic pattern, characterized by slow absorption, low blood concentration, and extremely slow excretion. All riminophenazines are soluble in fats, and in micronized form, are well absorbed by the intestine. Riminophenazines, once absorbed by the intestine, are carried by lipoproteins in the blood and ingested by macrophages. After continued oral administration, the macrophages appear as red-orange phagosomes. Therefore, the compounds have a diffusion that is mainly intracellular. They are stored in the body for a long time, and this confers some prophylactic action on them (219).

5.1.7.4 SARs. To obtain compounds with better pharmacodynamic and pharmacokinetic properties, which would be more useful than clofazimine in the treatment of mycobacterial infections, a number of riminophenazines were prepared and tested. All the derivatives with hydrophilic groups are either less active or inactive. Compound B-1912 (33)was selected for further investigation because it showed higher serum levels and lower tissues levels (except than in lipids) than clofazimine. In *M.* leprae infections experimentally induced in mice, it was shown that clofazimine and B-1912 have the same activity (234). Newer riminophenazines, such as B746 and B4157, showed increased antimycobacterial activity and produced less skin pigmentation, which is the main drawback of this group of compounds, and both need further investigation (235).

5.1.7.5 Adverse Reactions. In laboratory animals, clofazimine has low acute and subacute toxicity (236). In clinical use, treatment with clofazimine is not accompanied by relevant toxicity. The main and sometimes unacceptable side effect is a red-purple coloration of skin, particularly within skin lesions (231). Gastrointestinal intolerance may also occur.

5.1.8 Ethambutol

5.1.8.1 History. Extensive studies of the chemical and biological properties of compounds related to alkylenediamine were carried out after the discovery, during the screening of randomly selected compounds, of the antimycobacterial activity of *N,N'*-diisopropylethylenediamine (34) (237, 238). This compound was found to be active both in vitro and in *vivo*, with a therapeutic index of the same order of streptomycin.

$$RNHCH_2CH_2NHR$$

(34) $R = CH(CH_3)_2$

(35) $R = \underset{\displaystyle CH_2OH}{\overset{\displaystyle CHC_2H_5}{|}}$

(36) $R = \underset{\displaystyle CHO}{\overset{\displaystyle CHC_2H_5}{|}}$

(37) $R = \underset{\displaystyle COOH}{\overset{\displaystyle CHC_2H_5}{|}}$

5.1.8.2 SARs. Chemical modifications of the compound (34), attempted with the aim of obtaining the product with the highest therapeutic index, gave indications of the structural requirements for antimycobacterial activity. More relevant are the following: the presence of two basic amine centers, the distance between the two carbons, and the presence of a simple, small branched alkyl group on each nitrogen. A correlation between metal chelation of compounds of this structure and antimycobacterial activity suggested the use of synthesizing products with hydroxy substitution of the N-alkyl groups as more effective metal chelators and possibly more active antimycobacterial agents The most active of these derivatives was the dextro isomer of *N,N'*-bis-(1-hydroxy-2-butyl)ethylenediamine (ethambutol, 35). The *meso* isomer is less active and the *levo* almost inactive. Furthermore, hydroxy substitution on other alkyl groups (isopropyl, t-butyl) or in other positions of the butyl group gave inactive products. These data seem in contrast with the working hypothesis of a correlation between metal chelation and antimycobacterial activity. Various other modifications of the structure of ethambutol gave inactive products, with a few exceptions. The OCH,, OC_2H_5, and HNCH, derivatives have the same activity in *vivo* as the parent compound because dealkylation occur in the

body. In addition, the monohydroxy unsymmetrical analog has activity equal to ethambutol but is more toxic.

5.1.8.3 Activity and Mechanism of Action. Ethambutol inhibits in vitro the growth of most of the human strains of M. tuberculosis at concentrations around 1 μg/mL. Strains resistant to other antimycobacterial agents are just as sensitive to ethambutol. Among the other mycobacteria, M. bovis, M. kansasii, and M. marinum are usually susceptible. The MICs for M. avium intracellulare range between 0.95 and 15 μg/mL; 63% of strains is inhibited by 1.9 μg/mL or less (239, 240). Ethambutol is not active in the experimental mouse infection with M. leprae (177) and is not used in the treatment of human leprosy. The efficacy of ethambutol against M. tuberculosis in vivo was proved in various experimental models in animals and confirmed in the clinical trials in human tuberculosis.

The effect of ethambutol on mycobacteria is primarily bacteriostatic, with a maximum inhibitory effect at neutral pH. The primary mechanism of action is not understood. The growth inhibition by ethambutol is largely independent of concentration, being more related to the time of exposure. It seems that most of ethambutol taken up by mycobacteria has not direct role in growth inhibition, and there is no information on the subcellular components responsible for critical ethambutol binding (241,242). Treatment of mycobacteria with ethambutol results in inhibition of protein and DNA synthesis, and it was proposed that ethambutol interferes with the role of polyamines and divalent cations in ribonucleic acid metabolism (243–245). Other authors have found an inhibitory effect of ethambutol on phosphorylation of specific compounds of intermediary metabolism, under conditions of endogenous respiration (246). Other proposed that primary sites of inhibition include arabinogalactan biosynthesis (247) and glucose metabolism (248). However, further studies are necessary to clarify the primary action of the drug.

5.1.8.4 Pharmacokinetics. In current therapeutic use, the drug is administered orally at daily doses of 15–25 mg/kg, in combination with other antitubercular agents, to prevent emergence of resistant strains (249,250). The drug is well absorbed from the gastrointestinal tract. Except for the cerebrospinal fluid, it is widely distributed to most tissues and fluids. About one-half the ingested dose is excreted as active drug in the urine, where there are also minor quantities of two inactive metabolites: the dialdehyde (36) and the dicarboxylic acid (37) derivatives (251, 252).

5.1.8.5 Adverse Effects. Ethambutol is rather well tolerated. The main side effect of concern is ocular toxicity, consisting of retrobulbar neuritis with various symptoms, including reduced visual acuity, constriction of visual fields, and color blindness. Ocular toxicity seems to be dose-related (253). At a daily dose of 25 mg/kg, visual impairment occurs in about 3% of patients, rising to 20% at doses higher than 30 mg/kg per day.

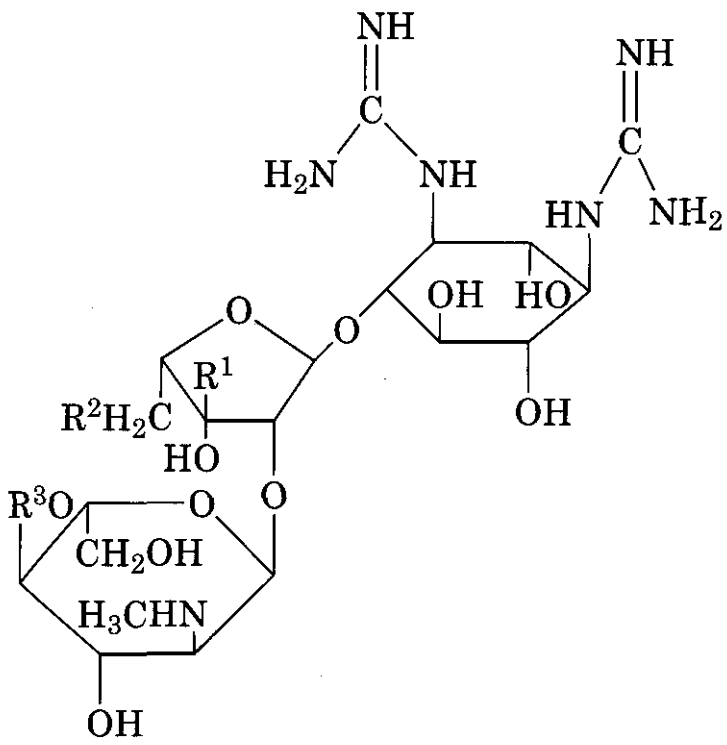
5.2 Antibiotics

5.2.1 Streptomycin

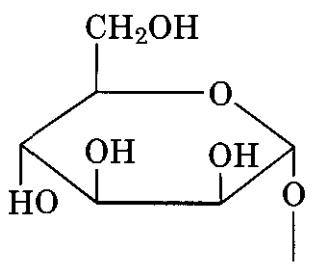
5.2.1.1 History. Streptomycin was discovered in 1944 as a fermentation product of Streptomyces griseus (254). It belongs to the family of aminoglycoside antibiotics, which includes kanamycin, gentamicin, neomycin, amikacin, nebramycin, paromomycin, ribostamycin, tobramycin, sisomicin, dibekacin, netilmicin, kasugamycin, and spectinomycin. In terms of chemical structure, they are aminocyclitols (cyclohexane with hydroxyl and amino or guanidino substituents) with glycosyl substituents at one or more hydroxyl groups. Streptomycin is an N-methyl-L-glucosaminidostreptosidostreptidine made up of three components: streptidine, streptose, and N-methyl-L-glucosamine (38). The intact molecule is necessary for antibacterial action.

Mannosidostreptomycin (39) is another antibiotic, produced together with streptomycin by S. griseus, which has not found clinical application because it is less active than streptomycin itself. Hydroxystreptomycin (40), produced by S. griseocarneus, has biological properties similar to those of streptomycin, with no advantages over it.

5.2.1.2 SARs. In the attempt to improve activity and/or decrease toxicity of streptomycin, some chemical modifications have been performed (e.g., on aldehyde or guanidino functions), which yielded generally less active



	R^1	R^2	R^3
(38)	—CHO	H	H



(39)	—CHO	H	

(40)	—CHO	—OH	H
(41)	CH_2OH	H	H

products. One chemical derivative of streptomycin, dihydrostreptomycin (**41**), obtained by catalytic hydrogenation of the carbonyl group of streptose, has almost the same antibacterial activity of the parent compound and investigators hoped that it would differ from the parent in having lower toxicity. Later clinical experience did not confirm this hope.

5.2.1.3 ***Activity and Mechanism of Action.*** Streptomycin is both bacteriostatic and bactericidal for the tubercle bacillus in vitro, according to the concentration of the antibiotic. Concentrations of streptomycin around 1 μg/mL inhibit the growth of M. tuberculosis *H37Rv*. NTM are not susceptible to streptomycin. The antibacterial activity of streptomycin is not restricted to M. tuberculosis but includes a variety of Gram-positive and Gram-negative bacteria. The most important clinical use of streptomycin is in the therapy of tuberculosis and it was the first really effective drug for this disease. Its importance declined after the introduction of other powerful oral antituberculous agents. Since the introduction of other broad-spectrum antibiotics, the use of streptomycin in the treatment of infections is limited to diseases in which other alternatives are lacking and the sensitivity of the infecting organism indicates the choice and eventually the use of this drug in combination with other antibiotics. Thus, it is still a drug of first choice for enterococcal endocarditis (in combination with penicillin or ampicillin), in brucellosis (in combination with tetracycline), in plague, and in tularemia.

The investigation on the mechanism of action of streptomycin has involved a number of elegant studies in microbiological chemistry and molecular biology that have led to a succession of hypotheses and to a continuous increase in knowledge not only of the mode of action of the antibiotic but also of the biology of the bacteria. After a series of preliminary hypotheses, it was finally ascertained that the drug is a specific inhibitor of protein biosynthesis in intact bacteria (255, 256). The ribosome, and particularly its 30S subunit, is the site of action of the antibiotic (257, 258), and after careful disassemble of 30S ribosomes, a protein designated P10 was determined to be the genetic locus responsible for the phenotypic expression of sensitivity and resistance and dependence on streptomycin (259). The antibiotic induces a misreading of the genetic code, demonstrated through studies of the erroneous incorporation of amino acids in cell-free ribosome systems (260). It was deduced that the misreading in vivo was the cause of the bactericidal effect of streptomycin, because it resulted in "flooding the cell" with erroneous, non-functional proteins. However, it was subsequently demonstrated that this could not be the case because in the intact bacteria the antibiotic inhibits the synthesis of proteins (261). The ultimate mode by which streptomycin exerts its bactericidal activity is not yet clear. Two hypotheses have been put forward: one suggesting that streptomycin specifically inhibits initiation of protein synthesis (262) (this is supported by the involve-

ment of protein P10, the site of action of streptomycin, in the initiation reaction) and the other suggesting that it inhibits peptide chain elongation, that is, the synthesis of peptide bonds at any time during the growth of the peptide chain (263,264). As noted before, sensitivity and resistance to and dependence on streptomycin all seem to be expressed in the ribosome and apparently are multiple alleles of a single genetic locus. Streptomycin-resistant mutant cells arise spontaneously in a bacterial culture, with a frequency of the order $1 \cdot 10^{-6}$ (265).

In the phenomenon of streptomycin dependence, bacteria require streptomycin to grow; these bacteria also arise by spontaneous mutation (266), and the mechanism of their behavior is also related to the reading of codons. This can be done correctly only in the presence of streptomycin, which overcomes an undiscriminating restriction (caused by mutation), leading to a mutant in which the ribosomal screen does not allow normal translation for growth (261–267). In addition, resistance to streptomycin can be transferred by means of R-factors or plasmids, namely, by extra-chromosomal DNA carrying multiple antibiotic resistance (268).

The mode of transmission of resistance is particularly frequent among enterobacteria. Enzymatic inactivation is a frequent cause of resistance to streptomycin in eubacteria. The aminoglycoside-inactivating enzymes are phosphotransferases, acetyl-transferases, and adenyl-transferases. Because they act by inactivating a chemical group that is common to different aminoglycosides, bacterial strains that produce only one of them can be resistant to all aminoglycosides possessing the same chemical group (cross-resistance). Streptomycin can be inactivated by some streptomycin-adenyltransferase and streptomycin-phosphotransferase, which usually do not affect other aminoglycosides except spectinomycin (172).

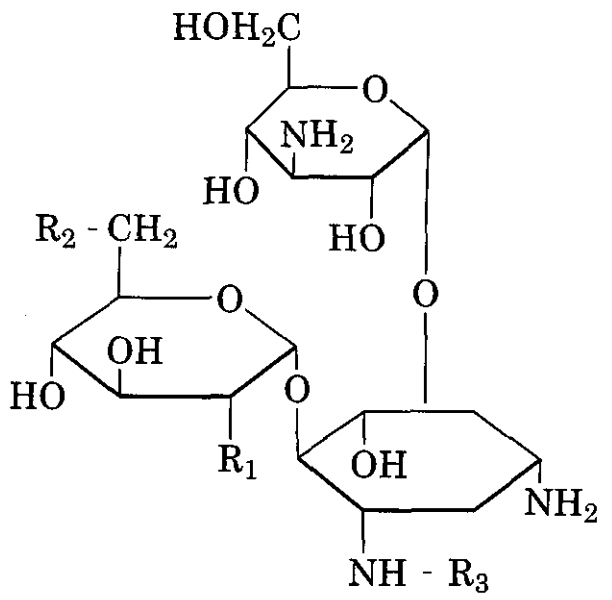
In mycobacteria, mutations in the *rpsL* gene, which encodes the ribosomal protein S12 have been shown to confer resistance to streptomycin. Analysis of the primary structure of the ribosomal protein S12 in M. tuberculosis has revealed that mutations in the gene replacing Lys^{43} or Lys^{88} by arginine are frequently associated with resistance to streptomycin (269–272). A second type of mutation conferring resistance has been identified in streptomycin-resistant strains of M. tuberculosis that have a wild-type *rpsL* gene. These strains have point mutations in the 16S rRNA clustered in two regions around nucleotides 530 and 915 (273,274). In those isolates with a wild-type 16S rRNA and *rpsL* gene, other mechanism of drug resistance can be hypothesized, such as modifications of other components of the ribosome or alteration in cellular permeability.

5.2.1.4 Pharmacokinefics. Streptomycin, like all other aminoglycoside antibiotics, is not absorbed from the gastrointestinal tract, and therefore, it must be administered parenterally. Serum peak levels are reached in 1–2 h, and the values are 9–15 μg/mL after administration of 1 g. Its half-life is 2–3 h. The serum protein binding of streptomycin is 25–35% (275). Streptomycin diffuses slowly into the pleura and better into the peritoneal, pericardial, and synovial fluids. It does not penetrate into spinal fluid, unless the meninges are inflamed. Urinary elimination is rapid, and 70% of the drug is excreted in unmodified form in the first 24 h.

5.2.1.5 Adverse Effects. The most important toxic effects of streptomycin involve the peripheral and central nervous system. The eighth cranial nerve is the most frequently injured by prolonged administration of streptomycin, especially in its vestibular portion, causing equilibrium disturbances to appear. Treatment with 2–3 g/day for 2–4 months produces this type of side effect in about 75% of patients, but the incidence is much less at doses of 1 g/day. Other side effects are hypersensitivity reactions and renal damage.

5.2.2 Kanamycin

5.2.2.1 History. Another aminoglycoside antibiotic, used in the therapy of tuberculosis and named kanamycin, was isolated in 1957 as a fermentation product of Streptomyces *kanamyceticus*. It consists of three components: kanamycin A, B, and C (42–44). Kanamycin A (42) is the largest part of the mixture (98%). Structural studies indicated that the molecule contains deoxystreptamine (instead of the streptidine present in the streptomycin molecule) and two amino sugars: kanosamine and



	R_1	R_2	R_3
(42)	OH	NH_2	H
(43)	NH_2	NH_2	H
(44)	NH_2	OH	H
(45)	NH_2	NH_2	$CO-CHOH-CH_2-CH_2-NH_2$

6-glucosamine. It is water soluble and stable at both acid and basic pH as well as at high temperature.

5.2.2.2 ***Activity and Mechanism of Action.*** Kanamycin has a quite broad spectrum of activity, including Gram-positive cocci and Gram-negative bacteria, as well as M. tuberculosis and some other mycobacteria. Its activity against M. tuberculosis is weaker than that of streptomycin (276). The bactericidal concentrations are close to the bacteriostatic ones, but they are hard to achieve in vivo (277). Kanamycin is used in therapy of infections caused by penicillin-resistant Staphylococcus aureus (now less frequently used because the availability of penicillinase-resistant penicillins and other antistaphylococcal antibiotics) or by Gram-negative bacilli, such as E. coli, Klebsiella, Enterobacter, and Proteus. It has no activity against Pseudomonas. It has been used in the therapy of tuberculosis, in combination with other antituberculous drugs. The common dose is 15 mg/kg per day, but a total dose of 1.5 g must not be exceeded.

The mechanism of action of kanamycin is similar to that of streptomycin, because it produces a misreading of the genetic code, interacting with 30S ribosomal subunit in more than one site (whereas streptomycin is bound only to one site), and inhibits protein synthesis (278–280). All aminoglycoside antibiotics that contain a 2-deoxystreptamine moiety cause miscoding. Kanamycin, like streptomycin and other aminoglycosides, blocks both the initiation and elongation of peptide chains. This mechanism was confirmed in mycobacteria. In vitro studies on cell-free preparations of M. bovis have shown that kanamycin inhibits polypeptide synthesis, followed by breakdown of polysomes and detachment of mRNA (281). A kanamycin-induced increase in ^{14}C-isoleucine incorporation by poly-U-directed ribosomes indicated a misreading of the genetic code, but this did not seem to be directly related to the bactericidal action of the antibiotic.

Resistance to kanamycin can be acquired in vitro in a stepwise fashion by subculturing bacteria in increasing concentrations of antibiotic. In addition to chromosomal resistance, resistance to kanamycin can be acquired by conjugation, through the transfer of extrachromosomal DNA, the so-called R-factors or plasmids, and coding aminoglycoside-inactivating enzymes (phosphotransferases, acetyltransferases, and nucleotidyltransferases). Kanamycin A can be inactivated by neomycin-kanamycin phosphotransferases I and II, kanamycin acetyltransferase, and gentamicin adenyltransferase. Cross-resistance will appear to any other aminoglycoside antibiotic that may be inactivated by the same enzyme (269). Cross-resistance is total with neomycin and paromomycin. With streptomycin, a "one-way resistance" is observed, namely strains resistant to kanamycin and neomycin are usually resistant to streptomycin, whereas streptomycin-resistant strains are usually susceptible to kanamycin and gentamicin. It has been suggested that this is caused by different sites of action of the antibiotics on the ribosomes (282). Thus, in therapy, it is advisable to administer streptomycin before kanamycin.

5.2.2.3 Pharmacokinetics. From a pharmacokinetic point of view, kanamycin behaves similarly to the other aminoglycosides: it is not absorbed when given by the oral route, but it is rapidly absorbed after intramuscolar administration, reaching high peak serum levels 2–3 h after administration (283). There is almost no serum protein binding (275). Diffu-

sion into cerebrospinal fluid is poor when meninges are normal but increases when they are inflamed. Kanamycin diffuses quite well into pleural, peritoneal, synovial, and ascitic fluids (284–285), but poorly into bile, feces, amniotic fluid, and so on. It is excreted by the kidney, mainly by glomerular filtration (50–80%) and in unmodified form.

5.2.2.4 Adverse Effects. Drawbacks to the use of kanamycin are its ototoxicity and nephrotoxicity (286). Cochlear and vestibular functions are damaged in about 5% of patients, but the percentage increases proportionally to the total dose administered. Thus, in prolonged treatments, as in the case of tuberculosis, patients must be closely followed. Nephrotoxicity can be prevented if dosage to patients with impaired renal function is reduced in accord with the decrease in creatinine clearance or the increase in creatininemia (284). Kanamycin can produce neurotoxicity, with curare-like effects caused by neuromuscular blockade.

5.2.3 Amikacin

5.2.3.1 History. Amikacin ***(45)*** is a semisynthetic analog of kanamycin, in which the C-1 aminogroup is amidated with a 2-hydroxy-4-amino-butirric acid moiety. The synthesis of amikacin was suggested by the observation that butirosin B (an antibiotic produced by B. ***circulans*** differing from ribostamycin only in having an a-hydroxy-y-aminobutyric acid substituent on the amine in position 1 of 2-deoxystreptamine) was active against ***Pseudomonas*** and other inactivating strains, unlike ribostamycin. The different aminoglycosides obtained by acylation with α-hydroxy-γ-aminobutyric acid were found to be insensitive to the enzymes phosphorylating the oxygen in position 3′, to those acylating the nitrogen in position 3, and to those determining the nucleotide attachment to the oxygen in position 2.

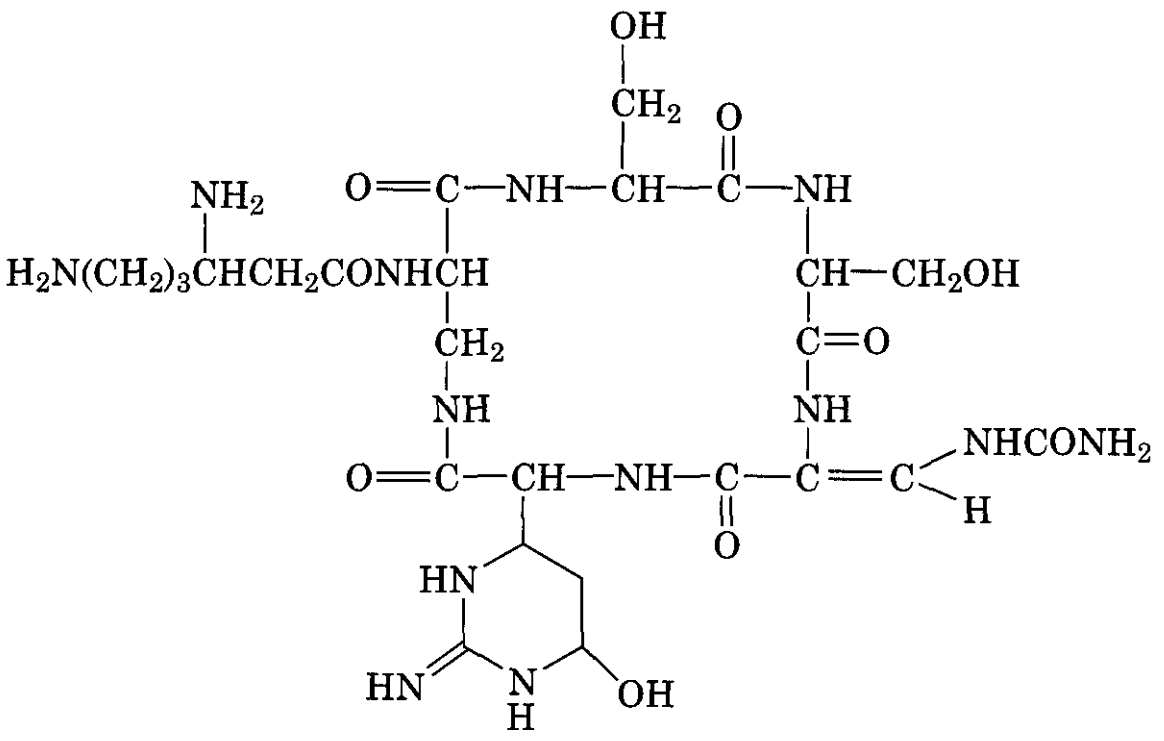
5.2.3.2 Activity. Amikacin, the derivative obtained by acylating the nitrogen on C-1 of kanamycin A with a-hydroxy-y- aminobutyric acid, is the aminoglycoside with the broadest spectrum of activity and can be used especially in cases of infections caused by bacteria resistant to other aminoglycosides. In fact, amikacin is not inactivated by many of the R-factor mediated enzymes that attack kanamycin. In particular, useful activity against ***Pseudomonas aeruginosa*** results. In ***in vitro*** and animal trials, amikacin is among the most active, if not the most active, aminoglycoside against M. ***tuberculosis*** (287).

Amikacin is not used for the initial treatment of susceptible tuberculosis, mainly because of its cost, but it seems to have merit as an alternative drug for the retreatment of resistant M. ***tuberculosis*** infections. Amikacin seems to have a role in the treatment of NTM infections, especially if the infections are caused by rapidly growing mycobacteria (M. ***fortuitum*** and *M. chelonae)* (288). It is one of the most bactericidal agent against M. ***avium*** complex both ***in vitro*** and in beige mouse models (289–290). In some clinical studies with AIDS patients, M. ***avium*** *intracellulare* complex bacteremia was cleared by combination of amikacin with other drugs (clarithromycin and ciprofloxacin) (291).

5.2.4 Viomycin and Capreomycin

5.2.4.1 History. Viomycin and capreomycin are two structurally similar polypeptide antibiotics. Viomycin (46) was discovered independently by two groups of investigators in 1951 (292, 293) from an actinomyces named ***Streptomyceus puniceus*** by one group and S. ***floridae*** by the other. Capreomycin is a polypeptide complex isolated in 1960 from ***Streptomyces capreolus*** (294) and the structure of the same components of the complex (**47**) indicated its similarity with viomycin.

5.2.4.2 Activity and Mechanism of Action. Viomycin is relatively more active against the mycobacteria than against other bacteria. It inhibits protein synthesis (295) but has little or no miscoding activity (279). Viomycin-resistant mutants isolated from M. ***smegmatis*** have altered ribosomes (296): one of these mutants had altered 50S subunits and others have 30S subunits. The genetic locus for viomycin-capreomycin resistance (*vic* locus) in *M. smegmatis* consisted of two groups: *vic A* and *vic* B. It is likely that alterations in the 30S subunit conferred by *vic* B and alterations in the 50S subunit conferred by *vic A* interact in response to viomycin (297). There is a one-way cross-resistance with kanamycin: viomycin-resistant strains may retain their susceptibility to kanamycin, but kanamycin-



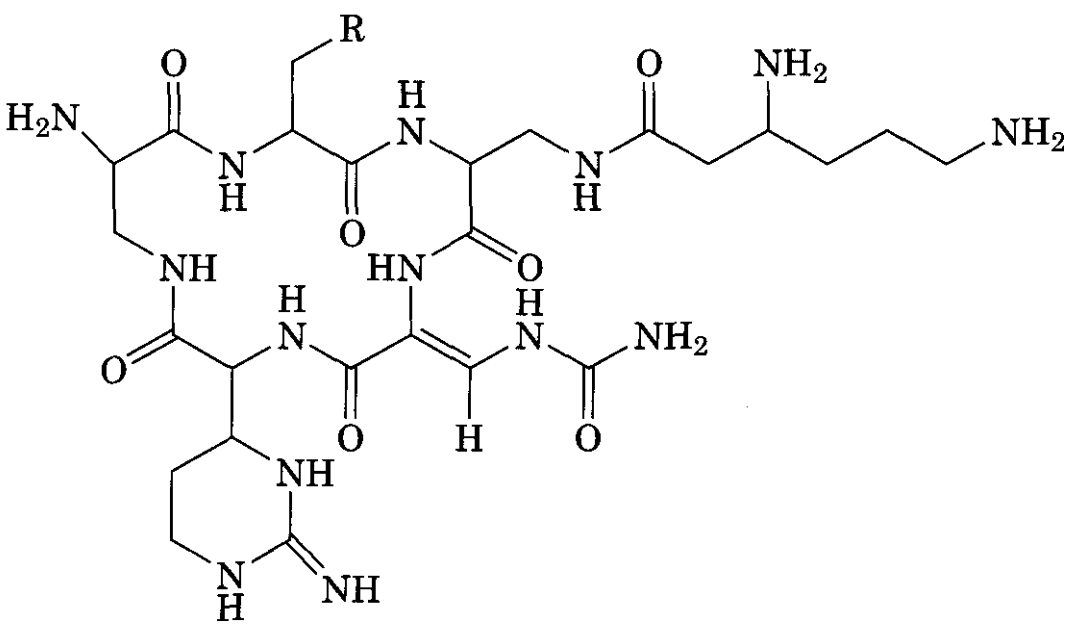
(46)

resistant strains are also resistant to viomycin. It is interesting to note that in M. smegmatis, the genetic locus for neomycin-kanamycin resistance (nek locus) is not linked to ***str*** locus (for streptomycin resistance) as in E. coli but is linked to *vic* locus (297).

Capreomycin is active only against M. tuberculosis and some other mycobacteria, particularly M. *kansasii*. Cross-resistance to kanamycin, neomycin, and viomycin has been described, and a phenomenon of partial "one-way resistance" to kanamycin has been demonstrated. Capreomycin-resistant strains are not always fully resistant to kanamycin, but kanarnycin-resistant strains are always resistant to capreomycin.

5.2.4.3 Pharmacokinetics. This type of antibiotics behaves like the aminoglycosides. They are not absorbed by the gastrointestinal tract. Peak serum concentrations of capreomycin are achieved 1–2 h after intramuscular administration of 1 g of the drug. The half-life is **3–*6*** h. The drug is eliminated unchanged in the urine.

5.2.4.4 Adverse Reactions. Side effects produced by viomycin are severe and frequent.



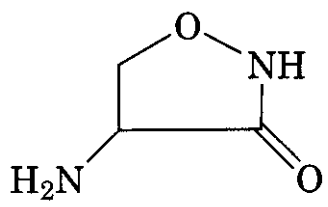
(47) R = H, OH

Vestibular and auditory impairment, renal damage, and disturbance in the electrolyte balance have a high incidence during viomycin therapy. For these reasons the drug is now seldom used.

Renal damage is the most consistent and significant toxic effect of capreomycin, which is also potentially toxic to the eighth cranial nerve. Capreomycin is usually reserved for retreatment regimens, when the primary antituberculous drugs cannot be used because of toxicity or the presence of resistant bacilli.

5.2.5 Cycloserine

5.2.5.1 History. D-Cycloserine was isolated in 1955 independently by several groups of workers from cultures of Streptomyces *garyphalus*, S. orchidaceus, and S. lavendulae (298–300). On the basis of degradation studies and physicochemical properties, the structure of D-4-amino-3-isoxazolidone (48) was assigned to this antibiotic (301, 302). The structure was confirmed by synthesis (303), and various methods of preparation have been reported subsequently, which have also been used to prepare L-cycloserine from L-serine.



(48)

5.2.5.2 SARs. No improvement on the antibacterial activity of D-cycloserine was obtained through chemical variation of its structure. Only the synthetic L-cycloserine possesses some antibacterial activity, but probably with a different mechanism of action.

5.2.5.3 Activity and Mechanism of Action. Cycloserine inhibits *M.* tuberculosis at concentrations of 5–20 μg/mL. Strains resistant to other antimycobacterial drugs have the same sensitivity to cycloserine. The antibiotic is also active in vitro against a variety of Gram-positive and Gram-negative microorganisms, but only in cultures media-free of D-alanine, which results to be an antagonist of the antibacterial activity of cycloserine.

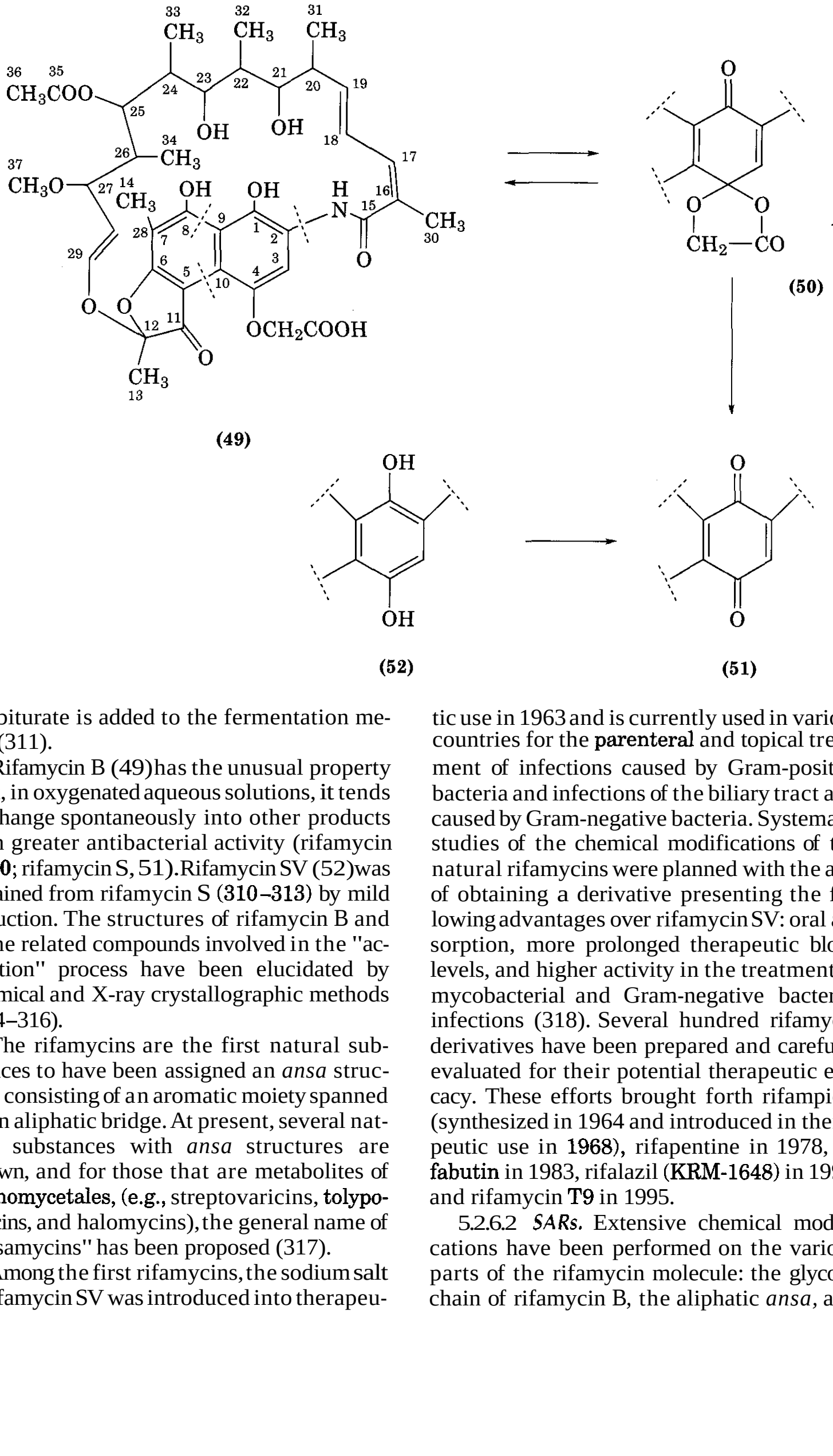
Concerning the mechanism of action of cycloserine, it has been proven in some bacterial species that this antibiotic interferes with the synthesis of the cell wall. In fact, cycloserine induces the formation of protoplasts in E. coli. Microorganisms treated with cycloserine accumulate a muramic-uridine-nucleotide peptide, which differs from that produced by penicillin in the absence of the terminal D-alanine dipeptide. The inhibition of alanine racemase, which converts L-alanine into D-alanine, is probably the primary action of cycloserine (304–306). The mechanism of action in mycobacteria is likely the same.

5.2.5.4 Pharmacokinetics. Cycloserine possesses different pharmacokinetic properties in the various animal species and this explains the different responses in animal infections. In *vivo*, cycloserine was ineffective against experimental tuberculosis in mice and marginally effective in guinea pigs, but some activity was found against the disease induced in the monkey. The drug is more effective in humans than in animals. When given orally to humans, cycloserine is well and quickly absorbed from the gastrointestinal tract and is well distributed in the body fluids and tissues. The usual dose for adults is 250 mg twice a day orally, always in combination with other effective tuberculostatic agents. About one-half of the ingested dose is excreted unchanged in the urine in 24 h. A part of the antibiotic is metabolized into products not yet identified.

5.2.5.5 Adverse Reactions. Cycloserine produces severe side effects in the central nervous system that can also generate psychotic states with suicidal tendencies and epileptic convulsions. Therefore, its use is limited only to cases in which other drugs cannot be used.

5.2.6 Rifamycins

5.2.6.1 History. The rifamycin antibiotics were discovered in 1959 as metabolites of a microorganism originally considered to belong to the genus Streptomyces and subsequently reclassified as a Nocardia (Nocardia *mediterranea*) (307–309) and more recently as *Amycolatopsis* mediterranea. The crude material extracted from the fermentation broths contained several rifamycins (rifarnycin complex) (310). Only rifamycin B was isolated as a pure crystalline substance, and it is essentially the only component found when sodium diethyl-



(49) (50) (52) (51)

barbiturate is added to the fermentation media (311).

Rifamycin B (49) has the unusual property that, in oxygenated aqueous solutions, it tends to change spontaneously into other products with greater antibacterial activity (rifamycin O, **50**; rifamycin S, 51). Rifamycin SV (52) was obtained from rifamycin S (310–313) by mild reduction. The structures of rifamycin B and of the related compounds involved in the "activation" process have been elucidated by chemical and X-ray crystallographic methods (314–316).

The rifamycins are the first natural substances to have been assigned an *ansa* structure consisting of an aromatic moiety spanned by an aliphatic bridge. At present, several natural substances with *ansa* structures are known, and for those that are metabolites of actinomycetales, (e.g., streptovaricins, tolypomycins, and halomycins), the general name of "ansamycins" has been proposed (317).

Among the first rifamycins, the sodium salt of rifamycin SV was introduced into therapeutic use in 1963 and is currently used in various countries for the parenteral and topical treatment of infections caused by Gram-positive bacteria and infections of the biliary tract also caused by Gram-negative bacteria. Systematic studies of the chemical modifications of the natural rifamycins were planned with the aim of obtaining a derivative presenting the following advantages over rifamycin SV: oral absorption, more prolonged therapeutic blood levels, and higher activity in the treatment of mycobacterial and Gram-negative bacterial infections (318). Several hundred rifamycin derivatives have been prepared and carefully evaluated for their potential therapeutic efficacy. These efforts brought forth rifampicin (synthesized in 1964 and introduced in therapeutic use in 1968), rifapentine in 1978, rifabutin in 1983, rifalazil (KRM-1648) in 1993, and rifamycin T9 in 1995.

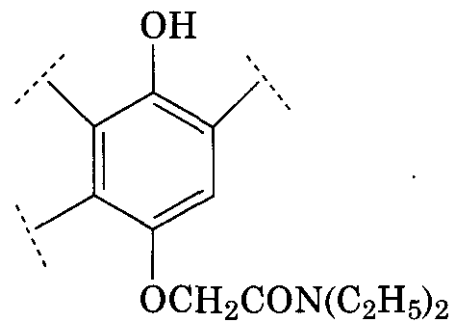
5.2.6.2 *SARs.* Extensive chemical modifications have been performed on the various parts of the rifamycin molecule: the glycolic chain of rifamycin B, the aliphatic *ansa*, and

the chromophoric nucleus. A great deal of information about the structure-activity relationships has been obtained (318–321). All rifamycins possessing a free carboxy group are partially or totally inactive because they do not enter into the bacterial cell. Requirements for activity seem to be the presence of two free hydroxyls in positions C21 and C23 on the *ansa* chain and of two polar groups (either free hydroxyl or carbonyl) at positions C1 to C8 of the naphtoquinone nucleus, together with a conformation of the *ansa* chain that results in certain specific geometrical relationship among these four functional groups.

This conclusion is based on the following data. First, substitution or elimination of the two free hydroxyls in position C21 and C23 gives inactive products. Inversion of the configuration at C23 leads to an inactive compound, and the inversion at C21 strongly reduces the activity (322, 323). Second, modifications of the *ansa* chain that alter its conformation (e.g., C16–C17 and C18–C19 monoepoxy and diepoxy derivatives) give inactive or less active products. Also, the stepwise hydrogenation of the ansa chain double bonds results in a gradual decrease in activity as a consequence of the increase in flexibility of the *ansa* diverging from the most active conformation. Third, the oxygenated functions at C1 and C8 must be either free hydroxyl or carbonyl to maintain biological activity. Fourth, the four oxygenated functions at C1, C8, C21, and C23 not only must be unhindered and underivatized, but must display well-defined relationships with one another. The absolute requirements for these four functions to be in a correct geometrical relationship suggest that they are involved in the noncovalent attachment of the antibiotic to the bacterial target enzyme. This contention is supported by the observation that in the active derivatives these four functional groups lie on the same side of the molecule and almost in the some plane with identical interatomic distances between the four oxygens (324), as seen in the spatial model derived from X-ray studies (325). However, conformational differences have been observed at the junction of the *ansa* chain to the naphtoquinone chromophore, according to the nature of the substituents in position 3 (326). Protonic nuclear magnetic resonance (^{1}H NMR) studies of rifamycin S have confirmed that the conformation of the molecule in solution corresponds well to that obtained in the solid state (327).

Finally, all modifications at C3 and/or C4 position that do not interfere with the previous requirements do not affect the general activity of the products (318–321).

A great number of active derivatives have been obtained by modifications on the glycolic moiety of rifamycin B and on position C3 and/or C4 of the aromatic nucleus of rifamycin S or SV, leaving unaltered the structure and the conformation of the *ansa* chain. Among the earlier derivatives of the glycolic side chain, the diethylamide of rifamycin B, rifamide (53), had a better therapeutic index

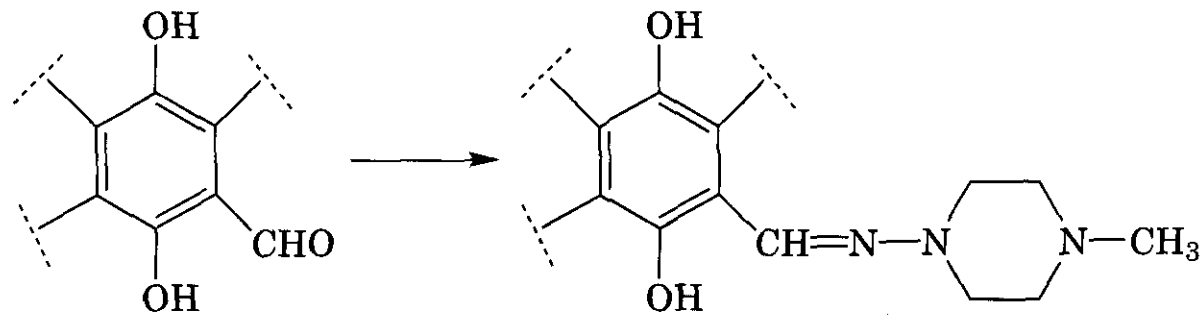


(53)

than rifamycin SV (328), but it still suffers from most of the limitations of use of rifamycin SV (329).

Chemical modifications of the chromophoric nucleus of rifamycin on C3 and/or C4 positions gave a large number of derivatives. The nature of the substituents at positions C3 and/or C4 influences the physicochemical properties of the derivatives, especially lipophilicity. The various derivatives show a minor degree of variation in antibacterial activity against intact cells, because the transport through bacterial wall and membrane is the major factor affected by these substituents (330). Other biological characteristics influenced by the various modifications of the positions C3 and/or C4 are the absorption from the gastrointestinal tract and the kinetics of elimination. Rifamycin derivatives with substitutions at position C4 include a series of 4-aminoderivatives. Modifications at positions C3 and C4 include rifamycins with heterocyclic nuclei fused on these positions (e.g., 3,4-phenazinerifamycins,

phenoxazinerifamycins, pyrrolerifamycins, thiazolerifamycins, and imidazorifamycins) with various groups on the heterocyclic nuclei. Rifamycins with substituents only in position C3 are represented by 3-thioethers, 3-aminomethylrifamycins, 3-carboxyrifamycins, 3-aminorifamycins, and 3-hydrazinorifamycins. When the 3-formyl-rifamycin SV (54) was prepared by oxidation of *N*-dialkyl-aminomethyl rifamycin SV (331), it was found that many of its *N,N*-disubstituted hydrazones had high activity, both in vitro and in *vivo*, against M. tuberculosis and Gram-positive bacteria, and moderate activity against Gram-negative bacteria. For some of these derivatives, the in *vivo* activity in animal infections was of the same order whether the products were administered orally or parenterally, indicating good absorption from the gastrointestinal tract (318). The hydrazone of 3-formylrifamycin SV



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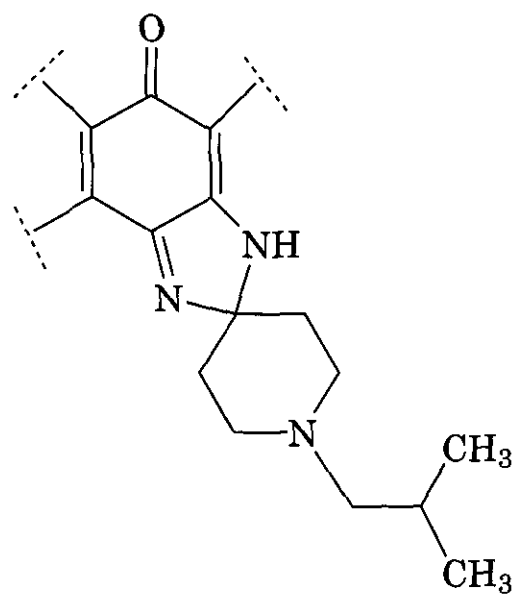

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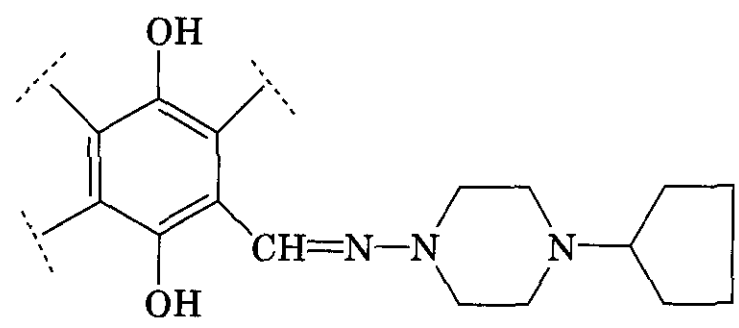

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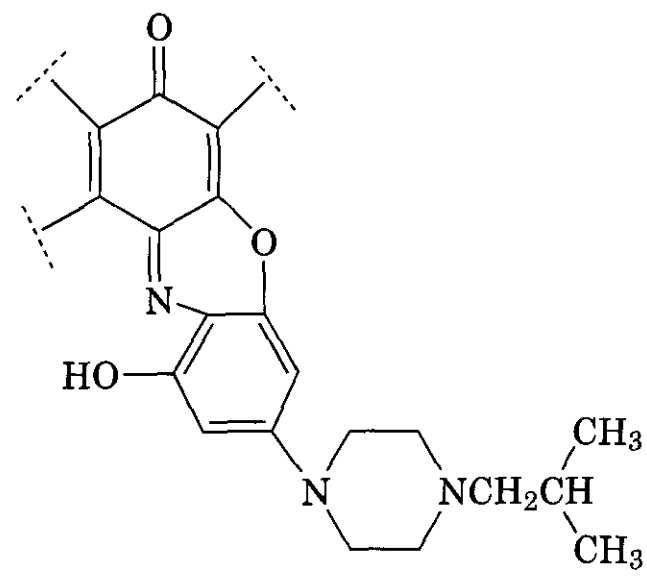

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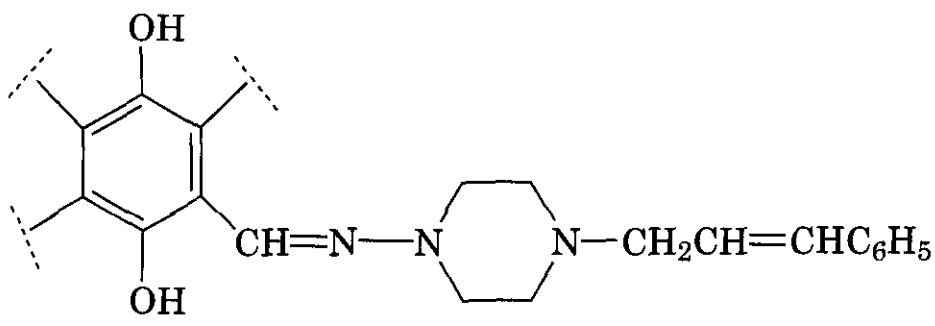


(59)

with *N*-amino-*N'*-methylpiperazine (rifampicin, 55) (318, 332) was the most active *in vivo* and was selected for clinical use.

The knowledge of the SARs in the rifamycins was the basis for further development of this family of antibiotics, leading to other products in clinical use [rifabutin (**56**), rifapentine (**57**), rifalazil (**58**), and rifamycin T9 (**59**)].

5.2.6.3 Mechanism of Action and Resistance. Studied mainly for rifampicin, the mechanism of antibacterial action is the same for all the active rifamycins and resides in the specific inhibition of the activity of the enzyme DNA-directed RNA polymerase (DDRP). The mammalian DDRP is resistant even to high concentration of rifamycins (333, 334). The inhibition of the action of the bacterial RNA polymerase by rifampicin is caused by the formation of a rather stable, noncovalent complex between the antibiotic and the enzyme with a binding constant of 10^{-9} M at 37°C (355). One molecule of rifampicin (mol. wt. 823) is bound with one molecule of the enzyme (mol. wt. 455,000) (336). The drug is not bound covalently to the protein because the complex dissociates in presence of guadinium chloride (337). The binding site of rifampicin to the RNA polymerase has been particularly well studied in E. *coli* (338–340). The inhibitory effect of rifampicin on DDRP as the cause of its bactericidal activity has been verified on several mycobacterial species, e.g., M. *smegmatis, M. bovis BCG,* and *M. tuberculosis* (341–342).

The DDRP is comprised of five subunits (a, a', β, β', and σ) and rifampicin binds to the β subunit. When rifampicin is bound to the enzyme, the complex can still attach to the DNA template and catalyze the initiation of RNA synthesis with the formation of the first phosphodiester bond, e.g., of the dinucleotide pppApU. However, the formation of a second phosphodiester bond, and therefore, the synthesis of long chain RNAs, is inhibited. The action of rifampicin is to lead to an abortive initiation of RNA synthesis (344, 345).

Resistance to rifampicin arises spontaneously in strains not exposed previously to the antibiotic at a rate of one mutation per 10^7 to 10^8 organisms. In E. *coli,* but also in M. *leprae* and in *M. tuberculosis,* resistance to rifampicin results from missense mutations in the *rpoB* gene, which encodes the β subunit of RNA polymerase. These mutations are all located in a short region of 27 codons near the centre of *rpoB* and consist predominantly of point mutations, although in-frame deletions and insertions also occur. In most of M. *tuberculosis* rifampicin resistant clinical isolates, changes have occurred in the codons for Ser 531, or His 526, or Asp 516 (346–351). The analysis of genetic alterations in the *rpoB* gene has been suggested for predicting rifampicin and other rifamycins susceptibility (352). In addition to the mechanisms caused by the *rpoB* mutations, the lower susceptibility of fast growing *Mycobacterium* spp. to rifampicin may be partly caused by antibiotic inactivation mechanisms, consisting in the 23-0-ribosylation of rifampicin (353).

Although the main mechanism of resistance is the modification of the target enzyme, some mutagenic treatments yield resistant mutants in which the RNA polymerase is still highly sensitive to the drug, but the rate of rifampicin uptake is reduced. The mechanism of this permeability mutation is not yet clear (354).

5.2.7 Antimycobacterial Rifamycins .

5.2.7.1 Rifampicin. Rifampicin (Rifampin, USAN, 55) is the 3-[(4-methyl-1-piperazinyl)iminomethyl]-rifamycin SV. It has an amphotheric nature (p*K*a 1.7, 7.9) and is soluble in most organic solvents, slightly soluble in water at neutral pH, but more soluble in acidic and alkaline solutions.

Rifampicin is active *in vitro* against *M. tuberculosis* at concentrations below 1 μg/mL in semisynthetic media. It is active against other Gram-positive bacteria at lower concentrations and against Gram-negative bacteria at concentrations of 1–20 μg/mL. Rifampicin is active at the same concentrations against strains resistant to other antibiotics and antimycobacterials.

The bactericidal activity of rifampicin is demonstrated at concentrations close to the static ones. It is possible to isolate strains resistant to rifampicin from mycobacterial cultures exposed to the antibiotic, but the frequency of resistant mutants to rifampicin in

sensitive populations of M. tuberculosis is lower than that to other antimycobacterial drugs (355).

Rifampicin is active against M. leprae, suppressing the multiplication and the viability of the bacilli in laboratory animal infections (128, 356–359). It is also active against many other mycobacteria, although at concentrations generally higher than those effective against M. tuberculosis. Among the various mycobacteria tested, M. kansasii and M. *marinum* are the most sensitive (360). There is no evidence of cross-resistance among rifampicin and other antibiotics or antituberculous drugs (361). Transfer of resistance to rifampicin could not be obtained.

Preliminary in *vivo* studies (362) showed that the antituberculous efficacy of rifampicin in experimental infections in mice was comparable with that of isoniazid and markedly superior to that of streptomycin, kanamycin, and ethionamide. In guinea pigs, it was comparable with streptomycin. Rifampicin was also remarkably active in experimental infections caused by Gram-positive and some Gram-negative bacteria.

The excellent antituberculous activity of rifampicin in *vivo* was confirmed by other experiments in many laboratories using various animal models (mice, guinea pigs, rabbits) and various schedules of treatment and criteria of evaluation (363–373). The overall results indicate that rifampicin has a high bactericidal effect and a therapeutic efficacy of the same order of isoniazid and superior to all the other antituberculous drugs. The combination of rifampicin plus isoniazid has been shown to produce a more rapid, complete, and durable sterilization of infected animals than other combination (366).

Many clinical trials have confirmed the efficacy of rifampicin in a variety of bacterial infections (374). In particular, rifampicin is largely used for the treatment of human tuberculosis both in newly diagnosed patients and in patients whose primary chemotherapy has failed. Normally, rifampicin is administered orally in a dose of 600 mg daily in combination with other antituberculous drugs. The short-course chemotherapy (SCT) of tuberculosis is a very effective treatment based on the combined administration of rifampicin with isoniazid and other antituberculous drugs for 6–9 months. In human leprosy (128, 356–359, 375, 376), treatment with rifampicin alone or in combination with other antileprosy drugs gave favorable results in almost all the patients. In particular, the variations of the morphological index, and when carried out, the mouse footpad inoculation, showed the rapid and constant bactericidal action of rifampicin. The same effect was observed in many patients who had become resistant to other antileprosy drugs.

5.2.7.1.1 Pharmacokinetics. After oral administration, rifampicin is well absorbed in animals and humans (377). After administration of 150 and 300 mg to humans, serum levels reach maximum values around h 2 and persist as appreciable values beyond h 8 and h 12, respectively. When the dose is increased, serum levels are high and long lasting. Generally, the serum levels found at the beginning of treatment are higher than the levels that gradually set in as treatment continues. This phenomenon occurs during the first few weeks of treatment (378,379). The half-life of rifampicin is around 3 h and increases in patients with biliary obstruction or liver disease (380–382). Rifampicin shows an extensive distribution in the tissues and crosses the blood-brain barrier. It reaches good antibacterial levels in cavern exudate and in pleural fluid. It penetrates inside the macrophages, where the tubercle bacillus, as an intracellular parasite, can live and multiply.

Rifampicin is eliminated through both the bile and the urine. It appears rapidly in the bile, where it reaches high concentrations that last even when the antibiotic is not measurable in the serum. After reaching the threshold of hepatic eliminatory capacity, biliary levels do not increase with an increased dosage, but serum and urinary levels do. In humans, rifampicin is mainly metabolized to 25-*O*-desacetylrifampicin (383), which is only slightly less active than the parent drug against M. tuberculosis, but considerably less active against some other bacteria. Both rifampicin and desacetylrifampicin are excreted in high concentrations in the bile. Rifampicin is reabsorbed from the gut, forming an enterohepatic cycle, but the desacetyl derivative is poorly absorbed and thus is excreted with the feces. Ri-

fampicin has a stimulating effect on microsomal drug-metabolizing enzymes (384), which leads to a decreased half-life for a number of compounds, including prednisone, norethisterone, digitoxin, quinidine, ketoconazole, and the sulfonylureas.

*5.2.7.1.2 **Adverse** Reactions.* Rifampicin has a low toxicity according to acute, subacute, and chronic toxicity studies in several animal species (385, 386). Results of animal and human studies showed no toxic effect on the ear and eye.

In humans, adverse reactions to daily rifampicin are uncommon and usually trivial. The most frequent ill effects are cutaneous reactions and gastrointestinal disturbances. Rifampicin can also disturb liver function, but the risk of its causing serious or permanent liver damage is limited, particularly among patients with no previous history of liver disease.

In the case of intermittent therapy, when the drug is given three times, or once a week, a high incidence of severe side effects (such as the "flu-like" syndrome and cytotoxic reactions) may result. These adverse reactions seem to be associated with rifampicin-dependent antibodies, suggesting an immunological basis. However, with proper adjustment of the size of each single dose, the interval between doses, and the length of treatment, it has been possible to develop intermittent regimens with rifampicin that are highly effective and acceptably safe (386).

5.2.7.2 *Rifabutin.* Rifabutin (56) belongs to the group of spiroimidazorifamycins obtained by condensation of 3-amino-4-iminorifamycin SV with N-butyl-4-piperidone (387–389). Rifabutin has an antibacterial spectrum similar to rifampicin, but seems to possess incomplete cross-resistance with rifampicin ***in uitro.*** In fact some strains resistant to rifampicin are still moderately sensitive to rifabutin. Another characteristic of rifabutin is that its activity against ***Mycobacterium auium*** complex is higher than that of rifampicin (390–394).

Rifabutin is rapidly absorbed by mouth, but its oral bioavailability is only 20%, and there are considerable interpatient variations. The elimination half-life is long (35–40 h), but as a result of a large volume of distribution, average plasma concentrations remain relatively low after repeated administration of standard doses. Binding to plasma proteins is about 70%. Rifabutin is slowly but extensively metabolized, possibly to more than 20 compounds in humans, the 25-desacetyl derivative being the main metabolite (395).

Rifabutin has proven value in preventing or delaying mycobacterial infections in immunocompromised patients (396). It has been approved in the United States and in other countries for the prevention of MAC infections in AIDS patients.

5.2.7.3 *Rifapentine.* Rifapentine (57) is the 3-(4-cyclopentyl-1-piperazinyl-iminomethyl)-rifamycin SV and therefore is an analog of rifampicin in which a cyclopentyl group substitutes for a methyl group on the piperazine ring (397). Its activity is similar to that of rifampicin, but is slightly superior against mycobacteria, including MAC (398, 408). It is more lipophilic and has a serum half-life about five times longer than rifampicin. This is because of its stronger binding to serum proteins than rifampicin (401, 402). Rifapentine is excreted primarily as intact drug in the feces; less than 5% of it and its metabolites are excreted in the urine. The primary metabolite in bile and feces is 25-desacetyl-rifapentine, with smaller amounts of the degradation byproducts, 3-formyl-rifapentine and 3-formyl-desacetyl-rifapentine, formed in the gut.

In experimental tuberculous infections, rifapentine administered once a week has practically the same therapeutic efficacy as rifampicin administered daily (397). It is in clinical trial as a drug for the therapy of tuberculosis and leprosy at a lower dosage and frequency of administration than rifampicin. However, relapse with rifamycin monoresistant tuberculosis occurred among HIV-seropositive tuberculosis patients treated with a once-weekly isoniazid/rifapentine continuation phase regimen (403).

5.2.7.4 Rifalazil. Rifalazil (previously known as KRM-1648) (58) is the 3'-hydroxy-5'-(4-isobutyl-1-piperazinyl) benzoxazinorifamycin, selected among various benzoxazine rifamycins (404–406). Rifalazil is more potent ***in uitro*** and ***in vivo*** against ***M. tuberculosis*** and ***M. auium*** complex than rifampicin. Rifalazil demonstrated excellent ***in vivo*** efficacy

against M. tuberculosis infections produced by rifampicin-sensitive organisms, having activity in the murine model superior to rifampicin (407). The potent antimycobacterial activity of rifalazil is largely because of its increased ability to penetrate the mycobacterial cell walls (408). The high tissue drug levels and long plasma half-life of rifalazil significantly contributes to its excellent in vivo efficacy. Various experiments with rifalazil in combination with isoniazid demonstrated its potential for short-course treatment of M. tuberculosis infections. Rifalazil plus isoniazid for a minimum of 10 weeks was necessary to maintain a non-culturable state through the observation period (409). Rifalazil in combination with pyrazinamide and ethambutol has sterilizing activity comparable with that of the combination isoniazid plus rifampicin, but significantly better with respect to relapse of infection (410). If rifalazil will be developed for human therapy, because of its remarkable activity, it can, in combination with isoniazid, significantly shorten the duration of therapy. This suggests that ultra–short-course therapy is an attainable goal.

5.2.7.5* Rifamycin *T9. Rifamycin T9 (59) is the 3-(4-cinnamyl-1-piperazinyl-iminomethyl) rifamycin SV, synthesized and evaluated for its antimycobacterial activity in the Chemical Pharmaceutical Research Institute of Sofia, Bulgaria. Investigations conducted by the developers of the drug indicated good therapeutic activity, lack of toxicity, and favorable pharmacokinetic and bioavailability in experimental animals. The in vitro activity of T9 against M. tuberculosis and MAC and its chemotherapeutic activity in experimental tuberculosis in mice gave encouraging results. The excellent in vitro, intracellular, and in *vivo* activities of T9, as well as its promising bioavailability, warrant its potential usefulness in the treatment of mycobacterial infections (411). Results of other studies show that T9 is twice as active as rifabutin and four times more active than rifampicin in inhibiting the growth of rifampicin-sensitive strains of *Mycobacterium* leprae. Furthermore, there is a demonstrated synergy between T9 and ofloxacin, suggesting that combination of T9 and ofloxacin would be an ideal formulation in multidrug regimen for leprosy (412).

5.3 Drugs Under investigation

In recent years the need of new drugs to meet the problems connected with the emergence of multidrug resistant (MDR) tuberculosis and mycobacteriosis particularly in AIDS patients, failing any really new antituberculous compound, has led to carefully evaluate the antituberculous activity of antimicrobial agents developed for their activity against common Gram-positive and Gram-negative bacteria.

Some promising drugs are present in the groups of fluoroquinolones, macrolides, and β-lactams (in combination with β-lactamase inhibitors), but also in a new series of products, such as azaindoloquinazolindione alkaloids and nitroimidazopyrans.

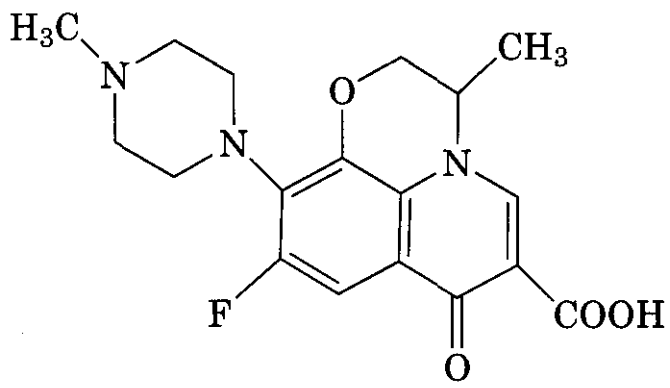
5.3.1 Fluoroquinolones

***5.3.1.1* Historical Development.** These products represent a development of the earlier analogs (nalidixic acid, oxolinic acid, pipemidic acid, and cinoxacin) in being more potent in vitro and in having broader antibacterial spectrum, which includes Gram-positive and Gram-negative organisms.

They have also improved pharmacokinetic properties; whereas the old agents distributed very poorly in body tissues and fluids, so that they can be employed only as urinary antiseptics, the new derivatives distribute much better in all body districts, penetrate cells, and can be efficaciously used in the treatment of systemic infections. Their oral bioavailability is excellent (413).

Key points in determining these characteristics are the attachment of a fluorine atom at C-6 and of piperazinyl or N-methylpiperazinyl groups at C-7 and of alkyl or cycloalkyl groups at *N*-1 of the 1,4-dihydro-4-oxo-3-quinolinecarboxylic acid (414).

***5.3.1.2* Antimycobacterial *Activity and Mechanism* of Action.** Derivatives recognized with activity against mycobacteria are ofloxacin (60) and ciprofloxacin (**61**), which have been studied at a larger extent (415–421), whereas sparfloxacin (419, 420), levofloxacin, which is the levoisomer of ofloxacin (421), lomefloxacin (422, 423), WIN 5723 (424), and AM-1155 (425) have been more recently submitted to investigation and are under development. Another fluoroquinolone, moxifloxacin, recently introduced in clinical practice, shows a good in



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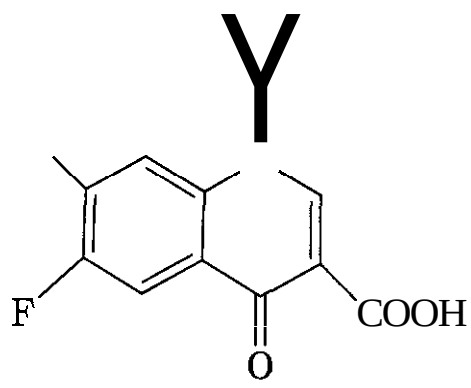

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vitro activity against M. tuberculosis and M. avium complex and does not cause phototoxicity (426).

MICs of ofloxacin and ciprofloxacin for M. tuberculosis range from 0.12 to 2 μg/mL. Both drugs are bactericidal, the MBC/MIC ratio being from 2 to 4. Levofloxacin, the levo-isomer of ofloxacin, is twice as active as the parent drug; the bactericidal concentration corresponds to MIC. Ciprofloxacin and ofloxacin are also active against M. avium complex. MICs of ciprofloxacin for 50% of strains range from 1 to 16 μg/mL and MIC for 90% from 2 to 16 μg/mL (with data from some series exceeding 100 μg/mL). MICs of ofloxacin are a little higher, ranging from 2 to 16 μg/mL for 50% of strains and from 8 to 16 μg/mL for 90% of strains (again with values from a few studies exceeding 100 μg/mL). The MBC/MIC ratio most commonly ranges from 1 to 8. In addition, ciprofloxacin and ofloxacin show a quite good in vitro activity against M. bovis, M. *kansasii* and M. fortuitum, but not against M. *chelonae*.

The targets of quinolone action in bacterial cell are topoisomerase II, a DNA girase that contains two A subunits (Gyr A) and two B subunits, encoded by the *Gyr A* gene. DNA girase functions within viable bacterial cell include introduction of negative supercoils and Iopoisomerase IV, involved with decatenation of linked DNA molecules. They are essential for DNA recombination and repair. The main effects of fluoroquinolones are the inhibition of DNA supercoiling and the damage to DNA, whose synthesis is rapidly interrupted. At high quinolone concentrations, RNA and protein synthesis is also inhibited and cell filamentation occurs. It has been suggested, however, that quinolones may have other effects in the bacterial cells, for instance, the formation of an irreversible complex of drug, DNA, and enzyme, functioningas a "poison." In fact, nalidixic acid reduces the burst size of bacteriophages T7 at permissive temperature in E. coli strain containing thermosensitive Gyr A subunits, but not at non-permissive elevated temperature, suggesting that the inhibitory action of the drug on phage T7 depends on DNA inhibition even if the girase function is not required for phage growth (427,428).

Moreover, quinolone concentrations that inhibit the DNA supercoiling and decatenating activity of purified DNA gyrase are several-fold higher than those required to inhibit bacterial growth. Again this discrepancy has suggested that other targets for quinolonesexist in bacterial cell and several interpretations of this behavior have been put forward.

Activity of fluoroquinolone on susceptible bacterial species is bactericidal, but the inhibition of DNA synthesis does not seem sufficient to explain bacterial killing. All steps of this effect have not yet been elucidated; cells in logarithmic phase of growth are rapidly killed and the rate of killing increases with the increase of drug concentration, up to a maximum above which, paradoxically, the killing is reduced (429–430). This effect was already observed with penicillin and is known as the "Eagle" effect (431) from the name of the author who first described it. It is possible that this effect is a result of the inhibition of protein synthesis occurring in presence of high concentrations of quinolones. This hypothesis is supported also by the observation that bacteria exposed to antibiotics that inhibits protein synthesis (chloramphenicol, rifampicin) or to aminoacid starvation are less efficiently killed by fluoroquinolones.

In vitro association with other antituberculous drugs has given variable results. Combination of ciprofloxacin with major antituberculous drugs did not show any synergistic effect, but only indifference against M. tuberculosis. Synergism between ciprofloxacin or ofloxacin and ethambutol could be put in evidence for some strains of M. avium. whereas the effect was less evident when rifampicin replaced ethambutol. The combination of sparfloxacin with ethambutol, but not with rifampicin, had a synergistic effect against M. avium. Triple combination of sparfloxacin, rifampicin, and ethambutol resulted in synergism (415, 432,433).

Triple combinations including isoniazid, rifabutine plus either ciprofloxacin, ofloxacin or norfloxacin, and isoniazid, rifapentine plus either pefloxacin or ciprofloxacin were the most active ones against resistant strains of M. tuberculosis. Rifampicin in combination with ciprofloxacin and amikacin in combination with isoniazid and ciprofloxacin gave the best results against M. *fortuitum* (433).

The synergistic interaction of antituberculous drugs in vitro, when obtained with in vivo achievable concentrations, may give some indications on the possible clinical efficacy.

Further information for the clinical application of fluoroquinolones can be drawn also from the activity of ofloxacin (434), ciprofloxacin (435), sparfloxacin (436), and levofloxacin (437), both singly or in combination with other antituberculous drugs in some experimental models of M. tuberculosis infection in mice.

Ofloxacin was shown to be highly active against M. leprae (MIC = 1.5 μg/mL), and the combination of ofloxacin and rifabutin or rifampicin has a synergistic effect (438). The combination of ofloxacin and rifabutin deserves further attention in the multidrug therapy of leprosy.

5.3.1.3 Clinical Uses in Mycobacterial Infection. Fluoroquinolones have been introduced in several multidrug regimens, particularly in retreatment regimens, of MDR tuberculosis both in immunocompetent and AIDS patients and in MAC disease. Ofloxacin, ciprofloxacin, and more recently, sparfloxacin have been administered either with ethambutol, pyrazinamide, or with isoniazid and rifabutin and in other combinations. Even if some results seem promising, no definite conclusion on the clinical value of these new approaches to treatment can be drawn.

5.3.1.4 Clinical Doses and Adverse Effects. The daily dosage varies according to the different derivatives as follows: ofloxacin, 400 mg b.i.d.; ciprofloxacin, 750 mg o.d. or b.i.d. or 500 mg t.i.d.; sparfloxacin, 200–400 mg o.d. and moxifloxacin, 400 mg o.d. (439,440).

Fluoroquinolones are usually well tolerated. The most common side effects are gastrointestinal reactions, central nervous disturbances, and skin and hypersensitivity reactions, particularly photosensitivity reactions (441). So far the quoted side effects have been observed during therapies of limited duration. Concerning the photosensitizing potential of the fluoroquinolones, it varies considerably between different agents. The likelihood of phototoxicity has probably been a limiting factor for the use of sparfloxacin in tuberculosis patients. There is an indication that a methoxy group at position 8 confers reduced phototoxicity, and this is the case of moxifloxacin. So far, however, no data support an increase of adverse events connected with the use of fluoroquinolones in the treatment of tuberculosis and mycobacteriosis.

Sporadic cases of hepatotoxicity have been reported, probably because of the association with other hepatotoxic drugs. It is suggested to monitor closely patients with concomitant liver impairment to stop medication if necessary (442,443).

5.3.2 Macrolides

5.3.2.1 Historical Development. Macrolides are a group of antibiotics characterized by a large lactonic structure of 12, 14, or 16 atoms. The lactone ring has hydroxyl, alkyl, and ketone groups in various positions. In the 16-membered ring macrolides, an aldehydic group can also be present. Sometimes two of three hydroxyl groups are substituted by sugars, which can be neutral (6-deoxy-hexoses), bound through a a-glycosidic linkage, or basic (3-amino-sugars) bound through a β-glycosidic linkage.

The first derivative introduced in therapy was erythromycin, which was isolated in 1952 from the fermentation broths of Streptomyces *erythreus* and followed by other natural deriv-

atives, such as spiramycin and oleandomycin, produced by other ***Streptomyces*** species. For some decades no other derivative was added to this antibiotic family until the 1980s, when josamycin and myocamycin, a semi-synthetic product, entered clinical use. Since then, new semi-synthetic derivatives have become available, namely roxithromycin, dirithromycin, flurithromycin, clarithromycin, and azithromycin. They belong to the 14-membered ring derivatives except the last one, which has a 15-membered ring with an N-methyl group between C9 and C10; for this reason, the name "azalides" has been proposed for azithromycin and other 15-membered ring macrolides (444).

Macrolides are usually administered by oral route, but because of their physico-chemical properties, they are poorly and erratically absorbed. Passage through the stomach can result in degradation and loss of activity. To obviate to this inconvenience, different salts and esters as well as adequate pharmaceutical formulations have been prepared (445). Once absorbed, they diffuse well in tissues and penetrate cells, so that they can be active in intracellular infections (446). They are metabolized in liver to a different extent according to derivatives, and because of their inducing activity on cytochrome P-450, they can interfere in the activity of other drugs metabolized by the same enzyme such as teophylline, antipyrine, and methylprednisolone (447). Macrolides are eliminated mainly by gastrointestinal route.

Among side effects, gastrointestinal disturbances prevail, and some derivatives show a certain degree of hepatotoxicity. However they are considered to be among the best-tolerated antibiotics (448).

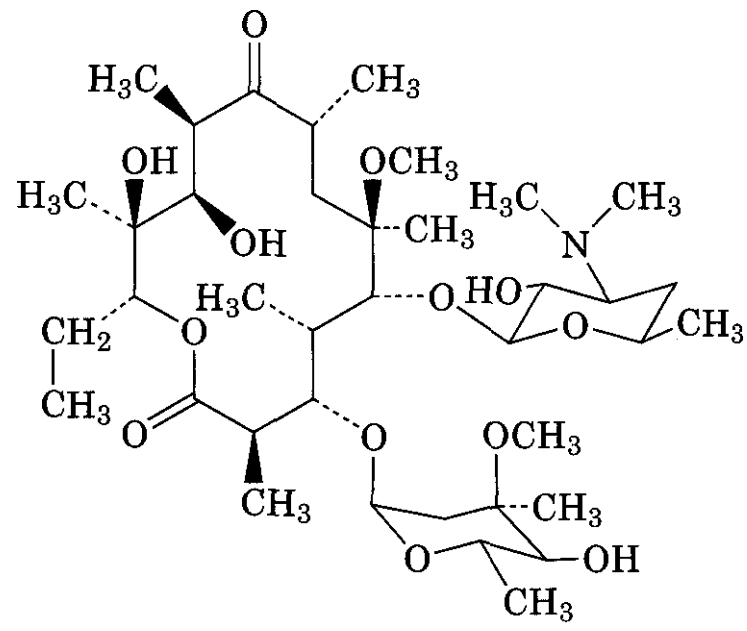
5.3.2.1.1 Antimycobacterial Activity. The general spectrum of activity of macrolides includes Gram-positive cocci, some Gram-negative species, and intracellular pathogens (449).

The most recent macrolide derivatives show better pharmacokinetic characteristics, particularly longer half-life, and higher tissue and intracellular concentration than old derivatives. In addition, they show better activity against some Gram-negative species, such as *H. influenzae*, and in addition, clarithromycin and azithromycin have some action against mycobacteria (449).

It was known that erythromycin could be active in infections caused by rapidly growing mycobacteria, M. ***fortuitum*** and ***M. chelonae,*** and probably also in ***M. smegmatis*** infection. New macrolides (clarithromycin, azithromycin, and roxithromycin) have a similar or higher activity (416,450).

At present, the interest is focused on determining which derivatives can have some clinically exploitable activity against MAC and M. ***tuberculosis.***

5.3.2.2 *Clarithromycin.* Clarithromycin (62), as well as its hydroxy-metabolite (14-hydroxy-clarithromycin), are active ***in vitro*** against



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MAC. However, MICs are quite variable from study to study (451–454). Several ***in vitro*** experiments were carried out in presence of human macrophages, where clarithromycin accumulates (455–466). MIC is 1 μg/mL and MBC range from 16 to 64 μg/mL (456): these values are similar to those obtained in broth cultures at pH 7.4 and lower than those found in broth culture at pH 6.8 and 5. Intracellularly, mycobacteria grow at pH 6.8 and pH 5 in phagosomes and phagolysosomes, respectively. The fact that MIC and MBC against them were lower than those found in broth cultures is an indirect indication of concentrations of clarithromycin exceeding those in extracellular medium. In fact it was previously demonstrated that clarithromycin accumulates in cells at concentration 10- to 16-fold higher than those in the extracellular fluid (457–459).

Moreover, it was showed that single 2-h pulsed exposure of macrophages infected with M. avium to clarithromycin at 3 μg/mL completely inhibited the intracellular bacterial growth during the first 4 days of observation. This finding suggests that in vivo, the intracellular bacteria can be inhibited after a short period of exposure to the high concentrations of drug that can be reached at the time of blood peak level, rather then to prolonged exposure to low concentrations (460).

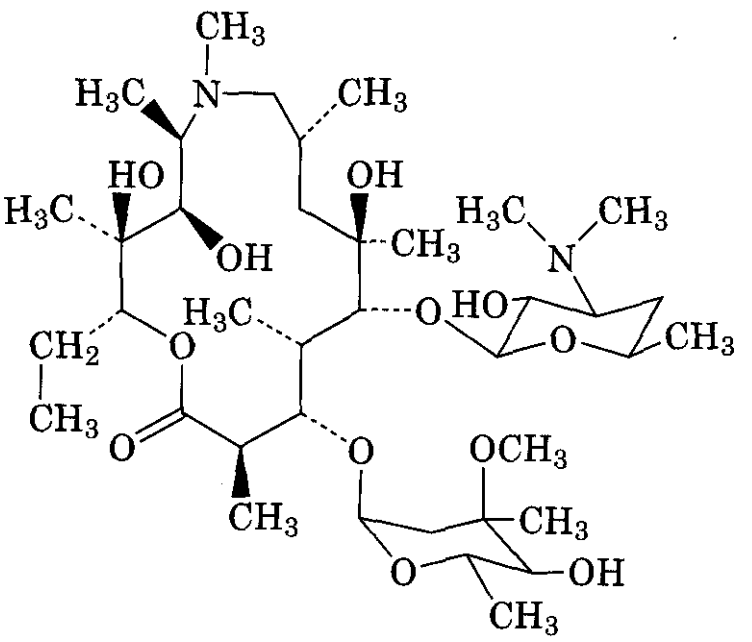
In vivo experiences confirmed the in vitro results; clarithromycin showed a good activity in the beige mouse model of disseminated M. avium infection, inducing a dose-related reduction in spleen and liver microbial cell counts for treatment with doses of 50, 100, and 200 mg/kg. Also, in patients with infection caused by M. avium, susceptible to 2 μg/mL or less. a dramatic decline in intensity of bacteremia up to a negative blood culture was achieved after 4–10 weeks of therapy (416, 453).

Combination of clarithromycin with amikacin, ethambutol, or rifampicin did not result in activity superior to that of clarithromycin alone, whereas the combinations with clofazimine or rifabutin were more active than clarithromycin alone. The triple combinations clarithromycin-rifampicin-clofazimine, clarithromycin-rifampicin-clofazimine, or clarithromycin-clofazimine-ethambutol were significantly more active than the macrolide alone (461).

Clarithromycin has been recommended by the U.S. Public Health Service Task Force on Prophylaxis and Therapy for MAC as a primary agent for the treatment of disseminated infection caused by M. avium. It should be used in combination with other antimycobacterial drugs that have shown activity against MAC, including ethambutol, clofazimine, and rifampicin (467).

The activity of some macrolides has been recently tested against M. *leprae* in vitro using ATP assay and in vivo using a food-pad model. Erithromycin was active in vitro but not in *vivo* (466, 468). Clarithromycin was active both in vitro (MIC = 0.1–2 μg/mL) and in vivo, demonstrating also a bactericidal activity (469). Similar data were obtained with roxithromycin. The acid stability of these new macrolides together with their long half-life, better penetration, and longer persistance in tissues are probably the reason for their activity, which deserves further investigation (466–469).

5.3.2.3 *Azithromycin.* Among the new macrolide derivatives, azithromycin (**63**) has a pe-



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culiar place because of its chemical structure, consisting of a 15-membered lactone ring, and its pharmacokinetic characteristics. In fact, blood levels are very low, whereas very high concentrations are reached in tissues and cells; in polymorphonuclear neutrophyles, they can be up to 200- to 300-fold higher than extracellular concentrations. From cells, the antibiotic is slowly released, a phenomenon that assumes a particular relevance at the infectious focus where PMN accumulate. Half-life is about 60 h (470,471). Because of these features, azithromycin can be administered for 3 days, assuring therapeutic tissue and cell concentrations for about 7–10 days (472–474). Azithromycin is endowed with good activity against some rapidly growing mycobacterial species and MAC. Its MICs on the latter species vary quite widely; the values range between 17 and 94 μg/mL (475, 476), but the MIC_{90} (62 μg/mL) exceeds concentrations in tissues after oral dosing (477–478). It is bactericidal against M. avium and M. xenopi in macrophages. The addition of another active antimycobacterial drug such as amikacin or rifabutin enhanced the intracellular killing (479). Uptake of azithromycin by macrophages was

increased by the addition of TNF or interferon-y, but it is not known if the stimulation of azithromycin uptake accounts by itself for the increased killing of the macrolide (480).

The activity of azithromycin against MAC was confirmed in infection of rats treated with cyclosporine (481) and in the beige mouse model of M. aviurn infection (476, 482). The drug was efficacious when administered intermittently. Azithromycin given in combination with rifapentine on a once weekly basis for 8 weeks showed promising activity (483).

Combination with amikacin and clofazimine (484) or with clofazimine and ethambutol enhanced the efficacy of treatment; combination with rifabutin did not seem to be significantly superior to rifabutin alone (482).

In the same experimental model in beige mice, azithromycin had activity comparable with clarithromycin and rifampicin against M. kansasii, M. xenopi, M. sirniae, and M. *malmoense*. The combinations with amikacin and clofazimine were more effective than the single drug (485).

Azithromycin was less active than clarithromycin and roxithromycin against M. *leprae* (118).

A few clinical trials have been carried out with azithromycin in M. aviurn infection in AIDS patients; after a few weeks of treatment (0.5–0.6 g once daily), a consistent reduction of bacteremia and improvement of clinical signs were observed (486). As already noted, macrolides cannot be employed alone in the therapy of mycobacteriosis; they must be included in polychemotherapeutic regimens.

5.3.3 β-Lactams and β-Lactamase Inhibitors. The resistance of M. tuberculosis to β-lactams has been attributed to the production of B-lactamases, with characteristics of both penicillinases and cephalosporinases (487).

One of the ways that was useful in overcoming the problem of β-lactamase resistance in other microorganisms has been to associate an inhibitor of the enzyme to penicillin, restoring the activity of the antibiotic susceptible to the enzymatic action. Inhibitors of β-lactamase such as clavulanic acid, sulbactam, and tazobactam have been associated to amoxycillin, ampicillin, ticarcillin, and piperacillin, and have been proven to be active not only in *vivo* but also in clinical setting, in the treatment of infections caused by p-lactamase–producing strains (488,489). A number of p-lactamases have been characterized; they have different properties and different susceptibilities to β-lactamase inhibitors (490). β-lactamase produced by M. tuberculosis is susceptible to clavulanic acid; therefore, interest has been focused to ascertain whether its combination with a penicillin derivative could have some activity against M. tuberculosis (491).In vitro, the combination amoxycillin-clavulanic acid was remarkably more active than amoxycillin alone and had bactericidal activity (492, 493).

Two patients with multidrug resistant tuberculosis and poorly responding to multidrug regimens including ethionamide, capreomycin, and cycloserine, in addition to streptomycin, ethambutol, or pyrazinamide, were successfully treated following the addition of amoxycillin-clavulanic acid to these regimens (494).

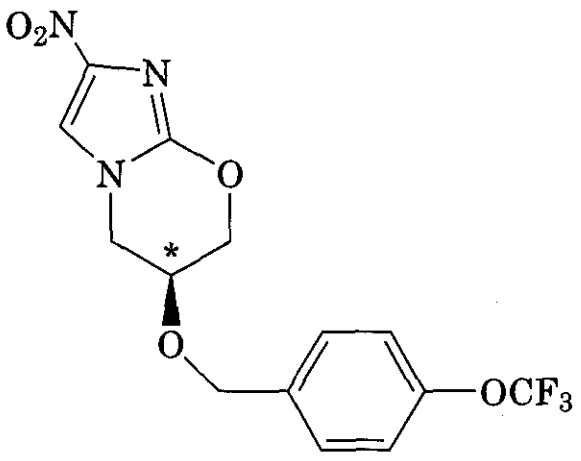
Imipenem and meropenem, two carbapenems highly resistant to β-lactamases, were shown to have antimycobacterial activity only when a peculiar technique of in vitro dosing (daily addition of the drug) was adopted, compensating for the loss of activity of the antibiotics during the test incubation period. In fact the instability of the two compounds, particularly of imipenem, in the culture medium can hide their antimycobacterial activity (495).

Other β-lactams resistant to the β-lactamase action should be investigated for activity against mycobacteria and for their possible inclusion in multidrug regimens.

5.3.4 Nitroimidazopyrans. It has been reported recently the synthesis and the biological properties of a series of compounds containing a nitroimidazopyran nucleus that possess antitubercular activity (496). After activation by a mechanism dependent on M. tuberculosis F 420 cofactor, nitroimidazopyrans inhibited the synthesis of proteins and cell-wall lipids. In contrast to current antitubercular drugs, nitroimidazopyrans exhibited bactericidal activity against both replicating and static M. tuberculosis. Structure-activity relationship studies focusing on antitubercular

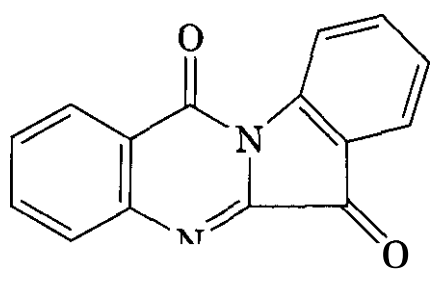
activity revealed substantial variety in the tolerated substituent at C3, but optimal activity was achieved with lipophilic groups. The stereochemistry at C3 was important for activity, as the S-enantiomers were generally at least 10-fold more active than the R-enantiomers.

Lead compund PA-824 (64) showed potent bactericidal activity against multidrug resistant M. tuberculosis and promising oral activity in animal infection models. The nitroimidazopyrans represent a class of antitubercular compounds that act by a new mechanism but share some interesting parallels and contrast with INH. Like INH, the PA-824 prodrug requires bacterial activation, albeit by a different F420 dependent mechanism. PA-824 also inhibit a step in the synthesis of cell-wall mycolates, but at a more terminal step than INH.



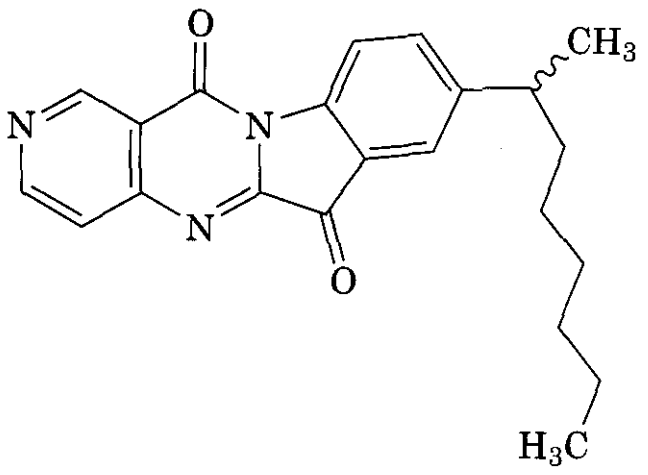
(64)

5.3.5 Tryptanthrin. The indolo-quinazolinone alkaloid tryptanthrin (65) has been known about since 1915. Isolated from a Chinese-Taiwanese medicinal plant, Strobilantes cusia, it was pursued intensively because of its very attractive in vitro properties, simple structure, and ease of synthesis. It retained its activity against M. tuberculosis H37Rv at concentration of 1 mg/L and against MAC at 2 mg/L, and it retained its activity against a panel of multiple drug-resistant strains.



(65)

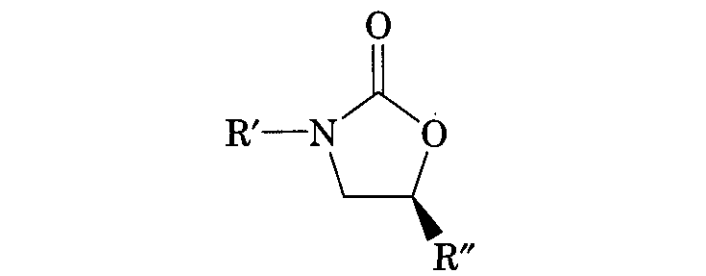
Extensive structure-activity studies have been performed on trytanthrin, with synthesis of many analogs using two parts of the molecule suitable for combinatorial chemistry. After examination of the various features of many analogs, PA-505 (the 2-aza-8-isooctyl analog of tryptanthrin (66)was considered the best and tested in detail. Its MIC against drug-sensitive and drug-resistant M. tuberculosis was 0.015 μg/mL and against MAC 19075 was 0.06 μg/mL. However, the in *vivo* activity in infected mice was unsatisfactory and the research is continuing on another series of derivatives (497).

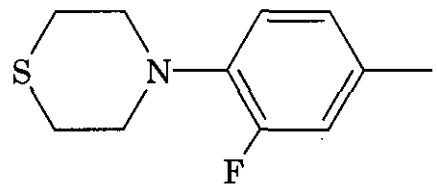
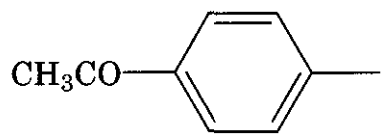


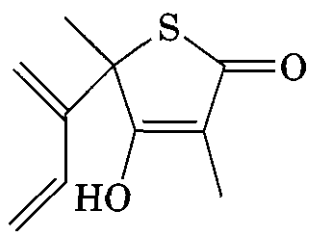
(66)

5.3.6 Oxazolidinones. The oxazolidinones represent a class of products, with a general skeleton indicated in (67), extensively studied for their activity against Gram-positive and Gram-negative pathogens resistant to several antibacterial drugs. Some of them have been selected for their effect against mycobacteria. In particular, U-100480 (68) and DuP-721 (69)showed very low MIC against drug-sensitive and drug-resistant M. tuberculosis isolates and deserve further development (498, 499).

5.3.7 Thiolactomycins. Thiolactomycins (TLM) (70)have been isolated as a metabolite of a soil Nocardia species. It is a unique thiolactone that exhibits antimycobacterial activity by specifically inhibiting fatty acids and mycolic acid biosynthesis. TLM targets two



	R′	R″
(67)	H	H
(68)		$NHCOCH_3$
(69)	CH_3CO 	$NHCOCH_3$



(70)

β-ketoacyl-carrier protein synthases, KasA and KasB, consistent with the fact that both enzymes belong to the fatty acid synthases type II system involved in fatty acid and mycolic acid biosynthesis. The design and synthesis of several TLM derivatives have led to compounds more potent both in *vitro* against fatty acids and mycolic acid biosynthesis and in *vivo* against M. tuberculosis. A three-dimensional structural model of Kas has been generated to improve understanding of the catalytic site of mycobacterial Kas proteins and to provide a more rational approach to the design of new drugs (500).

5.3.8 Rifazalil and Rifamycin T9. See Section 5.2.6.

6 RECENT DEVELOPMENTS AND PRESENT STATUS OF CHEMOTHERAPY

6.1 Tuberculosis

Chemotherapy has been the most potent and useful tool for modifying the evolution and prognosis of tuberculosis and has almost reached the goal of eradicating tuberculosis from some western countries. This positive tendency has been interrupted around 1985 when the incidence of tuberculosis started to increase for different reasons, outlined earlier. Moreover the rate of disease caused by resistant strains of mycobacteria, steadily falling in previous decades (being about 5% in industrialized countries), has risen to a varying extent according to countries and to socioeconomic status of some population classes. Data have not been systematically monitored in each country or region: therefore they have only an indicative value.

Recent data (1996–1999) reported by WHO and International Union Against Tuberculosis and Lung Disease, which undertook a global project on anti-tuberculous drug resistance surveillance in 35 countries, indicate that among patients not submitted to previous treatment, a median of 10.7% (range, 1.7–36.9%) of M. tuberculosis strains were resistant to at least one drug among the four first-line drugs taken into account (streptomycin, isoniazid, rifampicin, and ethambutol). The prevalence increased in Estonia from 28.2% in 1994 to 36.9% in 1998, and in Denmark from 9.9% in 1995 to 13.1% in 1998. In Western Europe, the median prevalence of primary multidrug resistance was less than 1%, whereas in Eastern Europe it was increasing, after a period of decline. In Estonia, it was 14.1% and in Latvia it was 9%. In the United States, the prevalence of primary resistance to any drug was 12.3%, whereas it was 1.6% for multidrug resistance. In patients who had prior treatment for more that 1 month, the prevalence of resistance for any drug ranged from 5.3% to 100% and that for MDR mycobacteria from 0% to 54% (501, 502). When there was a concomitant resistance to INH and RMP, the failure rate was over 50% in immunocompetent patients and about 100% with mortality of 50–90% in AIDS patients.

Data from many countries, China, India, and most African countries, are still lacking and are being collected together with those of other 40–50 countries. High rates of resistance were found in the countries of the former Soviet Union, The Domenican Republic, and Argentina. Of great concern is the very

high incidence of 89% of non-responding patients and 24% of patients with newly diagnosed tuberculosis among the prison population of some countries of the former Soviet Union, whose disease was caused by MDR tubercle bacilli. It is evident that there is an enormous threat for the transmission of the drug-resistant strains to the community (503–505).

The success of chemotherapy is strictly related to the use of drug combination (to avoid the emergence of resistance) and to the rigorous adherence to the prescribed therapy regimens. Unfortunately, despite the introduction of simpler and shorter course of antimycobacterial chemotherapy, the compliance of patients is poor, particularly in developing countries. In the attempt to avoid the disastrous consequences of this behavior, after limited experiences in same countries, by the early 1980s, WHO implemented the directly observed therapy short-course (DOTS), i.e., patients had to be administered drugs (standard drugs for 6 months) under the direct observation of a supervisor (health workers) along with other measures to favor the accomplishment of the therapy (506, 507). Undeniably, DOTS achieved remarkable successes in some countries such as China (508). The first experiences, however, have shown that, even if the rate of implementation increases, tuberculosis mortality will not be halved (509) before 2030, and that the DOTS program is unable to control tuberculosis in countries with high levels of HIV infection and MDR tuberculosis. In these settings, where the DOTs regimen is expected to be ineffective, a new control strategy has been proposed, the "DOTs-plus" program, introducing either standardized or individualized regimens with second-line drugs. Second-line drugs are expensive, and the organization of this program is complex. Therefore, it seems that "DOTS-plus" strategy will be hard to implement on a large scale in low socioeconomic countries where MDR tuberculosis is prevalent (510,511).

A turning point for the polychemotherapy of tuberculosis was represented by the introduction in clinical use of rifampicin in 1968. The addition of this potent drug to the association of the other active drugs already in use allowed the application of short-course treatment by reducing the period of treatment from 12–24 months to 6 months (or a maximum of 9 months). At present the choice of the therapeutic regimen is based on the knowledge of the epidemiology of resistant strains in the area where a patient is treated to apply the most adequate therapeutic regimen. This commonly must be prescribed on empirical basis, because the results of susceptibility tests require some weeks to be available: In the case of a presumably susceptible population of tubercle bacilli, an initial treatment with an association of three drugs is advised, whereas if a multiple resistance is suspected, a four- or five-drug regimen is recommended.

Drugs of first choice for the initial treatment are rifampicin, isoniazid, pyrazinamide, and ethambutol. Drugs should be administered daily for a 2-month period. In the meantime, the results of susceptibility tests should be available, which together with the clinical observation of the patient, will give indications for further treatment. A 4- to 6-month period follows in which drugs can be administered intermittently, two or three times a week. If the strain is susceptible to isoniazid and rifampicin, the maintenance treatment can continue with these two drugs (512,513). However, many other options are to be considered, according to patient condition and requirements. An array of regimens have been suggested and applied with success for maintenance period: one of the most commonly employed regimen includes isoniazid + rifampicin + pyrazinamide + streptomycin or ethambutol administered twice or thrice a week. The same therapeutic regimen can be followed in HIV patients, but it should be extended to at least 9 months, and a fourth drug should be added initially for severe disease. When isoniazid-resistant tuberculosis is suspected, a three-drug regimen can be adopted for the first 2 months (rifampicin + ethambutol + pyrazinamide) followed by 7 months of daily rifampicin and ethambutol. An alternative treatment is a four-drug regimen (rifampicin + isoniazid + pyrazinamide and ethambutol or streptomycin), applied for the first 2 months, followed by rifampicin + ethambutol twice a week for 7 months or more. For patients responding to treatment in which tubercle bacilli turn out to be susceptible to all

administered drugs, ethambutol (or streptomycin) can be withdrawn and isoniazid and rifampicin are continued for 4 or 6 months.

For patients living in areas where tuberculosis sustained by multidrug-resistant mycobacteria is widespread, a five-drug regimen is recommended initially, including both ethambutol and rifampicin. Successively, the treatment will be modified on the basis of results of susceptibility tests. In such patients, other antituberculous drugs, the second-line drugs, ethionamide, cycloserine, capreomycin, kanamycin, para-aminosalycilic acid, and sometimes clofazimine, are variably combined with each other and with first-line drugs with the aim to overcome resistance. The activity of other drugs showing in vitro activity against M. tuberculosis are under clinical investigation: amikacin, ciprofloxacin, and ofloxacin. Rifabutin has been included in some drug combinations, and it seems to have some efficacy also in the treatment of cases associated with low-level rifampicin resistance. Multidrug-resistant tuberculosis often requires prolonged treatment up to 24 months to reach sputum conversion (514–518).

The common dosages of antituberculous drugs and the schemas of some possible regimens are given in Tables 16.3 and 16.4.

The side effect of antituberculous therapy reflects the toxicity of the drug that is associated in the different combination regimens. Hepatotoxicity is quite frequent because drugs, and particularly the first-line drugs, are endowed with liver toxicity. Hepatitis with liver enzymes elevation can occur and it should be determined which is the responsible drug to discontinue it. Usually, after 1 week, liver enzymes return to baseline levels, and therapy can be resumed, but in some cases, the drug responsible of hepatotoxicity must be substituted with another better-tolerated agent. Other severe side effects are represented by hematologic derangements and neuropathy.

Antitubercolus drugs, particularly rifampicin and isoniazid, caused by the induction of citochrome P-450 system, may interfere with the metabolism of other drugs, metabolized through the same enzymatic pathway and the reverse can be true. In Table 16.5, the principal drug interactions of antituberculous drugs are indicated.

So far the prevention of TB has been put into practice by the vaccination with BCG since the late 1940s. The evaluation of its activity is still controversial (519). The explanation of the disparate results obtained in different countries has been attributed to variation between strains of BCG and/or to the different policy adopted for vaccination (vaccination has been prescribed to neonates, to school age children, to select high-risk groups, to tuberculin negative subjects). Repeated doses of BCG have been administered in countries of the ex-Soviet Union. Unfortunately there have been almost no comparative evaluations of the effectiveness of the different policies. It has been observed that poor results are obtained in populations that are exposed to many different "environmental" mycobacteria, which could itself provide a kind of "vaccination" so that BCG could not improve greatly the response. This assumption seems confirmed by the results of a large trial performed in 1968 in South India, where the exposure to environmental mycobacteria was high. Neither of the two vaccines tested that were obtained from different BCG strains offered protection against tuberculosis. On the other hand, in front of continue decline in TB incidence in the community, countries of northern Europe (Sweden and England) showed a trend to discontinue the routine use of BCG and to restrict its use to high-risk groups (TB contacts and recent immigrants).

At present, about 100 million children receive BCG annually throughout the world. Most countries now follow the policies of the Expanded Program on Immunization (EPI)/ Global Program on Vaccines of the WHO, which recommends only a single dose of BCG be given at birth or at the earliest contact with a health service.

Another form of TB prevention is chemoprophylaxis (520), i.e., the administration of isoniazid to individuals not yet infected (tuberculin-negative) but at risk to acquire tuberculosis because of close contacts with TB patients. Chemoprophylaxis should be also prescribed to infected subjects, i.e., tuberculin-positive (under 35 years of age), to prevent the progression of the infection to the active disease when they live or have close contacts with high-incidence groups (nursing home,

Table 16.3 Common Dosages of Available Antimycobacterial Drugs

	Dose and Route of Administration in Adults		
	Daily Dose	Twice Weekly Dose	Thrice Weekly Dose
First-line drugs			
Isoniazid (INH)	5 mg/kg os (max. 300 mg)	15 mg/kg os (max. 900 mg)	15 mg/kg os (max. 900 mg)
Rifampicin (RMP)	10 mg/kg os (max. 600 mg)	10 mg/kg os (max. 600 mg)	10 mg/kg os (max. 600 mg)
Rifabutin (RTB)	300 mg os	300 mg os	150 mg os
Ethambutol (EMB)	15–25 mg/kg os (max. 2.5 g)	50 mg/kg os	25–30 mg/kg os
Streptomycin (SM)	15 mg/kg IM (max. 1 g)	25.30 mg/kg IM (max. 1.5 mg)	25–30 mg/kg IM (max. 1.5 g)
Pyrazinamide	15–30 mg/kg os (max. 2 g)	30 mg/kg os (max. 2 g)	30 mg/kg os[a]
Second-line drugs[b]			
p-Aminosalicylic acid (PAS)	150 mg/kg os (max. 10–12 g)		
Ethionamide	15–20 mg/kg os (max. 1 g)		
Prothionamide	15–20 mgkg os (max. 1 g)		
Thioacetazone	150 mg os		
Capreomycin	15–30 mg/kg IM (max. 1 g)		
Viomycin	1 g IM		
Cycloserine	15–20 mg/kg os (max. 1 g)		
Kanamycin	15–30 mg/kg IM (max. 1 g)		

[a]Doses higher than 2 g are not well tolerated.

[b]Second-line drugs are seldom used at present. Some have been withdrawn from the market in many countries. The dosage in intermittent regimens is usually the same as for initial treatment.

Table 16.4 Tuberculosis Chemotherapy Regimens

	Regimen"	
Treatment (months)	Initial	Maintenance
8	2SHRZ	6HR
8	2HRE	$6H_3R_3Z_3E_3$ (or S)
6	2HRE	$4H_3R_3$
6	2HRZ (±S or E)	$4H_3R_3$
6	6HRS	
6	2HRZ	$4H_2R_2$.
6	$2S_3H_3R_3Z_3$	$2S_3H_3R_32H_3R_3$
6	$6H_3R_3Z_3$(S or E)	
6	$0.5SHRZ\ 1.5S_2H_2R_2Z_2$	$4H_2R_2$

"A numeral preceeding letters indicates the number of months that drug combination is given, e.g., 4RH indicates 4 months of daily isoniazid and rifampicin. A numeral in subscript following a letter indicates the number of doses per week in intermittent regimens, e.g., $5H_3R_3E_3$ indicates rifampicin, isoniazid, and ethambutol each given three times weekly for 5 months. If there is no subscript, the drug is given daily.

E, ethambutol; **H,** isoniazid; **R,** rifampicin (rifampin); **S,** streptomycin; Z, pirazinamide.

mental institution, correctional or long-term facilities, etc.). The recommended doses of INH are 10 mg/kg for children and adolescents and 5 mg/kg daily for adults (maximum daily dose = 300 mg). For subjects requiring DOT, a twice-weekly dose of 15 mg/kg to a maximum of 900 mg is suggested when daily DOT is not possible. If the contact is likely to be infected with INH-resistant strain, rifampicin should substitute INH in chemoprophylaxis at the dosage of 10 mg/kg up to 600 mg daily. The duration of the preventive therapy ranges from a minimum of *6* months up to 12 months.

6.2 Mycobacteriosis from NTM

These mycobacteriosis are particularly frequent in AIDS patients; about 90%of them are affected. The most frequent opportunistic infection in these patients is now caused by the

Table 16.5 Interaction of Some Antimycobacterial Drugs with Other Agents

Antimycobacterial Drug	Interaction with
Ethambutol	↑ Chloroquine, chloramphenicol, didanosine, disulfiram, isoprinosine, thiazide diuretics, zalcitabine
Isoniazid	↑ Anticoagulants, acetaminophen, barbiturates, carbamazepin, cyclosporine, corticosteroides, diazepam, didanosine, disulfiram, flucitosine, ketoconazole, methyldopa, phenitoin, rifampicin," pyrazinamide," theophylline, vidarabine, zalcitabine
Pyrazinamide	↑ Didanosine, isoniazid," ketokonazole, viridazole, thiazine diuretics
Rifampicin	↓ Analgesic, anticoagulants, anticonvulsant, azathioprine, barbiturates. beta-adrenergic blockers, chloramphenicol, oral contraceptives, corticosteroides, cyclosporine, dapsone, diazepam, digoxin, disopyramide, estrogens, haloperidol, methadone, protease inhibitors (saquinavir, ritonavir, indinavir, nelfinavir), quinidine, zidovudine
	↑ Didanosine, isoniazid," zalcitabine
Rifabutin	Less interaction than rifampicin.
	↓ clarithromycin, cyclosporine, protease inhibitors
Streptomycin	↑ Acyclovir, amphothericin, nephrotoxic beta-lactams, carboplatinum, cisplatinum, cyclosporine, loop diuretics, 5-fluorocytosine neuromuscolar blocking agents, NSAID, vancomycin

↑ Possibile increase of activity and/or toxicity.
↓ Possibile decrease of activity.
"Antimycobacterial drug that contributes to the increased hepatotoxicity of therapeutic regimens.

M. auium complex. The disease may be localized in lungs, lymph nodes, and skin while in patients with less >50 CD4/mm^3 the disease is disseminated. M. ***kansasii*** and ***M. xenopi*** cause chronic pulmonary infections, M. ***genauense*** is responsible for disseminated infections, and M. *haemophilum* causes septic lesions involving skin and joints. Except M. ***kansasii,*** NTM are scarcely susceptible to classic antituberculous drugs. Therapeutic regimens (521) applied in these infections are summarized in Table 16.6.

6.3 Leprosy

The therapy of leprosy has reached significant achievements since 1982, when WHO promoted new protocols of treatment, providing a short-term multidrug therapy (MDT) (522, 523). In this period, the estimates of the total number of leprosy cases in the world has been falling downward. This tendency has not to be attributed only to implementation of MDT but to a series of other factors, such the revision in the definition of patients. Patients who have completed their course of chemotherapy are no longer counted as registered cases even if they have residual disabilities. In addition, better living conditions and better nutrition greatly contributed to the decline of incidence of the disease in some countries. There has been a reduction of 42% in cases of leprosy in the world since 1985; the global estimates in 1991 were 5.5 million cases (of which 2 million were not receiving treatment) compared with 10–12 million in the past (522–524).

The regimens that have been followed according to WHO recommendations in developing countries are mainly two, one directed to treat paucibacillary disease and the other for multibacillary disease. The former consist in the administration of dapsone 100 mg daily plus rifampicin 600 mg monthly for 6 months, the latter in the administration of dapsone 100 mg plus clofazimine 50 mg daily plus rifampicin 600 mg and clofazimine 300 mg monthly for a minimum of 2 years (522, 523). Relapse rates have been considered low enough for WHO to recommend the reduction of the therapy to 1 year (525). In some cases a daily treatment with rifampicin and dapsone is privileged for 1 year in paucibacillary leprosy and for 2 years with the addition of clofazimine in multibacillary disease (522, 523).

On the basis of a multicenter trial, the WHO Committee on Leprosy concluded that single-lesion paucibacillary disease could be treated with a single dose of therapy consisting of 600 mg rifampicin, 400 mg ofloxacin, and 100 mg minocycline. This regimen seems to be as effective as the standard paucibacillary regimen previously approved by WHO (526), that is, dapsone 100 mg daily plus rifampicin monthly for 6 months. However the results are still under debate.

During therapy, about 25% of all borderline and lepromatous patients develop acute inflammatory reactions probably caused by the formation of immune complexes caused by the high antigen load released by dying mycobacteria, with a consequent secretion of TNF-α from macrophages (527). Reactions are of two main types, namely reversal reaction and erythema nodosum leprosum. The former consists in new and increased inflammation in preexisting skin lesions, with or without involvement of nerve trunks; the latter consists in the formation of small tender erythematous subcutaneous nodules, accompanied by fever, arthralgia, vasculitis, adenopathy, and further inflammatory reaction in other organs. These reactions are usually controlled with corticosteroids. In the most severe cases thalidomide, azathioprine, and cyclosporine have been employed, but because of serious side effects, their use has to be restricted and closely monitored (528).

A field which attracts a particular interest is the possibility to develop a vaccine to prevent the disease. Three vaccines have been prepared combining BCG with M. ***leprae*** or other mycobacterial species and are currently under investigation, but results of these trials will only be available in 8–10 years (529). A preliminary report on a trial performed in Venezuela with a vaccine constituted by BCG and killed M. ***leprae*** does not evidentiate any advantage over the use of BCG alone (530). In fact, BCG alone, particularly if repeated, has shown some protective activity against both tuberculosis and leprosy (531).

Table 16.6 Regimens for the Treatment and Prophylaxis of Some NTM Infections

Pathology	Therapy	Duration of Therapy
M. kansasii: pulmonary disease	Isoniazid 300 mg, + rifampicin 600 mg, + ethambutol 25 mg/kg for 2 months, (then 15 mg/kg), daily. Possible alternative drugs: clarithromycin, sulfamethoxazole, amikacin, newer quinolones, rifabutin	18 Months, with a minimum of 12 months sputum negativity
M. avium complex: pulmonary disease	Clarithromycin 500 mg, twice daily (or azithromycin 250 o 500 mg three time a week) + rifabutin 300 mg (or rifampicin 600 mg) + ethambutol 25 mg/kg daily for 2 months, then (15 mg/kg) + streptomycin 0.750-1 g three times a week for 2–3 months	Until culture negative for at least 12 months
M. avium complex: disseminated disease	Clarithromycin 500 mg twice a day (or azithromycin 250 mg three times a week) + rifampicin 600 mg (or rifabutin 300 mg + ethambutol 25 mg/kg for 2 months, (then 15 mg/kg) Streptomycin 0.75-1 g 2–3 times a week for the first 8 weeks should be considered if tolerated.	Until culture negative or lifelong
	Prophylaxis in adults with AIDS with CD4 < 50 cells: Rifabutin 300 mg daily or clarithromycin 500 mg twice daily; or azithromycin 1200 mg once weekly; or azithromycin 1200 mg once weekly + rifabutin 300 mg daily	Lifelong
M. avium, M. scrofulaceum, M. malmoense: cervical lymphadenitis	Possibly susceptible to clarithomycin, amikacin, ciprofloxacin, doxicycline, clofazimine. Surgical resection	4–6 Months
M. marinum: cutaneous diseases	Clarythromycin 500 mg twice daily; or minocycline or doxycycline 100 mg twice daily; or TMP/SMX 160/ 800 mg twice daily: or rifampicin 600 mg + ethambutol 15 mg/kg daily	3 Months
M. malmoense: pulmonary disease	Ethambutol, rifampicin, streptomycin (in combination according to susceptibility tests)	Variable
M. simiae: pulmonary and disseminated disease	Clarithromycin, ethambutol, rifabutin, streptomycin (4 drug in combination according to susceptibility test)	Variable

7 THINGS TO COME

What is the future of tuberculosis? What is the way to overcome the many problems that this disease still poses?

The future projections of the global tuberculosis epidemic, as estimated by WHO, are not optimistic. If effective national tuberculosis programs are not implemented, it is likely that the epidemic will worsen. Several factors,

Table 16.6 (Continued)

Pathology	Therapy	Duration of Therapy
Rapidly growing mycobacteria: M. fortuitum	Amikacin, tobramycin, cefoxitin, imipenem, newer macrolides, quinolones, doxycycline, minocycline, sulfonamides (2 drug combination therapy, according to susceptibility test)	6–12 Months
M. abscessus	Amikacin, cefoxitin, imipenem, newer macrolides (2 drug combination therapy, according to susceptibility test)	2-4 Weeks (curative course probably of 4–6 months)'

Adapted from American Thoracic Society (ATS), Diagnosis and treatment of disease caused by nontuberculous Mycobacteria, Am. *J. Resp. Crit. Care* Med., 156, 51 **(1997).**

mostly particular to developing countries, contribute to confirm this hypothesis: war, natural disaster, poverty, malnutrition, demographic changes, increasing antituberculous drug resistance, and expansion of HIV pandemic, especially in Southeast Asia. It is estimated that the global HIV-related tuberculosis cases will increase from **700,000** in **1995** to **1.4** million in **2000 (507, 532).**

However, some lines of research have to be pursued to find possibly innovative solutions to the problem of tuberculosis.

It is a common feeling and it seems to be a realistic approach that the global problem of tuberculosis will be solved when an efficacious vaccine is (will be!) available. A first goal is achieving a better evaluation of the effectiveness of **BCG** vaccine, determining the factors that influence it, and identifying the immunological correlates of its behavior in different populations, when the effectiveness of the vaccine is known. A better knowledge of its mechanism of action will allow some genetic modifications leading to an enhanced activity. This approach has been attempted, and encouraging results have been obtained. Strains of **BCG** secreting cytokines or overexpressing specific antigens showed enhanced immunogenecity **(533,** 534).

DNA vaccines have raised great interest; a recombinant antigen when administered as DNA vaccine is immunoprotective as the results obtained in animal models demonstrated **(535, 536).** That means that any of the **4000** genes identified in the M. ***tuberculosis*** genoma could be rapidly tested for their potential use as subunit vaccines. This possibility greatly enhances the projects for vaccine development **(537).** The steps for developing an improved tuberculosis vaccine have been indicated **(538).** More than **190** candidates vaccines have been screened in animal models **(539).**

There are already at least **40** potential vaccines that could be considered for clinical trials, but the enormous complexity and the cost of the organization of such trials delays or prevents their realization **(537).**

From the point of view of treatment, immunotherapy could offer a solution to the problem of MDR tuberculosis as well of other of mycobacterioses scarcely susceptible to the classical antituberculous therapy. Cytokines could be directly administered in patients systemically or by aerosol. Experiments that have been performed in animals and some limited trials in humans with **IL-2** and IFN-y seem to give some promising results **(540,541).**

The goal to be reached in the search of new antituberculous chemotherapeutic agents should be the development of drugs with novel mechanism of action and new targets, high antimycobacterial activity, improved pharmacokinetic characteristics, and better tolerability. Possibly, the ideal antimycobacterial agent should be capable to eradicate the infection by a very short course of therapy, better if by single-drug administration. This result has been practically reached by the use the single dose of the association rifampicin, ofloxacin, and minocycline in the treatment of certain forms of leprosy **(542).**

Improvements in the implementation of the DOT program, which in past years has been deceiving in many countries, will be of

value. It was estimated that 25% of the world's population may have access to DOTS in mid-2000, in comparison with about 10% in 1995. To further improve the results, it is necessary to increase substantially case detection and notifications. The WHO targets for TB control are 85% treatment success rate among smear positive cases and detection of 70% of all such cases. These targets had not been met by the start of the year 2000, but could be achieved by 2005. To achieve this program, it is necessary that the largest countries, particularly India, which have a high incidence rate of TB, extend the DOTS procedure to the whole population by 2004 (507, 532).

In the near future new and old tools (drugs, vaccines, diagnostic methods) can be combined and used in different ways to improve the outcome of tuberculosis, but it is necessary that the recent discoveries and hopefully some new therapeutic agents will provide us with the means to apply better strategies to the therapy and prevention of tuberculosis.

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CHAPTER SEVENTEEN

Antifungal Agents

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Contents

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1 INTRODUCTION

Of the five fundamental Kingdoms of Life, the Kingdom Fungi is arguably the most diverse and prevalent. Unlike the Kingdom Monera (containing bacteria), fungi are eukaryotic organisms whose cellular functions consequently resemble those of plants and animals more closely. Thus the issue of selectivity predominates in the quest for safe and effective chemotherapeutic remedies for diseases caused by fungi. As with all chemotherapy, there is a risk-reward ratio to be taken into account; in the context of fungal infections, this ratio may vary greatly, from minor irritations such as athlete's foot to life-threateningsystemic infections such as those caused by *Aspergillus fumigatus*. This chapter addresses medicinal aspects of the treatment of fungal diseases of all types, but because most recent research has been directed toward the treatment of systemic infections, emphasis is placed on this aspect.

1.1 Fungal Diseases and Pathogens

1.1.1 *Candida* spp. Invasive candidiasis is the most common nosocomial mycosis, perhaps because the causative organism is a component of the endogenous flora of the human alimentary tract. There has been debate over the significance of positive blood cultures (candidemia) in the progression of fungal disease. Given the high mortality rates (up to 75%) in cases of invasive candidiasis, the current consensus is that all high risk patients with candidemia should receive therapy (1).

C. glabrata, C. krusei, C. tropicalis, and *C.* parapsilosis have emerged in recent years as troublesome organisms, challenging the supremacy of *C.* albicans in candida infections. The most common manifestation of these infections, particularly in AIDS patients, is oral or esophageal candidiasis ("thrush": white plaques that cause pain or difficulty upon swallowing); studies suggest that up to 90% of those suffering from AIDS have had at least one such episode (2). Catheter-related candida infections are also very common in hospitalized patients and, if not treated adequately, may lead to disseminated disease in which virtually any organ may be affected. This is particularly a risk in patients undergoing treat-

Table 17.1 Common Fungal Infections[a]

Disease	Etiologic Agents	Main Tissues Affected
	Contagious, Superficial Disease	
Dermatophytoses (ringworm/tinea)	Epidermophyton, Microsporum, Trichophyton spp.	Skin, hair, nails
	Noncontagious, Systemic Diseases	
Aspergillosis	*Aspergillus* spp.	External ear, lungs, eye, brain
Blastomycosis	Blastomyces dermatitidis	Lungs, skin, bone, testes
Candidiasis	Candida spp.	Respiratory, gastrointestinal and urogenital tracts; skin
Chromomycosis	*Cladosporium, Fonsecaea,* and Phialophora spp.	Skin
Coccidioidomycosis	Coccidioides *immitis*	Lungs, skin, joints, meninges
Cryptococcosis	Cryptococcus neoformans	Lungs, meninges
Histoplasmosis	Histoplasma capsulatum	Lungs, spleen, liver, adrenals, lymph nodes
Mucormycosis	Absidia, *Mucor, Rhizopus* spp.	Nasal mucosa, lungs, blood vessels, brain
Paracoccidioidomycosis	Paracoccidioides brasiliensis	Skin, nasal mucosa, lungs, liver, adrenals, lymph nodes
Pneumocystosis	Pneumocystis carinii	Lungs
Pseudoallescheriasis	Pseudoallescheria boydii	External ear, lungs, eye
Sporotrichosis	Sporothrix schenkii	Skin, joints, lungs

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ment for leukemia, with prolonged periods of bone marrow dysfunction and neutropenia.

1.1.2 Cryptococcus neoformans, This organism, which survives in the feces of pigeons and is probably acquired in humans by aerosolization and inhalation, emerged at the height of the AIDS pandemic as a life-threatening pathogen. In recent years incidence of these infections has abated somewhat, but an episode of cryptococcal meningitis remains a serious cause for concern, and after therapy a life-long antifungal regimen is required in AIDS patients to prevent relapse.

1.1.3 Opportunistic Filamentous Fungi. These are a group of organisms that cause very serious infections, particularly in neutropenic patients. Infection attributed to ***Aspergillus*** spp. is the most common. After the inhalation of spores, pulmonary disease develops; in severe cases, the disease spreads to any of several other organs. Mortality in cases of invasive pulmonary aspergillosis remains high, despite some improvement in early diagnosis, and the need for effective, less toxic therapies remains acute (3). The situation is complicated by the incidence of pulmonary infection resulting from other moulds that may be refractory to agents that have some effect on ***Aspergillus*** spp.

1.2 Trends in Incidence of Fungal Infection

The greatest use of antifungal agents, by far, is in treatment of dermatophytic infections—dandruff, athlete's foot, and toenail infections, for example—that occur in otherwise healthy individuals. The incidence of such disease is relatively constant. Because these are infections of the skin, many of the available agents are applied topically, although a few notable oral alternatives are now available. Despite the relative triviality of the infections, however, their eradication is often problematic and requires many weeks or months of therapy.

Far more serious, but much less prevalent, is the morbidity caused by systemic mycoses. In populations with a fully functioning immune system, the incidence of invasive fungal infections is low, but in the immunocompromised host the situation is very different. Because the proportion of the population that is immunocompromised has increased sharply in recent years, so the incidence of life-threatening fungal infections has risen dramatically. Data from the National Nosocomial Infections Surveillance System conducted in the United States showed a 487% increase in candida bloodstream infections between 1980 and 1989 (4). The increase in numbers of immunocompromised patients is attributed principally to three factors: (*1*) advances in transplant technology, necessitating a period of deliberate immunosuppression to avoid complications resulting from rejection of the foreign organ; ***(2)***chemotherapeutic regimens in cancer that kill rapidly dividing cells such as key parts of the immune system; and **(3)** the spread of the AIDS pandemic. By the early 1990s, fungemia was recognized as the cause of 10% of nosocomial infections (5). The advent of highly active antiretroviral therapy (HAART) in the treatment of AIDS has ameliorated the number of fungal infections in this subpopulation, but fungal infections remain a significant cause of morbidity and mortality, particularly in the nosocomial setting (6).

1.3 Epidemiology of Resistance

As with all other areas of anti-infective drug therapy, the phenomenon of resistance to currently available antifungal therapies is a major concern, and the biochemical means by which such resistance to various drug classes is manifest in the clinic is discussed in section 3. However, it is appropriate at this juncture to compare and contrast the epidemiological circumstances by which such resistance develops in the antifungal (as opposed to antibacterial or antiviral) context.

A major cause of concern in antibacterial chemotherapy is the rapid spread of resistant genes within a bacterial strain (or even across species) by the exchange of genetic material on plasmids, a phenomenon that has been exacerbated in the West by the growth in air travel, ensuring that any mechanism by which a population of bacteria becomes resistant to a particular agent will be shared across much of the world in a relatively short time. Fortunately, in the fungal context (where life-threatening

systemic diseases are not contagious), the spread of resistance by such means occurs at a negligible rate. Here, there are two major ways in which resistance becomes manifest. The first arises when initial antifungal therapy (combined with a defective immune system) fails to eradicate the organism from the patient. Under these circumstances, repeated sublethal exposure to the drug allows a variety of resistance mechanisms to be induced, in much the same way as resistant strains are deliberately generated in the laboratory. The second mechanism for the emergence of infections that are refractory to therapy arises (like all resistance phenomena) from the use of antifungal agents: inevitably, any agent is less efficacious for some fungal species than for others. Thus, the use of any drug is bound to present an environmental stress on the most susceptible strains, allowing the less susceptible to become more prevalent. In the clinical context, the most obvious manifestation of this phenomenon has been the recent identification of Candida spp. that are intrinsically resistant to fluconazole in cases of invasive candidiasis (7). These are strains that ordinarily make up only a small proportion of the fungal burden, but emerge as predominant pathogens when the population of C. albicans is reduced through antifungal therapy.

Thus, although antifungal therapy is not bedeviled by the facile mechanisms for the spread of resistance that are such a problem in the antibacterial or antiviral contexts, nonetheless there are ways in which drugs become less efficacious over time, and the consequent clinical limitations should not be underestimated (5).

1.4 Diagnostic and Microbiological Issues

Although the pharmaceutical industry commonly classifies antifungal with antibacterial (and antiviral) research and development as a single therapeutic area (anti-infectives), there are some important clinical differences that affect the way in which antifungal agents are used, and that have a significant impact on the discovery process. First, considerably fewer clinical microbiological data are available to allow meaningful analysis of the pathogenic organisms involved. This is partly because less routine identification of fungal species (much less individual strains) is performed in the general hospital setting. The generation time of most fungi is longer than that of bacteria, and consequently the common practice is to rule out bacteria as origins of an infection and proceed to antifungal therapy on an empiric basis. In some extreme cases, such as for invasive aspergillus infections, the incubation time is comparable with the time it takes for the disease to progress from presentation with fever to death. This, combined with the reduction in postmortem clinical microbiological investigations, may contribute to significant underreporting of the incidence of serious systemic fungal infections in general.

Second, only recently have standard methods begun to emerge for routine susceptibility testing, further confounding attempts to interpret historical data in a comparative manner (8). The variable morphology of important pathogens (the dimorphism of Candida spp. between yeast and hyphal forms, or the vastly different morphology of *Cryptococcus* neoformans in vitro and in vivo, for example), the differing correlations of growth time in vitro with clinical outcomes across species, "trailing" effects with important classes of agents confounding the definition of a minimum inhibitory concentration (MIC), and difficulties in defining interpretative breakpoints for use in a predictive manner clinically, all combine to render the definition of broadly useful methods extremely problematic; the need for different methods for different organisms further increases the burden on clinical microbiology laboratories.

Given the limited success in defining microbiological susceptibility testing conditions that correlate with clinical outcomes, it is only to be expected that trends in the emergence of resistance may be hard to define. However, particularly for azoles, approximately 50% of azole-resistant isolates correlate with clinical failure (9, 10), and the increase in MIC upon sequential isolation from individual patients provides clear evidence of such trends (11).

In this context, it is not surprising that the field of antifungal research is littered with examples where tests in vitro were not adequate to predict effects in vivo, and many of these were false negatives, examples where the compounds turned out to be active in vivo despite

apparently poor ***in vitro*** antifungal activity. Two very prominent and significant classes of antifungals (the azoles and the echinocandins) were significantly underestimated by conventional ***in vitro*** methods; the use of pharmacokinetic and ***in vivo*** efficacy tests at an early stage in the discovery process is therefore particularly appropriate in this field of medicinal discovery (12).

1.5 Selectivity

The search for antifungal agents that are clinically useful may fairly be characterized as the search for selective ways to kill one eukaryotic organism within another, and for systemic mycoses, it is also fair to say that until recently clinicians have been forced to use agents whose mammalian toxicity has been the limiting factor in achieving successful outcomes. Indeed, amphotericin B, the gold standard of therapy for serious invasive mycoses for over 40 years, causes nephrotoxicity in over 80% of patients who receive it, and a primary concern in the design of therapeutic regimens (both in amount and frequency of dosing, as well as in the search for new formulations) is to avoid permanent defects in renal function (see Sections 3.1.4, 3.1.7, and 3.1.8).

The two primary differences between fungi and higher eukaryotes that have been exploited in the identification of antifungal agents with clinical utility are the constitution of the cell membrane and the architecture of the cell wall.

1.5.1 The Biosynthesis and Cellular Functions of Ergosterol. In virtually all pathogenic fungi, the principal sterol is ergosterol (Fig. 17.1), which differs from its mammalian counterpart cholesterol in several structural respects. Although none of the classes of currently available antifungal agents was discovered by a rational search based on this fact, a remarkable number of them turn out to depend on it for their utility.

The principal route for the biosynthesis of ergosterol from squalene in *C. albicans* is shown in Schemes 17.1 and 17.2. Not surprisingly, there are many parallels with the biosynthesis of cholesterol in mammals; even when the substrates differ, many of the chemical transformations are analogous. Furthermore, it is important to appreciate that the route to ergosterol in the latter stages is not identical in all fungal species, nor is there necessarily a unique path in each (13). For example, the order of methylenation at C_{24} and demethylations at C_4 and C_{14} may vary, as may the isomerizations and adjustments in oxidation state that lead thereafter to the appropriate installation of olefins. Thus inhibition of one of these later steps may cause not only the depletion of cellular reserves of ergosterol, but also the accumulation of aberrant sterols as intermediates in the pathway.

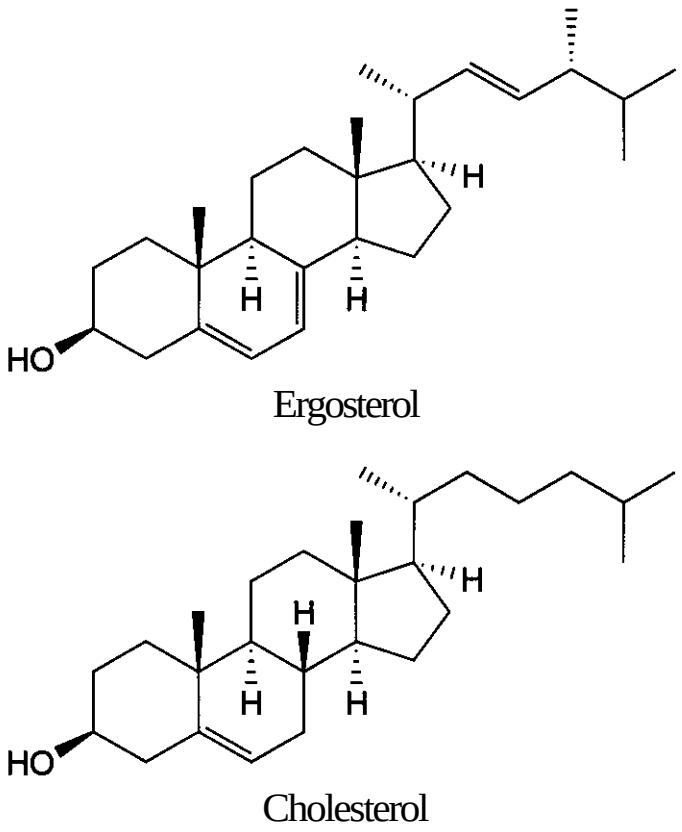
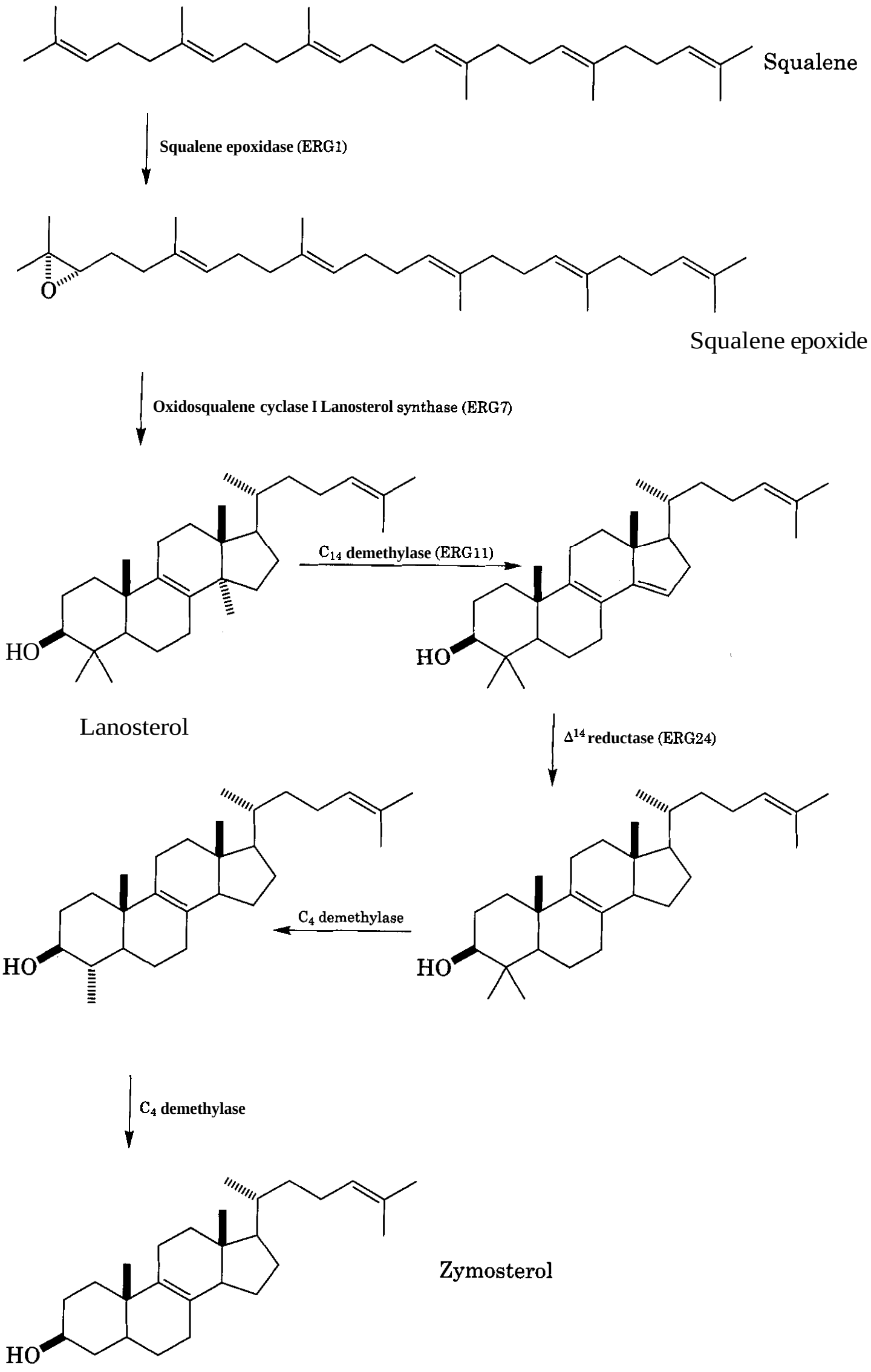
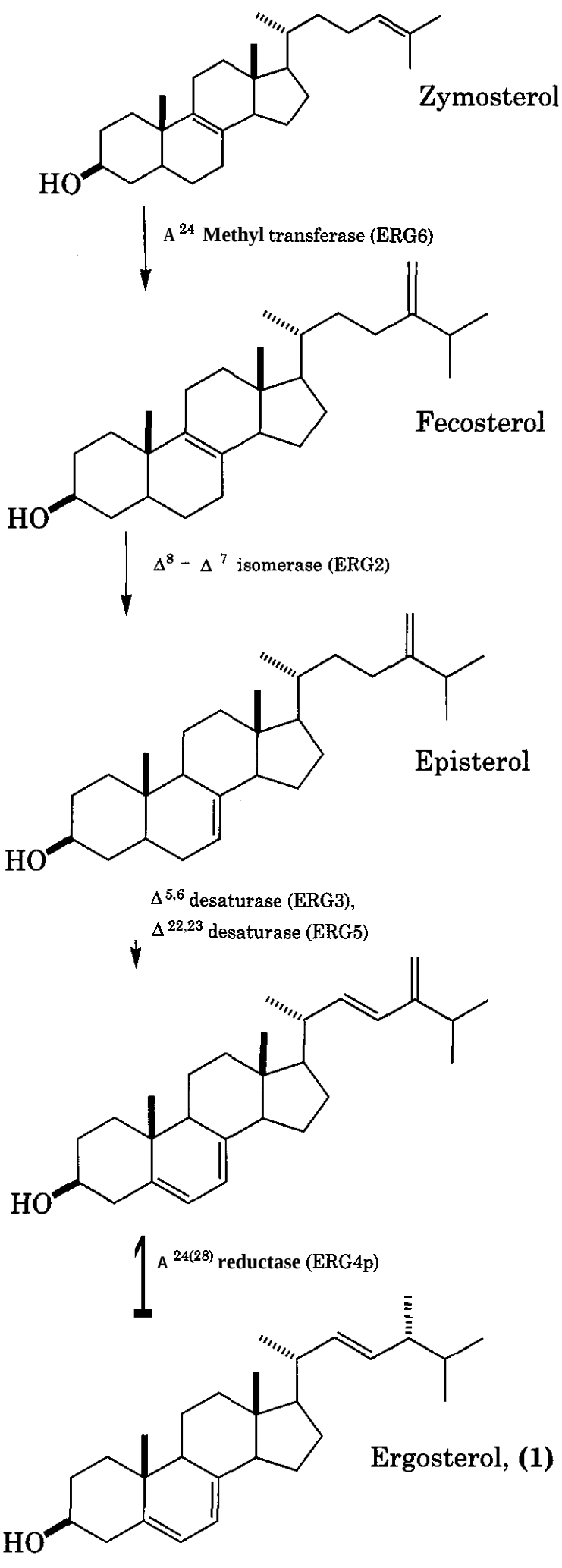


Figure 17.1. Ergosterol and cholesterol.

Ergosterol may constitute 10% of the dry weight of a fungal cell. Using *S. cerevisiae* as a convenient model organism and studying growth patterns using surrogate sterols, it has been shown to be important for the growth and survival of fungi in a variety of ways (14). At least four sterol functions have been implicated: sparking, critical domain, domain, and bulk (15). Of these, the sparking function (associated with cellular proliferation) is the most structurally demanding and requires a sterol with a planar a-face (i.e., no methyl group at C_{14}) and bearing a C_{5-6} olefin, and the bulk function is the least demanding, requiring only a sterol bearing a 3β-hydroxy group.



Scheme 17.1. Biosynthetic pathway from squalene to zymosterol. For further stereochemical detail, see Fig. 17.1.



Scheme 17.2. Biosynthetic pathway from zymosterol to ergosterol (in C. albicans). For further stereochemical detail, see Fig. 17.1.

1.5.2 The Fungal Cell Wall. The fungal cell wall is a complex structure external to the cell membrane containing a high degree of polysaccharide content. It is essential for fungal viability, maintaining organism osmotic integrity and shape during its growth. In pathogenic fungi, adhesion to the mammalian host appears to be regulated by the cell wall. Given that there are no mammalian counterparts to the fungal cell wall, compounds that target it should be selective and inherently fungicidal, making them quite attractive for clinical development. The clinical success of antibacterial agents such as the β-lactams that target the bacterial cell wall suggest that a functional equivalent in fungi would have good clinical potential (16).

The composition of the fungal cell wall varies among species but is generally composed of glucans, chitin, and mannoproteins. Chitin and glucan fibrils form the scaffolding, whereas embedded mannoproteins form the matrix that is responsible for cell wall porosity and hydrophobicity.

Glucans, which constitute nearly 60% of the fungal cell wall, are variously linked β-1,3-, α-1,3-, and β-1,6-glucose polymers composed of approximately 1500 monomer units with side chains averaging 150 monomer units in length. Glucan is evenly distributed over the entire cell surface but is primarily deposited at sites of new cell wall growth. Caspofungin (see Section 3.5), an inhibitor of β-1,3-glucan synthase, was recently approved to treat patients with refractory invasive aspergillosis. Other glucan synthase inhibitors of the candin class that are currently in clinical development include Micafungin (FK-463) and Anidulafungin (LY303366, V-002).

Chitin, a homopolymer of monomer units of *N*-acetyl-D-glucosamine, is a minor but essential cell wall component. Its proportion in the fungal cell wall varies among species (i.e., 2% in yeasts, higher in moulds). Chitin synthesis occurs on the cytoplasmic surface of the plasma membrane with the translocation of the linear polymer through the membrane to the cell surface. Chitin synthesis is competitively inhibited by the naturally occurring nucleoside-peptides polyoxins and nikkomycins (see Section 3.5.1.2).

Mannoproteins, which are O- and *N*-glycosylated proteins that constitute approximately 40% of the cell wall, are linked at their manno-oligosaccharide to β-1,3-glucan by a phosphodiester linkage. Because glycosylation of fungal mannoproteins is generally similar to that of mammalian mannoproteins, disruption of this target is considered less attractive.

2 CURRENT THERAPEUTIC OPTIONS

2.1 Current Drugs

The entries in the following tables are listed according to mode of action, with those affecting ergosterol biosynthesis or function (the vast majority) grouped together.

The pharmaceutical requirements (and markets) of agents for use in treating systemic and superficial cutaneous mycoses differ greatly. Consequently, compounds for these different uses are listed in separate tables below. Where significant use for a single agent is found in both contexts, it is listed in both tables.

2.1.1 Drugs for Treatment of Systemic Mycoses. See page 890.

2.1.2 Drugs for Treatment of Superficial Cutaneous Mycoses. See page 891.

3 ANTIFUNGAL CHEMICAL CLASSES

3.1 Polyenes: Amphotericin B and Nystatin

3.1.1 Overview and Mode of Action. Amphotericin B (Fig. 17.2), produced by *Streptomyces nodosus,* was discovered in 1955 by Gold and coworkers. It is the archetypal polyene antifungal and was the first systemic agent available to treat invasive fungal infections. The primary indications of its current therapy include invasive candidiasis, cryptococcosis, aspergillosis, and histoplasmosis. It is the treatment of choice for empirical therapy in febrile neutropenic patients.

The polyenes act by binding to ergosterol, a sterol present in the membrane of sensitive fungi. The interaction with ergosterol forms pores within the fungal cell membrane, resulting in leakage of cellular contents such as sodium, potassium, and hydrogen ions. Polyenes display little or no activity against bacteria, as these organisms lack membrane-bound sterols. Although amphotericin B binds approximately 10 times more strongly to fungal cell membrane components than to mammalian cell membrane cholesterol, the interaction with and disruption of mammalian cells by the compound can result in adverse side effects. Consequently, polyenes such as nystatin that have a higher affinity for cholesterol have correspondingly greater toxicities for mammalian cells.

3.1.2 Structure-Activity Relationship. Approximately 60 polyene antifungal compounds have been described to date. They are amphoteric, poorly soluble in water, and somewhat unstable because of extensive unsaturation. They consist of a large, macrocyclic core (38-membered in the case of amphotericin B), where a hydrophilic, polyhydroxylated chain (C_1–C_{13} in amphotericin B) is bound to a lipophilic polyene fragment (C_{20}–C_{33} in amphotericin B) by two poly-substituted alkyl links (C_{34}–C_{37} and C_{14}–C_{19} in amphotericin B, where the latter exists in a pyranose hemiacetal form). Diversity among the polyenes arises principally from the number and configurational arrangement of hydroxyl groups and double bonds in the macrocycle. X-ray crystallography has shown amphotericin B to be rod-shaped, with the hydrophilic hydroxyl groups of the macrolide forming an opposing face to the lipophilic polyene portion.

3.1.3 Resistance. Clinical resistance to polyene treatment remains rare; however, resistant strains have been isolated under laboratory conditions. These strains typically demonstrate alterations in the nature of the sterols or in the amount of sterols present in the membrane.

3.1.4 Side Effects. The clinical use of polyenes is limited, principally because of dose-limiting nephrotoxicity. The primary manifestations of nephrotoxicity include decreased glomerular filtration, loss of urinary concentrating ability, renal loss of sodium and potassium ions, and renal tubular acidosis. Ampho-

Table 17.2 Drugs for Treatment of Systemic Mycoses[a]

Chemical Class[b]	Generic Name	Trade Name	Originator	Formulations	Indications, Dose
Polyenes	Amphotericin B (AmB)	Fungizone	Bristol-Myers Squibb	Deoxycholate	Systemic fungal infections; no more than 70 mg over 2 days
	AmB Lipid Complex	ABELCET	The Liposome Company	Dimyristoyl phosphatidylcholine, dimyristoyl phosphatidylglycerol	Systemic fungal infections; 5 mg/kg/day
	AmB Colloidal Dispersion	Amphocil Amphotec	InterMune		Invasive aspergillosis; 3–6 mg/kg/day
	Liposomal AmB	Ambisome	Fujisawa	Liposomal membranes	Empiric therapy, 3 mg/kg/day; systemic fungal infections, 3–6 mg/kg/day
	AmB Oral suspension	Fungizone Oral Suspension	Bristol-Myers Squibb	Oral, 100 mg/mL suspension	Systemic fungal infections; 2 g/day
Azoles	Fluconazole	Diflucan	Pfizer	Oral, i.v.	Candidiasis, cryptococcal meningitis; up to 400 mg/day
	Itraconazole	Sporanox	Janssen	Oral, i.v.	Blastomycosis, histoplasmosis, aspergillosis; up to 400 mg/day
	Ketoconazole	Nizoral	Janssen	Oral	Candidiasis; 400 mglday
	Voriconazole	Vfend	Pfizer	Oral, i.v.	Acute invasive aspergillosis; 4 mg/kg/day i.v.[c]
Allylamines	Terbinafine	Lamisil	Sandoz (Novartis)	Oral	Candidiasis; 250 mg/day
Candins	Caspofungin	Cancidas	Merck	i.v. infusion	Refractory invasive aspergillosis; 50 mg/day
Others	Flucytosine	Ancobon	Roche	Oral	Candidiasis, cryptococcal meningitis; 50–150 mg/kg/day

[a]Reproduced with permission from www.dodorfungus.org.
[b]For structures, see relevant sections.
[c]FDA advisory committee recommendations; FDA approval still pending.

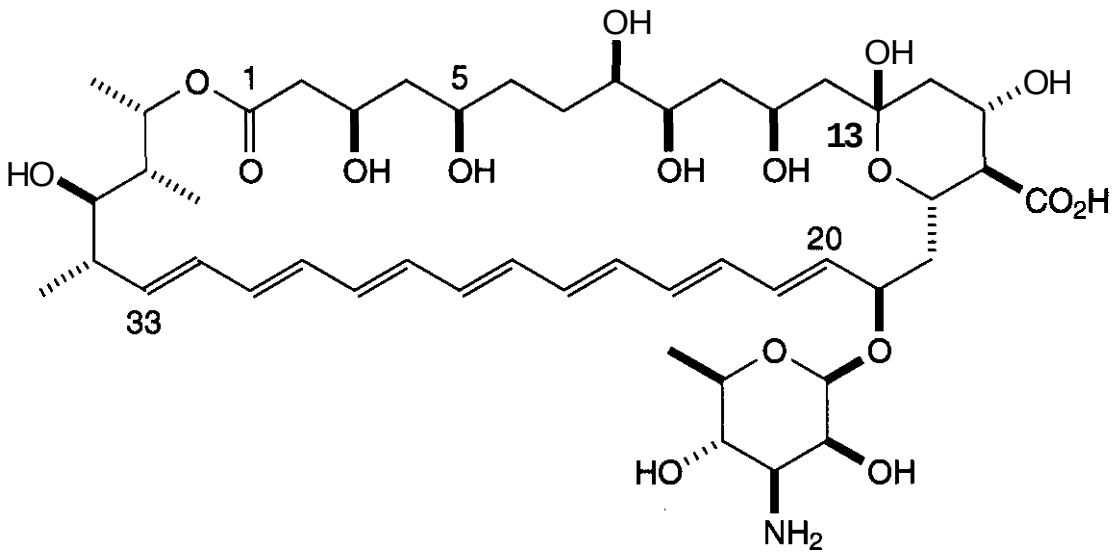
Table 17.3 Drugs for Treatment of Superficial Cutaneous Mycoses[a]

Chemical Class[b]	Generic Name	Trade Name	Originator	Formulations[c]	Indications[c]
Polyenes	Amphotericin B	Fungizone	Bristol-Myers Squibb	C, L, O	CC
	Nystatin	Various	Various	C, O, OS, P, VT, T	CC, OC, VC
Azoles	Butoconazole	Femstat	Syntex (Roche)	C	VC
	Clotrimazole	Various	Bayer	C, L, S, T, VT	D, CC, OC, VC
	Econazole	Spectazole	Janssen	C	D, CC
	Itraconazole	Sporanox	Janssen	S	D
	Ketoconazole	Nizoral	Janssen	C, S	D, CC
	Miconazole	Micatin, Monistat	Janssen	C, L, S, P, VS	D, CC, VC
	Oxiconazole	Oxistat	Roche	C, L	D, CC
	Sulconazole	Exelderm	Syntex (Roche)	C, S	D, CC
	Terconazole	Terazole	Janssen	C, VS	VC
	Tioconazole	Vagistat	Pfizer	C, VO	VC
Allylamines	Naftifine	Naftin	Sandoz (Novartis)	C, O, P	D
	Terbinafine	Lamisil	Sandoz (Novartis)	C, S	D
	Butenafine	Mentax	Kaken	C	D
Miscellaneous	Amorolfine	Loceryl	Roche	C??	D
	Ciclopirox	Loprox	Aventis	C, L	D, CC
	Griseofulvin	Various	Various	Oral	D
	Haloprogin	Halotex	Bristol-Myers Squibb	C	D, CC
	Tolnaftate	Aftate, NP-27, Tinactin, Ting	Schering-Plough	C, S, P	D
	Undecylenate	Cruex, Desenex	Fisons	C, P, O, S	D

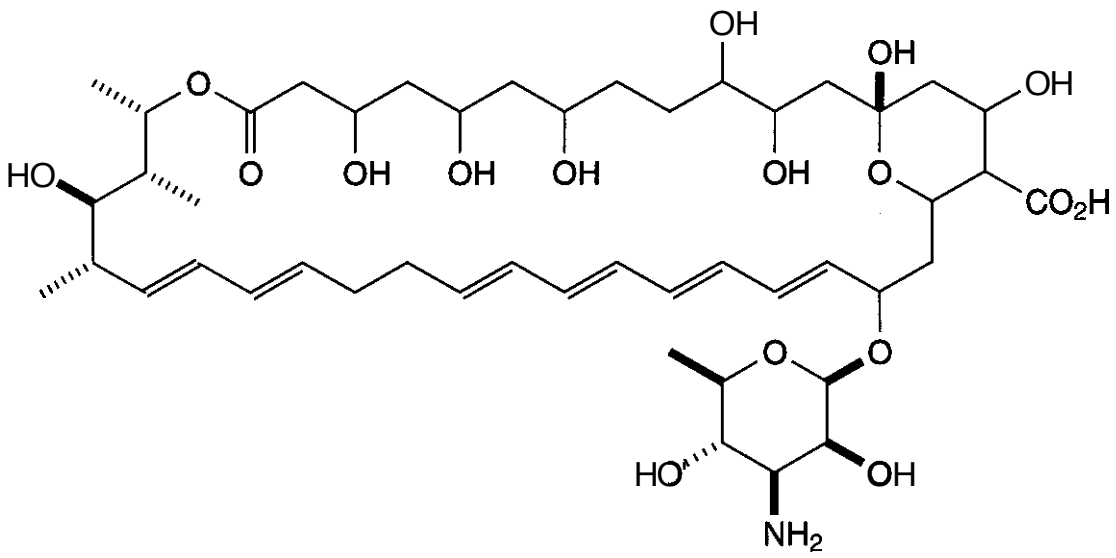
[a]Reproduced with permission from www.doctorfungus.org.

[b]For structures, see relevant sections.

[c]C, cream; L, lotion; O, ointment; OS, oral suspension; P, powder; S, solution/spray; VO, vaginal ointment; VS, suppository; T, troche; VT, vaginal tablet; D, dermatophytosis; CC, cutaneous candidiasis; OC, oropharyngeal candidiasis; VC, vulvovaginal candidiasis.



Amphotericin B



Nystatin

Figure 17.2. Structures of polyenes.

tericin B-induced renal impairment may result in the premature or.temporary cessation of treatment or a reduction in dosage, leading to a worsening of the infection or prolonged hospitalization. Complications in addition to nephrotoxicity include hypokalemia and anemia, caused by reduced erythropoetin synthesis.

3.1.5 Absorption, Distribution, Metabolism, Excretion (ADME). Amphotericin B is poorly absorbed when given orally, and therefore is administered only by intravenous injection. Serum concentrations of the compound plateau at 2.5 mg/L, with little increase for dosages >1 mg/kg/day. Side effects appear more related to the total cumulative dose than serum concentrations of the drug. The distribution of amphotericin B is believed to follow a three-compartment model, with a reported total volume of distribution of 4 L/kg; the compound is distributed to various organs of the body such as lungs, kidneys, and the spleen. Little of the agent penetrates into cerebrospinal fluid (CSF). The metabolism of the compound is not well understood, and the drug is eliminated primarily by way of renal and biliary routes.

3.1.6 Drug Interactions. Because multiple drug therapy is typically required for patients receiving amphotericin B, the risk of drug interactions is quite high. Caution is advised with concomitant use of other nephrotoxic agents such as aminoglycosides.

3.1.7 Polyene Liposomal Formulations. In an effort to overcome the side effects associated with the use of amphotericin B, a number of lipid based formulations have been developed, each with its own composition and pharmacokinetic behavior (17, 18). There are three currently available lipid formulations of amphotericin B that include: amphotericin B lipid complex (ABLC, Abelcet), amphotericin

Table 17.4 Lipid-Based Formulations of Amphotericin B[a]

Commercial Name	Lipid Formulation	Lipid Configuration	Vehicle Lipid
Abelcet	ABLC	Ribbonlike	DMPC, DMPG
Amphocil, Amphotec	ABCD	Disklike	Cholesteryl sulfate
AmBisome	Liposomal amphotericin B	Vesicle	HSPC, DSPG, cholesterol

[a]ABLC, amphotericin B lipid complex; ABCD, amphotericin B colloidal dispersion; DMPC, dimyristoyl phosphatidylcholine; DMPG, dimyristoyl phosphatidylglycerol; DSPG, distearoyl phosphatidylglycerol; HSPC, hydrogenated soy phosphatidylcholine.

B colloidal dispersion (ABCD, Amphotec), and liposomal amphotericin B (L-AMB, AmBisome) (Table 17.4). Comparative physicochemical properties of amphotericin B and its lipid formulations are shown in Table 17.4. Clinical use of lipid-based formulations of amphotericin B has shown that antifungal activity is maintained while the rate of nephrotoxicity is appreciably reduced. One of the limitations to the use of polyene liposomal formulations is their cost. Ongoing evaluation of these compounds should address the pharmacoeconomic issues by analyzing the cost-benefit ratios for their wider use.

3.1.8 Things to Come. Like amphotericin B, the use of nystatin has been limited because of its nephrotoxic side effects. Lipid formulations of nystatin have displayed decreased toxicity with the maintenance of antifungal activity; liposomal nystatin is a newer formulation whose pharmacokinetics, efficacy, toxicity, and comparative advantages to lipid formulations of amphotericin B are currently being evaluated (19–25).

3.2 Azoles

3.2.1 Overview and Mode of Action. Azoles have emerged as the preeminent class of antifungal agents for all except the most life-threatening infections, and even in these cases highly active new variants are being developed. The most significant agents in clinical use are shown in Fig. 17.3. Chlormidazole, miconazole, clotrimazole, and econazole are administered topically; ketoconazole, itraconazole, and fluconazole are useful in the treatment of systemic infections. The discussion of the pharmacology of various members of the class below is limited to systemic agents.

Azoles interfere with ergosterol biosynthesis by inhibiting demethylation at C_{14} of lanosterol (see Scheme 17.1), thereby causing accumulation of 14α-methylated sterols that disrupt the various sterol functions in the cell. Except at very high concentrations, the agents are fungistatic, a feature that is of some clinical concern in highly immunocompromised patients, where eradication of the pathogen is a very desirable outcome of therapy. The target protein (C_{14} demethylase, also known as CYP51) is a cytochrome P450 (CyP450) enzyme that achieves its effect by three sequential radical-mediated hydroxylations, and spectroscopic studies were used to show that the triazole or imidazole moiety in the inhibitor coordinates at the sixth ligand binding site of the ferrous ion (26). Because the protein is membrane bound, X-ray crystallographic studies have not been readily forthcoming, despite the many years of intensive research in this area. Very recently, however, the structure of a complex of a soluble CYP51 ortholog from *Mycobacterium tuberculosis* with fluconazole has been solved (27).

Despite the presence of an analogous enzyme for the C_{14}-demethylation of lanosterol in mammalian cells, the fungal enzymes are typically 100- to 1000-fold more susceptible to inhibition by azoles. For at least one agent, inhibition of the fungal enzyme was shown to be noncompetitive with respect to the substrate, whereas in the corresponding mammalian enzyme the inhibition was competitive (and therefore potentially reduced by increases in substrate concentration); this may provide a further explanation for the relative lack of effect in mammalian systems (28).

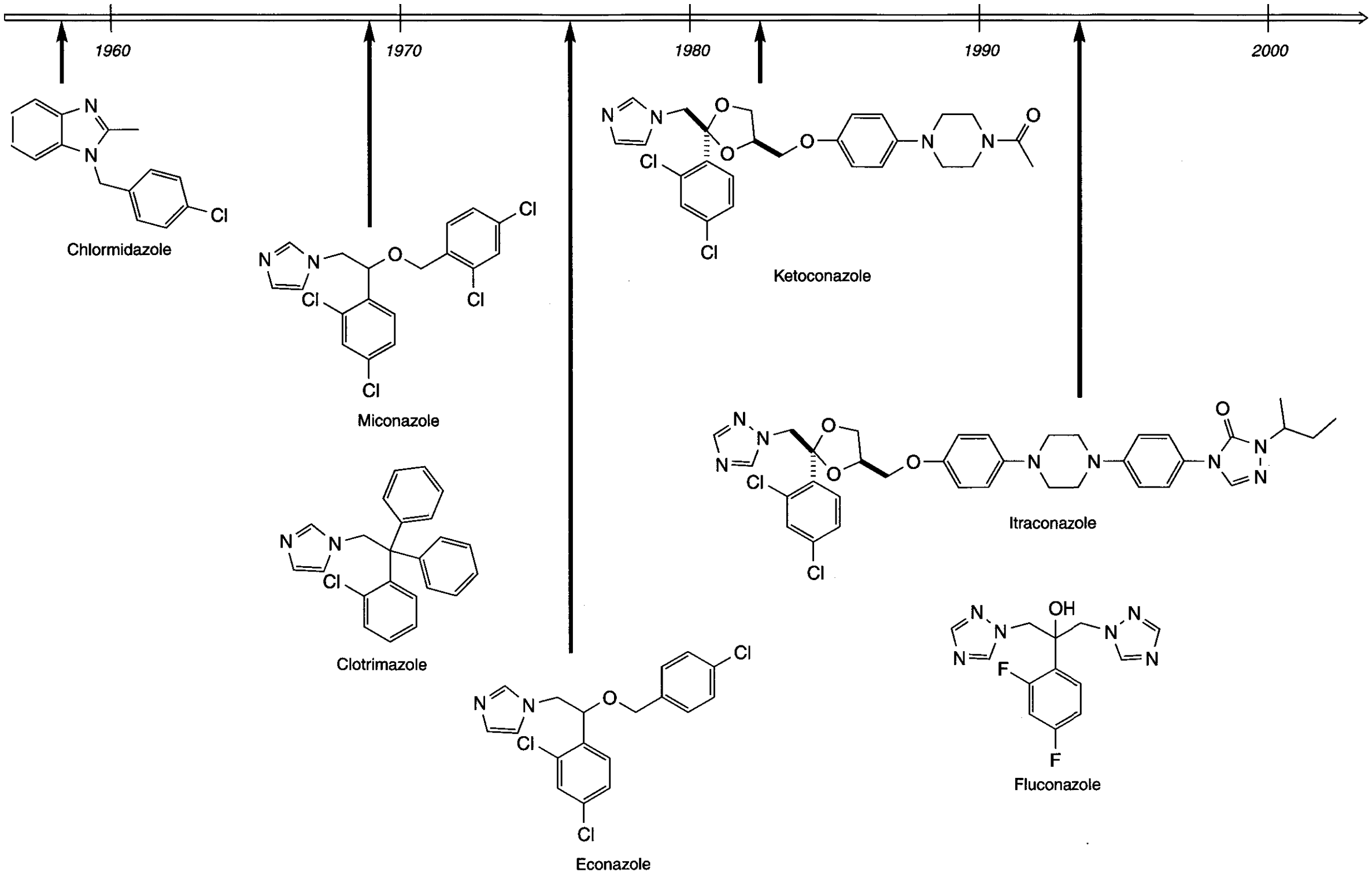


Figure 17.3. Chronology of discovery of clinically significant azoles.

3.2.2 History of Azole Discovery. The azoles were the first purely synthetic class of antifungal agents to be exploited clinically, and their discovery was facilitated by the search for agents for use in the control of fungi in an agrochemical context, where they have had an equally dramatic impact. Thus early antifungal screens soon established that simple imidazole derivatives had activity, building on the first (serendipitous) observation by Woolley in 1944 that benzimidazole affected fungal growth *in vitro* (29). Of the very many agents that have been reported, the chronology of discovery of the most significant clinically is highlighted below.

The first azole to become available for clinical use (as a topical agent) was chlormidazole (Fig. 17.3), introduced by Chemie Gruenenthal in 1958. It was followed in 1969 by Janssen's miconazole (30) and Bayer's clotrimazole (31), and econazole (30) was launched by Janssen in 1974. Even today, the latter three agents remain the mainstay of topical therapy for many dermatophytoses.

In the late 1970s and 1980s, the emphasis in research in anti-infectives was toward the identification of antibacterial agents, in that the clinical need was particularly evident in that area. The only significant event in the fungal context was the introduction by Janssen in 1981 of ketoconazole, the first azole with utility in the treatment of systemic mycoses (32). Like its forebears, this agent is an imidazole derivative, albeit of significantly greater structural complexity; the dioxolane ring was first incorporated in much simpler acetophenone derivatives that were reported as part of the miconazole discovery effort (33). It proved a significant advance in the treatment of serious fungal disease in that it provided an orally available, less toxic alternative to amphotericin B, and for nearly 10 years was the only azole available for this purpose. As such, it saw widespread use, despite limitations with respect to bioavailability and pharmacokinetic interactions (see below).

The finding that imidazole could be replaced by triazole as the C_{14}-demethylase heme-coordinating ligand was a major breakthrough because it provided both greater selectivity for the fungal enzyme and greater metabolic stability *in vivo*. The issue of selectivity was a particular concern, given the ubiquity of CyP450-mediated enzymes in mammalian systems and the attendant association of azoles with hepatic side effects and effects on hormone synthesis. Janssen's itraconazole made use of this key change (33), although the extended ketoconazole-like side chain rendered the compound extremely insoluble, presenting formidable challenges in identifying formulations that allowed parenteral administration or gave reliable oral bioavailability. Thus, despite the appearance on the market of an oral capsule form in 1992, an oral solution in cyclodextrin was not launched until 1997, and it took until 1999 for an intravenous formulation (in hydroxypropyl-β-cyclodextrin) to become available.

The discovery of fluconazole at Pfizer was an interesting study in the optimization of molecular properties important for good activity *in vivo*. As with most azole discovery programs at this time, a mouse survival model involving challenge with a lethal, systemic dose of C. *albicans* was being used at an early stage in the evaluation process to differentiate analogs. The use of the triazole moiety was based on the reduced propensity for first-pass metabolism, and the emphasis on analogs containing a tertiary alcohol (as opposed to dioxolanes or tetrahydrofurans) was also based on the superior activity observed *in vivo*. A conscious attempt was made to focus on polar analogs, particularly with regard to the side chain, thereby minimizing serum protein binding and maximizing the amount of unbound drug available at the site of infection; it was this consideration that led to the incorporation of a second azole moiety into the molecule. Finally, systematic variation of the halo-substituted aromatic group, combined with pharmacokinetic studies in mice, led to the selection of the 2,4-difluorophenyl analog, which was notable for its high urinary recovery (indicative of metabolic stability) (34). This discovery strategy has been fully vindicated: the solubility, safety, and predictable pharmacokinetics of fluconazole, launched in 1991, have driven its success as the world's predominant prescription antifungal agent.

3.2.3 Structure-Activity Relationship. From the many series of azoles that have been re-

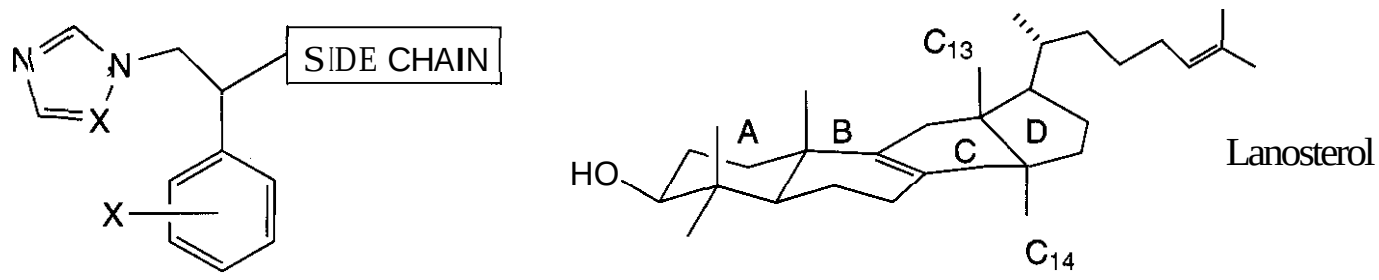


Figure 17.4. Azole pharmacophore.

ported by diverse groups, several common structural features emerge: an imidazole or triazole heme-coordinating group, a halo-substituted aromatic separated from the azole moiety by two atoms, and a side chain (see Fig. 17.4). The latter represents the feature of greatest diversity across the family.

Early postulates that the halo-substituted aromatic residue binds in the pocket normally occupied by the A/B-ring region of lanosterol (see conformational depiction in Fig. 17.4) now seem less likely in the light of recent modeling and crystallographic studies (27, 35); indeed, it may be that this residue binds in the same hydrophobic cleft as the C_{17} side chain of the substrate. The structural diversity and variable length of the side chains explored by various groups suggests that this part of the pharmacophore may extend beyond the substrate binding site, perhaps into the substrate access channel. This general binding model is further substantiated by the more recent discovery that incorporation of a methyl group β to the aromatic ring gives added potency (particularly against moulds), but only for one of the four possible stereoisomers (36). This may be because the methyl group occupies the binding pocket filled by the C_{13} methyl group on the β face of lanosterol; it is also likely that its presence favors conformations in which the side chain is antiperiplanar to the aromatic ring (37).

3.2.4 Resistance. Resistance to azoles has become a significant problem since their use in the treatment of systemic fungal infections has increased (38). Target modification is clearly a common contributor to clinical resistance to azole therapy and has been implicated directly for C. *neoformans* (39), *C. albicans* (40–42), *C. glabrata* (43), and by inference for other *Candida* spp. (44). Different mutations have been documented, making it difficult to rationally design new agents that are less prone to this resistance mechanism. Azole resistance also occurs when compensatory changes exist in other enzymes in the ergosterol biosynthesis pathway, notably inactivation of $\Delta^{5,6}$-desaturase. This leads to the build-up of nontoxic 14α-methylated steroids in fungal membranes. The change in membrane composition can lead to cross-resistance to polyenes (45, 46).

Probably the most prevalent cause of resistance to azole therapy is caused by reduction in intracellular drug concentrations ascribed to active efflux. In C. *albicans*, two types of efflux pump have been shown to be clinically relevant: a member of the Major Facilitator superfamily known as MDR1 (or BEN), and ATP-binding cassette (ABC)-type transporters CDR1 (47) and CDR2 (48). Fluconazole (Fig. 17.3) is a substrate for all three of these pumps, whereas other azoles are affected only by CDR1/2. Homologs of the ABC-type transporters have been shown to confer resistance in clinical isolates of *C. glabrata* (CgCDR1/2) (49, 50) and *A. fumigatus* (ADR1) (51). Tools for the convenient characterization of such resistance mechanisms in clinical isolates are becoming available (52).

3.2.5 Side Effects. The most common adverse effects of ketoconazole (Fig. 17.3) treatment are dose-dependent nausea and vomiting, which occur in approximately 20% of patients receiving a 400 mg daily dose. Better tolerance is achieved by administration with food. Hepatic abnormalities are common, with approximately 5–10% of patients experiencing a mild, asymptomatic elevation of liver transaminases, which return to normal after cessation of treatment. Liver function tests are indicated in patients with hepatic abnormalities. Hepatic insult by the azoles is mech-

anism based, and the selectivity improves from ketoconazole to fluconazole.

Itraconazole is typically well tolerated, with few limiting adverse effects in patients receiving up to 400 mg of the compound. Most adverse effects are transient and can be dealt with by reduction in dose.

Of the three systemic azoles, fluconazole demonstrates the least adverse effects. Daily doses up to 1200 mg are well tolerated. Marked increases in hepatic transaminases are seen with less than 1% of patients treated with fluconazole, and more severe hepatic toxicity or hepatitis is rare.

3.2.6 ADME. Ketoconazole was the first successful orally active mole useful for fungal infections. An acidic environment is required for its dissolution; therefore, its absorption is decreased in patients with decreased gastric acidity, such as those receiving antacids or H_2-histamine blockers. After oral doses of 200, 400, and 800 mg the peak plasma concentrations of the agent are approximately 4, 8, and 20 μg/mL, respectively. The half-life of ketoconazole increases with dose and can be as long as 8 h (after an 800 mg dose). The compound is extensively metabolized, with inactive metabolites excreted mainly through the bile. Concentrations of the active drug or its metabolites in the urine are low.

Like ketoconazole, itraconazole is soluble at low pH. Hence, its absorption is affected by patients in the fasting state or those individuals receiving concurrent treatment with H_2 receptor antagonists or antacids. Plasma levels vary widely among patients; however, clinically useful levels can be achieved when the compound is taken with food or an acidic drink. Peak plasma concentrations are reached 1.5–4 h after drug administration. The compound is extensively metabolized in the liver and is excreted as inactive metabolites into the bile and urine. One metabolite, hydroxyitraconazole, is as active as the parent. Although it is eliminated more rapidly, hydroxyitraconazole's maximum plasma concentration is approximately 2 times higher than that of the parent.

Fluconazole is well absorbed; its bioavailability is greater than 90%. Unlike ketoconazole and itraconazole, absorption is not affected by intragastric pH. The serum protein binding of fluconazole is 12%, whereas both ketoconazole and itraconazole are >95% bound. The volume of distribution of fluconazole approximates that of total body water and the drug penetrates well into nearly all body tissues, including the CSF. In further contrast to ketoconazole and itraconazole, fluconazole is relatively stable to metabolism; its terminal half-life ranges from 27 to 37 h. More than 90% of a therapeutic dose is excreted through the kidneys, 80% of which is recovered unchanged in the urine.

3.2.7 Drug Interactions. As a class, the azoles are notable for their propensity for drug-drug interactions. Most have measurable affinity for human CyP450 enzymes. Interactions of ketoconazole with other agents, however, are much more frequent than with newer compounds, especially fluconazole. Compounds such as rifampicin increase ketoconazole clearance through the induction of hepatic enzymes.

Itraconazole-induced inhibition of CyP450 enzymes may lead to potentially toxic concentrations of coadministered drugs. As with ketoconazole, compounds that affect the CyP450 enzyme systems can possibly increase or decrease the metabolism of the compound.

The types of drug interactions observed with fluconazole are generally similar to those of ketoconazole or itraconazole. but the number and severity of relevant interactions reported with fluconazole are much lower than those with the other two agents.

3.2.8 Things to Come. With the success of fluconazole and the increased incidence of fungal infections in general, there have been many attempts to develop novel agents with increased activity, particularly against organisms that cause serious disease in neutropenic patients, notably Aspergillus spp. The three azoles (Fig. 17.5) that are currently under FDA review or in late-stage development are reviewed briefly below.

3.2.8.1 Voriconazole. Voriconazole (Vfend), discovered by Pfizer and previously known as UK-109,496 (53), is under FDA review. Unlike fluconazole, this derivative shows potent activity against a wide variety of fungi, in-

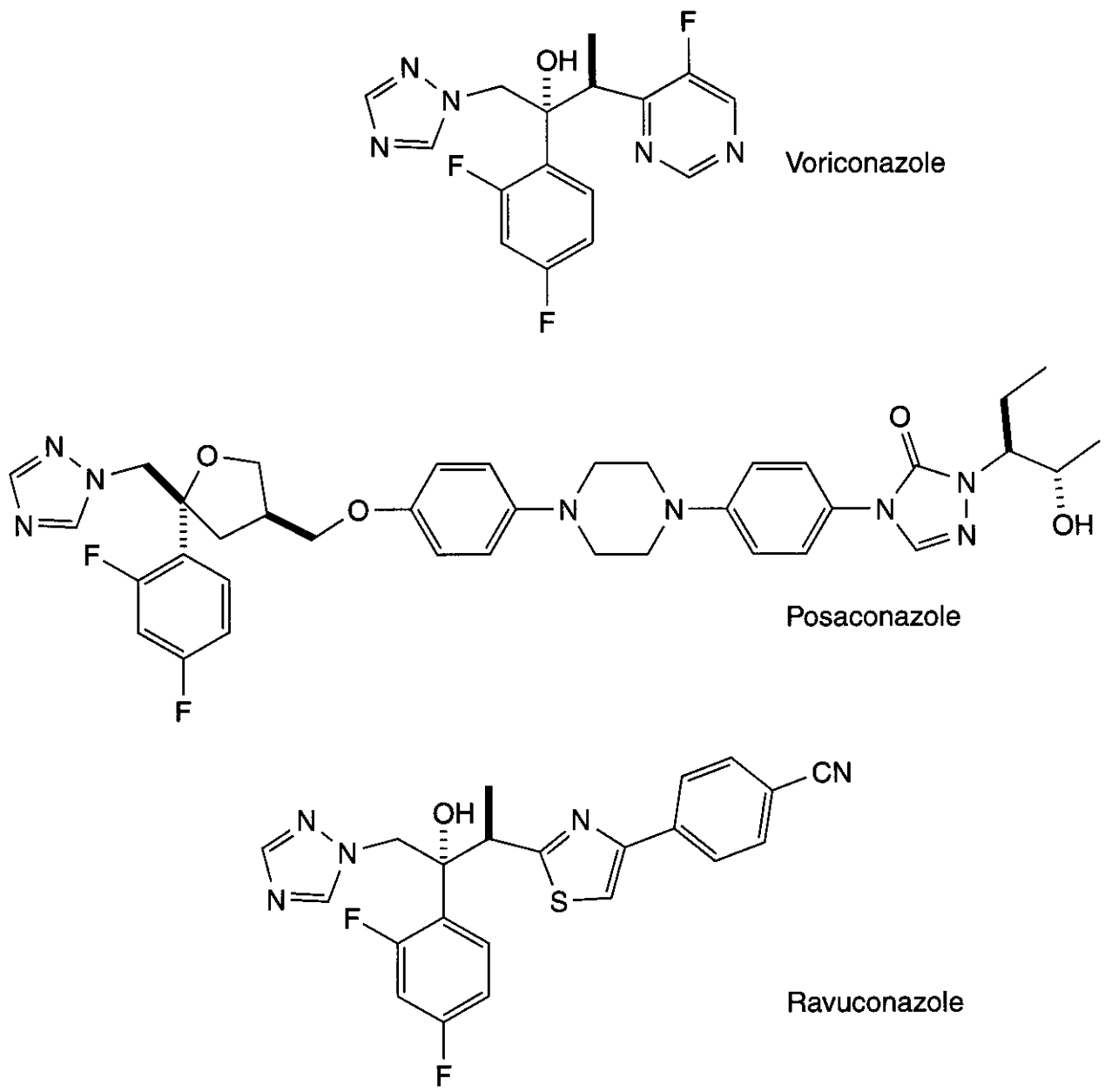


Figure 17.5. Azoles in advanced clinical development.

cluding all the clinically important pathogens (54–60). Voriconazole is clearly more potent than itraconazole against *Aspergillus* spp. (59) and is comparable to posaconazole and ravuconazole in its activity against *C. albicans;* however, it appears slightly more potent against *C. glabrata* (61). In general, *Candida* spp. that are less susceptible to fluconazole also exhibit higher MICs to voriconazole and other azoles (62). Despite this, the voriconazole MIC_{90} for 1300 bloodstream isolates was 0.5 μg/mL (61).

Of all the azoles in current use or under development, voriconazole most closely resembles fluconazole in its pharmacokinetic profile: in humans, bioavailability is high (up to 90%), protein binding (58%)and volume of distribution (2 L/kg) are low. The mean half-life of about 6 h is, however, considerably lower than that of fluconazole, which is attributable to extensive metabolism; pharmacokinetics are nonlinear, possibly suggesting saturable clearance mechanisms (63). In phase II clinical trials the compound was shown to be efficacious in acute or chronic invasive aspergillosis in both neutropenic and nonneutropenic patients (64, 65), and in oropharyngeal candidiasis (66). Voriconazole has also been shown to be a suitable alternative to amphotericin B preparations for empirical antifungal therapy in patients with neutropenia and persistent fever (67).

3.2.8.2 ***Posaconazole and Ravuconazole.*** The *in vitro* activity of posaconazole (Schering-Plough) (Fig. 17.5) appears similar to that of voriconazole and ravuconazole (55, 61, 68). Currently in phase III clinical trials, the compound has been shown to be efficacious in a wide variety of infections in animal models, including those caused by rarer pathogens (69–74).

Posaconazole is subject to enterohepatic recirculation and is eliminated primarily by way of the bile and feces (75, 76). The compound exhibits high oral bioavailability in animal models (77), but its low solubility precludes convenient formulation for intravenous use. A program aimed at identifying a more soluble prodrug has identified SCH 59884, in which the hydroxyl group of posaconazole is derivatized as a butyryl ester bearing a y-phosphate, as a potential candidate (78, 79). This agent is inactive in vitro, but is dephosphorylated in *vivo* to produce the active 4-hydroxybutyrate ester of posaconazole. This, in turn, is hydrolyzed to the parent compound in human serum.

Ravuconazole (Esai/Bristol-Myers Squibb) has in vitro activity that is broadly similar in spectrum and potency to that of voriconazole and posaconazole (55, 80–82). Like posaconazole, the compound exhibits good oral bioavailability and a long serum half-life in both rats and dogs (83, 84) (in contrast, the half-life of voriconazole is significantly shorter). The activity of the agent in animal models of systemic and pulmonary infections attributed to Candida, *Cryptococcus*, and Aspergillus spp. has been demonstrated (83, 85, 86).

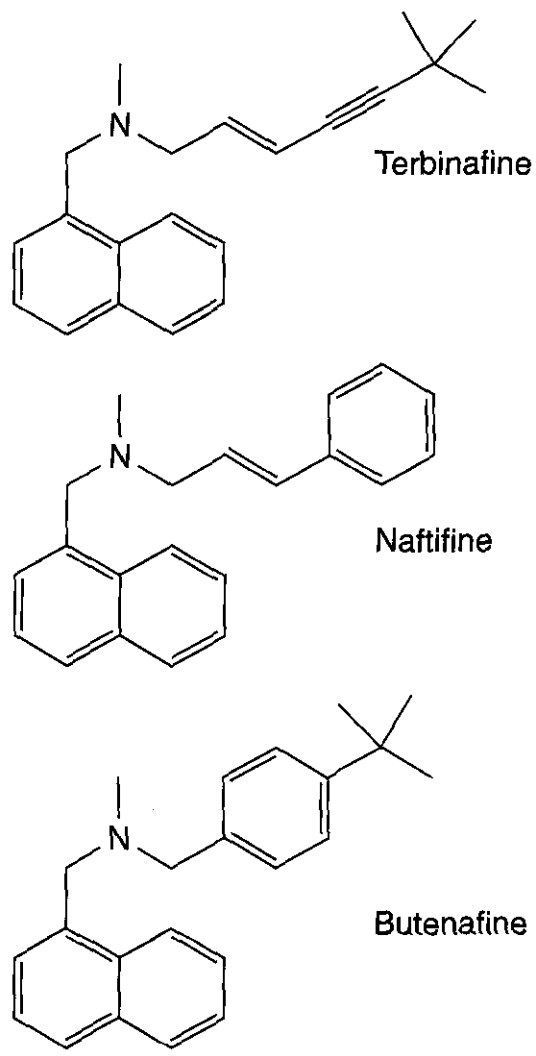


Figure 17.6. Allylamines.

3.3 Allylamines

3.3.1 Overview and Mode of Action. The allylamines are the most prominent of a number of antifungal classes (see also Section 3.5.1.1) that exert their activity by inhibition of squalene epoxidase; the intracellular accumulation of squalene that results is thought to be the primary cause of the fungicidal consequences of exposure to the drug (87). The predominant example of this class of antifungal agent is terbinafine (Fig. 17.6), which is one of the mainstays for the treatment of dermatophytosis. In the treatment of onychomycosis, in particular, it is safer, more efficacious, and requires shorter duration of therapy than griseofulvin (the previous standard, but inadequate, agent); its efficacy in this indication is enhanced by the propensity of the compound to accumulate in nails and hair. It has seen some use, particularly in combination with fluconazole, in the treatment of oropharyngeal infections arising from Candida spp. (88, 89). The other significant member of the class is naftifine, which is an older agent whose use has been in the topical treatment of ringworm (tinea cruris, tinea corporis) (90). Butenafine (91) is used for the same purpose.

The inhibition of squalene epoxidase by the allylamines is reversible and noncompetitive with respect to squalene, NADPH, and FAD (92), and the agents have no effects on other enzymes in the ergosterol biosynthetic pathway (93). In cell-free rat liver extracts, terbinafine has been shown to inhibit cholesterol synthesis, also by specific inhibition of squalene epoxidase, although the concentration required to do so is at least 1000-fold greater than that required in the analogous assay derived from fungal cells (93).

3.3.2 Structure-Activity Relationship. The moniker "allylamine" was originally applied to the class because the tertiary (*E*)-allylamine structural element was perceived to be essential for antifungal activity (94). In more recent studies, however, it has been shown that this feature can be replaced by a suitably substi-

tuted benzylamine, or by a homopropargylamine. Thus, the structural requirements for potent activity are represented in the broadest sense by two lipophilic domains linked to a central polar moiety by spacers of appropriate length; for good activity, the polar moiety is a tertiary amine, and one of the lipophilic domains consists of a bicyclic aromatic ring system such as naphthalene or benzo[*b*]thiophene (95).

3.3.3 Resistance. Clinical resistance to terbinafine has not yet been documented. However, it has been shown that inhibitors of CDR-type pumps in C. *albicans* can reduce the MIC to terbinafine (96); thus, the potential for the emergence of efflux-mediated resistance is established. Allylamines are widely used in the agrochemical context, and resistance has been documented in the corn pathogen *Ustilago* maydis (97), in which both decreased intracellular concentrations and decreased affinity for the target enzyme were implicated.

3.3.4 Side Effects. In general, the adverse events after oral terbinafine treatment are mild and transient. Rare cases of liver failure have occurred with the use of the compound; however, in the majority of the cases reported, the patients had an underlying hepatic condition.

3.3.5 ADME. Terbinafine is well absorbed (>70%) after oral administration, and the bioavailability of about 40% is attributable largely to first-pass metabolism. Nonetheless, after a single oral dose of 250 mg, peak plasma concentrations of only 1 μg/mL are achieved, despite >99% plasma protein binding. Such relatively low plasma concentrations are ascribed to the extensive distribution to the sebum and skin with a terminal half-life of 200–400 h, reflecting the slow elimination of the compound from the body. Terbinafine is extensively metabolized, although none of the metabolites observed to date possesses antifungal activity. Approximately 70% of the administered dose is eliminated in the urine.

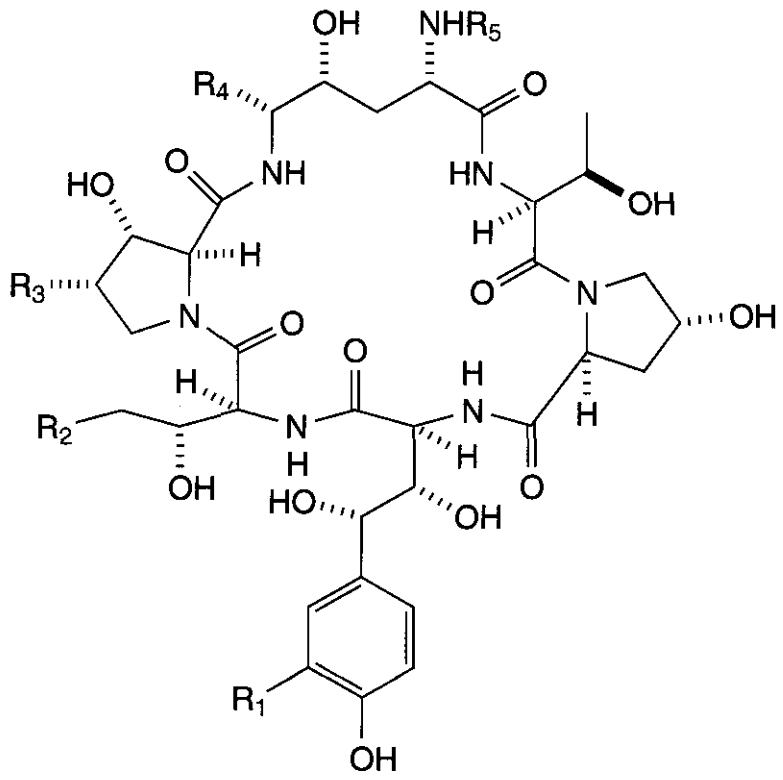
3.3.6 Drug Interactions. Drug-drug interactions conducted in healthy volunteers showed that terbinafine does not affect the clearance of antipyrine or digoxin but does decrease the clearance of caffeine by 19%. Terbinafine clearance is increased 100% by rifampin, a CyP450 enzyme inducer, and decreased by a factor of 3 by cimetidine, a CyP450 inhibitor.

3.3.7 Things to Come. The growing awareness of toenail onychomycosis should be a catalyst for the clinical use of terbinafine. Several large open-labeled, multicenter trials of terbinafine treatment have been conducted and continue to demonstrate the efficacy of the compound in the treatment of this particular infection (98). The potential utility of the agent in the treatment of systemic infections has also recently been explored (89).

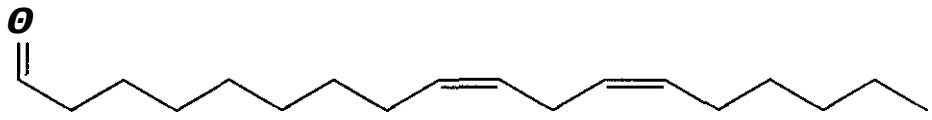
3.4 Candins

3.4.1 Overview and Mode of Action. Echinocandins, natural products discovered in the 1970s, and the related pneumocandins, discovered in the 1980s by researchers at Merck, are presumed to act as noncompetitive inhibitors of (1,3)-β-D-glucan synthase, an enzyme complex that forms glucan polymers in the fungal cell wall [16, 99, 100; see Section 1.5.2]. There are at least two subunits of this enzyme: one a catalytic subunit in the plasma membrane, the other a GTP-binding protein that activates the catalytic subunit. Analysis of various resistant mutants of S. cerevisiae led to the cloning of the echinocandin target gene *ETG1*. The gene encodes for a 215-kDa protein that contains 16 putative transmembrane domains. Whereas disruption of *ETGl* alone does not lead to lethality in S. cerevisiae, disruption of *ETGl* together with a homologous gene, *FKS2*, leads to fungal cell death (100).

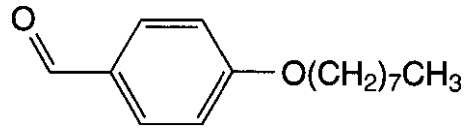
Derivatives of the echinocandins and pneumocandins have been explored, and three prominent analogs have emerged. One of these, Cancidas (caspofungin acetate, MK-0991), was approved in early 2001 for the treatment of refractory invasive aspergillosis in patients who do not respond or cannot tolerate other therapies such as amphotericin B, lipid formulations of the polyenes, and/or itraconazole. A summary of the pharmacology,



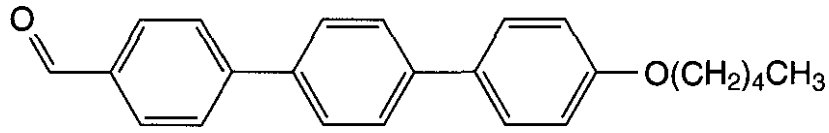
Echinocandin B: R_1,R_2 = H, R_3 = CH_3, R_4 = OH, R_5 =



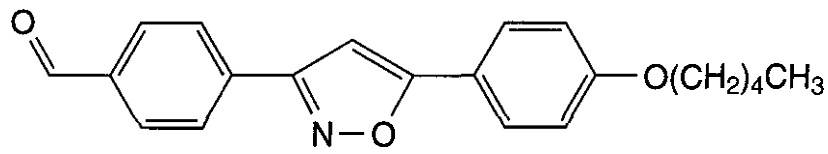
Cilofungin: R_1,R_2 = H, R_3 = CH_3, R_4 = OH, R_5 =



Anidulafungin: R_1,R_2,R_3 = H, R_4 = OH, R_5 =



Micafungin: R_1 = $OSO_3^-Na^+$, R_2 = $CONH_2$, R_3 = Me, R_4 = OH, R_5 =



Caspofungin: R_1,R_3 = H, R_2 = CH_2NH_2, R_4 = $NH(CH_2)_2NH_2$, R_5 =

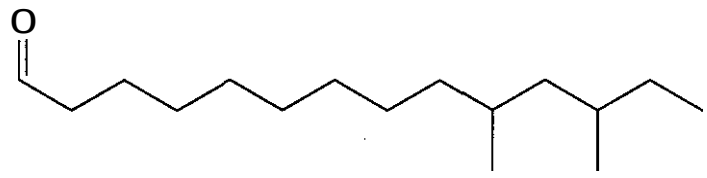


Figure 17.7. Notable candins.

pharmacokinetics, clinical efficacy, and adverse effects of caspofungin has been reported (101).

Caspofungin and the two related compounds in clinical development (anidulafungin and micafungin, see below) have potent activity against a variety of *Candida* and Aspergillus spp., including azole-resistant strains.

3.4.2 Structure-Activity Relationship. Echinocandin B (Fig. 17.7) is the archetype of the family of glucan synthase inhibitors. The structural features common to these lipopeptides include a cyclic peptide composed of six amino acids, one of which (an ornithine) is acylated at the a-N with a fatty acid containing 14–18 carbons. The peptide is cyclized by an aminal linkage connecting 3-hydroxy-4-methylproline to the δ-amino group of dihydroxyornithine. This group is readily cleaved at basic pH, resulting in ring opening and complete loss of antifungal activity.

The clinical limitations of echinocandin B include poor solubility, lack of oral bioavailability, and a propensity to lyse red blood cells

in vitro. Because chemical modifications implicated the lipophilic acyl tail in the lytic potential of echinocandin B, various analogs of the natural product were prepared in which the cyclic peptide nucleus was acylated with modified fatty acid tails. Antifungal activity was maintained in these compounds when a lipophilic tail of at least 12 carbon atoms in length was incorporated, with C_{18} unbranched fatty acids being optimal. Cilofungin (Fig. 17.7), echinocandin B with a *p*-octyloxybenzoyl side chain, was prepared in this fashion and chosen for clinical evaluation, but its development was halted because of its poor solubility, narrow spectrum of activity, and nephrotoxicity (thought to be attributed to the dosing vehicle).

A related analog (anidulafungin, LY303366, V-002), containing the echinocandin B nucleus with a terphenyl side chain with a tail of five carbon atoms, is in late-stage clinical development (102). Recent structure-activity relationship (SAR) studies with anidulafungin have focused principally on (1) improving the water solubility of the compounds by introducing phosphonate and phosphate ester prodrugs on the phenolic hydroxy group (103), and *(2)* addressing the instability of the compound under strongly basic conditions by incorporating nitrogen-containing ethers at the hemiaminal hydroxy group (104).

In general the echinocandins display poor intrinsic water solubility; however, the isolation of the natural product FR901379 was notable in that it was the first example of an intrinsically soluble echinocandin-like cyclic hexapeptide. Side-chain modification of this natural product led to the discovery of micafungin (FK-463), which is in late-stage clinical trials (105). The target compound was prepared by condensation of an active ester of 4-(5-(4-pentoxyphenyl)isoxazol-3-yl)benzoic acid with the cyclic peptide nucleus, obtained by enzymatic deacylation of the natural product.

The related pneumocandins differ in structure from the echinocandin B class of lipopeptides by the replacement of threonine with hydroxyglutamine, along with a modified proline in place of the 3-hydroxy-4-methylproline in the cyclic peptide nucleus. The glutamine residue provides a handle for chemical modification to improve both activity and solubility. The lipid tail of pneumocandin B_0, a component of the pneumocandins produced by the fungus Glarea lozoyensis, has 10,12-dimethylmyristate in place of echinocandin B's linoleoyl side chain. In contrast to echinocandin B, pneumocandin B_0 did not cause lysis of red blood cells. The aim of early medicinal chemistry focused on the improvement of solubility. One strategy employed a prodrug approach, the best candidate from which was the phosphate derivative of pneumocandin B_0, L-693,989 (106). In an effort to identify an intrinsically water-soluble and less labile analog, further chemical modification focused on the hemiaminal and the hydroxyglutamine residue (107). This effort ultimately afforded caspofungin (Fig. 17.7).

Various programs have focused on identifying new glucan synthase inhibitors that may overcome several of the problems associated with the echinocandins and pneumocandins. For example, cyclic amino hexapeptides of echinocandin B have been reported (108), as well as the discovery of novel classes of (1,3)-β-D-glucan synthase inhibitors (109).

3.4.3 Side Effects. Adverse effects after caspofungin treatment have been minimal and are typically related to histamine-mediated symptoms such as rash, facial swelling, and/or the sensation of warmth. A more complete side-effect profile of caspofungin will emerge as its use becomes more widespread.

3.4.4 ADME. Caspofungin is not orally bioavailable and is therefore administered parenterally. After a 1-h intravenous infusion, the compound is extensively distributed into tissues, and is slowly metabolized by phase I transformations such as hydrolysis and N-acetylation. Elimination of caspofungin and its metabolites occurs equally through the feces and urine; only a small percentage of the parent compound is detected in the latter.

3.4.5 Things to Come. As noted above there are two compounds in addition to caspofungin that are in late-stage clinical trials:

3.4.5.1 Anidulafungin (LY303366, V-Echinocandin, V-002). This agent is a pentyloxyterphenyl side-chain derivative of echi-

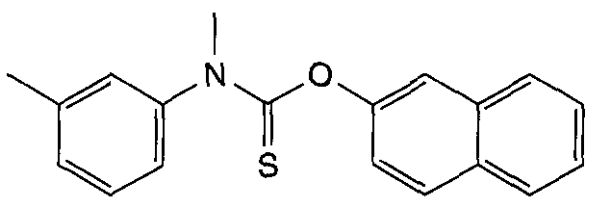
nocandin B (Fig. 17.7). The agent is in late-stage phase II and early phase III clinical trials (110). Like caspofungin, the compound is active *in vitro* against *Candida* and *Aspergillus* spp. (111) and its potent activity in animal models of disseminated candidiasis and pulmonary aspergillosis has been described (112–114). Anidulafungin is also active in animal models of esophageal candidiasis and aspergillosis using organisms that are resistant to fluconazole and itraconazole, respectively (115, 116). The pharmacokinetics of the compound in healthy and HN-infected volunteers after single-dose administration have been reported (117).

3.4.5.2* Micafungin *(FK-463). Micafungin (Fig. 17.7), like anidulafungin and caspofungin, has potent in *vitro* activity against a variety of *Candida* (MIC range ≤ 0.004–2 μg/mL) and *Aspergillus* (≤0.004–0.03 μg/mL) spp. (118–121), and is also active in a number of animal efficacy models (122–124). The compound has favorable pharmacokinetics and was well tolerated in a single-dose phase I study in healthy volunteers (125). In a phase II study in an AIDS population, micafungin was effective in improving or clearing the clinical signs and symptoms of esophageal candidiasis at 12.5, 25, and 50 mg once daily for up to 21 days (126). In addition, once-daily dosing for 14-21 days revealed no safety-related concerns.

3.5 Miscellaneous

3.5.1 Other Inhibitors of Ergosterol Biosynthesis

***3.5.1.1* Thiocarbamates.** The clinically most prominent class of antifungal ergosterol biosynthesis inhibitors not mentioned above are the thiocarbamates. Like the allylamines, these are reversible, noncompetitive inhibitors of squalene epoxidase (127), with inherent selectivity for the fungal enzymes over mammalian (128). The most significant member of the class is tolnaftate (Fig. 17.8). Unlike terbinafine, its spectrum is restricted primarily to dermatophytes; the lack of effect on *Candida* spp. is attributed to poor penetration of the cell envelope (128). Consequently, it is not used in the treatment of systemic disease and its pharmacokinetic properties dictate topical administration for the treatment of skin infections, such as athlete's foot, where cure rates are around 80% (cf. 95% for miconazole) (90).



Tolnaftate

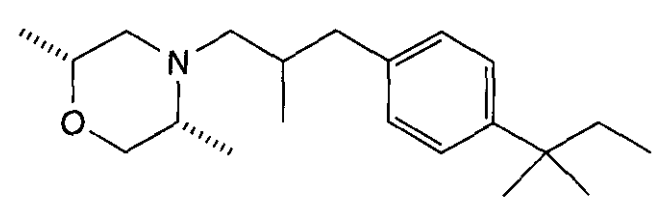
Figure 17.8. Tolnaftate.

***3.5.1.2* Morpholines.** Discovered in the 1970s, amorolfine (Fig. 17.9) has seen some use topically in the treatment of nail infections. Like fenpropimorph (Fig. 17.9), which is a prominent antifungal agrochemical, the compound inhibits both Δ^{14} reductase and Δ^{7}-Δ^{8} isomerase. Antifungal activity is attributable primarily to inhibition of the former, which is an essential enzyme in S. *cerevisiae* (129). The toxicity of amorolfine precludes its systemic use (127).

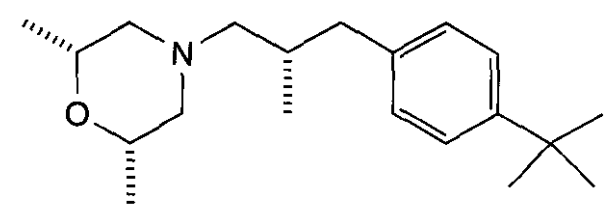
3.5.1.3* Other *Azoles. The structures of azoles listed in Table 17.3, but not discussed in the text, are shown in Fig. 17.10.

3.5.2 Flucytosine.

***3.5.2.1* Overview and Mode of Action.** Flucytosine (Fig. 17.11) is a fluorinated pyrimidine related to the anticancer agent fluorouracil. It was originally developed in 1957 as an antineoplastic agent, but reports in 1968 of its being used to treat candida and cryptococcal infections in humans led to its use as an antifungal agent. As with many other antifungal compounds, *in vitro* susceptibility testing of flucytosine has correlated poorly with



Amorolfine



Fenpropimorph

Figure 17.9. Amorolfine and fenpropimorph.

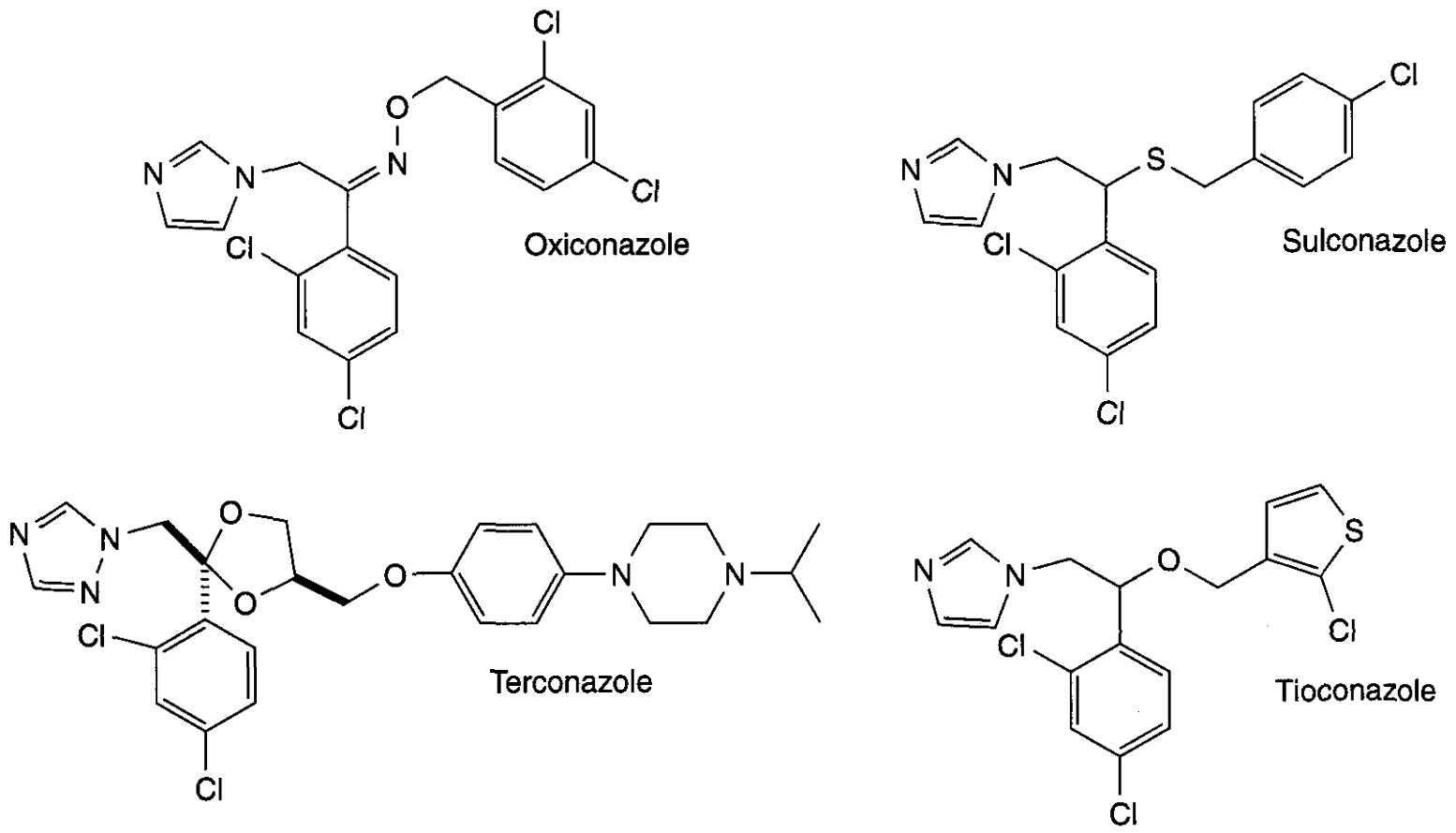


Figure 17.10. Other azoles for topical use.

clinical outcome. Therapeutically, flucytosine is used predominantly in combination with amphotericin B to treat cryptococcal meningitis and infections caused by Candida spp. In these cases, lower doses of amphotericin B can be used.

Susceptible fungi, such as Crytococcus *neoformans* and Candida spp., deaminate flucytosine by way of cytosine deaminase to 5-fluorouracil, which is ultimately converted into metabolites that inhibit DNA synthesis (Scheme 17.3). 5-Fluorouracil is also incorporated into fungal RNA, thereby disrupting transcription and translation. Selectivity is achieved because mammalian cells are unable to convert flucytosine to fluorouracil.

3.5.2.2 Resistance. Resistance to flucytosine is common and can be caused by the loss of the permease that is necessary for compound transport or decreased activity of either cytosine deaminase or UMP pyrophosphorylase (130). Strategies to prevent the emergence of resistant isolates have focused on maintaining sufficient drug concentration at the infection site as well as combining the compound with other antifungal agents such as amphotericin B.

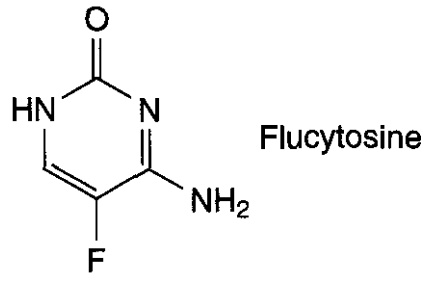
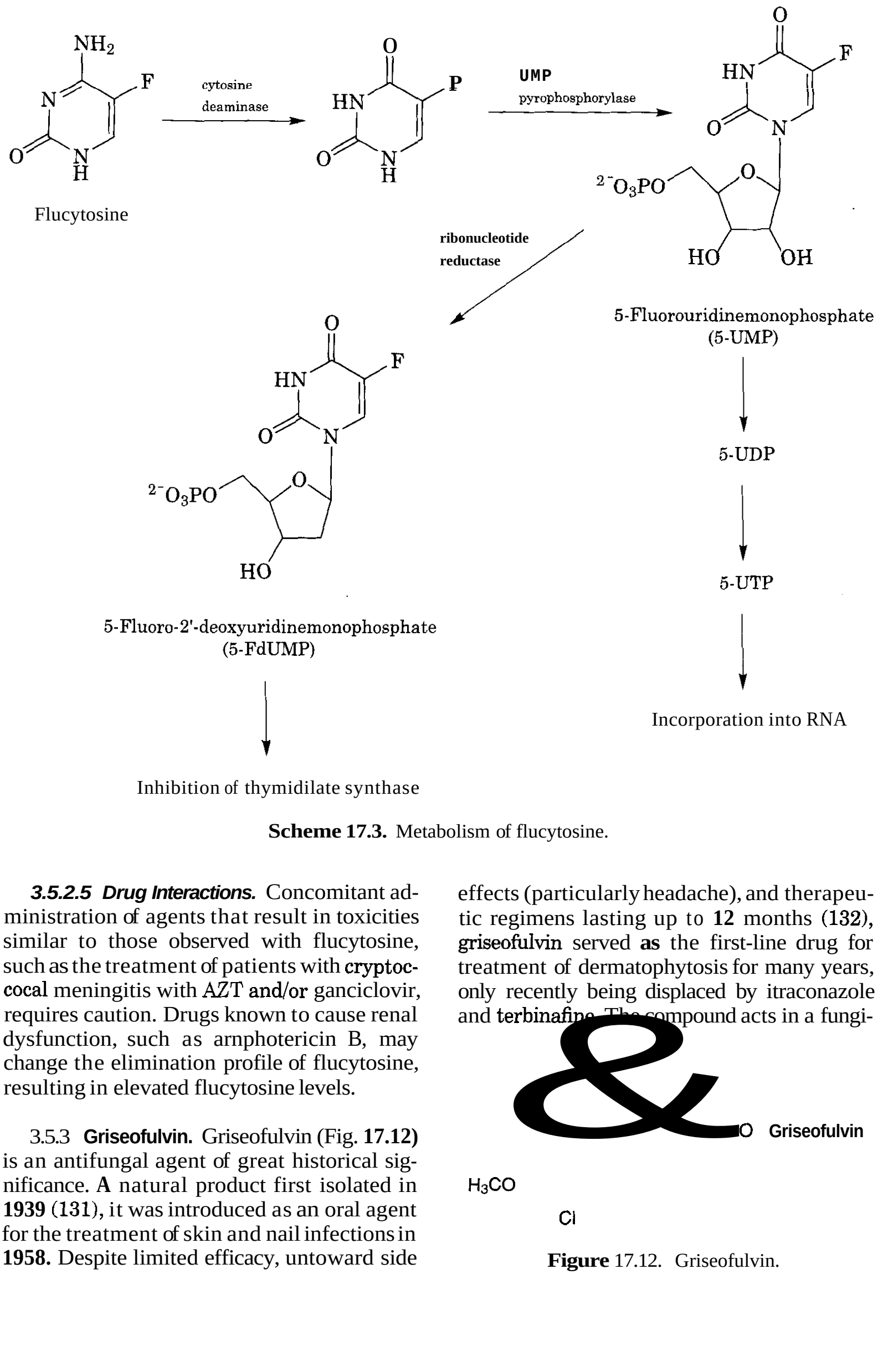


Figure 17.11. Flucytosine.

3.5.2.3 Side Effects. The most serious adverse effects associated with flucytosine therapy are hematological, manifested as leukopenia and thrombocytopenia. Patients that are more prone to this complication, such as those having an underlying hematologic disorder, require careful monitoring after treatment. The hematological toxicities are thought to result from the conversion of flucytosine to the antimetabolite 5-fluorouracil by bacteria in the host.

3.5.2.4 ADME. Approximately 80–90% of a dose of flucytosine is absorbed after oral administration. It is widely distributed in the body with a volume of distribution that approximates total body water. Flucytosine levels in the CSF are approximately 80% of the simultaneous serum levels. Approximately 80% of a given dose is excreted unchanged in the urine, with the half-life of the drug in healthy individuals ranging from 3 to 6 h.

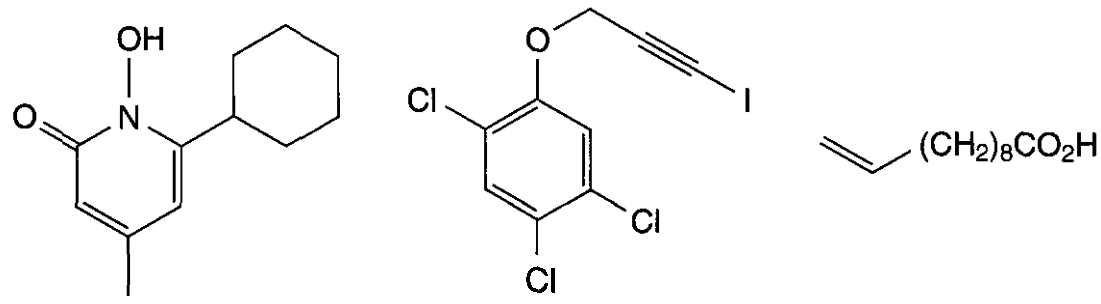


Scheme 17.3. Metabolism of flucytosine.

3.5.2.5 Drug Interactions. Concomitant administration of agents that result in toxicities similar to those observed with flucytosine, such as the treatment of patients with cryptococcal meningitis with AZT and/or ganciclovir, requires caution. Drugs known to cause renal dysfunction, such as arnphotericin B, may change the elimination profile of flucytosine, resulting in elevated flucytosine levels.

3.5.3 **Griseofulvin.** Griseofulvin (Fig. **17.12)** is an antifungal agent of great historical significance. **A** natural product first isolated in **1939** (131), it was introduced as an oral agent for the treatment of skin and nail infections in **1958.** Despite limited efficacy, untoward side effects (particularly headache), and therapeutic regimens lasting up to **12** months (132), griseofulvin served **as** the first-line drug for treatment of dermatophytosis for many years, only recently being displaced by itraconazole and terbinafine. The compound acts in a fungi-

Figure 17.12. Griseofulvin.



Ciclopirox Haloprogin Undecylenate

Figure 17.13. Other topical agents.

static manner by inhibiting the formation of microtubules in the process of cell division (133).

3.5.4 Other Topical Agents in Clinical Use. The structures of other agents referred to in Table 17.3, but not discussed in the text, are shown in Fig. 17.13. Ciclopirox is used as the ethanolamine salt and is a broad-spectrum agent with some antibacterial activity (134). Haloprogin (135, 136) also has antibacterial activity. Undecylenate is commonly marketed as the zinc salt.

3.5.5 Other Classes of Medicinal Interest. There are very many natural products and totally synthetic materials that have antifungal activity in uitro, but only a few in which the mode of action has been determined in sufficient detail to justify a focused medicinal development program. Those that have not been described above are discussed briefly in this section.

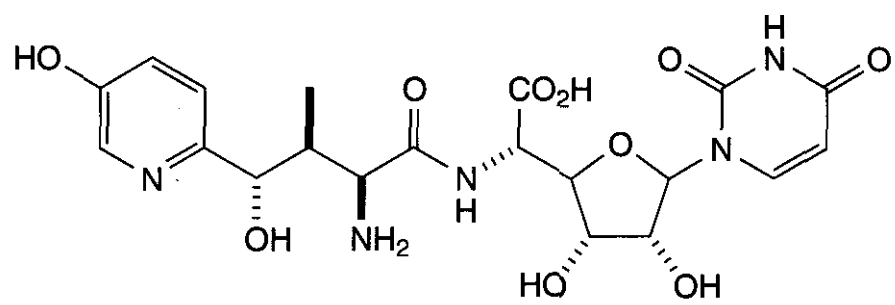
3.5.5.1 Polyoxins and Nikkomycins. These are naturally occurring nucleoside peptide antibiotics that inhibit chitin synthase, an enzyme that catalyzes the polymerization of *N*-acetylglucosamine, a major component of the fungal cell wall (see Section 1.5.2). A comprehensive review of synthetic efforts and subsequent biological studies on these agents has been reported (137).

Nikkomycin Z (Fig. 17.14) (138), the most advanced of these agents, has demonstrated additive and synergistic interactions with either fluconazole or itraconazole against C. *albicans* and C. neoformans in vitro (139). Marked synergism was also observed with nikkomycin Z and itraconazole against A. *fumigatus*. Nikkomycin Z is active against the less common endemic mycoses such as histoplasmosis, where pronounced synergistic interactions with fluconazole have been observed both in *vitro* and in *vivo* (140). An apparent drawback of nikkomycin Z is that it appears to inhibit only weakly the most abundant chitin synthase found in fungi.

A series of nikkomycin analogs has recently been prepared; among them, an analog containing a phenanthrene moiety at the terminal amino acid possessed potent antichitin synthase activity (141). A structurally distinct chitin synthase inhibitor, Ro-09-3143 (Fig. 17.15) arrests cell growth in C. albicans (142).

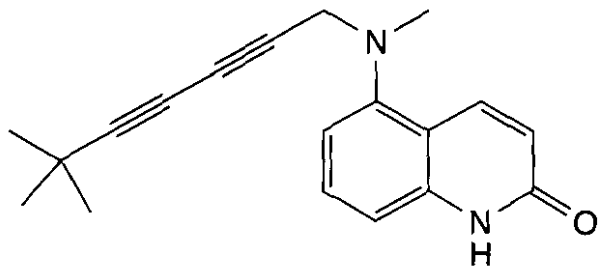
3.5.5.2 Aureobasidins. Aureobasidin A (Fig. 17.16), a cyclic depsipeptide produced by *Aureobasidium pullulan*, inhibits inositol phosphorylceramide synthase (IPC synthase), an enzyme essential and unique in fungal sphingolipid biosynthesis (143). Target modification and efflux have both been implicated as a mode of resistance to the compounds (144, 145).

The syntheses of Aureobasidin A and several related cyclopeptide derivatives have been reported (146). Aureobasidin derivatives with modifications at amino acid position 6, 7, or 8 were prepared as part of a study to elaborate the SAR of the natural product. Whereas



Nikkomycin Z

Figure 17.14. Nikkomycin Z.



Ro-09-3143

Figure 17.15. Ro-09-3143.

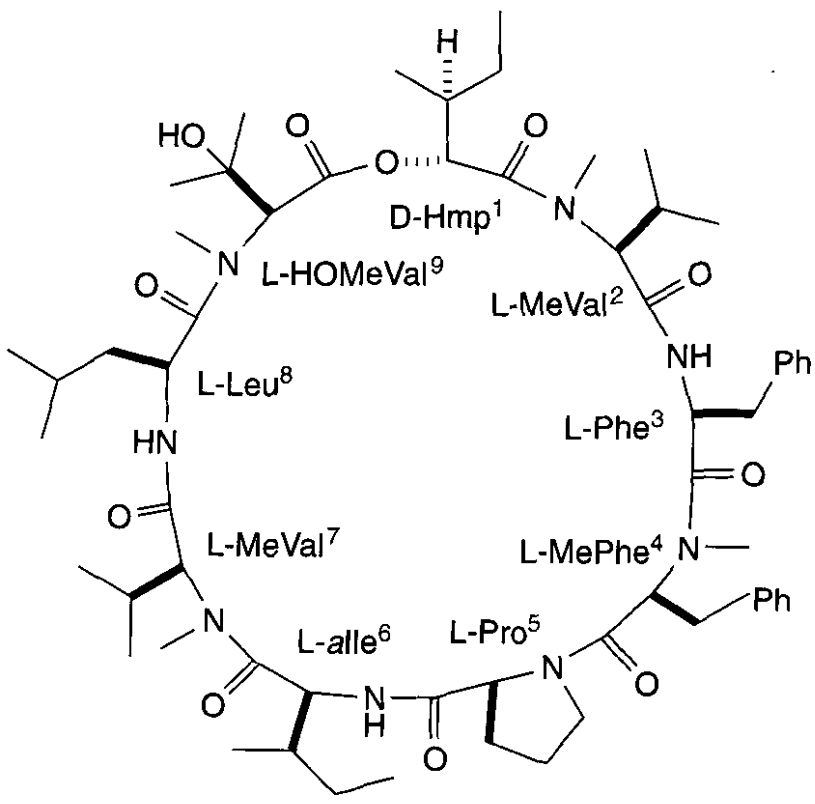
analogs having L-glutamic acid at position 6 or 8 showed weak activity, esterification of the y-carboxyl group with benzyl or shorter alkyl (C_4–C_6) alcohols significantly enhanced the potency (147). Introduction of a longer C_{14} alkyl chain resulted in total loss of antifungal activity. SAR for the inhibition of the ABC-transporter P-glycoprotein (P-gp) by various aureobasidins has recently been disclosed. Although several compounds demonstrated potent inhibition of this efflux pump, the P-gp activity and the SAR for antifungal activity were not correlated (148).

3.5.5.3 Sordarins. Both fungal and mammalian cells require two proteins, elongation factors 1 (EF1) and 2 (EF2), for ribosomal translocation during protein synthesis. Fungi also have a third elongation factor, EF3, which is not present in mammalian cells, and as such is a potential target for antifungal drug development. A family of selective EF2 inhibitors, derived from the tetracyclic diterpene glycoside natural product sordarin (Fig. 17.17), has been identified and shown to possess activity in vitro against a wide range of pathogenic fungi, including Candida spp., *Cryptococcus* neoformans, and P. carinii (149–153). Target modification has been implicated as a mode of resistance to the sordarins (151).

Using a sordarin derivative, the localization of EF2 in the 80S ribosome fraction of Saccharomyces cerevisiae has been determined (154). In addition, the presumed binding site of sordarin in C. albicans has been identified by use of a photaffinity label of a sordarin derivative (155).

In *vivo*, sordarin derivatives have shown efficacy against systemic infections in mice caused by fluconazole-sensitive and -resistant Candida albicans, with ED_{50} values ranging from 10 to 25 mg/kg (156). The activities of the sordarins in experimental models of aspergillosis and pneumocystosis have also been reported (157). The toxicological properties of the new sordarin derivatives have been evaluated in several in vitro and in vivo preclinical studies (158, 159). Overall, the compounds have demonstrated no evidence of genotoxicity in the Ames test, are not clastogenic in cultured human lymphocytes, and are well tolerated in rats and dogs.

Modification of the sugar unit affords the tetrahydrofuran derivative GM 237354 and structurally related analogs (150). Researchers at Merck have also reported the prepara-



Aureobasidin A

Figure 17.16. Aureobasidin A.

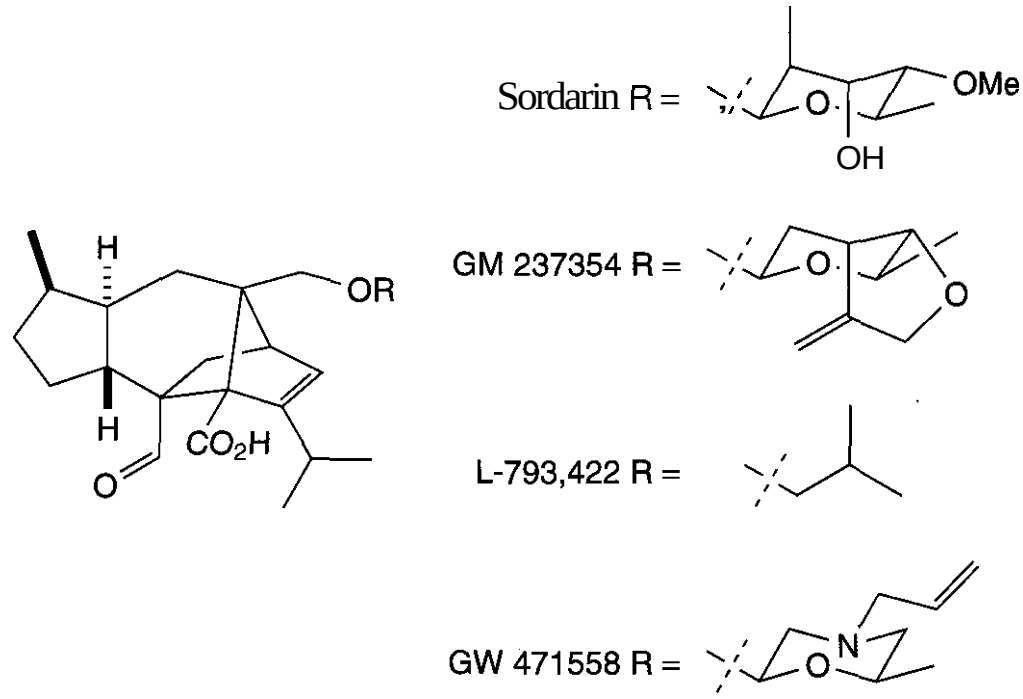


Figure 17.17. Sordarin and notable derivatives.

tion and evaluation of a variety of alkyl-substituted derivatives such as L-793,422 (160). Compounds of this type clearly demonstrate that a certain degree of lipophilicity on the side chain is important for optimal antifungal activity. Biological studies have confirmed that L-793,422 and GM 237354 share the same mode of action (161).

An enantio-specific synthesis of the monocyclic core of sordarin has been achieved through the conversion of (+)-3,9-dibromocamphor into a 1,1,2,2,5-penta-substituted cyclopentane bearing all of the key functionalities present in the natural product (162). In addition, preliminary reports describing the SAR of the sordarin class of antifungal agents have recently appeared (163,164).

Recently, efforts have been directed toward the identification of new sordarin agents that display improved activity against *Candida* spp. other than *C. albicans,* and that possess improved pharmacological properties such as increased efficacy and decreased toxicity. To that end, a new class in which the traditional sugar moiety is replaced by a 6-methylmorpholin-2-yl group with N-4' substituents, known as the azasordarins, has been identified. These compounds, highlighted by GW 471558 (Fig. 17.17), have the advantage of beingeasier to synthesize from fermentation-derived starting materials than from the parent class (165,166).

3.5.5.4 Pradimicins and Benanomycins. These compounds are dihydrobenzonaphthacene quinones conjugated with a D-amino acid and a disaccharide side chain (167). They bind to cell wall mannoproteins in a calcium-dependent manner that causes disruption of the plasma membrane and leakage of intracellular potassium. Spectroscopic studies on the interaction of BMS 181184 (Fig. 17.18), a water-soluble pradimicin derivative, suggest that two molecules of the compound bind one Ca^{2+} ion, and each compound binds two mannosyl residues (168,169). BMS 181184 possesses activity toward *Aspergillus* spp. *in vitro,* but is less potent than itraconazole or amphotericin B (170). In a model of invasive pulmonary aspergillosis in persistently neutropenic rabbits, daily doses of 50 and 150 mg/kg of BMS 181184 were as effective as amphotericin B at 1 mg/kg/day (171).

3.5.5.5 N-Myristoyl Transferase Inhibitors. N-myristoyl transferase (NMT) is a cytosolic enzyme that catalyzes the transfer of myristate from myristoylCoA to the N-terminal glycine amine of a variety of eukaryotic proteins, thereby facilitating protein-protein or protein-lipid interactions involved in intracellular signal transduction cascades. The enzyme has been shown to be essential for the viability of both *C. albicans* and *Cryptococcus neoformans* (172,173).

An approach to inhibit NMT by exploiting the peptide binding site has been reported. Remarkably, it proved possible to mimic four terminal aminoacids (ALYASKLS-NH_2) of a weak octapeptide inhibitor of the substrate by use of an 11-aminoundecanoyl motif. Initial optimization of this lead gave a highly potent

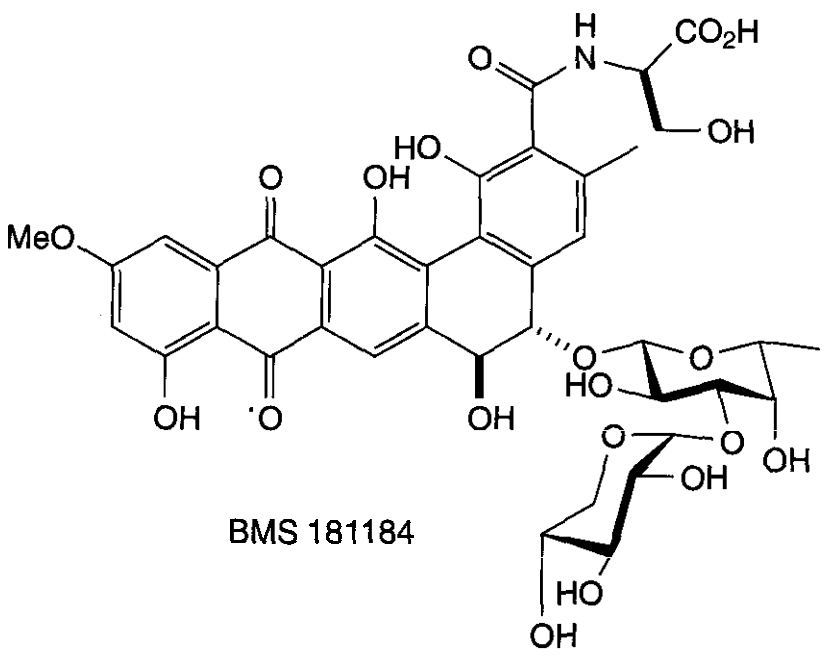


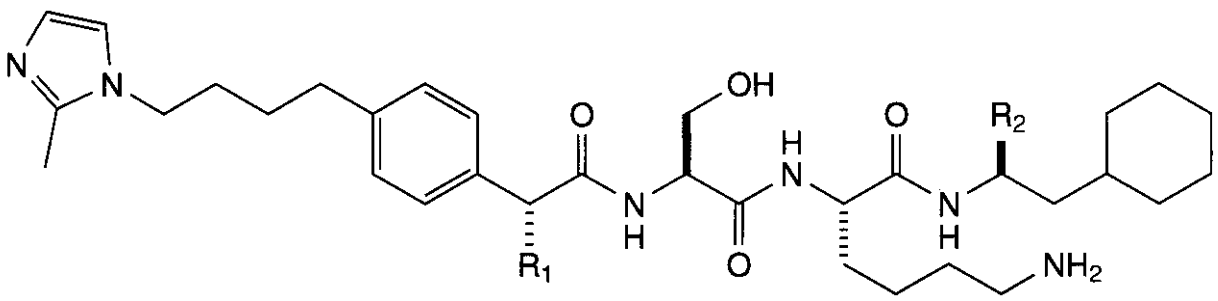
Figure 17.18. BMS **181184.**

and selective agent **(1,** Fig. 17.19), which was shown to be a competitive inhibitor of *C. albicans* NMT with respect to the octapeptide substrate GNAASARR-NH„ with a $K_{i(app)}$ value of 70 n*M*, and to exhibit 400-fold selectivity over the human enzyme (174). However, no whole-cell activity was observable. Addition of a second carboxylic acid (2) ameliorated the potency against the enzyme, but gave a compound with weak fungistatic activity, as did replacement of the remaining peptidic residues (**3**) (175). The crystal structure of an inhibitor in this series bound to NMT from *S. cerevisiae* has been published (176).

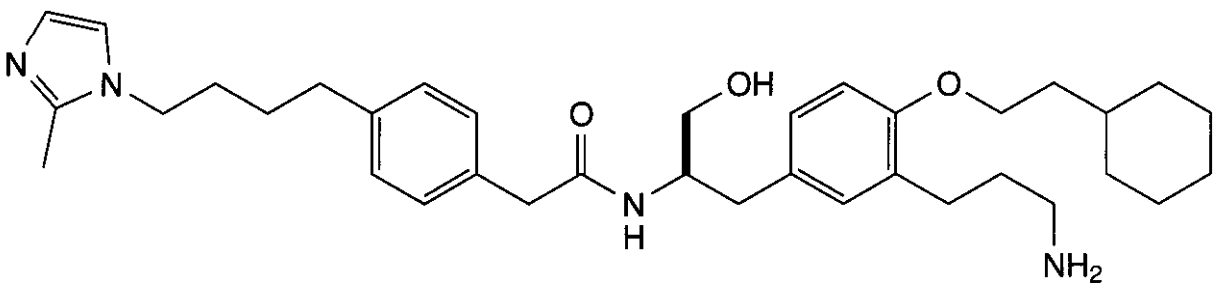
Two alternative structure-based drug design approaches, based on a lead identified through high throughput screening, have also been reported. In the first, a compound with weak but selective activity (4, Fig. 17.19; IC_{50} = 0.98 μM; 200-fold selectivity over the human enzyme) (177) was refined to afford highly potent agents with activity against *C. albicans* both *in vitro* and in a rat model of systemic candidiasis (5, **6)** (178). Like compounds 1–3, these inhibitors interact with the key carboxylate residue (Leu451) implicated in the acyl transfer reaction.

In the second structure-based drug design approach (179, 180), early leads based on screening hits that were competitive with the peptide substrate (e.g., 7, Fig. 17.19; IC_{50} = 0.5 μM) were potent enzyme inhibitors but lacked whole-cell antifungal activity. Based on the premise that this was attributed to excessive hydrophilicity, lipophilic substituents were added to the primary amino group. This effort culminated in the identification of a highly potent compound (8; IC„ = 86 n*M*; *C. albicans* MIC 0.09 μg/mL) that was also shown to be fungicidal. A surprising lack of activity against *Aspergillus fumigatus* was attributed to a single change (Phe to Ser) in the binding pocket of the benzothiazole-carboxamide, and this was corroborated by site-directed mutagenesis studies in *Candida*.

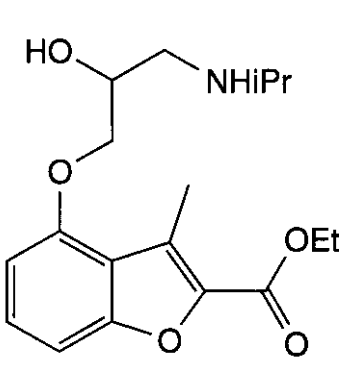
3.5.5.6 Fungal Efflux Pump Inhibitors. It will be apparent from the above discussions on resistance that the inhibition of efflux pumps in pathogenic fungi would be expected to have a significant effect on the susceptibility of several clinically problematic *Candida* spp. toward several classes of antifungal drugs. The first reports of inhibitors of ABC-type pumps in *C. albicans* and *C. glabrata* have recently appeared (181). The agents lack antifungal activity and were characterized by their ability to increase intrinsic susceptibility to known pump substrates (azoles, terbinafine, rhodamine 6G), but not to agents not subject to efflux (amphotericin B). In a fluorescence assay, the compounds were shown to increase intracellular accumulation of rhodamine 6G. Such compounds can reverse CDR-mediated azole resistance in *C. albicans* (64- to 128-fold reduction in MIC of fluconazole or posaconazole) and reduce intrinsic resistance in *C. glabrata* (8-to 16-fold reduction in MIC). A representative of the class, milbemycin a-9 (MC-510,027, Fig. 17.20), was shown to dramatically reduce the MIC_{90} of a broad panel of clinical isolates of *Candida* (182).



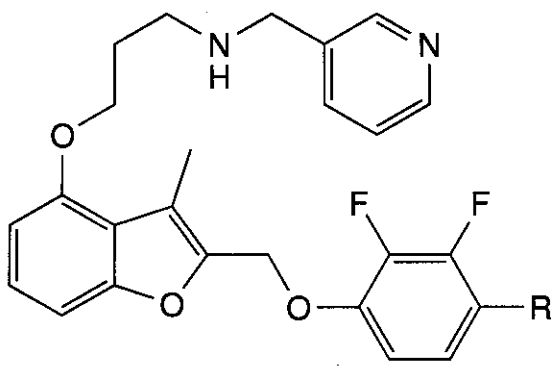
(**1**), R_1 = Me, R_2 = H; (**2**), R_1 = H, R_2 = CO_2H



(**3**)

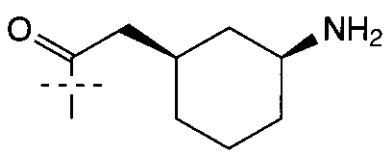


(**4**)



(**5**), R = H; (**6**), R = F

(**7**), R =



(**8**), R =

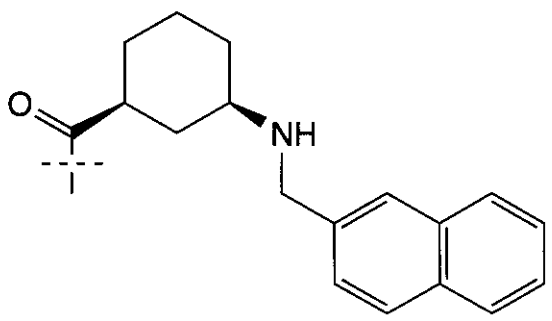
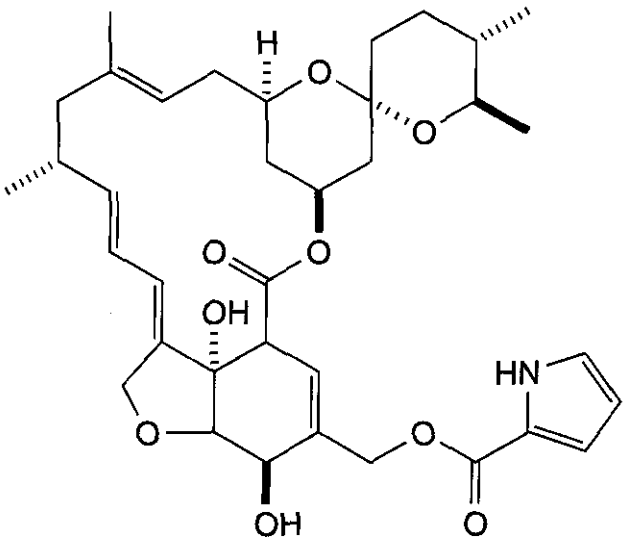


Figure 17.19. NMT inhibitors.

4 NEW TRENDS IN ANTIFUNGAL RESEARCH

The sequencing of microbial genomes is revolutionizing the discovery of novel antifungal drugs, providing the tools for the rational identification of novel targets and compounds. High throughput genomic sequencing, combined with fragment assembly tools, has delivered **a** cornucopia of sequence information to assist in the search of new targets.



MC-510,027

Figure 17.20. MC-510,027.

Genomic information, combined with the ability to selectively delete or modify genes of interest, is proving useful in evaluating the selectivity of a target and its essentiality for growth. In addition, comparative genomics allows the identification of potential targets shared across fungal species. For instance, entire biochemical pathways can be reconstructed and compared in different pathogens. Sequence comparisons may also provide some indication of potential mammalian toxicity if proteins of similar sequence exist in mammalian sequence databases.

Numerous databases. available over the Internet and easily downloaded onto local servers, are now available that contain both sequence and functionality information. In addition. certain commercial databases are available for nonexclusive use by commercial subscribers.

Overall, the genomic revolution is expected to have a profound impact on antifungal drug discovery, with the potential for the identification of new agents with novel mechanisms of action **(183).**

5 WEB SITES AND RECOMMENDED READING

5.1 Web Sites

A number of very informative Web sites serve as excellent starting points for further information on various aspects of the material covered in the chapter. The following are good sources for general information:

- http://www.doctorfungus.org/ This site is maintained principally by clinicians and others at the University of Texas Medical School in Houston, and is an excellent and authoritative source, particularly on clinical and microbiological aspects of fungal disease. The site also contains links to the manufacturer's Web sites for clinically significant antifungal agents.
- http:/lwww.aspergillus.man.ac.uk/This site is associated with the center of clinical expertise in systemic aspergillosis at the University of Manchester, UK, and is designed to provide information on pathogenic ***Aspergilli*** for clinicians and scientific researchers.
- http://www.mic.ki.se/Diseases/cl.html This site, based in Sweden's Karolinska Institute, is an excellent source of background information on both bacterial and fungal diseases, and contains many links to other informative sites.
- http://genome-www.stanford.edu/Saccharomyces/ This is a scientific database of the molecular biology and genetics of the yeast ***Saccharomyces cerevisiae*** (baker's yeast), which serves as a genetically manipulable surrogate model for pathogenic organisms. It contains many links to other sources of proteomic and biochemical information concerning this organism.

5.2 Other Texts

Several recent reviews on clinical aspects of fungal disease and chemotherapeutic options

are available, and are cited at particularly relevant points above. In particular, the reader's attention is drawn to the following texts:

- V. L. Yu, T. C. Merigan, and S. L Barriere, Eds., *Antimicrobial Therapy and Vaccines*, Williams & Wilkins, Baltimore, 1999. This book provides comprehensive information on clinical and microbiological aspects of antifungal chemotherapy.
- J. Sutcliffe and N. H. Georgopapadakou, Eds., *Emerging Targets in Antibacterial and Antifingal Chemotherapy*, Chapman & Hall, London, 1992. Although nearly a decade old, this book contains a series of chapters that concisely cover the clinical context and, particularly, biochemical aspects of current antifungal agents.

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CHAPTER EIGHTEEN

Antimalarial Agents

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Contents

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"If we take as our standard of importance the greatest harm to the greatest number, then there is no question that malaria is the most important of all infectious diseases (1)."

"Ah, poor heart! he is so shaked of a burning quotidian tertian, that it is most lamentable to behold (2)."

1 INTRODUCTION

Malaria is one of the most serious, complex, and refractory health problems facing humanity this century. Some 300–500 million of the world's people are infected by the disease, presenting over 120 million clinical cases annually. It is estimated that between 1.5 and 2.7 million people die from malaria every year, either directly or in association with acute respiratory infections and anemia, and up to 1 million of those deaths are among children younger than 5 years old. Malaria is a leading cause of morbidity and mortality in the developing world, particularly in tropical Africa, and it remains an outstanding tropical disease control priority.

1.1 The Disease

Human malaria is caused by four species of protozoan parasites of the Plasmodium genus. These are Plasmodium falciparum, P. *vivax*, P. *ovale*, and P. malariae, each of which presents slightly different clinical symptoms. P. falciparum is the most widespread of the four geographically and the most pernicious, causing the majority of malaria-related morbidity and mortality. Other Plasmodia species specifically infect a variety of birds, reptiles, amphibians, and mammals.

Parasites are transmitted from one person to another by an insect vector, the female anopheline mosquito. In most malarious areas, several species are able to transmit the parasite and the exact species responsible vary from region to region. Male mosquitoes do not transmit the disease. These mosquitoes are present in almost all countries in the tropics and subtropics, and they bite during nighttime hours, from dusk to dawn. It has been demonstrated that transmission can occur from transfusion of infected blood or from mother to child in utero, although these instances are very rare when compared with mosquito inoculation. The parasites develop in the gut of the mosquito and are passed on in the saliva when an infected mosquito bites a person. Uninfected mosquitoes become infected by taking a blood meal from an infected human. The parasites are carried by the blood to the victim's liver. After 9–16 days, the parasites have multiplied greatly and then return to the blood and penetrate the red cells. Inside erythrocytes, the parasite begins its replication cycle, a cycle of differing duration depending on the infecting species. Rupture of the infected erythrocyte and release of the merozoites begins a new cycle of red cell infection and parasite replication.

The signs and symptoms of malaria illness are variable, but most patients experience fever. Other symptoms often include headache, back pain, chills, muscle ache, increased sweating, malaise, nausea, and sometimes vomiting, diarrhea, and cough. Early stages of malaria may resemble the onset of the flu. Between paroxysms, the patient may remain febrile or may become asymptomatic. Early in an infection, the cyclic patterns of fever may not be noticeable, but later, a clear cyclic trend with symptoms recurring at regular intervals occurs. Of the four species of parasite, only falciparum malaria can progress rapidly to the cerebral stage, where infected red cells obstruct the blood vessels in the brain. Cerebral malaria is a medical emergency best managed in an intensive care unit. Untreated cases can progress to coma, renal failure, liver failure,

pulmonary edema, convulsions, and death. Although infections with P. *vivax* and *P. ovale* often cause less serious illness, parasites may remain dormant in the liver for many months, causing a reappearance of symptoms months or even years later.

Malaria is diagnosed by the clinical symptoms and by microscopic examination of the blood. Stained thick and thin blood smears are used to diagnose malaria and to quantify the level of parasitemia. Giemsa-stained thin smears are used to differentiate between the species of parasite. Clinical symptoms are an inaccurate means of diagnosis by themselves, although in the absence of adequate laboratory facilities, as is the case in many malarious regions, it is the only means available. Malaria can normally be cured by antimalarial drugs. The symptoms quickly disappear once the parasites are killed. The standard measures of clinical antimalarial drug efficiency are fever clearance time and parasite clearance time. In certain geographic regions, however, the parasites have developed resistance to antimalarial agents, particularly chloroquine. Patients in these areas require treatment with newer, often more expensive drugs.

In the last several years, it has been noted that microscopic examination of blood is an inadequate method for detecting low levels of parasitemia. The lack of sensitivity seldom affects treatment and diagnosis in acute cases but it does limit understanding of the degree to which malaria is chronic. In an endemic area, polymerase chain reaction studies revealed that more than 90% of the exposed population at any one time was chronically infected with P. falciparum **(3).**

In treating malaria, curing patients is often difficult to define. The relief of symptoms of a malaria attack is a "clinical cure." Should parasites remain, even after symptoms have resolved, either in blood cells or in liver tissue, then recrudescences and/or relapses may result in the re-establishment of the infection. A "radical cure" is when the parasites are completely eliminated from the body so that relapses cannot occur. Obviously, a radical cure is the ideal therapeutic endpoint.

The choice of antimalarial agent(s) for treatment in each particular case is determined by a multiplicity of factors including the parasite species causing the infection, the acquired immune status of the patient, the susceptibility of the parasite strain to antimalarial agents, the facilities and resources available for health care, and the genetic make-up of the patient. Rapid onset and a relatively long duration of antimalarial action to cover three to four parasite life cycles are deemed essential for radical therapy. During pregnancy, women are at high risk of death from falciparum malaria. Also at risk are children who are prone to severe attacks until they develop partial immunity. Non-immune travelers to malarious areas are similarly vulnerable.

1.2 The Parasite

The four species of human malaria parasites are evolutionarily, morphologically and clinically distinct. P. *vivax*, P. malariae, and P. *ovale* are closely related on an evolutionary basis to a number of simian malarias. When comparing small subunit ribosomal RNA gene sequences, P. *vivax* is closer to P. fragile, a parasite of toque monkeys, than to either P. *ovale* or P. malariae. It has been suggested that P. *malariae* was derived from a West African chimpanzee malaria. And a plasmodium of New World monkeys, P. brasilianum, may in fact be P. malariae that has adapted to a new host over the last few hundred years. These parasites most likely arose alongside the primate hosts an estimated **30** million years ago. P. falciparum seems more closely related to avian malarias and is of more recent origin (4, 5), perhaps corresponding to the rise of agriculture (6).

Recently, a new species, dubbed P. *vivax*-like parasite, has been described that infects humans (7). Its morphological characteristics show it to be similar to P. *vivax*, but analysis of its DNA indicates that the sequence for the circumsporozoite protein (CS) gene is quite different from that of P. *vivax*. Rather, its CS gene seems to be identical to that of a parasite isolated from toque monkeys, P. *simiovale*. The CS protein is the major surface protein of the sporozoite stage of the parasite and has been studied as a source of malaria vaccine antigens.

The life cycle of the parasite in both mosquitoes and humans is complex (Fig. 18.1) as is

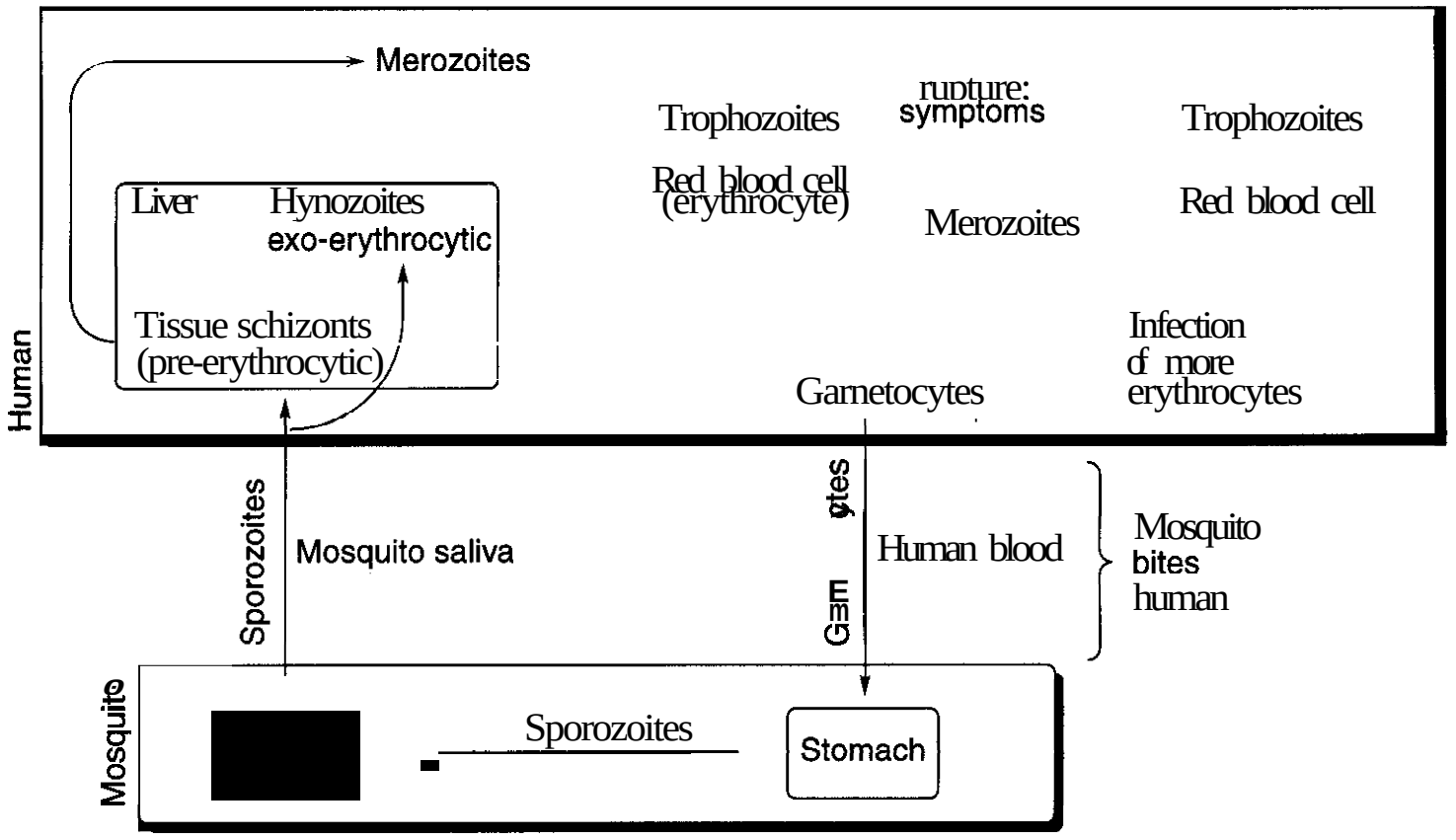


Figure 18.1. Plasmodia Life-Cycle.

the terminology of the various parasite development stages (8).When an infected mosquito bites, **sporozoites** are injected into the blood stream of the human victim and then travel to liver tissue where they invade **parenchymal** cells, a process involving receptor ligand mediated adhesion. During development and multiplication in the liver, known as the **pre**-erythrocytic stage, the host is asymptomatic. After a variable period of time, 5–7 days for **falciparum**, 6–8 days for **vivax**, 9 days for ovale, and 12–16 days for **malariae**, **merozo**ites (5000–40,000 per **sporozoite**) are released from the liver and the parasites take up residence in the red blood cells (erythrocytic stage) again by way of a receptor mediated process. In **non-relapsing** malarias (**falcipa**rum and malariae), no parasites are left in the liver; the infection moves entirely into the blood stage. In relapsing malarias, some of the **merozoites** (or **sporozoites**) differentiate into a dormant non-dividing stage (hypnozoites), providing a reservoir of parasites in the liver that can be activated for up to 5 years after the initial infection. Invasion of the red cell by a **merozoite** results in the development of the **trophozoite** stage. The parasite feeds on the protein portion of hemoglobin and a waste product, hemozoin, accumulates in the host cell cytoplasm. After the parasite undergoes nuclear divisions, the erythrocyte bursts and **merozoites**, parasite waste, and cell debris are released. The presence of the debris is the cause of the episodes of fever and chills associated **with** malaria. The **merozoites** released by the red cell rupture go on to infect more erythrocytes. Time intervals between cell rupture (fever), infection of other erythrocytes, and then their rupture (new bouts of fever) **are** characteristic of the parasite species. **A** few **merozoites** become differentiated into male and female gametocytes, forms that are dormant in humans. When a mosquito takes a blood meal from the infected human, the gametocytes begin sexual reproduction in the digestive track of the mosquito. Ultimately, **sporozoites** form and reside in the mosquito saliva, ready for a new round of infection.

In discussing malaria, the terms recrudescence and relapse are used to described the return of disease symptoms from different reservoirs of residual parasites. When a patient has been symptom-free for a period of time greater than the usual periodicity of the paroxysms and then clinical **symptoms** of malaria return, the situation is termed "recrudescence" if the re-established infection is a result of **surviving** erythrocytic forms of the parasite. If symptoms reappear because of the continuing presence of parasites in liver tissue, the

term "relapse" is used. Because only P. vivax and P. ovale have hypnozoites that reside in the liver, these are the true relapsing malarias.

Plasmodium vivax, benign tertian malaria, or simply tertian malaria, presents acute symptoms that recur every second day; each erythrocytic cycle is completed within 41–45 h. Infections with P. *vivax* were termed benign, or uncomplicated, in distinction from the severe morbidity associated with P. *falciparum* infections. The pathology of vivax infections is almost invariably transient and with no detectable tissue-damaging consequences, and vivax parasites do not sequester to any detectable degree at any stage in their development. As a true relapsing malaria, vivax malaria is characterized by a prolonged or secondary tissue development stage that can remain dormant for very long periods in the liver. Relapse can occur months or even years after clearance of the initial blood-stage infection and different strains have characteristic periods between primary infection and late relapses. This allows vivax to remain endemic in areas that experience cold winters with periods of no chance for transmission by mosquito. Treatment with drugs that kill only the erythrocytic forms of the parasite will not effect a radical cure of a vivax infection. Vivax is widely spread geographically with the notable exception of tropical Africa. This has been explained by the absence of a red cell surface antigen in most black Africans that vivax requires for cell penetration (9, 10).

Plasmodium ovale is much less widespread than the other three species, with patchy distributions where it does occur in tropical Africa and in islands of the western Pacific. A relapsing malaria that is rarely the cause of morbidity even in children, its features are similar to that of vivax with an erythrocytic cycle of 49–50 h. Ovale and vivax overlap very little geographically. P. vivax and P. ovale infect only young erythrocytes. The fever of P. malariae or quartan malaria recurs every third day or 72 h and is noted for persistent reappearance of symptoms. Even so, it is not a true relapsing malaria; P. malariae has extremely long-lasting erythrocytic forms that can persist in an infected host for decades at very low parasite densities. Mature red blood cells are the target of *P.* malariae infection. Its geographic distribution is broad but irregular.

The three species of malaria above cause a comparatively mild form of the disease. Given the selectivities for age of the erythrocyte infected, the degree of total parasitemia is limited. Red cells are destroyed in the peripheral capillaries and anemia results. Although in non-immune children and travelers these symptoms can be quite severe, the global incidence of mortality from vivax, ovale, and malariae is extremely small. The possibility of relapse or deep recrudescence, however, makes the treatment and monitoring of patients critical.

In contrast, P. falciparum, subtertian malaria, or tropical malaria can lead to serious and life-threatening conditions when untreated. Erythrocytes of all ages can become infected by P. falciparum, and thus, a high percentage of red cells can become parasitized. The severe pathology associated with falciparum malaria involves the adhesion of parasitized erythrocytes to the capillary endothelium. The cytoadherence seems to involve parasite-derived proteins that are presented in the membranes of infected red cells (10, 11). The resulting sequestration in the post-capillary microvasculature leads to nearly continuous aggravation of the endothelial tissue in every organ affected and often irreversible tissue damage. Microcirculatory arrest occurs and when this happens in the brain, the condition is termed cerebral malaria; delirium, coma, convulsion, and death may ensue. Falciparum malaria is characterized by erythrocytic cycles of 48 h, but it does not relapse because it forms no hypnozoites in the liver. It is the most serious form of the infection, most widespread geographically, and accounts for the vast majority of malaria deaths. In areas of intense transmission, persons may be infected by more that one of these species at a time, causing complications in treatment.

Humans are not the only vertebrates that are parasitized by Plasmodia species. Several other parasites and their hosts have been used extensively for research in malaria. P. berghei, P. vinckei, and P. yoelii are useful models for malaria in mice and rats, while P. *cynomolgi* and P. knowlesi are studied in various monkey species. The human parasites can be studied

in owl monkey (aotus) and in the splenectomized chimpanzee. Many avian and reptilian malarias are known, but their value as experimental models of human malaria is not as great as the rodent and primate malarias.

The most common method for evaluating the antimalarial activity of drugs and experimental compounds is the microdilution technique introduced by Desjardins and co-workers in 1979 (12) and modified by Milhous et al. (13). Cultured intra-erythrocytic asexual forms of P. *falciparum* are treated with serial dilutions of compounds to be tested. Inhibition of uptake of [G-^{3}H]hypoxanthine by the parasites serves as the indicator of antimalarial activity. [2,8-^{3}H]Adenosine may also be used as the radiolabel in these assays (14). For some types of studies, standard cultures are often unsuitable because of the high proportion of uninfected erythrocytes. A new procedure for producing highly concentrated cultures of P. *falciparum* from the ring stage provides a new tool in malaria research (15).

1.3 Parasite Biochemistry and Genetics

The biology, biochemistry, and genetics of Plasmodia are topics of wide interest. The information from those fields that more directly impacts on the design of new antimalarial agents is presented briefly.

For the majority of their life cycle in humans, malaria parasites live in red blood cells. Within the erythrocytes, the parasites feed on hemoglobin, digesting the protein as a source of amino acids and releasing heme [Fe(II)protoporphyrin IX]. Disruption of hemoglobin catabolism results in parasite death. Hemoglobin digestion by P. *falciparum* proceeds by an ordered metabolic pathway (16). The initial events are the endocytosis of hemoglobin from the host cytoplasm and transport to the parasite's food vacuole. The tetramer structure of hemoglobin does not spontaneously dissociate in the food vacuole. Ample evidence exists that malaria parasites degrade hemoglobin in a stepwise fashion, a process mediated by a series of proteases (17, 18). Initial cleavage of native hemoglobin occurs specifically at 33Phe-34Leu of the α chain mediated by an aspartic protease, plasmepsin I, which has been cloned and characterized from P. *falciparum* (19, 20). Cleavage at this site can be expected to unravel the tetramer, making it susceptible to further proteolysis. The plasmepsin I gene has striking homology to human renin and cathepsin D. A second aspartic protease, plasmepsin II, prefers to act on acid-denatured globin but also has some overlap of specificity with plasmepsin I. The biosynthesis of the plasmepsins has been explored (21). The crystal structures of recombinant plasmepsin II (22) and plasmepsin II complexed with a known aspartic protease inhibitor, pepstatin A, are available (23). The active sites of plasmepsins cloned from P *falciparum*, P. *vivax*, and *P. malariae* have been compared (24). The literature on plasmepsins I and II from P. *falciparum* has been reviewed (25). The third enzyme in the degradation pathways is a cysteine protease, falcipain. Falcipain cleaves denatured protein but does not act on intact hemoglobin. A fourth enzyme, falcilysin, a novel metallopeptidase, acts downstream of the others (26). In addition to a role in globin degradation, these enzymes may function in dissociation of the hemoglobin tetramer and the release of heme from globin (27).

The other by-product of hemoglobin metabolism is heme. Between 25% and 75% of the erythrocyte hemoglobin is digested during growth of the parasite in the red cell (17). Because erythrocytes contain 310–350 mg/ml of hemoglobin, the concentration of released heme is $\sim$20 mM. However, if one considers that the heme would be released in the small volume of the parasite's food vacuole, the concentrations could reach 200–500 mM (28). The heme then undergoes an autoxidation to produce toxic hematin [aquaFe(III)protoporphyrin IX]. The parasite detoxifies hematin by conversion to an insoluble material known as hemozoin or malaria pigment. An X-ray diffraction study has shown that hemozoin is chemically and structurally identical with β-hematin, a synthetic product (29). The structure of β-hematin (and hence of hemozoin) was shown to be a crystalline dimer of hematin in which two protoporphyrin systems coordinate with one another by association of the propionate side-chains with the Fe(III) center of the opposite hematin (30). This is in contrast with an earlier theory that hemozoin was a polymer, rather than a dimer, of hematin.

The mechanism of hemozoin/β-hematin formation in *vivo* or in *vitro* remains unclear, even though a number of researchers have explored this question. Early workers suggested than the process was enzyme catalyzed (31) and it was reported in 1992 that cell-free preparations from infected erythrocytes caused hematin to precipitate in its insoluble form (32, 33). Extensive efforts to identify an enzyme responsible for this process, a "heme polymerase," have not been successful. Others have suggested that the process of hemozoin formation is not dependent on protein and that it was an essentially spontaneous, physicochemical process (34), although subsequent workers were not able to detect autocatalysis (35). A lipid catalyst has also been proposed (36, 37). Finally, histidine-rich proteins such as Pf-hrp-2 (P. falciparum histidine-rich protein 2) may play a role in hemozoin formation (38, 39); specifically, they may serve as initiators or scaffolds for hematin binding before sequestration as hemozoin (40). New studies have indicated that the mechanism of the chemical formation of β-hematin can best be understood as a biomineralization process, a view that may shed light on the biological formation of hemozoin (41).

Malaria parasites evade the human immune system through a process of continuous variation in a specific protein, erythrocyte membrane protein 1 (EMP-1) (42–45). During infection of the red blood cell, P. falciparum synthesizes EMP-1 that presents on the surface of the infected cell. EMP-1 serves to bind infected cells to blood vessels in the brain and in other organs. The presence of the EMP-1 protein would also be expected to notify the immune system of an infectious agent present in the cell. But, the parasite carries as many as 150 genes for EMP-1, each encoding a slightly different protein. New variants of EMP-1 allow the parasite to avoid destruction by immune processes. Studies have estimated that about 1 in 50 of each new generation of parasites secretes a different EMP-1 protein.

The conventional view is that Plasmodia use *de* novo folate synthesis because they lack folate salvage pathways. Inhibitors of key enzymes in the folate pathway, especially dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS), have been shown to be clinically valuable antimalarials. A more complex picture is emerging, however, because it has been shown that some strains of *P. falciparum* are able to use exogenous folate, thus circumventing any blockage to *de* novo synthesis provided by antimalarial drugs.

Plasmodia synthesize dihydrofolate by a pathway unique to microorganisms. Para-aminobenzoic acid (PABA) is linked with a pteridine to form dihydropteroate by the enzyme dihydropteroate synthetase (DHPS), an enzyme not present in mammals. Then, conjugation of dihydropteroate with glutamate forms dihydrofolate (dihydropteroylglutamate). In contrast, mammalian cells obtain dihydrofolate through reduction of dietary folic acid. Sulfonamides and sulfones, inhibitors of DHPS, are selectively toxic to the parasite and relatively safe in the human host.

Malaria parasites are unable to use preformed pyrimidines using "salvage pathways" as mammalian cells do. Rather, plasmodia synthesize pyrimidines *de* novo. An important enzymatic target is dihydroorotate dehydrogenase (DHOD), which catalyzes the conversion of dihydroorotate to orotate, an intermediate in the pyrimidine biosynthetic pathway. Some compounds with antimalarial action such as atovaquone have been found to be inhibitors of DHOD. In a step further along in pyrimidine biosynthesis, tetrahydrofolate is a required cofactor. Compounds that inhibit dihydrofolate reductase (DHFR) effectively cut off the supply of tetrahydrofolate. Thus, compounds such as pyrimethamine and proguanil that inhibit DHFR are effective antimalarial agents.

There exist other metabolic pathways in Plasmodia that are potential sites for drug action. The shikimate pathway, a series of enzymatic conversions that produces aromatic cofactors and aromatic amino acids, was detected in apicomplexan parasites including plasmodia (46). Because the shikimate pathway is absent in mammals, inhibition of enzymes in this pathway provides an excellent prospect for drug design efforts. Plasmodia, as with other members of the phylum *Apicomplexa*, has been shown to possess an unusual organelle, a plastid that seems to have been acquired from algae at some point in its evolutionary history (47–49). Replication of this

apicomplexan plastid, termed the apicoplast, is essential for parasite survival (50). An apicoplast metabolic pathway has been identified that is not found in animals. the mevalonate-independent pathway of isoprenoid biosynthesis (51), which is also known as the 1-deoxy-D-xylulose 5-phosphate (DOXP) pathway. Sequence data provided by the malaria genome project suggested the presence of two genes encoding important enzymes from the DOXP path, DOXP synthase and DOXP reductoisomerase.

Erythrocytes infected by plasmodia suffer oxidant damage from the parasite; the parasite causes measurable oxidation to the host red blood cell (52). The cell may be placed under oxidant stress from parasite-generated oxidants and from a weakening of the defense mechanisms of the cell itself. Increases in methemoglobin formation and lipid peroxidation have been documented in infected cells.

Two of the 14 chromosomes of the P. *falciparum* genome, roughly 7% of the total genome, have been sequenced. The sequence of chromosome 2 was published in 1998 (53) and that of chromosome 3 in 1999 (54). Approximately 215 protein-coding genes were identified on chromosome 2 and 209 on chromosome 3, giving an estimate of 6500 genes. Sequencing the P. *falciparum* genome required overcoming the technical difficulties of high A + T content. A genome-wide high-resolution linkage map of P. *falciparum* has been published (55) as has a shotgun optical map (56). The WHO maintains a database of gene protein information on malaria parasites (57). It is anticipated that new targets for drug development will arise from examination of the gene sequence.

1.4 Global Incidence

Malaria is endemic in 101 countries and territories; 45 of these are in Africa, 21 in the Americas, 4 in Europe, 14 in the Eastern Mediterranean, 8 in Southeast Asia, and 9 in Western Asia. There are small pockets of transmission occurring in a further 12 countries (Table 18.1). Although P. *falciparum* is the predominant parasite, falciparum malaria occurs only sporadically or does not exist in 19 of those countries. For comparison, in 1955, there were 140 countries or areas where malaria was endemic. Global statistical information on malaria is presented in Table 18.2 and is available from the WHO web site (58).

An indication of the magnitude of the health problem that malaria poses can be obtained from WHO's Malaria Fact Sheet (60):

- Malaria is a public health problem for more than 2400 million people, 40% of the world population (Table 18.2).
- There are an estimated 300–500 million clinical cases/year.
- 1,086,000 deaths were reported as attributable to malaria in 1999 (61).
- Approximately 90% of both malaria incidence and mortality occur in sub-Saharan Africa.
- The mortality is estimated to be greater than 1 million; the vast majority of these are young children in Africa, especially in rural areas with limited health care available.
- One child dies every 30 s or 3000 children per day under the age of 5 years.
- In affected countries, 3 of 10 hospital beds are occupied by malaria victims.

Historically, malaria was endemic throughout much of the continental United States; an estimated 600,000 cases were reported in 1914. During the late 1940s, a number of factors combined to successfully interrupt malaria transmission including improved socioeconomic conditions, water management programs, insect-control efforts, and case management approaches. Since then, surveillance has been maintained to detect reintroduction of transmission. In recent decades, almost all cases of malaria in the United States were imported by travelers from regions of the world where malaria transmission is known to occur. In 1997, the CDC reported 1544 cases of malaria in the United States, the highest overall incidence since 1980 and the highest number of civilian cases since 1968. This figure represents a 10.9% increase over 1996. Of these cases, only five were acquired in the United States. Six persons died of malaria (62).

Africa has the highest levels of endemicity in the world; in very large areas transmission is intense and perennial. In areas with alti-

Table 18.1 Countries Where Malaria Transmission Occurs (59)

Afghanistan[a,f]	Armenia[b]	Angola[c,f,g]
Argentina[b]	Belize[a,']	Azerbaijan[a,d]
Bangladesh[f,g]	Bolivia[a,f,g]	Benin[c,f]
Bhutan[f,g]	Burkina Faso[c,f]	Botswana[c,f]
Brazil	Cameroon[c,f,g]	Burundi[c,f]
Cambodia[c,f,g,h]	Chad[c,f]	Cape Verde[d]
Central Afr. Rep.[c,f,g]	Comoros[c,f]	China
Colombia[f,g]	Côte d'Ivoire[c,f,g]	Congo[c,f]
Costa Rica[b]	Djibouti[c,f]	Dem. People's Rep. Korea[b,d]
Dem. Rep. Congo[c,f,g]	Egypt[d]	Dominican Repepublic[c,d,e]
Ecuador[f]	Eritrea[c,f]	El Salvador[b,d]
Equatorial Guinea[c,f,g]	Gabon[c,f,g]	Ethiopia[c,f]
French Guiana[c,i]	Ghana[c,f]	Gambia[c,f]
Georgia[b]	Guinea-Bissau[c,f]	Guatemala[a]
Guinea[c,f]	Honduras[a]	Guyana[e]
Indonesia[f,g,j]	Iran, Islamic Rep.[f]	India[e]
Kenya[c,f,g]	Lao People's Dem. Rep.[c,f]	Iraq[b]
Madagascar[c,f]	Malawi[c,f,g]	Liberia[c,f,g]
Mali[c,f]	Mauritania[c]	Malaysia[c,d,f]
Mayotte[c]	Mexico[b]	Mauritius[b]
Mozambique[c,f,g]	Myanmar[c,f,g]	Morocco[b,d]
Nepal[a,e]	Nicaragua[a,']	Namibia[c,f]
Nigeria[c,f]	Oman[d,f]	Niger[c,f]
Peru[f,g]	Papua New Guinea[c,f,g,j]	Pakistan[e]
Rwanda[c,f,g]	Philippines[e]	Paraguay
Senegal[c,f]	Sao Tome & Principe[c,f]	Republic of Korea[b,d]
Somalia[c,f]	Sierra Leone[c,f]	Saudi Arabia[c,f]
Sudan[c,f]	South Africa[c,f]	Solomon Islands[c,f]
Syrian Arab Republic[b]	Suriname[c,f,k]	Sri Lanka[a,f]
Togo[c,f]	Tajikistan[b,f]	Swaziland[c,f]
Vanuatu[c,f,g,j]	Turkey[b]	Thailand[f,g,h,i,k]
Yemen[c,f]	United Arab Emirates[d]	Turkmenistan[b]
	Venezuela[e]	United Rep.
	Zambia[c,f]	Vietnam[c,f,g]
		Zimbabwe[c,f]

"Predominately P. *vivax;* [b]exclusively ***P.*** *vivax;*'predominately *P.* ***falciparum;***[d]limited; [e]no resistance reported; [f]chloroquine-resistant falciparum reported; [g]sulfadoxine/pyrimethamine resistance reported; [h]mefloquine resistance reported; [i]multi-drug resistance reported; [j]chloroquine-resistant vivax reported;'lessened sensitivity to quinine reported.

tudes over 1500 m and rainfall below 1000 mm/year, endemicity decreases, and the potential for epidemic outbreaks increases. Ecological, demographical, and meteorological factors including quasi-cyclic occurrence of heavy rains have led to epidemics or serious exacerbations of endemicity, especially in Botswana, Burundi, Ethiopia, Kenya, Madagascar, Rwanda, Sudan, Swaziland, Zaire, and Zambia.

Excluding Africa, of the total number of cases reported annually to the WHO, more than two-thirds are concentrated in only six countries: India, Brazil, Sri Lanka, Afghanistan, Vietnam, and Colombia. In other parts of the world, the distribution of malaria varies greatly from country to country and from region to region within countries. For example, in India, the majority of reported cases occur in only a handful of states. Three states of the Amazonian Basin in Brazil account for close to 80% of all cases while representing only 6.1% of the country's overall population.

Inadequate and irregular reporting, particularly in areas known to be highly endemic and often out of the reach of established health services, make it difficult to obtain accurate information on the incidence of malar-

Table 18.2 Reported Cases of Malaria, Endemic Countries Only, 1982-1997 (58)

Year	World Total (Countries Reporting)	Africa	Americas	Asia	Oceania
1982	14,530,337 (92)	8,045,746 (39)	713,878 (21)	5,580,381 (29)[a]	190,332 (3)
1983	9,024,790 (80)	4,351,945 (26)	829,546 (21)	3,606,587 (30)	236,712 (3)
1984	10,514,053 (83)	5,483,965 (29)	929,891 (21)	3,849,728 (30)	250,469 (3)
1985	19,271,828 (91)	14,557,717 (37)	909,162 (21)	3,556,744 (30)	248,205 (3)
1986	23,829,689 (90)	18,567,724 (36)	948,906 (21)	4,088,518 (30)[a]	224,541 (3)
1987	26,633,220 (90)	21,294,523 (36)	1,016,327 (21)	4,058,717 (30)	263,653 (3)
1988	30,887,818 (91)	25,602,604 (37)	1,118,132 (21)	3,992,351 (30)	174,731 (3)
1989	38,634,970 (90)	33,284,881 (36)	1,111,732 (21)	4,013,520 (3)	224,837 (3)
1990	27,238,125 (85)	21,903,265 (32)	1,055,897 (21)	4,029,017 (29)	249,946 (3)
1991	27,208,754 (86)	21,570,180 (33)	1,230,155 (21)	4,163,317 (29)	245,102 (3)
1992	26,689,817 (84)	21,371,102 (33)	1,186,053 (20)	3,939,559 (28)	193,103 (3)
1993	32,657,473 (86)	27,603,152 (35)	982,040 (20)	3,868,984 (28)	203,297 (3)
1994	38,433,333 (88)	32,619,436 (36)	1,113,407 (21)	3,935,507 (28)	764,983 (3)[b]
1995	27,378,851 (85)	21,512,357 (35)	1,279,651 (20)	4,409,796 (27)	177,047 (3)
1996	24,273,550 (76)	18,096,602 (24)	1,138,453 (20)	4,877,033 (29)	161,462 (3)
1997	18,715,601 (73)	12,260,126 (22)	1,054,230 (20)	5,288,916 (28)	112,329 (3)

[a]Not all cases from China confirmed by laboratory tests.
[b]Not all cases from Papua New Guinea confirmed by laboratory tests.

ial disease in many areas. Under ideal circumstances, each case would be confirmed clinically by microscopic examination of a blood smear. In practice, malaria is most often defined in association with disease symptoms rather than with microscopic confirmation. And priority is given to reporting of severe and complicated cases and malaria deaths rather than total number of cases. Especially in Africa, strict reporting is fragmentary and based on clinical signs and symptoms.

1.5 Resistance

Drug resistance in malaria has been defined as the "ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to, or higher than, those usually recommended but within the limits of tolerance of the subject" (63). Later modification of this definition stated that "the form of the drug active against the parasite must gain access to the parasite, or the infected blood cell, for the duration of time necessary for its normal action." The modified definition makes clear that for parasites to be termed resistant, the drug must be bioavailable. The phenomenon of drug resistance has become common in many infectious diseases: tuberculosis, staphylococci, streptococci, HIV, and syphilis. Drug resistance was perhaps first recognized in the treatment of malaria. Early in this century, it was noted that some cases of malaria responded much more poorly to quinine, the only drug available at the time, and that larger doses of drug had to be administered to effect a cure. At present, the increasing occurrence of chloroquine-resistant strains of P. ***falciparum*** has inspired global drug-design and development efforts in an attempt to identify new agents for the treatment of resistant strains of Plasmodia. The sensitivity of a parasite strain to a drug can be classified into four general groups: sensitive (S), in which clearance of asexual parasitemia occurs within 7 days of the initiation of treatment without subsequent recrudescence; slightly resistant (RI), in which asexual parasitemia is cleared as in sensitive cases but recrudescence follows; moderately resistant (RII), in which the level of asexual parasitemia is reduced markedly but not cleared fully; and highly resistant (RIII), in which little or no reduction in asexual parasitemia occurs. A higher resistance to a particular agent is not necessarily correlated with higher virulence. A single isolate of P. *falciparum* from an infected individual consists of parasites with differing drug responses.

For descriptions of the history of the emergence of malarial drug resistance, see Ref. 64.

The overall picture is that malaria parasites have developed resistance to each and every therapeutic agent in areas where drug selection pressure has been applied. Drug pressure is considered to be the main causative factor of selecting and propagating resistant P. *falciparum* in a particular locality or area. Chloroquine has been the agent of choice against P. falciparum for decades. When first brought into wholesale use for mass chemotherapy in the 1950s, its effectiveness led to the hope of global malaria eradication. Such broad usage for chloroquine, however, induced resistance beginning in South America and Southeast Asia. Even so, for compounds such as chloroquine, prolonged drug exposure seems to be required before resistance began to develop (65). Chloroquine resistance emerged much later in Africa than in South America and Southeast Asia. The first case from East Africa was documented in non-immune travelers returning from that area in 1979 (66). Since that time, chloroquine resistance has spread rapidly in Africa, and it is estimated that more than 60% of the P. falciparum strains that infect non-immune travelers are resistant to chloroquine. Significantly, chloroquine still largely retains is therapeutic efficacy in semi-immune populations. Among the countries where falciparum malaria exists, only those of Central America have not recorded the resistance of falciparum malaria to chloroquine. An excellent set of maps tracking the development of chloroquine resistance globally is shown in Ref. 63.

The resistance of P. *vivax* to chloroquine is not uncommon in Indonesia and Papua New Guinea, although the extent of the spread is not known. There have been sporadic reports of chloroquine-resistant vivax from Myanmar, Thailand, Borneo, India, and Brazil (67). Although not widespread as yet, primaquine-resistant strains of P. *vivax* have been reported (68, 69), although others have raised concerns about the definition of primaquine resistance (70).

Chloroquine is not the only agent that has lost effectiveness against P. falciparum because of the development of drug resistance. Resistance to sulfadoxine/pyrimethamine has developed in Southeast Asia, South America, and Africa. For the dihydrofolate reductase inhibitors proguanil and pyrimethamine, resistance can be induced by a single large dose. In Thailand, there are indications that more than 50% of cases in certain border areas no longer respond to mefloquine therapy, while the sensitivity to quinine is also diminishing in areas of Thailand and Vietnam. In cases of resistance, quinine, sulfadoxine/pyrimethamine, mefloquine, and now the artemisinin-type agents, are being used therapeutically.

Thailand has served as a laboratory for the development of drug-resistant *P.* falciparum (71). Beginning in the early 1950s, resistance to pyrimethamine arose as a result of its use in prophylaxis and in presumptive treatment (treatment pending laboratory diagnosis). Initial experiences with emerging chloroquine resistance involved recrudescence on administration of standard doses in the late 1950s, although its took until the mid-1970s until chloroquine resistance became so frequent and problematic that alternate therapies were introduced. Sulfadoxine/pyrimethamine as a routine treatment was useful only for a short time before resistance emerged. By 1982, the recommended treatment regimen was changed again to a 7-day course of quinine/tetracycline. The inconvenience and poor compliance associated with a 7-day course necessitated the move to the triple combination mefloquine/sulfadoxine/pyrimethamine in 1985. Treatment failures with mefloquine were observed beginning in 1991.

Multi-drug resistance may be defined as resistance of P. falciparum to more than one operational class of antimalarial agents. Clinically, the problem of multi-drug resistant strains is limited to the Indochina Subcontinent, with some foci in Sabah, Malaysia, Papua New Guinea, and West Irian, Indonesia. Some sporadic occurrences of multi-drug resistant organisms have been seen in tropical Africa and South America. The border areas of Thailand with Cambodia and Myanmar are the areas of hardcore multi-drug resistance. P. falciparum in these areas are resistant to chloroquine and other 4-aminoquinolines, sulfadoxine/pyrimethamine, and quinine (if used as a single agent).

Given these observations, resistance to any new therapeutic agent can be expected, and

strategies are being developed to ensure an appreciable effective lifetime for each new drug. One important tactic has been the employment of combination therapy (72); the useful lifetime of pyrimethamine has been extended by an estimated 15 years because of its use in combination with sulfadoxine (73). For a discussion of the factors that influence the emergence of resistance and a consideration of the outcomes when antimalarial drugs are used in combinations, see Refs. 72, 74. Many therapies are being studied with the new artemisinin agents in combination with older agents. Another tactic is the avoidance of initiating widespread therapy with new agents in areas where other, older agents remain effective. The hope is that the newer agents might be held in reserve until absolutely necessary. Researchers have been studying the dynamics involved in the emergence of drug-resistant malaria strains and are attempting to develop models that will assist in the creation of strategies for deployment of newer agents in the field (75).

Current efforts at analyzing the molecular mechanisms of resistance are offering new approaches for drug resistance (76, 77). Mutations in key molecular targets had been identified for antifolate resistant strains. Mechanisms that confer resistance to chloroquine seem to be more complex, but new information is helping to clarify the variables. Rapid efflux of chloroquine from resistant parasites can be reversed by co-administration of certain agents. Additional research on the molecular basis of resistance may provide improved therapeutic strategies for use in resistant organisms.

1.6 Immunity and Prophylaxis

The average age of first infection with malaria is less than 1 year old for persons in most endemic areas. Estimates are that between 75,000 and 200,000 infant deaths each year are associated with malaria infection in pregnancy (78). Exposure does not induce lifelong immunity to further malaria infections. As children age, they acquire a functional immunity that provides fewer clinical attacks and lessened clinical symptoms. Adults can obtain sufficient immunity so that a substantial reduction in infection rates is observed. Asymptomatic parasitemia may occur among persons who have been long-term residents of malaria-endemic areas. As a result, children have the highest levels of mortality and morbidity resulting from malaria. It had long been assumed that these data were the result of a high transmission rate of the parasite. New studies have indicated, however, that most endemic areas contain parasites with a high degree of antigenic diversity (79). Each infection does in fact provide immunity to that particular strain; new infections are caused by parasites with different antigenic profiles. A calculation of transmission rates incorporating these considerations yields numbers an order of magnitude lower than previously estimated.

Pregnant women have a unique susceptibility to malaria, such that "maternal malaria," a distinct clinical entity, causes serious pregnancy-related complications in endemic areas. Increases in miscarriage, premature delivery, retardation of fetal growth, anemia, low birth weight, and mother and infant mortality rates are observed when the mother contracts malaria during pregnancy. Adult women acquire the same degree of immunity to the common strains of parasites as do adult men; however, after becoming pregnant, this acquired immunity diminishes markedly. With successive pregnancies, the loss of immunity is less pronounced. To explain the apparent loss of acquired immunity in pregnancy, researchers studied the binding of infected red blood cells to placental tissue (80). A subpopulation of P. *falciparum* parasites preferentially binds to placental tissue and multiplies there. The binding site in placenta normally binds chondroitin sulfate A (CSA). The severity of the infection must be caused by the initial appearance of placental tissue that harbors the infection. As the woman develops immunity to this subpopulation of parasites, the frequency and severity of malaria in a second or third pregnancy is lessened. With the rising incidence of HIV/AIDS in Africa and concern about maternal transmission of the virus, the problems of simultaneous multiple drug therapies emerge. For a review of the therapeutic and safety issues in the concurrent use of anti-HIV agents with antimalarial agents in pregnant patients, see Ref. 81.

Glucose-6-phosphatedehydrogenase (G6PD) deficiency is a genetic condition most prevalent among persons living in malaria-endemic areas. The most common African form of G6PD deficiency was associated with a 46–58% reduction in the risk of severe malaria (82). Parasites inflict oxidant damage to the erythrocytes that are infected and erythrocytes that are deficient in G6PD are especially susceptible to oxidant damage. In addition, it has been shown that phagocytosis of parasiticized G6PD-deficient erythrocytes occurs earlier than does phagocytosis of infected normal erythrocytes, perhaps accounting for the observed protective effect (83).

Other inherited red cell disorders contribute an immunity to malaria infections, at least partially as a result of sensitization to oxidant stress. These include the well-known example of heterozygous hemoglobin S (sickle cell trait) (84) as well as thalassemia (85), persistence of fetal hemoglobin, and hemoglobin E. α-Thalassemia seems to increase susceptibility to malaria in early childhood and then offers immunity later in life (86). An explanation of the effect involving hemoglobin/membrane interactions had been proposed (87). Southeast Asian ovalocytosis is another genetic disorder that confers marked protection against cerebral malaria (88). It is caused by a deletion in the gene for the erythrocyte membrane band 3. The band 3 protein is responsible to the cytoadherence of parasitized cells. The mutation occurs in high frequency in the western Pacific.

Non-immune travelers, with no prior exposure to malaria, are at greater risk of acquiring serious infections. If the infection presents clinical symptoms only after return from a malarious area, there may be a delay in diagnosis by physicians unfamiliar with the disease. Travelers to endemic areas should seek advice about the use of chemoprophylactic regimens and stand-by treatment. Further protection is gained by preventing exposure to mosquitoes using insect repellents on clothes and skin and staying indoors at night. For reviews on malaria chemoprophylaxis, see Refs. 89, 90.

Chemoprophylaxis demands a nearly impossible level of safety, especially when used over long periods. Drugs are given to healthy individuals, most often to prevent a disease of low probability. The most successful agent to date has been chloroquine, with a 50-year safety record that is matchless among anti-infective agents. Malaria prophylactic regimens are generally highly efficacious and low in cost, especially when compared with the costs of treatment including hospitalization. Even so, compliance has remained an issue among U.S. travelers to malarious areas; only 21% of travelers used recommended malaria prophylaxis appropriate for their area of travel in 1997 (62). International travel recommendations are available from both the WHO (59, 91) and the CDC (92, 93).

1.7 Vector Control

Systematic malaria control began after the discovery of the malaria parasite by Laveran in 1880 (for which he was awarded the Nobel Prize for medicine in 1907), and the demonstration by Ross in 1897 that the mosquito was the vector of malaria (94). Attempts to control mosquitoes have paralleled therapeutic and chemoprophylactic approaches in battling the disease (95). In 1955, the World Health Assembly initiated a malaria eradication program. The stated goal was the interruption of malaria transmission by reducing mosquito populations that fed on humans; complete elimination of the vector was not attempted. Household spraying with DDT was the main tactic. The approach was most successful in temperate regions where transmission was unstable. However, in some areas when spraying was halted, mosquito populations returned but were now resistant to DDT.

An alternative to reducing or eliminating vector populations is the prevention of contact between humans and mosquitoes. The use of insecticide-impregnated bed nets seems to be effective in limiting malaria in a number of different areas: the Solomon Islands, where malaria prevalence rates are among the highest in the world (96); Guatemala (97); Kenya (98); Tanzania (99); and The Gambia (100). Continual governmental support for such programs will be needed if any long-term benefits are to be ultimately accrued from such an approach. The CDC gives detailed instructions for travelers about avoiding contact with potentially infectious mosquitoes, including the use of bed nets and insect repellents (92).

A recent response to vector control has been studies of the replacement of normal mosquito populations with strains that cannot support normal parasite development (101). Success will require identification of the mosquito genes that inhibit parasite growth, the introduction of such genes in the mosquito genome, and dispersing the trait through natural populations. Efforts to sequence the ***Anopheles*** gambiae genome are underway (102). Fortunately, there are a number of points in the parasite life cycle within the mosquito that provide opportunities for manipulation.

It has been suggested both in the scientific literature and in the popular press that certain tropical diseases, especially those that involve a mosquito vector, are likely to increase in incidence with the increase in global temperatures. However, a statistical study incorporating a number of critical factors including temperature did not forecast global malaria expansion (103).

1.8 Economic and Political Issues

Today, malaria is becoming a greater health problem than before in many parts of the world. Epidemics are occurring in areas where transmission had been eliminated. These outbreaks are generally associated with deteriorating social and economic conditions, and many victims are in underprivileged rural populations. Demographic, economic, and political pressures compel entire populations (seasonal workers, nomadic tribes, and farmers migrating to newly developed urban areas or new agricultural and economic developments) to leave malaria-free areas and move into endemic zones. These people are often non-immune and at high risk of severe disease. Unfortunately, these population movements and the intensive urbanization are not always accompanied by adequate development of sanitation and health care.

Malaria is now mainly confined to the poorer tropical areas of Africa, Asia, and Latin America, but the problems of controlling malaria in these countries are aggravated by inadequate health structures and poor socioeconomic conditions. Moreover, in many areas, conflict, economic crises, and administrative disorganization result in the disruption of health services. As a result, control efforts are interrupted and more people are put at risk. The absence of adequate health services frequently results in a recourse to self-administration of drugs often with incomplete treatment. This is a major factor in the increase in resistance of the parasites to drugs.

Malaria thus has disastrous social consequences and is a heavy burden on personal and national economic development. On the level of the individual, an episode of the disease was estimated to cost US$8.67 in Ghana (reported in 1997), although costs would vary depending on the costs of the drugs (104). Short-term costs—loss of work time, losses associated with child morbidity and mortality, costs of treatment and prevention—are only part of the story. A high proportion of the cost of malaria care is the opportunity cost to the caretakers. Children in particular may suffer from chronic anemia and malnutrition as a result of repeated bouts of malaria, leading to altered physical and cognitive development.

In the aggregate, costs are staggering. According to 1997 estimates, direct and indirect costs of malaria in sub-Saharan Africa exceed $2 billion (60). In contrast, the average cost for each nation in Africa to implement programs to control malaria is estimated at $300,0001 year or approximately US$0.06 per person for a country of 5 million persons.

Areas with malaria are almost exclusively poor, with low rates of growth (105). But is this a cause or an effect? Successful elimination of malaria has historically required well-organized and well-financed programs. Even so, some of the most effective control efforts have used few resources beyond labor. Sustaining such efforts over long periods has not been successful in many locations, however. Endemic countries may find trade impeded, lowered interest in international investment and commerce, and fewer opportunities for tourism. Evidence suggests that overall economic development is hindered by malaria. In places where malaria has been eradicated, economic growth accelerated after eradication. Growth rates in endemic countries are over 1% per year less than that in comparable states without malaria. Over a period of decades, the economic burden can become immense (106). And so while countries do not

become prosperous by controlling malaria alone, it is certainly made easier.

A final issue is that of the economics of drug development of antimalarial agents and drugs to treat parasite diseases more generally. Because the populations affected are found mostly in developing countries with limited health-care resources, pharmaceutical companies have little financial incentive to invest in the development of new agents to treat these diseases (107).

On May 13, 1998, the Director General of the WHO announced the Roll Back Malaria Campaign with the goal of cutting global incidence by 50% by 2010. Another 50% reduction was targeted for 2015. Details of the program may be found at WHO web sites (108) as well as reports on the progress made to date (109, 110). As part of the Roll Back Malaria effort, a program called Medicines for Malaria Venture has been established (111).

2 ANTIMALARIAL AGENTS FOR CHEMOTHERAPY AND PROPHYLAXIS: CURRENT DRUGS IN USE

The therapeutic agents in use against malaria are summarized in Table 18.3. The available drugs represent a wide variety of structural types and modes of action. One of the drugs, quinine, has been in use for hundreds of years. Several were discovered and approved very recently. Some find uses in other infectious diseases while many are specifically used in malaria. The agents may be used in treatment or for chemoprophylaxis; some are valued in both applications. Each of the drugs is described in detail below.

2.1 Quinine and Quinidine

Quinine (1) may be claimed without exaggeration as the drug to have relieved more human suffering than any other in history (112). For 300 years, it was the only known effective treatment for a life-threatening infectious disease. Only a handful of other treatments, emetine for amoebic dysentery, mercury for syphilis, chaulmoogra oil for leprosy, and herbal anthelmintics, were effective as specific anti-infective agents until this century. The fanciful story of the miraculous cure of the Countess of Chinchón, wife of the Viceroy of Peru, by administration of a native remedy produced from tree bark is charming but very far from fact. For a scholarly discussion of the early history of cinchona, see Ref. 113. Tree bark from the cinchona tree, Cinchona *officinalis* and other Cinchona species, a native plant from South America, was the source for an effective treatment of recurrent fevers. In the 19th century, the active principles, the cinchona alkaloids, quinine (1), quinidine (2), cinchonine (3), and cinchonidine (4), were isolated and purified. The formal synthesis of quinine by Woodward and Doering in 1944–1945 was a landmark in modern synthetic chemistry (114, 115). The first stereoselective total synthesis of quinine was recently reported by Stork and co-workers (116). Included in their paper is a brief history of synthetic efforts toward quinine.

The principal areas producing cinchona are central Africa, India, and Indonesia. Commercial formulations of quinine have approximately 10% dihydroquinine as an impurity. The two compounds have nearly equivalent antimalarial activity, however, so that efficacy is not affected. The preparation Quinimax is a mixture of cinchona alkaloids, predominately quinine, and is reported to be more effective than quinine alone.

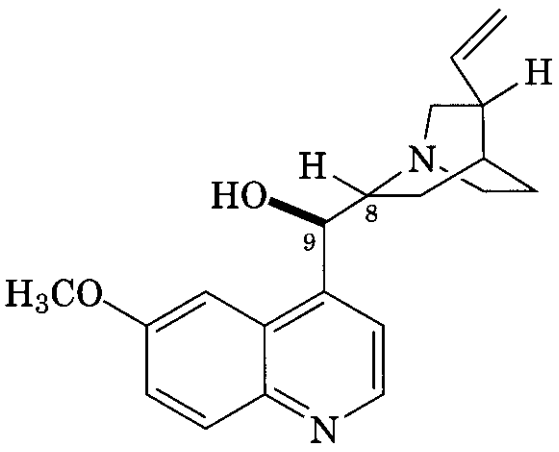
The stereochemical differences among the cinchona alkaloids result in differences in potency, and the stereoelectronic features have been examined (117). Conformational differences between the diastereomers apparently lead to differing ability to form critical hydrogen bonds. Quinidine is two- to threefold more active than quinine in both chloroquine-sensitive and chloroquine-resistant strains of P. falciparum (118). Likewise, cinchonine is more active than cinchonidine in vitro (119). The differences in activity based on stereochemistry are greater for those compounds like quinine and quinidine, which have a rigid quinuclidine moiety, than for synthetic compounds such as mefloquine, which bear a piperidine ring (120).

The main metabolite of quinine is 3-hydroxyquinine, which is produced by the action of CYP4503A4 (121). Certain drugs were found to inhibit the metabolism of quinine, including tetracycline, doxycycline, omeprazole,

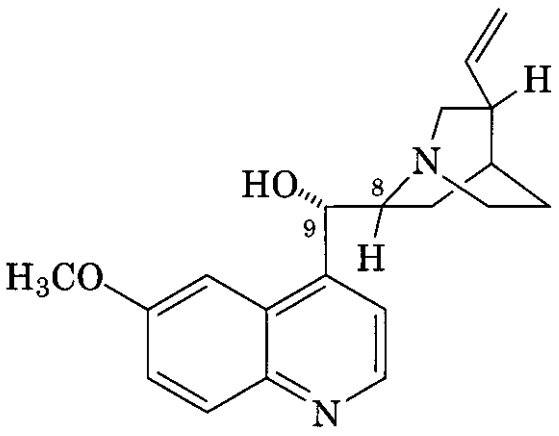
Table 18.3 Drugs Currently in Use

Generic Name	Structure	Trade Names	Year	Originator	Chemical Class	Dosage Forms
Quinine	**1**	Quinamm			Quinoline alcohol	Many sizes capsules and tablets, IV form no longer available in U.S.
Quinidine	**2**				Quinoline alcohol	IV solution
Chloroquine	**5**	**Aralen,** Avloclor, Nivaquin, **Resochin**			4-Aminoquinoline	500-mg Tablet (300-mg base), oral
Hydroxychloroquine	**6**	Plaquenil			4-Aminoquinoline	200-mg (155-mg base) Tablet oral
Mefloquine	**10**	Mephaquine	1985	Mepha	Quinoline alcohol	250-mg Tablet, oral
		Lariam	1989	**Hoffmann-LaRoche**		
Halofantrine	**13**	**Halfan**	1988	Smith **Kline** & French	Phenanthrenemethanol	250-mg Tablet, oral
Primaquine	**14**				8-Aminoquinoline	26.3-mg (15-mg base) Tablet, oral
Bulaquine	**21**	Aablaquine	2000	Central Drug Research Institute	8-Aminoquinoline	Aablaquine = bulaquine + chloroquine combination pack
Proguanil	**23**	Paludrine			Biguanide	100-mg Tablet oral
Chlorproguanil	**24**				Biguanide	
Pyrimethamine	**27**	Daraprim			Pyrimidine	
Sulfadoxine	**31**				Sulfanilamide	
Pyrimethamine/ sulfadoxine		Fansidar			Combination	500 mg Sulfadoxine, 25 mg pyrimethamine tablet oral
Mefloquine/ pyrimethamine/ sulfadoxine		Fansimef			Combination	
Dapsone	**33**				Sulfone	
Pyrimethamine/dapsone		**Maloprim** Deltaprim			Combination	25 mg **Pyrimethamine,** 100 mg dapsone tablets
Trimethoprim	**28**				Pyrimidine	
Sulfamethoxazole	**34**	Gantanol			Sulfanilamide	500-mg Tablet
Sulfamethoxazole/ trimethoprim		Bactrim **Cotrim**			Combination	
Sulfisoxazole	**35**	Gantrisin			Sulfanilamide	

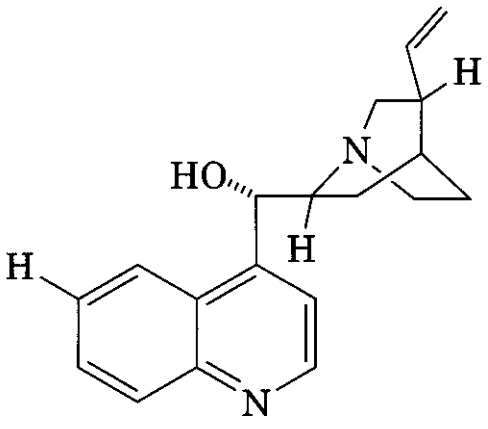
Sulfalene	**32**				Sulfanilamide	
Artemisinin	**42**		1987	Ping Hau Sau Research Group	Artemisinin	
Artemether	**50**	Paluther	1994		Artemisinin	
Arteether	**51**	Artemotil	2000	Central Drug Research Institute	Artemisinin	
Dihydroartemisinin	**47**				Artemisinin	
Artesunate	**48**	**Arsumax** Plasmotrim	1996		Artemisinin	
Atovaquone	**68**	Mepron Wellvone	1992	**Wellcome**	**Hydroxynaphthoquinone**	250-mg Tablet, also IV solution
Pyronaridine	**70**			China	**4-Aminobenzo[*a*]**1,5-naphthyridine	
Amodiaquine	**72**				4-Aminoquinoline	
Tetracycline	**76**	Achromycin and many others			Tetracycline	
Doxycycline	**77**	Vibramycin **Doxcyclin**		Pfizer	Tetracycline	100-mg Tablet, oral
Azithromycin	**78**	**Sunamed** Zithromax	1988	Pliva Pfizer	Macrolide	
Clindamycin	**83**	Cleocin			Linosamide	
Ciprofloxacin	**79**				Fluoroquinolone	
Atovaquone/proguanil		Malarone	1997	**GlaxoSmith Kline**	Combination	250 mg Atovaquone, 100 mg **proguanil** oral
Lumefantrine **(benflumatol)**	**69**				Fluorene alcohol	
Lumafantrine/ artemether **(coartemether)**		**Coartem Riamet**	1992 1999	China Novartis	Combination	



(1)
quinine
(–)-8S, 9R



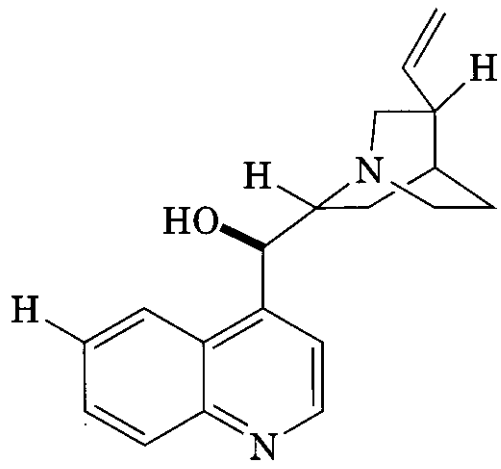
(2)
quinidine
(+)-8R, 9S



(3)
cinchonine

ketoconazole, troleandomycin, and erythromycin (122, 123). In contrast, chloroquine, pyrimethamine, proguanil and its metabolites, norfloxacin, and dapsone did not appreciably inhibit quinine metabolism.

Since the development of potent synthetic agents, quinine had fallen into disuse as a first-line antimalarial. The small difference between therapeutic and toxic doses is a decided drawback, and longer treatments over several days are required for good cure rates. But with the advent of multi-drug resistant strains, quinine has returned as an important agent in severe cases involving drug-resistant parasites. It is almost always used in combination with tetracycline. Effective treatment usually involves a 7-day course of the combination (124).Therefore, it is not as convenient as single-day treatments, and compliance can become a problem. In addition, tetracycline cannot be given to children.



(4)
cinchonidine

Quinine has been examined in combinations with other agents. A study suggested that co-administration of allopurinol, a potent inhibitor of purine biosynthesis, brings about faster eradication of P. *falciparum* and clinical remission than with quinine alone (125). Combinations of omeprazole, a proton pump inhibitor, with quinine were synergistic *in vitro* (126).

Several studies have examined the viability of rectal routes of quinine administration. Intra-rectal formulations may be given in the presence of vomiting and nausea and may be especially important in areas where the safety of parenteral administration cannot by assured. Quinine or Quinimax given by rectal administration was a viable alternative to parenteral routes (127,128). Despite the benefits of rectal administration, concerns remain about the low melting point of the vehicle, especially for use in warm climates.

There is an increased interest in the use of Quinimax as an alternative to quinine. Like quinine, 7-day dosing regimens are required for acceptable cure rates (129). The pharmacokinetics of Quinimax suppositories were

studied in children **(130). A** comparison of intra-rectal, intramuscular, and intravenous routes of administration showed that peak plasma concentrations and areas under the plasma concentration-time curve were similar; the time to peak plasma concentration was shorter for the intra-rectal route, however.

When used for monotherapy, sensitivity to quinine was declining over time; in Thailand, for example, the cure rates were **94%** in **1978–1979, 86%** in **1979–1980,** and **76%** in **1980–1981 (131).** By combining quinine with tetracycline, the cure rates were improved to >95%. Tetracycline serves to increase the plasma quinine levels above those seen with equivalent dosing of quinine alone. In a study involving highly drug-resistant parasites, clinical response to quinineltetracycline did not decline between **1981** and **1990.** African strains of P. falciparum are generally susceptible to quinine, although a few reports indicate a lessening of its effectiveness **(132, 133);** clinical cases of strains resistant to quinine have been documented in East Africa **(134)** and Brazil **(135).** In the Brazil study, data from the sequence analysis for the pfmdrl gene indicated that the mechanism for quinine resistance may differ from that of either chloroquine or mefloquine. Some researchers have gathered evidence that mutations in Pghl, the protein product ofpfmdrl, can confer resistance to quinine **(136).** Interestingly, there seems to be an inverse relationship between chloroquine resistance and quinine and mefloquine resistance in P. falciparum.

The clinical pharmacokinetics of quinine have been reviewed **(121,137).** Quinine disposition is linear over the dose range of 250–**1000** mg as single oral doses **(138).** Profiles were found to be similar following either oral administration or intravenous infusion to patients with acute falciparum malaria **(139).** Intramuscular quinine also gave predictable profiles in a population pharmacokinetic study of children with severe malaria **(140).** Details of the pharmacokinetic interactions of quinine with other antimalarial agents such as mefloquine, sulfadoxine/pyrimethamine, antibiotics, and primaquine have been reported **(137).**

The mechanism of action of quinine may involve inhibition of heme degradation, discussed in detail in the section on chloroquine. Quinidine inhibits hemozoin formation with an IC_{50} value of **90** mM, quinine of **300** mM, and epiquinine of >5 mM, the order that corresponds to their antimalarial activity **(32).** Quinine binds to both serum albumin and $\alpha 1$-acid glycoprotein (AAG) in human plasma **(141).** In malaria patients, the plasma concentrations of AAG are increased and the amount of quinine binding to AAG is consequently higher than in non-infected subjects. In addition, the hepatic disposition of both quinine and quinidine is altered in malaria patients from that seen in uninfected subjects **(142, 143).**

Efforts have been made in the last decade to clarify issues related to appropriate dosage regimens for quinine. Given that the main therapeutic use of quinine and quinidine in malaria is in severe cases of falciparum malaria, the rapid establishment of effective drug concentrations is essential. The use of a loading dose of quinine or quinidine has become standard practice, even in children where faster recovery from coma and clearance of parasitemia were observed **(144).** Both drugs have a low therapeutic index and concerns about toxicity intrude. Children younger than **2** years old represent a large percentage of the cases of severe malaria in Africa. Pharmacokinetic studies in children with severe malaria indicate that younger children may be more susceptible to quinine toxicity than older children (145).

The symptom complex called cinchonism is associated with the use of the cinchona alkaloids. Excess quinine or quinidine can induce the symptoms consisting of hearing loss, tinnitus, visual disturbances, rashes, vertigo, nausea, vomiting, and central nervous system changes including headache, confusion, and loss of consciousness. Tinnitus is one of the early signs of quinine toxicity and occurs in nearly all persons whose plasma concentrations of drug are in excess of **5** mg/l. In sensitive individuals, a single dose can cause problems; more commonly, persons experience cinchonism after a prolonged course of treatment. The symptoms resolve when administration of the drug ceases. However, several cases of irreversible sensorineural hearing loss and tinnitus have been reported.

Avoidance of cardiotoxicity is a primary consideration in risk-benefit assessments for using cinchona alkaloids in severe malaria. Quinine may cause myocardial depression, prolongation of QTc interval on the electrocardiogram, and peripheral vasodilatation. It may also cause initial generalized stimulation of the central nervous system leading to fever, delirium, and increased ventilatory rate. Quinine cardiac effects are amplified by concurrent administration of prochlorperazine, a resistance-reversing agent (146).

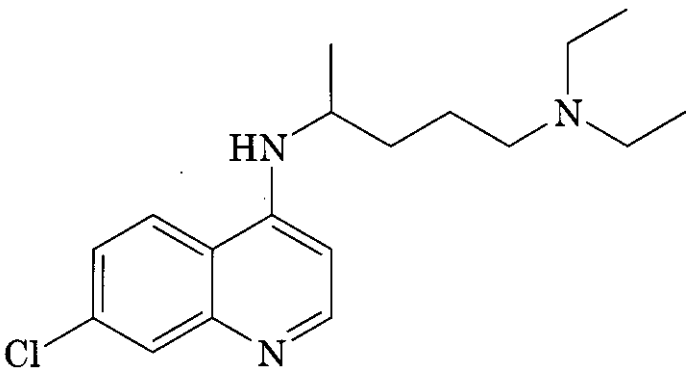
Quinidine has more potent intrinsic antimalarial properties than quinine. Since 1991, quinidine gluconate has been the only parenteral antimalarial available for use in the United States. It is indicated for the treatment of patients with life-threatening falciparum malaria. As newer anti-arrhythmics have replaced quinidine for many cardiac indications, some health care facilities have dropped quinidine gluconate from their formularies. The limited availability of and delays in obtaining quinidine gluconate have contributed to adverse patient outcomes (147). The pharmacokinetic data available for quinidine have been reviewed (121). Quinidine has more pronounced cardiac effects than quinine and has a greater tendency to induce hypotension. Given the low therapeutic index of quinidine, careful monitoring of cardiac function is required.

Quinine, like many other antimalarial agents, is photosensitizing; these properties have been reviewed (148). Electron paramagnetic resonance (EPR) studies indicated that oxygen and carbon-centered free radicals were formed during photolysis.

Hypoglycemia is another importance complication associated with cinchona use. Both quinine and quinidine stimulate the release of insulin from the pancreas. The degree of hyperinsulinemia varies greatly from patient to patient, and pregnant women are particularly susceptible to hypoglycemia resulting from excess insulin. Blood glucose levels should be monitored during quinine treatment. Children with severe malaria often present with hypoglycemia as well, even without prior quinine administration.

2.2 Chloroquine

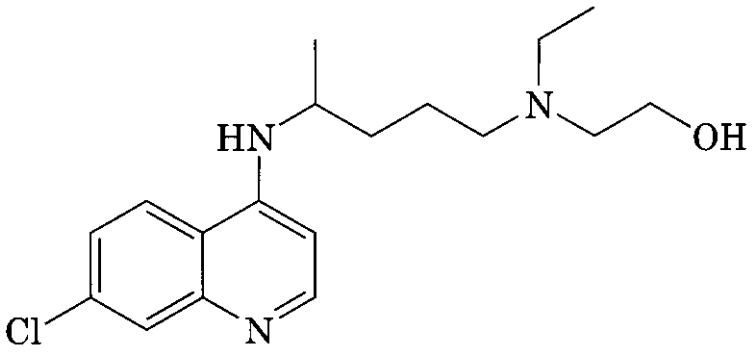
Chloroquine (**5**), the main drug among the 4-aminoquinoline class, is one of the most successful antimicrobial agents ever produced



(5)
chloroquine

and has had an enormous positive impact on human health. Chloroquine was synthesized in 1934. but at the time it was considered too toxic for human use based on studies of avian malarias and on limited clinical trials. At the outbreak of the Second World War, chloroquine was revisited and its wide-scale use in malaria treatment and prevention began. After decades of use as a first-line therapeutic and prophylactic agent, it still finds use in certain parts of the world more than 65 years after it was first prepared.

Chloroquine remains the favored agent for susceptible malaria. Hydroxychloroquine **(6)** is also active and available, although much



(6)
hydroxychloroquine

less widely used, except in the United States. Commercially available chloroquine is racemic. The main metabolite is desethylchloroquine, which is equally active as chloroquine in sensitive-strains of P. falciparum. In resistant strains, the metabolite is significantly less active. Chloroquine acts on the intra-

erythrocytic stages of the parasite life cycle; at therapeutic concentrations, it has no effect on the sporogonic, exoerythrocytic, and mature sexual stages. Chloroquine was found to increase the gametocytogenesis *in vitro* of *P. falciparum* (149, 150); this observation may explain the relative ineffectiveness of chloroquine therapy to reduce malaria transmission, because there is a positive relationship between garnetocyte densities and infectivity to mosquitoes. A review of the pharmacokinetics of 4-aminoquinolines as well as information about pharmacokinetic interactions of chloroquine with other antimalarial agents may be found in Ref. 137. The clinical pharmacokinetics of chloroquine have been reviewed (121, 151), and some reviewers have specifically examined the pharmacokinetics of chloroquine against P. *vivax* (152, 153). The pharmacodynamics of chloroquine in severe malaria has been studied (154). Chloroquine in pregnancy is discussed in Ref. 155. Chloroquine was found to be mutagenic but not clastogenic using mammalian *in vivo* assays (156).

P. ovale and *P. malariae* are fully susceptible to chloroquine in all geographic areas at present. For more than 40 years, chloroquine had been nearly 100% effective in treating blood stage infections of *P. vivax*. Although drug resistance of P. *falciparum* is an expected reality of antimalarial therapy, the resistance of P. *vivax* to drugs is comparatively recent and has been documented in Papua New Guinea (157), Myanmar (158), Sumatra (159), Sulawesi (160), Irian Jaya (161, 162), and South America (163). Further studies are needed to find alternatives to chloroquine for treatment and prophylaxis of vivax malaria.

P. falciparum, on the other hand, is susceptible to chloroquine in only a few areas, and those are likely to decrease with time (see Section 1.5). As an example of the consequences of increasing resistance, a prospective study of malaria mortality from 1984 to 1995 in Senegal suggested that the emergence of chloroquine resistance was accompanied by an increase in the risk of malaria death, especially among children (164). In sub-Saharan Africa, many countries are faced with increasing levels of chloroquine resistance. Some have already replaced chloroquine as the first-line therapy while others are assessing the current status of drug resistance and making national policy-level decisions. Standardized protocols and processes of making such decisions have been suggested (165).

Chloroquine is one of the most widely available and widely consumed drugs. It has been estimated that self-medication may account for as much as one-half the consumption of antimalarial drugs, most often chloroquine. Self-medication, inadequate dosing, and subtherapeutic blood levels are frequent and are believed to be predominate factors contributing to the development of chloroquine resistance. In Africa, many patients presenting for treatment have evidence of recent intake of chloroquine. A high prevalence of sub-curative chloroquine in the blood of Nigerian children and considerable chloroquine resistance were confirmed (166). One study has shown a beneficial effect on clinical outcome from chloroquine self-medication perhaps because of the inhibitory action of chloroquine on cytokine production, particularly tumor necrosis factor or nitric oxide, both of which are implicated in the pathogenesis of cerebral malaria (167), although other studies have not documented this phenomenon (168).

Even though chloroquine has been used for years as a therapeutic and prophylactic agent, improvements in the dosing regimens are continuously sought. In chemoprophylaxis, lower doses of chloroquine taken daily were shown to provide adequate drug concentrations that were as good as higher doses weekly or twice weekly (169). Compliance may be better with a daily tablet either as a single agent or in a combined formulation with proguanil. A prophylactic regimen of a loading dose followed by weekly chloroquine provided protection in the first week of dosing and better compliance; no post-exposure dosing was needed (170). Use of chloroquine prophylaxis in pregnancy provided a protective effect for the fetus such as a lower risk of neonatal infection, higher birth weights, and fewer perinatal deaths (171). The side effects associated with chloroquine use are blurring of vision, vertigo, ocular toxicity, pruritis, nausea, and headache. Hypotension and cardiovascular toxicity have been seen in children who were treated by intramuscular

injection. The drug can induce cardiac conduction disturbances, especially in extended use (172).

Over the years many proposals have been put forward on the mechanism of action of chloroquine and related antimalarials. A summary of these may be found in Refs. 28, 173, and 174. Most researchers in the field have focused attention recently on the inhibition of hemozoin formation by quinoline agents. For a brief discussion of the parasite's pathway for the metabolism of hemoglobin and the production of hemozoin, see Section 1.3.

Considerable evidence supports the proposition that inhibition of hemozoin formation is central to the mechanism of action of quinoline antimalarial agents (174–176). Much of the data on the effect of chloroquine on hemozoin formation was acauired when hemozoin/β-hematin was believed to be a polymer of hematin. Previous literature therefore describes the action of chloroquine as inhibiting heme "polymerization." Now that it has been shown that hemozoin is a crystalline dimer of hematin rather than a polymer (30), it would be more appropriate to discuss chloroquine's action on hemozoin formation. hematin dimerization, or heme sequestration.

The location of action of chloroquine is the parasite food vacuole, an acidic organelle wherein hemoglobin is digested. Chloroquine accumulates in the vacuole with an absolute dependence on the existence of a pH gradient between the acidic vacuoles and the extracellular medium, demonstrated in P. *falciparum*-infected human erythrocytes (177, 178). At pharmacological concentrations of chloroquine (10^{-9} M), drug concentrations in the food vacuole can reach millimolar levels at physiological pH. Compounds that inhibit the degradation of hemoglobin were found to be antagonistic to the action of chloroquine, quinine, amodiaquine, mefloquine, and halofantrine (179)supporting the view that this group of antimalarials is acting downstream of hemoglobin digestion in the food vacuole. That site of action appears to be the heme or hematin released by hemoglobin degradation. Chloroquine binds with high affinity to free heme (180), an observation that led to the original proposal that chloroquine action is mediated by binding to heme. Details of the binding of chloroquine with hematin have been studied using isothermal titration calorimetry (181). In addition, chloroquine accumulates bound to hemozoin and this binding is dependent on the presence of free heme (182). The binding of chloroquine/heme to hemozoin is saturable, specific, and has high affinity (39). A range of quinoline structures can compete for the binding, suggesting a common mode of action involving drug-heme-hemozoin interactions. Saturable chloroquine uptake at equilibrium is caused solely by the binding of chloroquine to hematin rather than to active uptake (183). In a study of chloroquine analogs, the structural features of binding to hematin was explored (184, 185). An electron withdrawing group at the 7-position of the quinoline nucleus, in particular a chloro group, was required for inhibition of both hemozoin formation and parasite growth.

The mere binding of chloroquine to hematin may prevent the sequestration of hematin into hemazoin (36, 186, 187). Hemozoin formation in not mediated by a classical enzymic process and no protein is required (34). The inhibition of both spontaneous and parasite extract-catalyzed β-hematin formation by chloroquine and other 4-aminoquinolines has been confirmed in **vitro** by a number of researchers (34, 187–189). Compounds with little or no antimalarial activity such as 9-epiquinine did not affect β-hematin formation. Heme sequestration activity may be caused, however, by the presence of preformed hemozoin in parasite extracts that act as nucleation sites for further dimerization (34, 190). The antimalarial action of a variety of quinoline agents correlates very well with their ability to inhibit hemozoin formation (174). The antimalarial activity of chloroquine and other quinolines has been shown to be a function of both the accumulation of the drug to appropriate concentrations at the site of action. the food vacuole, and interference with the process of hemozoin formation (191). By delaying the sequestration of heme, chloroquine may allow either hematin or a hematin-chloroquine complex to exert toxic effects (192–196).

However, there is good evidence (38) that heme binding proteins may also play a role, serving as a structural focus for the initiation of heme sequestration. P. *falciparum* histi-

dine-rich protein-2 (Pfhrp-2) is a major heme-binding protein in the parasite (40). There seems to be at least 18 heme binding sites on Pfhrp-2, perhaps associated with a recurrent hexapeptide repeat unit. Pfhrp2 promotes the conversion of heme to β-hematin under conditions that mimic the parasite food vacuole (197). Chloroquine was shown to inhibit the binding of heme to Pfhrp-2, although there was no detectable interaction of chloroquine with the protein in the absence of heme (40). Perhaps binding of heme to Pfhrp-2 is a necessary preliminary step before dimerization to hemozoin.

This may not be the complete picture however. Association of the aminoquinolines with hematin was shown to be necessary but not sufficient for inhibiting formation of β-hematin. Further, inhibition of β-hematin formation was necessary but not sufficient for antiparasitic activity (185). And inhibition of hemozoin formation may not be the only affect that chloroquine has on malaria parasites. There is evidence that chloroquine also interferes with the degradation of heme by peroxidation processes and that this might contribute to the observed lethal effects of the drug (198).

Given chloroquine's long history as a safe, well-tolerated, effective (until relatively recently), and affordable drug, understanding the details of the mechanisms of parasite resistance to chloroquine is critical. In chloroquine-resistant P. falciparum, the accumulation of chloroquine inside the parasite food vacuole is reduced (178, 182, 183, 199). This has been attributed to a rapid efflux of drug rather than decreased uptake. Verapamil inhibits multi-drug resistance in mammalian tumor cells and was found partially to reverse chloroquine resistance in malaria parasites as well (200). This action is mediated by P-glycoprotein in the multi-drug resistant tumor cases, a protein coded by the gene mdr. Reversal of resistance is specific for genetically resistant parasites and does not result simply from a change in the pH gradient from external medium to vacuole (201). A number of other agents have been shown to have similar resistance-reversing behavior (202–205). A few studies demonstrated benefits from combining chloroquine with a resistance reversal agent in animal models (206) and in the clinic (207). For a review of the reversal of chloroquine resistance, see Ref. 208. Highly-resistant lines of P. berghei, however, were associated with a loss of drug potentiation by verapamil (209), suggesting that parasites are able to avoid the activity of the reversing agents.

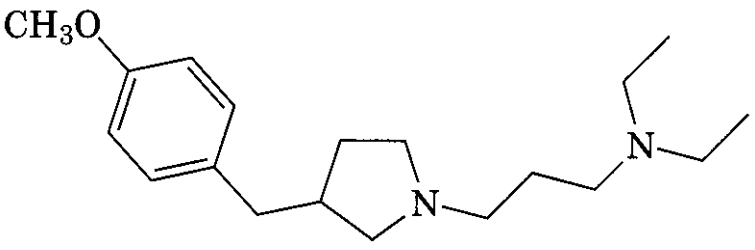
The parasite version of mdr, the P. falciparum multi-drug resistance gene pfmdrl, which encodes a P-glycoprotein homologue (Pgh1), has been proposed as the determinant of chloroquine resistance, and an association has been reported between resistance to chloroquine and amplification or mutation of pfmdrl (210, 211). The chloroquine-resistance phenotype was dissociated from inheritance of thepfmdrl gene, located on chromosome 5, in genetic studies, however (212–215), suggesting that other factors were most likely involved in the molecular mechanism of resistance. Some studies found an association between pfmdrl mutations and chloroquine resistance in field isolates (216–220) while others did not (221–228). It was shown recently that chloroquine-sensitive P. falciparum parasites that acquired pfmdrl mutations in transformation experiments did not become resistant to chloroquine (136). A genetic cross allowed the mapping of the locus for chloroquine resistance to a segment of chromosome 7. One gene on chromosome 7, cg2, and its polymorphisms were highly associated with chloroquine resistance (229–232), but allelic modification experiments failed to establish cg2 as playing a role in chloroquine resistance (233).

Recently, a new gene of interest, *pfcrt*, was identified near cg2 on chromosome 7 (234), a location that had been implicated earlier in chloroquine resistance (235). This gene encodes PfCRT, a transmembrane protein that seems to function as a transporter in the parasite food vacuole. Point mutations in pfcrt were completely associated with chloroquine resistance in vitro in laboratory lines of P. *falciparum* from Africa, South America, and Asia (234). One mutation, a replacement of lysine with threonine at position 76, was present in all resistant isolates and absent from all sensitive isolates tested in vitro. Transformation experiments demonstrated that mutant forms

of pfcrt were able to confer chloroquine resistance in clones originally sensitive to chloroquine. These studies point to a key role for the pfcrt T76 mutation in in vitro chloroquine resistance (236).

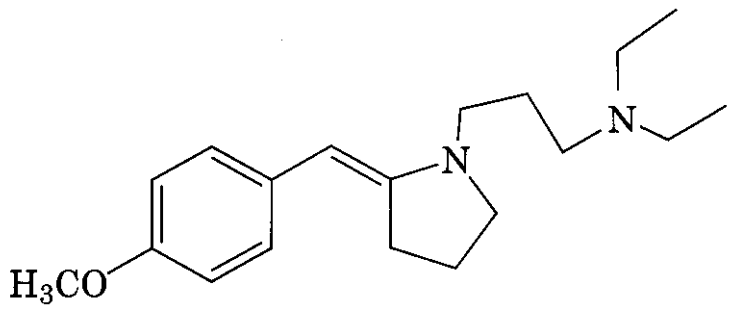
Many researchers then began to examine the role of pfcrt mutations in clinical cases of chloroquine resistance. There was a significant association, often complete, between the T76 mutations ofpfcrt with in vitro and in vivo response in field isolates from Mali (237), Sudan (238), Uganda (239), Cameroon (240), Mozambique (241), Nigeria (242), Indonesia (243), Laos (243), Papua New Guinea (244), and non-immune travelers returning to France (245). Where the correlation was less than perfect, researchers suggested this as most likely caused by the presence of multiclonal infections. Mutations in pfcrt, pfmdrl, andpfdhfr (the dihydrofolate reductase gene) were frequent in Ghana and often occurred together (246). A number of haplotypes of pfcrt in the region of residues 72–76 were identified in isolates from Papua New Guinea and all contained threonine at residue 76 (244). Thepfcrt T76 mutation has been suggested as a molecular marker for chloroquine resistant P. falciparum (237). Recent field studies showing the role of mutations in pfcrt in chloroquine resistance for P. falciparum have been reviewed (247).

Another class of agents does not restore chloroquine accumulation but acts by some other mechanism to reverse chloroquine resistance (248,249). The prototype of this group is CDRI 87/209 (7). It is only weakly anti-plas-



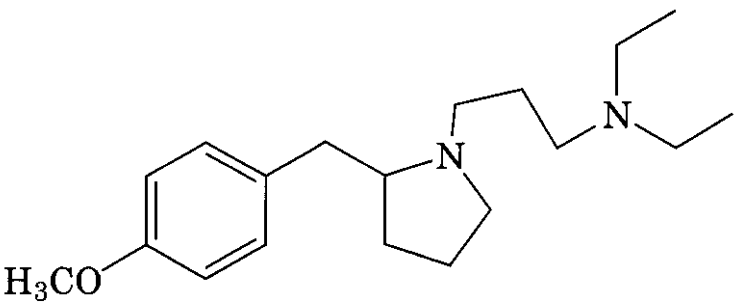
(7)
CDRI *871209*

modicidal alone, but an increase in drug susceptibility was observed only in resistant parasites and not in sensitive strains. Rotationally constrained analogs of CDRI 871209 were prepared and evaluated (250). A possible mode of action for the most active compound



(8)

in the series (8)relates to the heme degradation pathway and more specifically to heme oxygenase of the chloroquine-resistant parasite. Drug treatment leads to increased concentration of heme in the food vacuole and to increased levels of a heme-chloroquine complex resulting in toxicity to the parasite. Along similar lines, a pyrrolidino alkaneamine, WR268954 (9), is active in vitro as a chloro-



(9)
WR268954

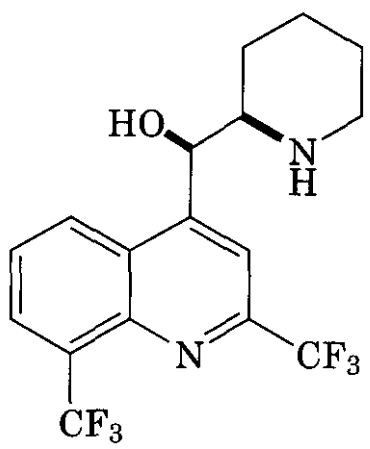
quine resistance modulator (251). Besides increasing the effects of chloroquine in resistant strains, the effects of quinine were also increased; mefloquine resistance was not affected.

Efforts continue to identify new aminoquinolines that would be effective against chloroquine-resistant strains and to clarify the structural features needed for good activity (173, 252, 253). The length of the chain between the aminoquinoline and the terminal tertiary amine was found to be critical. Replacement of the 7-chloro group of chloroquine with a 7-bromo or 7-iodo group and altering the side-chain length yielded aminoquinolines with good activity against resistant strains in vitro (254). Another strategy was to link two chloroquine moieties by bisamide units (255). Although the observed in vitro activity was not particularly high, enhanced activity against chloroquine-resistant

strains was noted. Strategies to prolong the usefulness of chloroquine have included exploring some metal-chloroquine combinations. A complex of gold with chloroquine was found to be active in vitro against chloroquine resistant strains of P. falciparum and in vivo against P. berghei in mice (256). Chloroquine analogs incorporating a ferrocene unit have been prepared and evaluated for antimalarial activity (257–259); the metallocene compounds had promising antimalarial activity in *vivo* in mice and in vitro against chloroquine-resistant strains of P. falciparum. Complexes of chloroquine with either rhodium or ruthenium were synthesized and evaluated for antimalarial activity (260). The complex with ruthenium was more active than chloroquine against P. berghei and against chloroquine-resistant P. falciparum and had low toxicity.

2.3 Mefloquine

In 1963 the Malaria Research Program was re-established at the Walter Reed Army Institute of Research. The stated goal was the identification and development of new antimalarial agents with particular regard for those compounds that would be effective against drug-resistant, especially chloroquine-resistant, strains of P. falciparum. Mefloquine (10), a 4-quinolinemethanol structurally related to quinine, was a product of that effort and was selected from a group of nearly 300 quinoline methanol agents because of its high activity in animal models. A brief history of the route that led to mefloquine is presented in Ref. 261. Mefloquine was first marketed in 1985 and is perhaps the most studied of all the antimalarial agents. Several excellent and comprehensive reviews of mefloquine have appeared (261–264). A review of physical and pharmaceutical properties includes spectroscopic data (265). The relationship between electronic structure and antimalarial activity for mefloquine and some substituted derivatives has been explored (266,267).



(10)
mefloquine

The synthesis of racemic mefloquine (WR142,490) was first reported in 1971 along with isomers that differed in the position of the trifluoromethyl groups around the quinoline system (268). The 2,8-bis-(trifluoromethyl) arrangement proved to be the most active of the series. Although compounds that possessed an aryl group at the 2-postion of the quinoline ring were found to have augmented antimalarial activity, this structural feature also conferred unacceptable levels of phototoxicity. The replacement in mefloquine of the 2-aryl group with the 2-trifluoromethyl group allowed the compound to retain high activity without photo-toxicity. Hydrogen bonding between the amine and the hydroxyl is critical in the active conformations of mefloquine and related structures. A detailed discussion of structure-activity relationships (SAR) in arylmethanols can be found in Ref. 264. Attempts at improving the synthetic pathway to mefloquine have included the use of a Wittig rearrangement (269), through a cyanohydrin intermediate (270), and by way of a sulfoxide-Grignard (271). Nor-mefloquine, in which the piperidinyl ring has been replaced by a pyrrolidinyl ring, displayed ED_{50} values equivalent to those of mefloquine (272). An interesting analog of mefloquine in which the piperidine ring is replaced by a quinuclidine ring was reported, but no biological data were given (273).

Mefloquine is orally active and effective against the intra-erythrocytic stages of the plasmodium life-cycle. It has no effect on mature gametocytes. Although mefloquine does cause recognizable changes in pre-erythrocytic forms, the effects are insufficient to prevent further development of the tissue parasite. Thus, mefloquine is not a causal prophylactic agent (274). It is a relatively slow-acting drug with greatest activity against the more mature blood stages. Mefloquine ap-

proaches the ideal antimalarial agent in that, in areas with low levels of resistance to mefloquine, a single dose of the agent is effective in accomplishing a cure. A decided drawback is that mefloquine costs about 100 times as much as chloroquine.

The mechanism of action of quinoline-containing compounds has been the focus of much research. Despite extensive scrutiny, the exact mode of action of mefloquine is not known. Its effects are confined to the pathogenic blood stages of the parasite. Most recent studies support the proposal that mefloquine interferes with the parasite's ability to detoxify heme. Based on ultra-structural changes observed in vitro, the parasite food vacuole is the primary target of mefloquine action and the affinity of mefloquine for free heme is high. Like chloroquine, mefloquine has been demonstrated to inhibit heme sequestration. For a more complete discussion, see Section 2.3.

Mefloquine is metabolized in the liver and excreted in bile and feces, predominately. The main metabolite of mefloquine is carboxymefloquine, a 4-carboxylic acid derivative. This metabolite is present in plasma in concentrations that exceed that of the parent drug by up to threefold. Carboxymefloquine seems to be inactive against P. falciparum in culture (275). Quinine and ketoconazole were found to inhibit the metabolism of mefloquine in human liver microsomes (276).

In two studies, the enantiomers of mefloquine [(+)-11*R*,2'*S* and (−)-11*S*, 2'*R*] were found to be equally active in vitro against P. falciparum (277) and P. yoelii (278). In another, the enantiomers of mefloquine and its threo isomer (WR 177,602) displayed up to a twofold difference in potency (120). The pharmacokinetics of the mefloquine enantiomers in whole blood were observed to be stereoselective both in adults (279) and in children (280), although uptake occurs in a non-stereospecific manner (281). The half-life of the (−)-enantiomer was significantly longer than of the (+)-enantiomer. Brain penetration of (+)-mefloquine is much higher than that of the (−)-enantiomer (282).

Several reviews of mefloquine pharmacokinetics have appeared (137,262,283,284). All the pharmacokinetic studies have been carried out with oral dosage forms because there is no parenteral formulation available. Generic mefloquine tablets were found not to be bioequivalent to the reference tablet in one study (285,286). The drug shows marked differences in pharmacokinetics between healthy volunteers and patients and between some ethnic groups. Absorption of mefloquine is generally good and is enhanced by food (287). The elimination half-life of mefloquine is relatively long (14–28 days), and a single day treatment is generally effective. The long half-life also presents the opportunity for resistant strains to arise. The drug does not seem to accumulate with repeated administration.

In animal studies, mefloquine displays low toxicity. In humans, however, the incidence of minor adverse effects seems to be high. A series of non-serious adverse effects have been noted with mefloquine treatment including vomiting, dizziness, nausea, anorexia, dermatological conditions (288), and general malaise (289–291). Early vomiting was correlated with poor treatment outcome, despite re-administration of the drug. Splitting the dose of mefloquine reduced the incidence of adverse effects. Travelers seem to report adverse effects from mefloquine more often than do malaria-experienced populations. The safety of mefloquine in pregnancy has been reviewed (155). In a retrospective study of mefloquine treatment in pregnancy along the western border of Thailand, it was found that women who received mefloquine during, but not before, pregnancy had a significantly greater risk of stillbirth than those exposed to other treatments or those who had no malaria (292). The association remained even after adjusting for all identified confounding factors; mefloquine was not associated with abortion, low birth weight, neurological retardation, nor congenital malformation.

Serious neurological or psychiatric reactions (hallucinations, neurosis, affective disorders, anxiety, and depression) have been associated with mefloquine use (293). Most of the reports relate to its prophylactic use; however, the incidence of serious reaction is higher when mefloquine is used for treatment rather than prophylaxis. The risk of serious neuropsychiatric reaction was seven times higher if mefloquine was given to treat a recrudescent infection that had originally been treated with

mefloquine. In one case, neuropsychiatric symptoms were presented after a single dose of mefloquine (294). A combination of mefloquine prophylaxis and ethanol consumption resulted in two episodes of severe psychiatric disturbance in one individual (295). Seizure has also been reported as resulting from mefloquine prophylaxis (296). The drug clearly should not be used in patients with a history of neuropsychiatric disturbances.

To delay the development of resistance to mefloquine, it was recommended that mefloquine be used only in combination. In particular, the combination of mefloquine with pyrimethamine and sulfadoxine (Fansimef) has been widely used in Southeast Asia. Initial clinical trials confirmed the efficacy of the triple combination in preventing and treating drug-resistant falciparum malaria and in delaying the development of mefloquine resistance (297). There seemed to be no pharmacokinetic interaction between the three components (298), and the pharmacokinetics have been reported (299, 300). Unfortunately, the strategy has failed and over a 6-year period, during which mefloquine was used only in the triple combination, resistance to mefloquine increased rapidly. Given the possibility of severe dermatological reaction to the pyrimethamine/sulfadoxine components and the apparent lack of therapeutic advantage at present over mefloquine alone (301) or pyrimethamine/sulfadoxine alone (302), the triple combination is no longer recommended for treatment. Even so, a trial in Nigeria found a low dose regimen of mefloquine/pyrimethamine/sulfadoxine to be significantly more efficacious than chloroquine alone, which is perhaps not surprising given the level of chloroquine resistance in that area (303).

Mefloquine combinations with a variety of artemisinin-derived agents have been the subject of study especially in areas where multi-drug resistant strains of P. falciparum are notoriously difficult to treat. The fast-acting artemisinin derivative clears a large percentage of parasites quickly and then the longer circulating mefloquine can act on any remaining parasites. The combination increases cure rates in falciparum patients, reduces transmission, and may slow the development of resistance. Pharmacokinetic studies support the in vitro and in *vivo* synergistic antimalarial activity of artemisinin derivatives and mefloquine (304). Artesunate (305, 306), artemether (307), artemisinin (308), and dihydroartemisinin (309) have all been used in combination with mefloquine (310, 311). Two-day courses of treatment have better success than single-day regimens. In many cases, the overall efficacy of the combination was better than with mefloquine alone or than other therapies such as quinine/tetracycline. In studies of the pharmacokinetics of artemisinin derivatives with mefloquine, the bioavailability of mefloquine was reduced in the presence of either artemether or artesunate (312, 313). A sequential schedule of dosing, an artemisinin followed by mefloquine, may be necessary for maximum efficacy. Other mefloquine combinations include those with tetracycline and with pyrimethamine/sulfadoxine/tetracycline (314, 315).

Mefloquine is the only newer antimalarial that is recommended for use as a prophylactic agent. In travelers and malaria-naive persons temporarily exposed to the risk of drug-resistant falciparum malaria, weekly mefloquine seems to be the agent of choice. In a comparison of mefloquine with chloroquine/proguanil for prophylaxis, several studies found both regimens to be well-tolerated and effective. A higher withdrawal rate was noted by some researchers for those taking mefloquine, presumably caused by side effects (316), whereas others detected no significant difference in side effects (317) or in compliance (318) between the groups. None of the subjects developed serious neuro-psychosocial reactions in one report (317), whereas another observed significant differences (319). In an assessment of the risks of mefloquine prophylaxis, one author concluded that mefloquine is the most effective chemoprophylactic drug for much of the tropics, that it is as well-tolerated as less-effective alternatives, and that it rarely causes of life-threatening adverse effects (320). As with mefloquine treatment, mefloquine prophylaxis is clearly contraindicated in persons with a history of psychiatric illness or seizures.

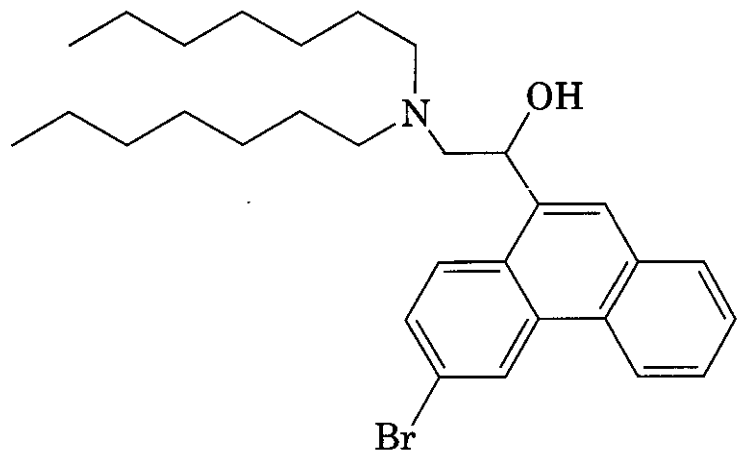
As with all drugs studied, stable resistance to mefloquine could be induced in sensitive

strains by applying continuous drug pressure (321). Mefloquine resistance as a clinical problem had been confined to specific areas of Southeast Asia. Up to a 10-fold decrease in sensitivity to mefloquine was observed since 1984 on the Thai-Myanmar border (322). In these multi-drug resistant areas of Thailand, mefloquine prophylaxis was not 100% effective, however, and continued monitoring of the levels of mefloquine sensitivity in such areas will be important (323). Although mefloquine continues to provide a high cure rate in Brazil, cases of emerging resistance have been documented (324). In contrast, mefloquine exhibited 100% growth inhibition against wild isolates from Tanzania in an in vitro study (325), and it was effective in areas with high levels of resistance to chloroquine and pyrimethamine/sulfadoxine (326). When resistance emerges, increasing the dose of mefloquine will bring temporary respite but will undoubtedly be associated with increased adverse effects. There is some evidence that resistance to mefloquine can be conferred by mutations in Pghl, the protein product of pfmdrl (136) or by an increase in pfmdrl copy number (327).

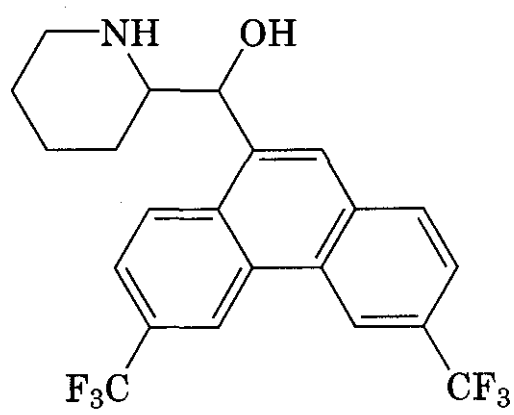
selection for mefloquine resistance in P. falciparum may be linked to amplification of the *pfmdr1* gene (210,211,328) and to cross-resistance to halofantrine and quinine (222, 329, 330), although the data are mixed. In some studies, a clear association is found between increased pfmdrl gene copy number (327), pfmdrl sequence polymorphism (331), or mutations in Pghl, the protein product of pfmdrl, (136) and mefloquine resistance. But results from other studies suggest that amplification and over-expression of pfmdrl are not involved in the increased mefloquine resistance phenotype (332). But, none of these factors is a prerequisite for mefloquine resistance and other mechanisms must also be able to mediate resistance to mefloquine (333). The exact relationship of pfmdrl to mefloquine resistance and any clinical significance thereof are not known.

2.4 Halofantrine

Phenanthrenemethanols were first discovered to possess antimalarial activity during the drug discovery efforts of World War II. It was not until 20 years later that further studies were undertaken on this class of compounds. The first two members of the series that were examined in depth were WR33,063 (11) and



(11)
WR 33,063



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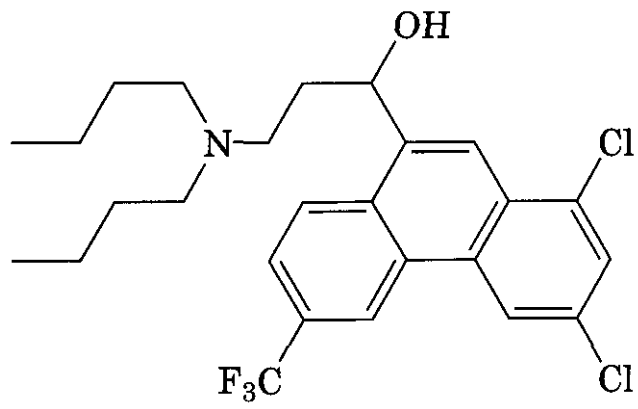
WR 122,455

WR122,455 (12), both of which displayed good activity in chloroquine-resistant strains, but high cure rates required multiple doses over several days. Halofantrine (13, WR 171,669),



(13)
halofantrine

originally synthesized by Colwell et al., (334) was the third synthetic phenanthrenemethanol considered. In structure-activity relation-

ship studies, it was determined that the dibutylaminopropanol side-chain was optimal for the substituted 9-phenanthrene system. Initial human trials confirmed that halofantrine was a promising new agent for the treatment of malaria (335). A study of the molecular electronic properties of carbinolamine antimalarial agents, including halofantrine, indicated that potent compounds share specific molecular electronic properties regardless of the type of aromatic ring and of the nature of the amine side chain, whether cyclic or aliphatic (266). Reviews of the antimalarial activity, pharmacokinetic properties, and therapeutic potential of halofantrine have appeared (283, 336–340).

Halofantrine was placed onto the market in 1988 by Smith, Kline & French, with its first introduction being in Ivory Coast. Halofantrine is marketed as the racemate; (−)-halofantrine is the *S* isomer as determined by X-ray (341). The separated enantiomers display similar antimalarial activity against P. berghei in mice and in *vitro* (277,342). Metabolic studies have shown that on incubation of (±)-halofantrine with either rat liver homogenate (343) or in humans (344), (−)-halofantrine was metabolized preferentially. The primary metabolite is N-desbutylhalofantrine, either enantiomer of which is pharmacologically active in vitro (345,346). Significant differences were noted in the plasma concentrations of the enantiomers of halofantrine; (+)-halofantrine and its N-monodesbutyl metabolite had higher values than (−)-halofantrine and the corresponding metabolite (347). Minor metabolites have also been identified (348). Halofantrine is metabolized primarily by CYP3A4 but CYP3A5 is also involved; inhibitors or other CYP3A4 substrates, such as ketoconazole, might serve to increase halofantrine concentrations, and consequently, the associated cardiotoxicity (349). Quinine and quinidine, when incubated with halofantrine in human hepatic microsomes, inhibited its metabolism (350). One the other hand, halofantrine is an inhibitor of CYP2D6 activity in healthy Zambians reinforcing a concern over concomitant dosing with agents that are metabolized significantly by CYP2D6 (351).

Halofantrine is schizontocidal with selective activity against the erythrocytic stages of Plasmodia, especially the more mature parasite stages. The drug accumulates in parasitized red blood cells with concentrations up to 60% higher than in uninfected cells; erythrocytes with mature parasites showed the highest accumulations (352). There is no observed action on gametocytes (353) nor hypnozoites.

Clinical trials with halofantrine have confirmed the efficacy of the drug against multidrug resistant strains of P. falciparum and against P. *vivax*; in the few cases of P. *ovale* and P. malariae in which halofantrine has been used, good results were obtained with no recrudescence (338). The relative efficacy of halofantrine compared with mefloquine varies with the test system but the effective doses in humans in sensitive strains were similar. The precise dosage regimen seems to be of critical importance. A standard 1-day regimen of halofantrine, even with larger doses, was inadequate therapy for falciparum malaria in multi-drug resistant areas such as the Thai-Burmese border (354). Single-dose treatments, even with larger doses, were plagued by recrudescence. On the other hand, multidose regimens have been studied with good results. One recommended halofantrine regimen is 24 mg/kg followed by a second treatment 7 days later, but cardiac complications are more common after the second treatment. Lowering the follow-up dose to 250 mg, which may reduce the risk of cardiac side effects, was shown to be effective in non-immune people with mild-to-moderate falciparum malaria (355). Treatments 7 days apart offer problems with compliance, however. In a randomized, comparative dose-finding trial in Colombia, the best cure rates were obtained with three doses of 500 mg of the hydrochloride at 6-h intervals (356); the study was carried out on patients from an area known to contain multidrug resistant P. falciparum. A combination of halofantrine with primaquine was significantly more effective against falciparum malaria than a chloroquine/primaquine combination and similarly effective against vivax in Irian Jaya, Indonesia (357). Fever cleared much faster and was associated with a more rapid and significant decline in malaria-related physical complaints.

As is the case with all antimalarial drugs, the incidence of resistance and cross-resis-

tance is important (330). In one study, halofantrine displayed activity against lines that were primaquine-, cycloguanil-, pyrimethamine-, and menoctone-resistant (358). But parasites resistant to quinine, chloroquine, mefloquine, and amodiaquine showed resistance to halofantrine as well. An artemisinin-resistant organism has a somewhat reduced response to halofantrine. In an in *vitro* study of P. falciparum sensitivity in Burkina Faso, resistance to halofantrine was discovered in 1% of the isolates in 1995 and 9.6% in 1996 (133). A single halofantrine resistant isolate was also resistant to chloroquine in 1996. A fourfold decrease in halofantrine sensitivity was noted in Gabon from 1992 to 1994 in P. falciparum isolates (359) while halofantrine remained effective in Nigeria in an area where chloroquine sensitivity continued to drop (360). In one set of experiments, researchers uncovered evidence that mutations in Pghl, the protein product of *pfmdr1*, can confer resistance to halofantrine (136).

A disturbing trend has been observed, however, in locales with highly multi-drug resistant strains. Some strains seem to have diminished sensitivity to halofantrine without drug pressure, that is, in areas where the drug had not been used before. Resistance to halofantrine in P. falciparum has been linked to the amplification of the pfmdr 1 gene. Pfmdrl, is found to have increased gene copies and to be overexpressed in association with halofantrine- and mefloquine-reduced susceptibility (222,329). In contrast, another study supplied evidence that resistance to halofantrine (and mefloquine) was related to reduced drug accumulation within the parasite that occurred without overexpression of the *pfmdr* protein product (361).

There is some evidence to suggest that halofantrine interacts with ferriprotoporphyrin IX in much the same way as chloroquine, although the data are conflicting (338). One study demonstrated the formation of a complex between halofantrine and FP, whereas another failed to observe it. The morphological changes seen in mouse red blood cells infected with P. berghei and treated with halofantrine are similar to those observed with quinine and mefloquine, although halofantrine also induces mitochondria1 damage. Alternatively, the main action of halofantrine may involve inhibition of a proton pump present at the host-parasite interface; halofantrine was observed to inhibit glucose-dependent proton efflux from P. *berghei*–infected erythrocytes.

Halofantrine is a potent and selective inhibitor of the catalytic subunit of cyclic AMP-dependent protein kinase. No inhibition was observed with other protein kinases and halofantrine; antimalarial agents such as chloroquine, pyrimethamine, and artemisinin did not display the effects of halofantrine (362). It is not clear whether the kinase activity is related to antimalarial action, to cardiac side effects, or to neither.

One drawback of halofantrine, a highly lipophilic molecule, is its poor and variable absorption. Drug absorption is improved by food intake (363–365), and the aqueous solubility of halofantrine increases with addition of caffeine or nicotinamide (366). Even so, the practicality and advisability of administering food to very ill persons is questionable. The drug is extensively bound to lipoproteins in human serum (367) and it perturbs lipid bilayers (368). The distribution of halofantrine between lipoprotein fractions, both pre-prandial and post-prandial, has been explored (369, 370). The IC_{50} value of halofantrine was shown to be increased by increasing concentrations of triglycerides (371). The data suggest that altered plasma lipoprotein profiles could influence the pharmacodynamic profile of halofantrine. The authors also argue for care in the interpretation of drug sensitivity data, especially when monitoring the development of drug resistance based on unknown lipoprotein profiles.

When treatment failures have been observed, several reasons have been cited although complete assessment has not been made. One factor associated with treatment failure is low blood concentrations of the parent compound and of the active metabolite, desbutylhalofantrine caused by poor absorption. The possibility of resistance or decreased susceptibility cannot be ruled out, however. Some cross-resistance between halofantrine and mefloquine has been observed; in cases of recrudescence, re-treatment with halofan-

trine was less effective after failure of mefloquine. Even in these cases, halofantrine treatment was more effective than re-treatment with mefloquine.

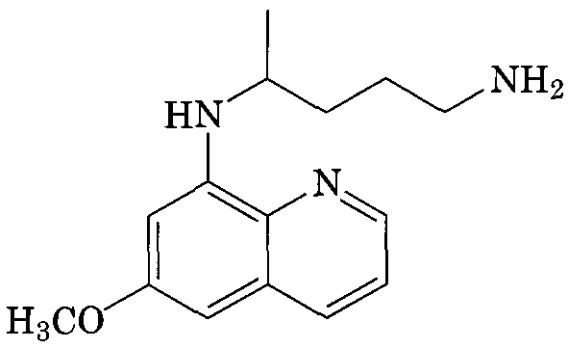
Halofantrine was found to be generally well tolerated with less central nervous system or upper gastrointestinal side effects than mefloquine. The most commonly reported adverse effects were abdominal pain, pruritis, vomiting, diarrhea, headache, and rash. Nevertheless, serious concerns have been raised about cardiac effects with halofantrine treatment (372,373). In a group of children being treated with halofantrine, serious cardiac side effects were noted (374, 375). Because the observed electrocardiographic effects are dose-dependent (376), extra caution is needed at the higher dose used in conjunction with multidrug resistant strains and after mefloquine failure. Mefloquine caused significant changes in the clearance of high dose halofantrine (377), necessitating caution when treating persons who may have been on mefloquine prophylaxis before treatment with halofantrine. Given the long biological half-life of mefloquine, the effect may be exacerbated by drug interaction between halofantrine and mefloquine. Some have suggested that electrocardiography be performed on patients before initiating halofantrine treatment to identify those persons at the greatest risk (378). Cardiotoxicity of the (+)-isomer of halofantrine is greater than for the (−)-compound. This may reflect a more general pattern seen in the greater cardiotoxicity of quinidine over quinine. Mapping of the one chiral center in halofantrine with the secondary carbinol of quinine/quinidine suggests a similar spatial organization (341).

No evidence of mutagenic, teratogenic, or genotoxic effects or of effects on fertility was observed in animal toxicity studies with halofantrine (155,379). Embryotoxicity could only be demonstrated at doses that caused maternal toxicity. No cases of adverse outcomes of human pregnancy on accidental exposure to halofantrine have been reported.

Halofantrine is currently recommended only for the treatment of malaria. It is not recommended for use as a suppressive prophylactic to minimize the selection of drug resistance and because of inadequate long-term toxicity data. In addition, halofantrine has no action on tissues stages and is not recommended as a causal prophylactic. Given the emerging picture of potentially serious complications, the appropriate use of and prescription guidelines for halofantrine have appeared (380). It has been recommended that halofantrine be restricted to use in cases of suspected chloroquine-, quinine-, or pyrimethamine/sulfadoxine-resistant malaria and that it should not be employed for general use (381). As with other newer antimalarial agents, warnings have been sounded about the use of halofantrine for monotherapy. The cost of halofantrine is about 100 times that of chloroquine.

2.5 Primaquine

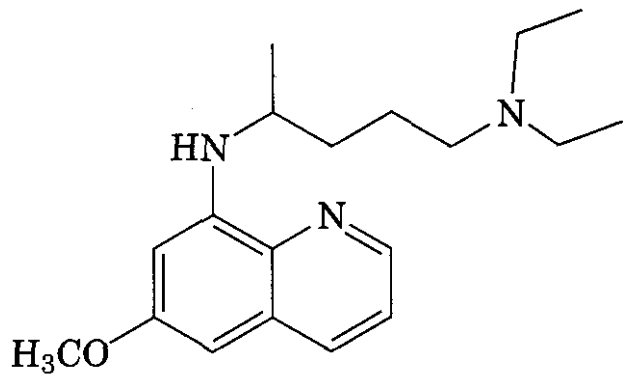
P. vivm and *P. ovale* are the true relapsing malarias in humans, P. *vivax* being by far the more common. Dormant hypnozoites can reside in the liver for months and years after an initial infection, providing a reservoir of parasites that are the cause of malaria relapses. Radical cure of a vivax or ovale infection must involve not only removal of erythrocytic forms of the parasite but of the remaining liver forms as well. Agents that act on liver forms are termed tissue schizontocides and only one drug, primaquine (**14**), an 8-aminoquinoline,



(14)
primaquine

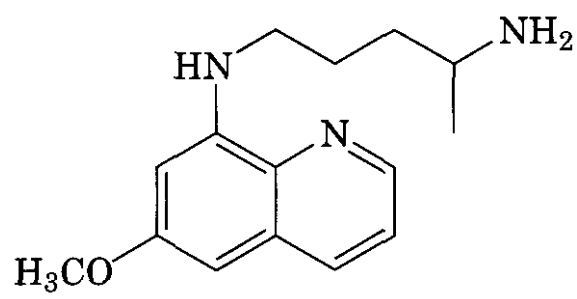
is currently available to effect such a radical cure.

The prototype 8-aminoquinoline was pamaquine (15, plasmochin), introduced in 1925. The action of pamaquine was clearly different from that of quinine, especially in its ability to prevent relapses. At the beginning of the Second World War, pamaquine was the only antirelapse drug available. Therapy for vivax malaria involved a combination of pamaquine with quinine or quinacrine. Because of human



(15)
pamaquine

toxicity, the use of pamaquine declined, and other 8-aminoquinolines were synthesized. Of these, primaquine (WR 2975, 14) has been used extensively as the anti-relapse drug of choice. d-, *l*-, and dl-Primaquine are essentially identical in curative properties against P. cynomolgi (382). Quinocide (**16**), an isomer



(16)
quinocide

of primaquine, has been used extensively in Eastern Europe and the former Soviet Union.

Primaquine has a multiplicity of antimalarial activities (339). To clear hypnozoites from the liver and to effect a radical cure of relapsing malarias, primaquine is given daily for 14 days in a usual dose of 15 mg. The standard treatment often involves a combined regimen of chloroquine with primaquine. Chloroquine is given to eradicate the asexual erythrocytic stages of the parasite. In addition, primaquine is active against the primary exoerythrocytic forms and could be used as a causal prophylactic, although until recently it was believed that the drug was too toxic for such prolonged use. A single dose of primaquine (35–45 mg base) is effective against gametocytes of P. falciparum, blocking infection of the vector. For this purpose, it is often given with either mefloquine or quinine to further interrupt transmission of the infection. Finally, primaquine is active (as metabolites) in inhibiting the development of sporozoites in already infected mosquitoes.

Given that primaquine has been in use for many decades, it comes as no surprise that parasites resistant to it have been reported (68, 383). For example, between 1977 and 1997, the efficacy of a 5-day primaquine regimen in India declined from ~99% to 87%, although a 14-day regimen provided complete clinical cure with no relapses in a 6-month follow-up period (384). Unlike the situation with chloroquine and the 4-aminoquinolines, defining resistance to primaquine is problematic (70). Different strains of P. vivax display widely differing degrees of susceptibility to primaquine, a phenomenon of long-standing, not believed to be a function of varying drug pressure. Strains from New Guinea (the Chesson strain) are the least sensitive to primaquine, whereas those from Vietnam occupy an intermediate position, and the Korean strains are most sensitive. The interval between primary infection and relapse also varies with differing strains of P. vivax. The Chesson strain relapses rapidly, at intervals of 28 days; strains from southern China may relapse on an approximately annual basis. Recent studies have suggested that different populations of hypnozoites may have different susceptibilities to primaquine (385). So the incidence of primaquine-refractive strains may reflect increasing resistance or it may be the natural heterogeneity of P. vivax. Furthermore, primaquine in general is not as effective against erythrocytic parasites as chloroquine and others. When symptoms reappear, it may be a result of recrudescence or re-infection as well as primaquine resistance, and separating these possibilities is often not possible. Unlike the situation with P. falciparum, molecular events involved in resistance of P. vivax to chloroquine do not seem to be associated with mutations in *pfcrt* (236,386). For a review on drug-resistant P. vivax, see Ref. 67.

Although primaquine has activity against the blood forms of the parasite, the dose required for monotherapy was considered too toxic for widespread use. However, much of the evidence for this toxicity resulted from data acquired with other, less active, and more toxic 8-aminoquinolines, pamaquine and pentaquine. The intrinsic activity of primaquine

against P. *vivax* is certainly lower than that of all other commonly used agents except pyrimethamine/sulfadoxine when evaluated based on parasite clearance times (387). Similar results were observed with primaquine against P. falciparum (388). Therefore, for therapy, primaquine is combined with a faster acting agent, often chloroquine. With declining sensitivity of vivax to chloroquine, other agents have been studied as partners with primaquine including Fansidar and artesunate (389).

The causal prophylactic activity of primaquine is well established. Even so, because of problems with toxicity, real or perceived, routine use of primaquine for chemoprophylaxis had not been promoted. The diminishing efficacy of chloroquine and other agents is prompting a re-evaluation of the prophylactic use of primaquine (90). Primaquine prophylaxis has been shown to be safe and effective against both P. falciparum and P. *vivax* in Colombian soldiers (390) and in non-immune travelers to Africa (391). It is more effective and better tolerated than prophylactic chloroquine (392, 393). In some cases the rates of protection were better for primaquine than for either mefloquine or doxycycline. The lack of effect of chloroquine is perhaps not surprising given the prevalence of resistance in the areas where these studies were carried out. Indeed, in a study of a prophylactic combination, results showed that the addition of chloroquine did not increase the prophylactic efficacy of primaquine (394). More surprising was the lack of drug-related adverse side effects. Incidence of methemoglobinemia after 50 weeks of daily primaquine prophylaxis were comparable with that seen in a standard 14-day course (395). Administration of the primaquine doses with food may have limited the adverse side effects that had been reported with extended primaquine use. If daily primaquine taken during the period of exposure completely prevents the establishment of liver forms of the parasite, continuation of suppressive treatment after leaving a malarious area would not be required. This would represent an advance in prophylaxis caused by poor compliance with the usual post-exposure regimen.

Primaquine is rapidly absorbed from the gastrointestinal tract reaching maximal concentrations 1-3 h after administration. The distribution of primaquine in blood involves extensive binding to glycoprotein in plasma (396). For reviews, see refs. 137,339, and 397. The pharmacokinetics of primaquine have been studied in both healthy volunteers (398) and in patients. The clearance of primaquine is reduced significantly during P. falciparum infection (399).

Metabolism of primaquine occurs rapidly in the liver, and the elimination half-life is 6 h. In rat liver homogenate, P450 and MAO enzyme systems contributed almost equally to primaquine metabolism (400). The metabolic profile seems to be balanced between activation to various hydroxylated derivatives (401), which may be responsible for the observed pharmacological and toxicological properties of the drug and conversion to the inactive carboxyprimaquine metabolite. Carboxyprimaquine, resulting from oxidative deamination of the side-chain, is the only metabolite of primaquine formed in human liver in *vitro* (402). The half-life of carboxyprimaquine is longer than that of primaquine, and its concentrations are much higher than primaquine itself in plasma. Carboxyprimaquine accumulates on daily dosing with a 14-day course of treatment. A number of hydroxylated metabolites of primaquine have been identified in animal models, although their isolation from humans remains to be accomplished. It has long been recognized that primaquine metabolites have significant effects on the oxidation state of the infected erythrocyte. Studies on 5-hydroxyprimaquine, 5-hydroxy-6-demethylprimaquine, and 5,6-dihydro-8-aminoquinoline have demonstrated the ready oxidation of these metabolites under physiological conditions (403, 404). A proposed mechanism of action for the 8-aminoquinolines involves the oxidation of such metabolites to a quinone-imine derivative, which may mimic ubiquinone. Analogs have been designed in which the metabolic pathway is blocked by a fluoro group at the 5 position (405). Metabolism of primaquine by human liver microsomes is inhibited by ketoconazole, a known inhibitor of cytochrome P450 isozymes. Quinine, artemether, artesunate, halofantrine, and chloroquine did not alter the metabolism of primaquine; only mefloquine had a slight inhibitory effect on

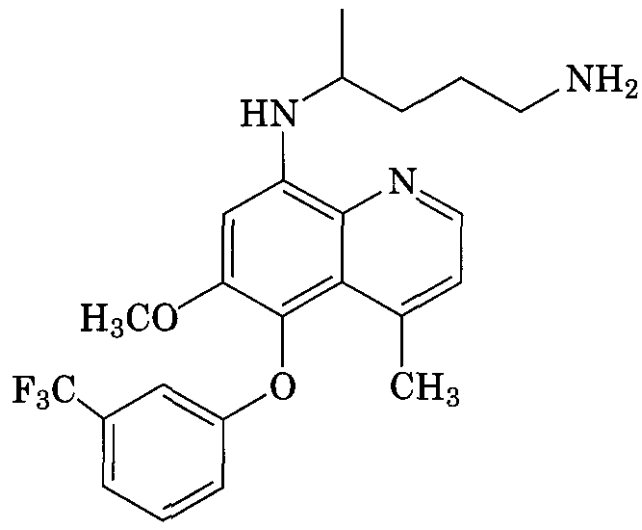
metabolite formation (402). Primaquine and some of its newer analogs induce changes in metabolic activity, particularly in CYP1A1 gene expression (406).

The most serious adverse effect associated with primaquine is intravascular hemolysis. Primaquine, when incubated with red blood cells, induces extensive cell lysis and oxidation of oxyhemoglobin to methemoglobin. The two toxic effects however are not causally related; in the presence of a stable nitroxide radical, cell lysis was prevented even though hemoglobin oxidation was enhanced (407). Other studies have also examined the formation of and biological results from radicals generated by primaquine and its metabolites (408–411). These results support the hypothesis that free radicals and redox-active species are involved in the cellular damaging processes induced by primaquine. Hydrogen peroxide has been suggested as the potential toxic product formed from oxidation and redox cycling of primaquine metabolites (404,412).

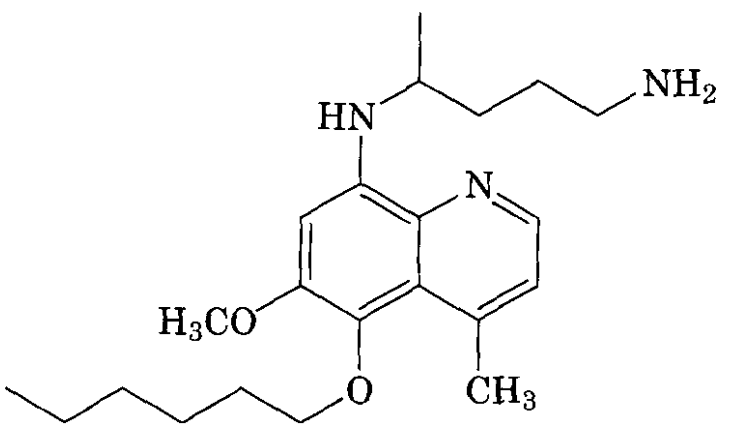
Persons who are deficient in the enzyme glucose-6-phosphate dehydrogenase (G6PD) are particularly at risk of hemolysis from primaquine treatment. G6PD plays an important role in the redox processes of the erythrocyte. The conversion of glucose-6-phosphate to 6-phosphonoglucon-D-lactone by G6PD produces NADPH from NADP+. NADPH is used by the erythrocyte to reduce the oxidized form of glutathione back to the reduced form. The reduced glutathione acts as a "sulfhydryl buffer" to maintain cysteine residues of hemoglobin and other proteins in the reduced state. Insufficient levels of the G6PD result in inadequate amounts of NADPH, making erythrocytes especially susceptible to damage from oxidants. As noted above, primaquine generates a number of toxic reactive oxygen species. Clearly, in patients with severe G6PD deficiency, primaquine should be avoided for both treatment and prophylaxis (413).

Methemoglobin formation is another predictable dose-related adverse effect. The usual 14-day curative regimen of primaquine has been reported to lead to a number of other symptoms including anorexia, nausea, cyanosis, epigastric distress, abdominal pain and cramps, malaise, dark urine, vomiting, and vague chest pains. Abdominal cramps are common when primaquine is taken on an empty stomach, but the discomfort is lessened markedly when it is given with food. Even standard doses of primaquine are to be avoided in pregnancy to minimize the hemolytic effects; the fetus is relatively G6PD-deficient (155). Primaquine was found to induce mutations in a standard assay (414).

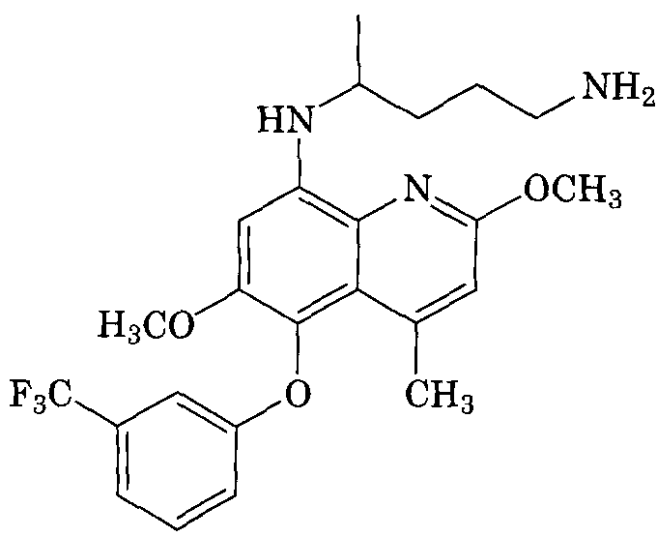
Two excellent reviews cover the general structure-activity relationships in the 8-aminoquinoline group (415, 416). A number of studies have been carried out with the aim of identifying drugs to replace primaquine as the agent of choice for radical cure of relapsing malarias; for a summary of the structural types of interest, see Ref. 417. In particular, a reduction in the toxicity associated with primaquine has been a goal. Early on, 4-methylprimaquine was shown to have superior activity to primaquine with less toxicity. Similarly, 4-ethyl and 4-vinyl primaquine retained the radical curative activity of primaquine and had better therapeutic indices (418). Further modification by the introduction of an aryloxy group at the 5-position of the quinoline enhanced antimalarial activity (419–421). The best of this series were fluorine-containing compounds that were more active than primaquine. The U.S. Army has been involved in research on a number of anti-relapse agents. Among these are WR225,448 (17), WR242,511 (18), and WR238,605 (19). All are modified 8-aminoquinolines clearly related to the primaquine structure. Although WR225,448 had good activity against the exoerythrocytic parasite and effective doses much lower



(17)
WR225448



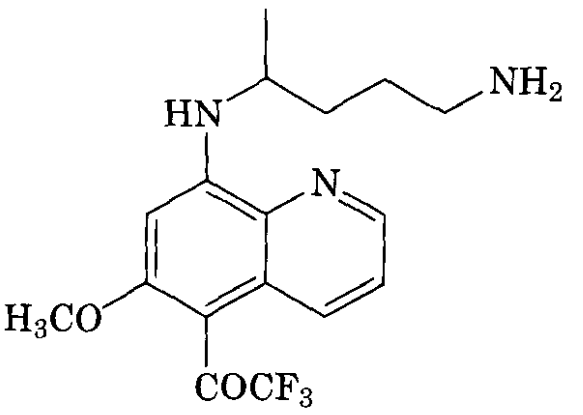
(18)
WR242511



(19)
WR 238,605
tafenoquine

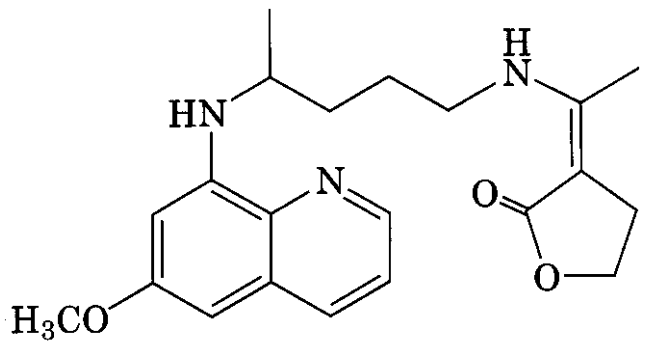
than those of primaquine, the liver toxicity was high (422). The pharmacokinetics of WR242,511 have been studied, and a significant lag was observed between in the appearance of drug in the plasma and the onset of methemoglobinemia. Even so, methemoglobin formation remained a major side effect (423). More promising is WR238,605, now called tafenoquine (424, 425). It is effective against pre-erythrocytic stages, drug-sensitive and multi-resistant erythrocytic stages, gametocytes, and hypnozoites (P. cynomolgi), with activity and safety profiles both significantly better than primaquine. Studies of tafenoquine prophylaxis have demonstrated that it was effective and well tolerated, and the duration of protection afforded by the drug is long (426). In a pharmacokinetic study, tafenoquine was administered in single oral doses; it was found to be well tolerated, had a long absorption phase, and was slowly metabolized with at„ of 12 h and an elimination half-life of 14 days (427). These workers concluded that the safety, efficacy, and pharmacokinetic properties make the drug an excellent candidate for use as a prophylactic, radical curative, and terminal eradication drug. Tafenoquine is now in phase II clinical studies in semi- and non-immune people.

Another strategy for developing agents with high activity and lower toxicity than primaquine has involved connecting amino acids or peptides to the free amine group of primaquine or primaquine analogs. 4-Methylprimaquines that had an alanine or a lysine conjugated to the free amine were more active and less toxic than primaquine (428). A peptide derivative of primaquine was shown to have equivalent antimalarial activity as the parent compound and less toxicity (429). Dipeptide derivatives of primaquine in which the oxidative deamination of primaquine was blocked were found to be gametocytocidal to P. berghei (430). The most active member of this group was gly-gly-PQ. 5-Trifluoroacetylprimaquine (M8506, **20**) was shown to be a causal prophylactic agent, equivalent in activity to primaquine in P. yoelii–infected mice (431). As a tissue schizonticide, it is more effective than primaquine in P. cynomolgi–infected rhesus monkeys and better tolerated than primaquine in mice, rats, and dogs.



(20)
M8506

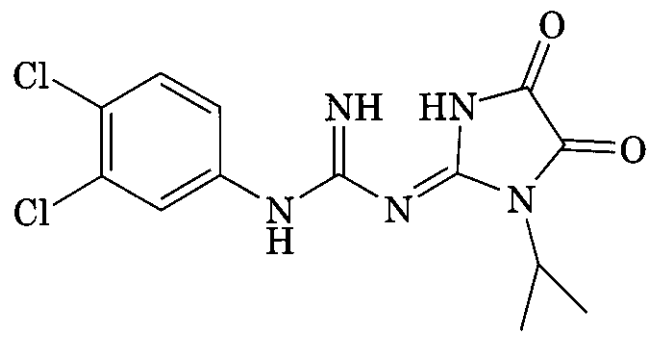
A prodrug of primaquine, CDRI compound 80153 (21, bulaquine), has been shown to possess radical curative and causal prophylactic action against P. cynomolgi in rhesus monkeys (432). In addition, this compound caused less methemoglobinemia than did primaquine (433, 434), had very low toxicity (435), and had lessened effects on hepatic enzyme levels



(21)
CDRI 80/53, bulaquine

when compared with primaquine (436). Clinical trials have shown that bulaquine was an effective alternative to primaquine in the treatment of vivax malaria (437). Bulaquine has been approved for use in India.

As a result of extensive screening efforts, a number guanylhydrazones were found to have causal prophylactic activity in rodents. One example of this group, WR182,393 (22) has



(22)
WR182393

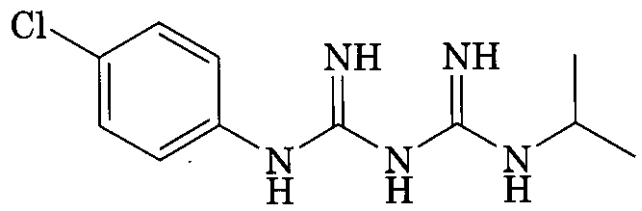
been shown to have both causal prophylactic and radical curative properties against a primate relapsing malaria (438). It is the first non-8-aminoquinoline to display both properties.

2.6 Antifolates

A number of agents have been discovered that affect antimalarial activity through inhibition of the parasite dihydrofolate pathways. These agents are divided into two types: inhibitors of dihydrofolate reductase (DHFR) and those that inhibit dihydropteroate synthetase (DHPS).

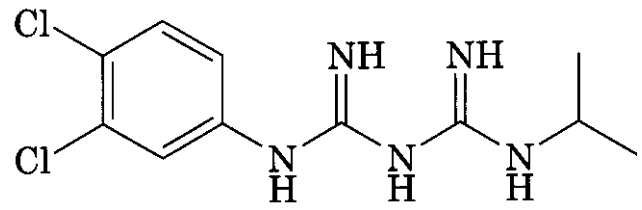
The pharmacokinetics of antifolates have been reviewed (137); this reference also has information about pharmacokinetic interactions of antifolates with other antimalarial agents.

2.6.1 Proguanil and Chlorproguanil. Studies in the synthesis of pyrimidine derivatives led British researchers at ICI to examine the antimalarial activity of biguanides. Proguanil (chloroguanide, Paludrine, 23) was the most



(23)
proguanil

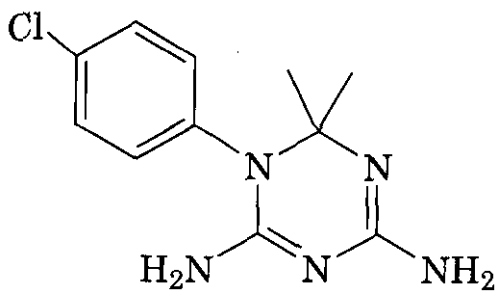
active of a series of these compounds screened in the 1940s. Proguanil proved to be an excellent causal prophylactic in falciparum malaria as well as a satisfactory suppressive of vivax malaria, although the drug is not active against hypnozoites (439). A related compound, chlorproguanil (24, Lapudrine) has



(24)
chlorproguanil

been used occasionally, and recent studies suggest that it may be a good alternative to proguanil in areas of drug resistance (440). Proguanil is a tissue schizonticide, active against pre-erythrocytic liver stages, and a sporontocide. Because of their slow action, the availability of faster-acting agents, and the fact that resistance seemed to develop quickly in some strains, these biguanides have not been used for monotherapy. During the 1980s, proguanil was temporarily removed from the list of recommended prophylactic agents because of what seemed to be wide-spread resistance in P. falciparum. A combination of proguanil with chloroquine was found to be an effective prophylactic regimen (441,442).

It has long been thought that the antimalarial activity of proguanil resided exclusively in a cyclized metabolite, cycloguanil (25); proguanil would then be a prodrug form of cycloguanil (443). A small percentage of



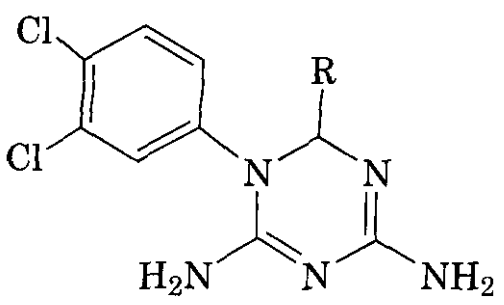
(25)
cycloguanil

proguanil is converted into an N-dealkylated product, 4-chlorophenylbiguanide, which is not active against malaria. Some confusion has arisen over the question of whether the antimalarial activity associated with proguanil is caused entirely by the metabolite cycloguanil or if the parent compound possesses some inherent activity itself. Some studies have reaffirmed that the activity associated with proguanil administration is completely accounted for by cycloguanil (444), whereas others find intrinsic efficacy against falciparum and vivax independent of cycloguanil (445). The activity of cycloguanil is not affected by physiological concentrations of folic acid or folinic acid in human serum, suggesting that the drug may have additional or different sites of action from inhibition of folate pathways (446). Clinical pharmacokinetic studies of proguanil and cycloguanil have been reported (283,447,448). Proguanil lacks serious side effects, is not mutagenic (449), and there is no evidence of any toxic effects of prophylaxis with proguanil during pregnancy from over 40 years of use (155). Cycloguanil acts specifically on P. falciparum DHFR and seems to have no other significant target; the action of proguanil may include sites of action other than on DHFR (450,451).

The enzyme that effects the conversion of proguanil to cycloguanil is a cytochrome P450, specifically CYP2C19. It has been shown that the ability to carry out the metabolic conversion varies widely among individuals and among ethnic groups (452, 453). Between 2% and 3% of Caucasian populations have a relative inability to convert proguanil to cycloguanil; the numbers may be as high as 20% in Oriental subjects and 25% in Kenyans. An association between CYP2C19 mutants and poor metabolism of proguanil was demonstrated (454). Compounds that inhibit CYP2C19, such as omeprazole and fluvoxamine, were shown to inhibit proguanil metabolism (455,456). In Asia and Africa, the variable metabolism of proguanil may have decided clinical importance. There was a significant correlation between breakthrough parasitemias and the proguanil/cycloguanil ratios in subjects who were taking proguanil prophylactically (457). A possible solution would be the development of cycloguanil itself as an antimalarial agent.

Proguanil, or more precisely cycloguanil, acts as a mimic of dihydrofolate. The drug competitively inhibits the parasite enzyme dihydrofolate reductase (DHFR, tetrahydrofolate dehydrogenase). Plasmodia require a tetrahydrofolate cofactor to synthesize pyrimidines de *novo* because they have no salvage pathways for pyrimidines. The binding of the drug to the plasmodial enzyme is several hundred times that of binding to the mammalian enzyme. In strains resistant to proguanil/cycloguanil, a mutated DHFR protein is observed that displays a reduced affinity for the normal substrate and for the inhibitor. These strains are hypersensitive to the sulfonamides and sulfones (inhibitors of DHPS), which also reduce the availability of dihydrofolate. Thus, a synergistic effect is observed between inhibitors of DHPS and inhibitors of DHFR; combinations have proven to be particularly useful in chemoprophylaxis. See Section 2.7.2 for information on mutants of plasmodial DHFR and resistance to antifolates.

Based on a three-dimensional homology model of plasmodial DHFR, analogs of cycloguanil were designed and evaluated against both wild-type DHFR and a mutant strain (458). A lead compound (26) was identified that was equally active with cycloguanil in inhibition of wild-type DHFR and 120 times

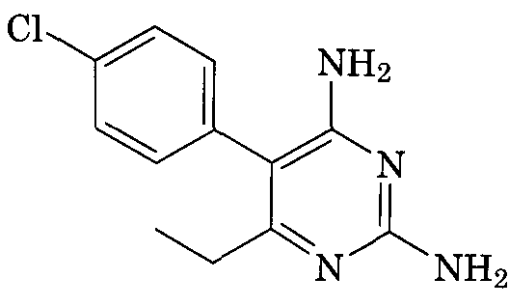


(26)
R = CH_3, Ph

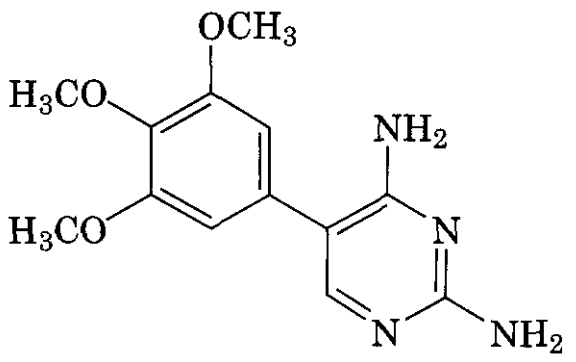
more effective than cycloguanil against mutated enzyme. Several of the compounds were active both in enzyme assays and against P. falciparum clones in *vitro*.

Proguanil and chlorproguanil have been use in combination with a number of drugs. In particular, the combination of proguanil with atovaquone (Malarone) has been extensively studied (see Section 2.8.1).

2.6.2 Pyrimethamine. Pyrimethamine (Daraprim, 27) was developed by Burroughs Wellcome in 1950. The researchers there were investigating folic acid antagonists for use as anticancer agents. A rather tenuous structural similarity between these antifolates and proguanil led them to examine pyrimidines for antimalarial activity. As was later shown, the structural resemblance between cycloguanil, the active metabolite of proguanil, and pyrimethamine is striking. Pyrimethamine is eliminated primarily by oxidation to pyrimethamine 3-N-oxide; the P450 isozyme responsible for the oxidation is not known. Despite its wide margin of safety, pyrimethamine is no longer used as a single agent because of the ease with which resistance develops to the drug when administered alone. In addition to combinations with sulfadoxine, pyrimethamine has been studied with artemether. In a 3-day regimen, the combination was more effective than artemether alone, even in an area of pyrimethamine resistance (459). When given during pregnancy, there was no increase in the observed rate of stillbirth, neonatal death, or malformations with pyrimethamine prophylaxis (155), and the drug is not mutagenic (449). A related agent, both structurally and in action, is trimethoprim (28). It is used much less often in the treatment of malaria and always in combination.



(27)
pyrimethamine



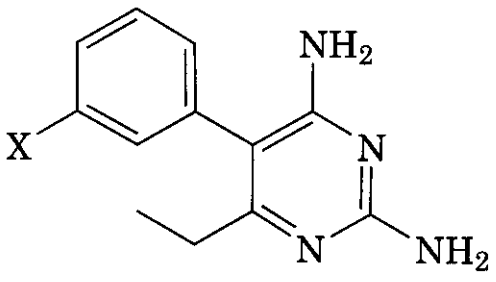
(28)
trimethoprim

Although proguanil and pyrimethamine both act through inhibition of the parasite DHFR, it is clear that some strains resistant to one of these agents can retain sensitivity to the other. In attempting to understand the reasons for the rapid development of resistance to pyrimethamine and the occasional lack of cross-resistance, researchers began to examine the DHFR protein in detail. It was recognized that the DHFR activity was linked on a single protein to the thyrnidylate synthetase (TS) enzyme (460,461). This observation led ultimately to the cloning of plasmodial DHFR. Analysis of the DHFR proteins from resistant strains of P. falciparum has revealed a structural basis for resistance of antifolate agents (462–465). Mutations in the DHFR gene of P. falciparum correlate with antifolate resistance in lab and field isolates.

Cycloguanil resistance involves two point mutations: alanine to valine mutation at position 16 and serine to threonine at position 108. When position 108 is taken by asparagine rather than either serine or threonine, pyrimethamine resistance is conferred. Those strains with the highest levels of pyrimethamine resistance also incorporate a cysteine to arginine mutation at amino acid 59. Significant cross-resistance to both drugs is seen in parasites with a DHFR protein that has asparagine at 108 as well as leucine replacing isoleucine at 164. Using this information, polymerase chain reaction assays can be used in surveillance of drug resistance in the field (466, 467). Such surveillance has led to the acquisition of a sizable quantity of data on DHFR mutants and patterns of resistance

(466, 468–473). Mutations at 108 are the most common; only one example has been reported of a mutant DHFR that was wild-type at 108 (474). Other changes that have been reported occur at amino acids 16, 50, 51, 59, 140, and 164. Although most studies of antifolate resistance have focused on P. falciparum, work has also been done on DHFR-TS from P. vivax. Point mutations in the vivax enzyme at positions analogous to those seen for P. *falciparum* were a major determinant of antifolate resistance in P. vivax (475). Studies have been carried out of the conformations of antifolate drugs and possible modes of binding to DHFR (476).

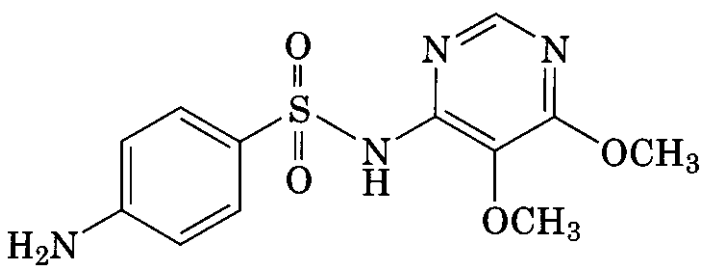
Two analogs of pyrimethamine were designed and tested for activity against mutant recombinant plasmodial DHFR. CC83 (29) and SO3 (30) were active against wild-type



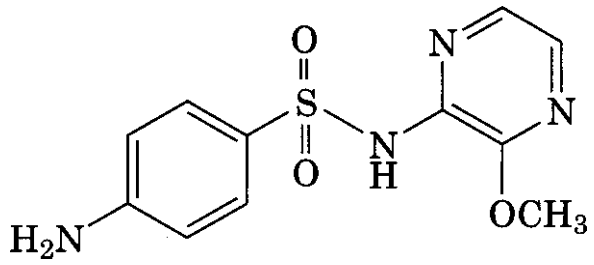
(29)
CC83, X = OCH_3
(30)
SO3, X = Cl

DHFR as well as showing good inhibition of a double mutant (477). The inhibitors retained activity in *vivo* against P. berghei in infected mice.

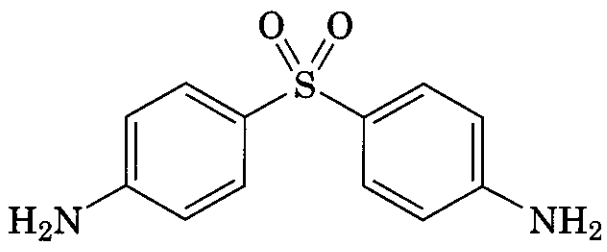
2.6.3 Sulfonamides and Sulfones. On discovery that sulfanilamide had appreciable antimalarial activity, a number of related sulfonamides were tested for activity. Two longer acting agents, sulfadoxine (31) and sulfalene



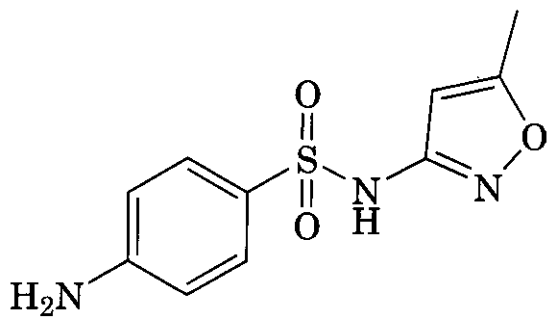
(31)
sulfadoxine



(32)
sulfalene

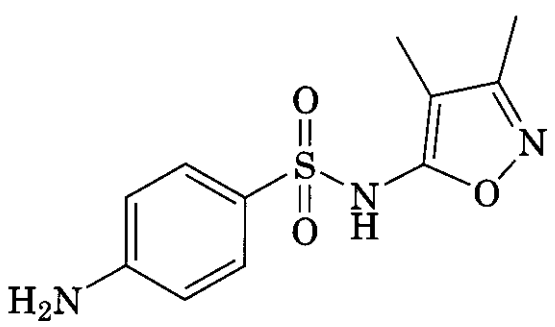


(33)
dapsone



(34)
sulfamethoxazole

(32), were identified as the most promising members of the group. These studies led to the assessment of dapsone (33), a sulfone drug, used for leprosy. Sulfamethoxazole (34) and sulfisoxazole (35) are other examples of this class of agents.



(35)
sulfisoxazole

Sulfonamides and sulfones are selectively toxic to malaria parasites because they inhibit a plasmodial enzyme, dihydropteroate synthetase (DHPS), that is not present in mam-

mals. DHPS catalyzes the combination of PABA with a substituted pterin. The product, dihydropteroate, is ultimately converted into dihydrofolate. The folate co-factors are necessary in the biosynthesis of pyrimidines. Sulfa drugs compete with PABA and are converted into non-metabolizable adducts; the result is a depletion of the folate cofactor pool. A recent study has noted that several sulfa drugs exert toxic effects on the parasite *in vitro* at concentrations that are two to three orders of magnitude lower than the concentrations needed to inhibit the isolated enzyme (479). The results suggest that (1) sulfa drugs are concentrated by the parasite, (2) partial inhibition of DHPS may be lethal, or (3) sulfa drugs actually act by some other mechanism than the inhibition of DHPS. The general paradigm that holds that plasmodia are unable to use exogenous folate may well be a simplification; folic acid is known to antagonize the antimalarial effects of sulfonamide drugs *in vitro* (480).

The DHPS coding sequence has been determined in P. *falciparum* (481). It forms part of a longer sequence that also specifies the preceding enzyme in the plasmodial folate pathway, 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase. Parasite lines resistant to sulfadoxine were found to possess mutations in the DHPS sequence. Those with high-level resistance carried either a double mutation at both Ser-436 and Ala-613 or a single mutation at Ala-581. Polymerase chain reaction systems have been developed to further document mutations in DHPS and to track clinical failures as a result of sulfadoxine resistance (482).

Sulfadoxine is not mutagenic nor genotoxic (449). Neither the long-acting sulfonamides (sulfalene, sulfamethoxine, sulfadoxine) nor dapsone in low doses cause teratogenicity (155). Dapsone is excreted in breast milk in sufficient quantity to cause toxicity, however; breastfeeding is not recommended during dapsone therapy. Sulfonamides as a class have been associated with cutaneous reactions and the longer-acting agents seem to be more likely to generate severe reactions. When sulfadoxine alone has been used at dosages higher than that for weekly malaria chemoprophylaxis, the incidence of severe reaction approached 1 in 10,000 (483).

2.6.4 Combinations. A number of combinations involving both DHFR and DHPS inhibitors have been studied: sulfadoxine/pyrimethamine (SP, Fansidar), pyrimethaminel dapsone (Maloprim), dapsone/chlorproguanil (484), sulfisoxazole/proguanil (485), sulfamethoxazole/trimethoprim, and sulfalenel trimethoprim. Inhibition at both points in the parasite's folate pathway provides a potentiation of the antimalarial action.

The most widely used combination has been sulfadoxine/pyrimethamine. The actions of the two drugs have been shown to be synergistic (480). The clinical pharmacokinetics of the combination have been reported (283,339, 486). In many places in Africa, SP has begun to replace chloroquine as the drug of choice in falciparum malaria unless the patient displays sensitivity to sulfonamides. In a number of areas where chloroquine resistance is prevalent, SP has proven to be effective such as in India (487), Côte d'Ivoire (488), Malawi (489), Pakistan (490), and Colombia (491). Increasing SP resistance has been documented in Kenya (492) and Tanzania (493), however. Perhaps not surprisingly, a high proportion of ineffectiveness has been reported with SP in P. *vivax* in patients infected in the Thailand-Myanmar border region, an area of intense multi-drug resistance (389). Given the problem of resistance in Africa, studies have looked at combining SP with chloroquine in Gambian children. The triple combination was more effective than SP alone in controlling symptoms in the first few days after treatment (494). SP has also been combined with artesunate in Gambian children, leading to faster resolution of fever and parasitemia than with SP alone (495,496).

SP has been used both in a prophylactic regimen as well as for treatment. But severe and even fatal skin reactions have been observed when SP was used for prophylaxis (483, 497). Cases were reported of American travelers using SP for malaria prophylaxis suffering toxic epidermal necrolysis, erythema multiforme, and Stevens-Johnson syndrome. No other risk factors could be identified. Based on these outcomes, the routine use of SP as prophylaxis is not recommended unless persons are at highest risk of acquiring chloroquine-resistant P. *falciparum*. In therapy, however,

SP is still recommended as presumptive self-treatment for travelers to areas of where the presence of chloroquine-resistant P. *falciparum* is documented. Of course, no one with a history of sulfa drug allergy should take SP.

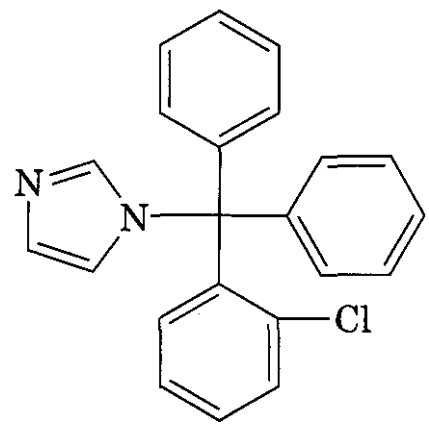
Despite recommendations against using SP during pregnancy (155), studies have shown a beneficial outcome. Treatment of pregnant women with SP, either on a monthly basis or with two prenatal doses, was efficacious in preventing placental malaria in Africa (498). No significant differences were seen between HIV positive and HIV negative women in terms of drug tolerability and adverse side effects (499). Birth weights were higher and fetal growth was improved (500). The risk of severe anemia to the mothers was decreased (501).

The combination of proguanil with dapsone has been used in malaria prophylaxis against chloroquine-resistant falciparum malaria for over 20 years (502). The pharmacokinetic effects of proguanil on dapsone and dapsone on proguanil have been examined. Proguanil was found not to alter the plasma levels of dapsone and its metabolites in healthy individuals (503). The elimination half-life of proguanil was found to be slightly longer in the presence of dapsone; other pharmacokinetic parameters were not affected (504). In a multi-drug resistant area (Thai/Myanmar-Cambodia border), proguanil combined with dapsone offered no significant advantage as a chemoprophylactic agent over pyrimethamine/dapsone against falciparum malaria (505). Proguanil/dapsone was much more effective in preventing cases of vivax malaria, however. The combination of chlorproguanil with dapsone has also received favorable report (506).

The combination of pyrimethamine with dapsone has not been as widely used. It was shown to be more efficacious than chloroquine in Tanzanian children both for treatment (507) and for prophylaxis (508), although dermatological side effects were noted when it was used prophylactically (509). Some have argued that the dapsone/proguanil combination is superior to other antifolate pairs based on pharmacokinetic profiles (504). The elimination half-lives of dapsone (23 h) and proguanil (15 h) are sufficiently similar so that on discontinuation of administration, suboptimal concentrations of the drug combination in the blood are possible only briefly. In contrast, dapsone (27 h) with pyrimethamine (83 h) or sulfadoxine (204 h) with pyrimethamine (112 h) have markedly different half-lives. This leads to one drug remaining in the blood for long periods without the potentiating effects of the combination. Selective resistance to the longer-lived agent may well result.

Chlorproguanil (Lapudrine) with dapsone is a relatively new combination of antifolate drugs (510) and has been termed "lapdap." For a summary of clinical trials using lapdap, see Ref. 511. The combination is inexpensive, comparable to SP, safe, and effective in uncomplicated falciparum malaria in certain areas (512). In other locations, however, such as in Thailand where multi-drug resistant parasites are prevalent, short-course treatments with lapdap were not effective either for therapy (513) or as prophylaxis (514).

In areas where both malaria and acute respiratory infection in children are endemic, the combination of clotrimazole **(36)** may be

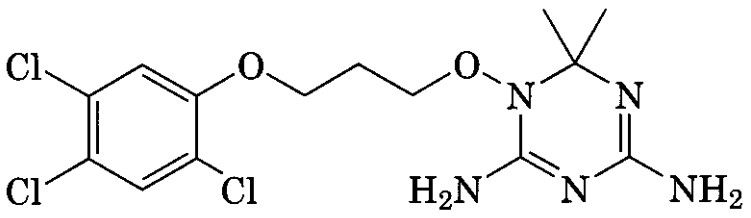


(36)
clotrimazole

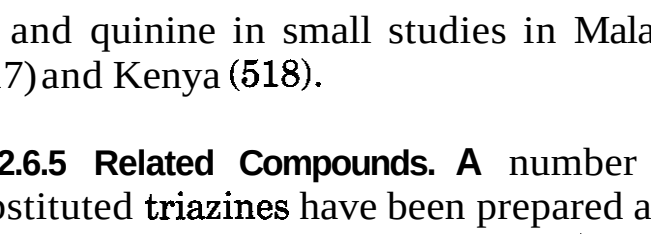
indicated. Clotrimazole has IC_{50} values higher than SP but is active at doses commonly used in treating bacterial infections (478,515,516). There is need for further *in vivo* and clinical studies to assess the usefulness of the combination. Cotrifazid, a fixed combination of rifampicin, isoniazid, sulfamethoxazole, and pyrimethamine, was shown to be effective against malaria in humans (517). The combination was effective either as a first-line treatment or after drug failures of SP, quinine, or

SP and quinine in small studies in Malawi (517) and Kenya (518).

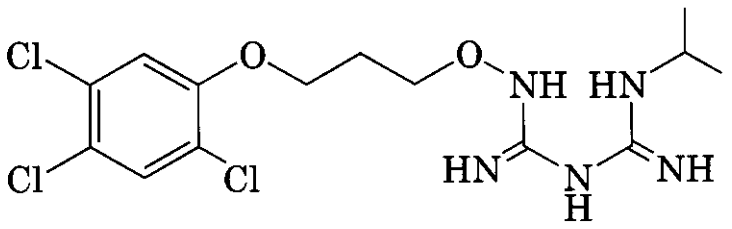
2.6.5 Related Compounds. A number of substituted triazines have been prepared and evaluated with some enthusiasm. WR99210 (**37**), a triazine analog of proguanil, was synthesized and studied in the 1970s and early 1980s (519). WR99210 is remarkably active against resistant and sensitive P. falciparum clones and isolates and lacks cross-resistance with cycloguanil and pyrimethamine, suggesting that the mechanism of WR99210 differs from the other antifolates (520). WR99210 seems to act exclusively on the parasite DHFR displaying no inhibitor effect on the human enzyme (450). Clinical trials uncovered severe gastrointestinal symptoms, however, and development of the compound was dropped. A related compound, clociguanil (38, BRL 50216), had a promising activity profile, but its biological half-life was short in human trials and there was no demonstrable benefit over proguanil or pyrimethamine (521). In 1993, the biguanide version of WR99210, PS-15 (39, WR250417), was synthesized and studied (522,523). Like proguanil, PS-15 is an inhibitor of DHFR and is metabolized in *vivo* to WR99210. In vitro tests against drug-resistant clones of P. falciparum demonstrated that PS-15 was more active than proguanil, and the metabolite WR99210 was more active than cycloguanil. PS-15 was more active than either proguanil or WR99210 in monkeys infected with multi-drug-resistant P. falciparum. When administered orally to mice with P. berghei, PS-15 was also less toxic than proguanil. In studies designed to assess the chemoprophylactic efficacy of PS-15, neither the parent compound nor the cyclized metabolite prevented primary infection in monkeys (524). PS-15 does not seem to be cross-resistant with other DHFR inhibitors. Studies of combinations of PS-15 with atovaquone, sulfamethoxazole, or dapsone in monkeys demonstrated that the combinations were effective. The combination with atovaquone was particularly promising (525). Additional compounds in this series such as PS-26 (**40**) and PS-33 (41) were prepared to identify compounds that retained good activity without the problematic 2,4,5-trichlorophenoxy moiety of PS-15 (526).



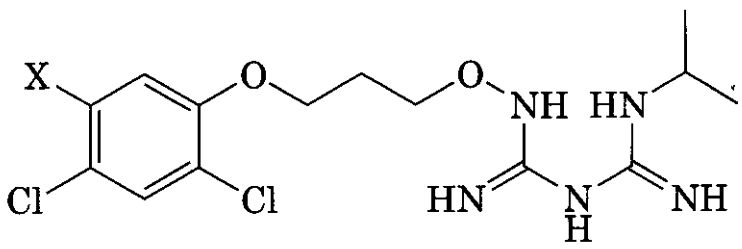
(37)
WR99210



(38)
clociguanil



(39)
PS-15

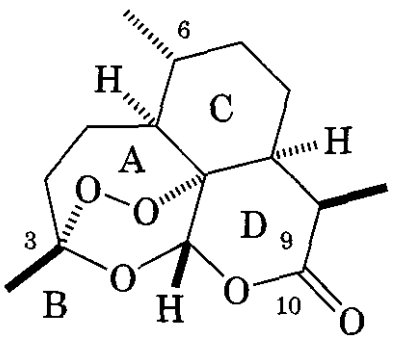


(40)
PS-26, X = Cl
(41)
PS-33, X = H

2.7 Artemisinin and Its Derivatives

2.7.1 Background, Isolation, and Chemistry. The weed Artemisia annua (sweet wormwood, sweet annie) has been used for many centuries in Chinese herbal medicine as a treatment for fever and malaria (527). In 1971, Chinese chemists isolated from the leafy portions of

the plant the substance responsible for its reputed medicinal action (528–530). The compound, artemisinin (42, qinghaosu, artean-



(42)
artemisinin

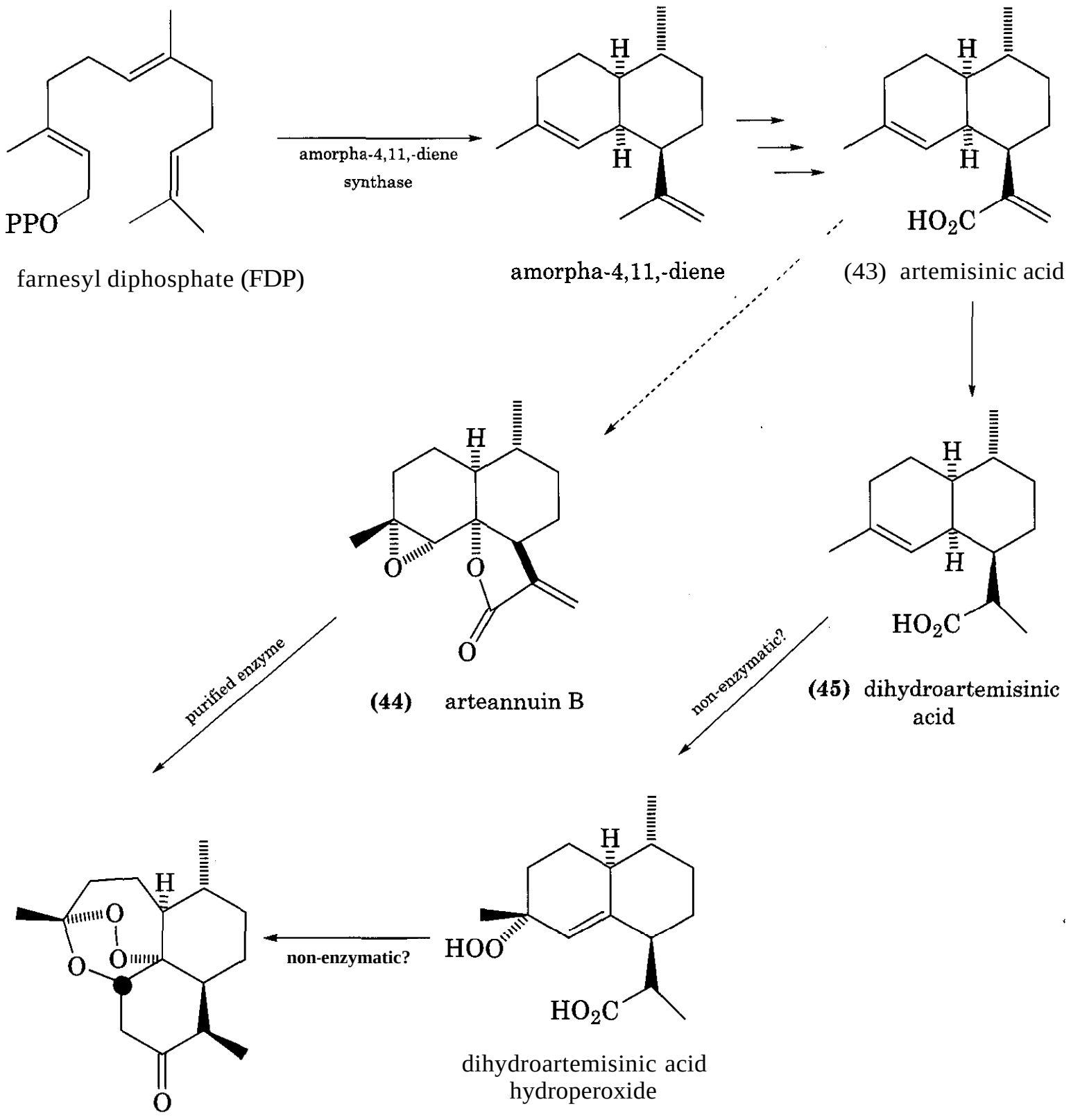
nuin), was determined by X-ray crystallography to be a sesquiterpene lactone bearing an endoperoxide (531). A number of very fine reviews have appeared on artemisinin and its derivatives (527–529, 532–539).

Early results from China that several thousand malaria patients, including those with chloroquine-resistant strains of *Plasmodium falciparum*, were successfully treated with artemisinin (539–541) sparked world-wide interest in the study and development of artemisinin-related compounds. By this writing, the number of persons treated with artemisinin will number in the hundreds of thousands. The practical value of artemisinin itself as a therapeutic agent has been limited by several factors, however. Artemisinin has a high rate of recrudescence, poor oral activity with high doses required (542), a short half-life in plasma (543), and limited solubility in both water and oil (544). In an effort to circumvent these therapeutic and pharmaceutical problems, chemical modifications of artemisinin as well as the synthesis of large numbers of analogs have been extensively pursued.

A. *annua* is being grown commercially and processed for its artemisinin (42) and artemisinic acid (43) in both China and Vietnam (545). The botany, horticulture, and agricultural production of artemisinin have been reviewed (546,547). Artemisinin is extracted at low temperature from the aerial portions of the plant. The yield of artemisinin is dependent on the plant strain, the stage of development, soil conditions, and the growing environment. The maximum yield observed is ~1% of the dry plant weight, but values of 0.01–0.5% are more common, especially in plants collected from the wild. Values will also vary depending on whether the plant material assayed was predominately leaves or included stems, which have a lower artemisinin content. Attempts at the improved production of artemisinin by plant cell or tissue culture have not been particularly successful (548). A. *annua* is not the only natural source of artemisinin; small amounts have also been isolated from A. *apiacea* (549) and A. *lancea* (550).

Sesquiterpenoids are known to be synthesized from farnesyl diphosphate (FDP) mediated by a sesquiterpene cyclase (or synthase). Scheme 18.1 outlines a proposed biosynthetic sequence for artemisinin. Amorpha-4,11-diene has recently been isolated from *A. annua* extracts and is suggested as an intermediate in the biosynthesis (551). A key biosynthetic enzyme, amorpha-4,11-diene synthase, has been cloned, expressed, and characterized from A. *annua* (552). A protein accomplishing the transformation of artean-nuin B (**44**), the most abundant cadinane from A. *annua*, to artemisinin has been purified to homogeneity from *A. annua* (553). From these studies, it is clear that at least some artemisinin is biosynthesized by an enzyme-mediated peroxidation. Although the conversion of amorpha-4,ll-diene to artemisinic acid is, at present, undocumented, the remaining steps through dihydroartemisinic acid (45) have been replicated under conditions that mimic those of the plant (554, 555). The steps from dihydroartemisinic acid to artemisinin may be non-enzymatic in the plant (555).

Artemisinin has been prepared by total synthesis in several laboratories (537, 539, 556). Schmid and Hofheinz, of the Hoffmann-LaRoche Company, prepared artemisinin starting from (−)-isopulegol (557). The key step was a photo-oxygenation of a methyl vinyl ether followed by acidic ring closure to give the endoperoxide linkage. The synthesis established the absolute stereochemistry of artemisinin. Avery and co-workers used (*R*)-(+)-pulegone to set the necessary stereochemistry and incorporated the peroxide group by ozonolysis of a vinylsilane in their total synthesis (558, 559). Using similar methods, Avery's group also prepared (+)-9-desmethylartemisi-



farnesyl diphosphate (FDP)

amorpha-4,11,-diene

(43) artemisinic acid

(44) arteannuin B

(45) dihydroartemisinic acid

dihydroartemisinic acid hydroperoxide

(42) artemisinin

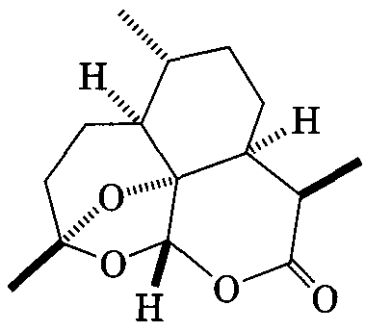
Scheme 18.1. Proposed biosynthetic pathways for artemisinin.

nin (559) and (+)-6,9-didesmethylartemisinin (560). Zhou et al., of the Shanghai Institute of Organic Chemistry, began their synthesis from natural artemisinic acid (561). Later, they synthesized artemisinic acid from 10*R* (+)-citronellal (562), thus bridging the gap for the totally synthetic material (563–565). A group at the University of Alberta reported a total synthesis of (+)-artemisinin starting from (−)-β-pinene (566). Syntheses of specifically deuterated (567) and ^{14}C-labeled artemisinin has been reported (568, 569). Other noteworthy syntheses have been provided (570–574). Syntheses of artemisinin and its derivatives and analogs have been reviewed (534, 535, 575,576).

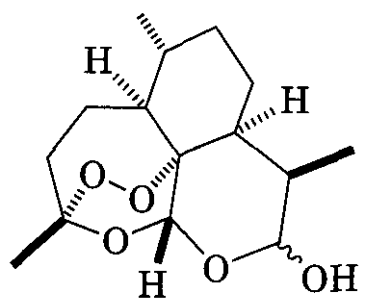
A number of constituents of *A. annua* other than artemisinin have been exploited in developing semi-syntheses of artemisinin and its related compounds. Among these, artemisinic acid (43) is 8-10 times more abundant than is artemisinin (547, 577) and can be isolated

from the plant source without chromatography (578). Partial syntheses of artemisinin from artemisinic acid or dihydroartemisinic acid (45) have been reported by Roth and Acton (579, 580), Ye and Wu (574), Haynes and Vonwiller (581), and Lansbury and Nowak (582,5831, who also used arteannuin B (44) as a precursor for artemisinin. Although some very interesting chemistry has resulted from these approaches, the most economical source for artemisinin, both for use itself and for conversion into arteether, artemether, and artesunate, will continue to be *A. annua*.

A fine summary of the chemistry, stability, and reactions of artemisinin is found in (529). The molecule itself is surprisingly robust. It is stable at temperatures above its melting point (156–157°C) (584) and at reflux in neutral solvents (bp < 100°C) for several days. Selective reduction of artemisinin is possible: catalytic hydrogenation or reaction with triphenylphosphine produces deoxyartemisinin (46) and borohydride reduction provides dihydroartemisinin (47) (585). Although the structure of artemisinin was originally determined by X-ray analysis (531), a more recent X-ray study has provided improved atomic coordinates and a comparison of conformations between artemisinin, dihydroartemisinin, and artemether (586). ^{13}C data for a number of sesquiterpenoids and analogs related to artemisinin have been reported (587). Guidelines for assigning stereochemistry in synthetic trioxanes based on 1D and 2D NMR data have been compiled (588).



(46)
deoxyartemisinin



(47)
dihydroartemisinin

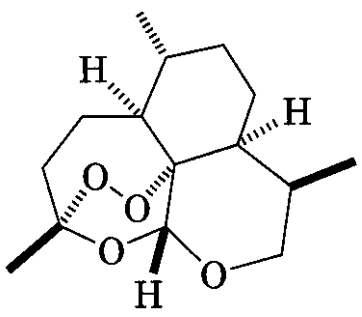
2.7.2 Structure-Activity Relationships. The artemisinins, although fabulously successful, do have drawbacks. Artemisinin has limited availability from the plant, relatively low potency, poor oral activity, poor oil and water solubility, and a short half-life. These issues and concern over neurotoxicity in some derivatives have resulted in a continuing search for improved compounds. A plethora of analogs and derivatives of artemisinin have been prepared and evaluated for antimalarial effect. Activity data on these compounds have been compiled in several excellent review articles (534, 535, 539, 575, 589). The study of these compounds has led to the development of some general structure-activity relationships (534,575, 590).

The endoperoxide is essential for activity; deoxyartemisinin (**46**), in which the dioxy bridge has been reduced to mono-oxy, is completely inactive (528,529,540,591). A number of synthetic peroxides have been screened for activity (592), and some very simple peroxides such as hydrogen peroxide and tert-butyl hydroperoxide, although only weakly active, have been found to suppress parasitemias of several Plasmodia *in* vivo (593). The presence of an endoperoxide is not sufficient on its own to confer activity, however (594,595); ascaridole, a peroxy natural product, is not active at the maximum tolerated dose (596). Indeed, some bicyclic 1,2,4-trioxanes structurally unrelated to artemisinin do not possess appreciable activity (596–598). Clearly the peroxide grouping is necessary but not sufficient for activity.

These results have led researchers to speculate on what additional criteria need to be met in defining the antimalarial pharmacophore. Some have suggested that activity resides in the array of endoperoxide-ketal-acetal-lactone functionalities (599). The additional oxygen substituent at the 5 position of the 1,2,4-trioxane has been identified as a possible critical feature (598). A strong connec-

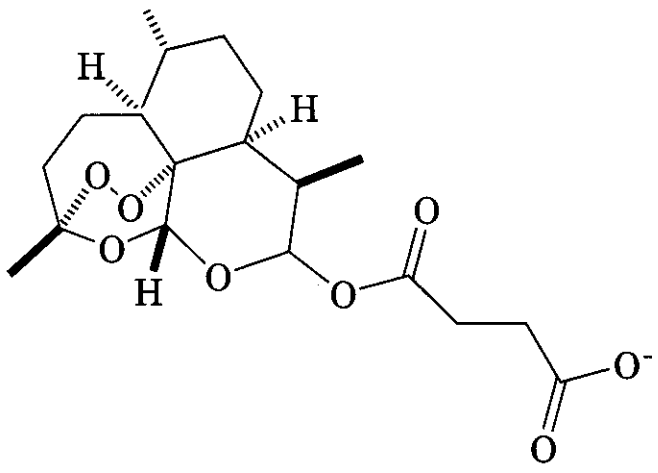
tion between the lipophilicity of a compound and its activity has led researchers to propose that the position of the peroxide bond relative to hydrophobic groups might be important (600). The investigation of 1,2,4-trioxanes as the central pharmacophore has borne promising preliminary results. The structural requirement of a 1,2,4-trioxane is not absolute, however (601,602). Activity has been demonstrated in bicyclic peroxy compounds far removed from the artemisinin structure.

It is quite clear that modifications of dihydroartemisinin (47) and deoxoartemisinin (48) are well tolerated, and many derivatives



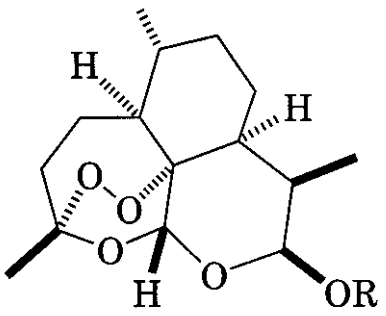
(48)
deoxoartemisinin

and analogs of these types have been prepared. Both compounds are more potent than artemisinin itself. Among the agents presently in clinical use, dihydroartemisinin derivatives account for all of them except artemisinin itself [dihydroartemisinin (**47**), artesunate (**49**),

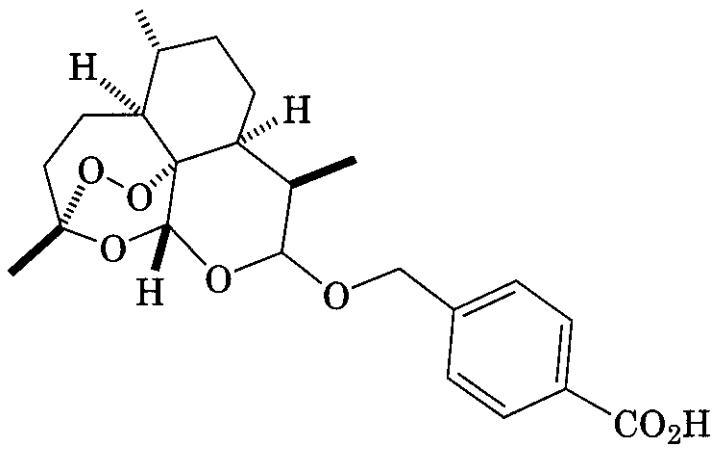


(49)
artesunate

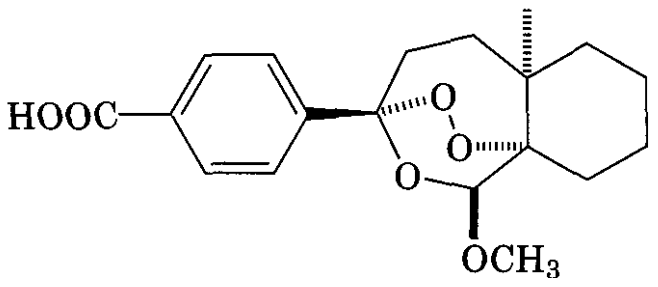
artemether (**50**), arteether (**51**), and artelinic acid (**52**)]. It was realized quite early in structural studies that, in general, the ether, ester, and carbonate derivatives of dihydroartemisinin demonstrate appreciable antimalarial activity (603). One conclusion is that increasing polarity and water solubility of an artemisinin-type analog decreases the antimalarial activity. An example is that carboxylate esters have significantly greater antimalarial activity than do the corresponding carboxylic acids in one series of dihydroartemisinin derivatives (604). The lesson from many analog studies seems to be that lipophilicity is an important factor in maintaining and increasing antimalarial activity, even though it might also contribute to the toxicity associated with some analogs. In contradiction of this conclusion, one notes that artesunate (49) and artelinic acid (**52**), both water-soluble derivatives of dihydroartemisinin, are very active. One particular emphasis has been to identify water-soluble compounds with better stability that artesunate, the only currently available artemisinin drug that can be given intravenously. Several new families of active compounds, including those which are glycosylated (605) or containing amino groups (605–607), have been identified, but none has been of sufficient interest as yet for further development. A



(50) R = OCH_3
β-artemether
(51) R = OEt
β-arteether



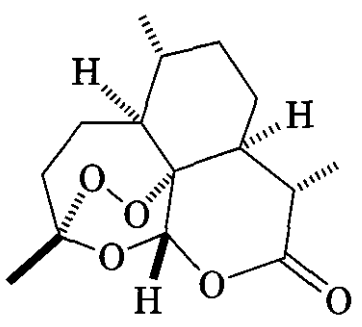
(52)
artelinic acid



(53)

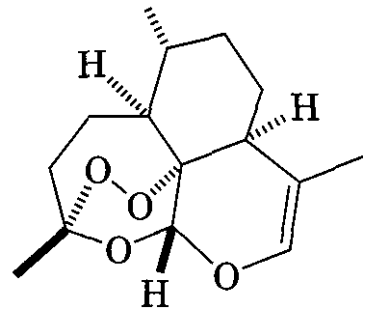
more promising example, however, is (**53**), which incorporates a phenylcarboxylate at the 3 position (608).

The oxygen functionality on the D ring, either alkoxy or carbonyl, is not required for antimalarial activity. **A** change in the D ring size, from 6 to 7, results in a decrease in activity perhaps as a result of conformational changes (609). The addition of alkyl groups at position 9 on the artemisinin system provided a number of active analogs of **deoxoartemisinin** but was not uniformly advantageous to antimalarial activity (610). Introduction of functional groups at this site was similarly shown to be of little value (611). Smaller groups, methyl, ethyl, and propyl, can increase potency, but the effect drops off rapidly. The most lipophilic analogs are not the most active (600). Substitution and stereochemistry at position 9 does impact antimalarial activity. 9-Epi-artemisinin (54) and isoartemisitene

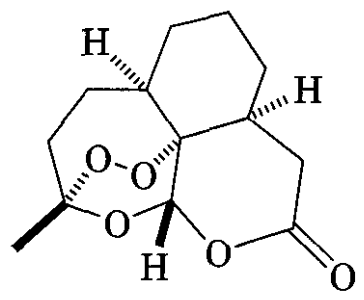


(54)
9-*epi*-artemisinin

(55) are approximately an order of magnitude less active than artemisinin (612, 613). The methyl group in 9-epi-artemisinin may be situated such that steric hindrance of the peroxide bond results. 6,9-Didesmethylartemisinin (56) was reported to have significant activity in vitro (614). So while many changes at position 9 result in active compounds, not all variations lead to good activity. An intact D ring does not seem to be required, but those com-

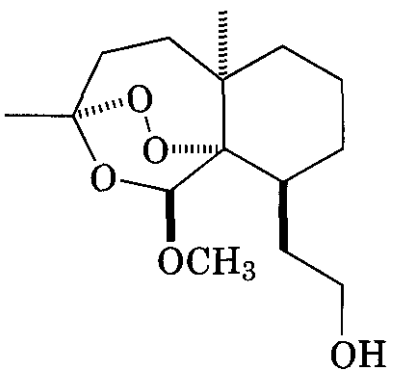


(55)
isoartemisitene



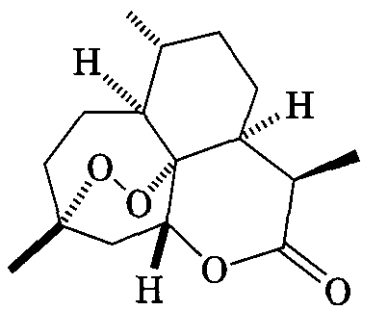
(56)

pounds with an alkoxy group on a bicyclic trioxane tend to be more active than those without. **A** series of compounds based on alcohol structure (57) were synthesized and evaluated



(57)

for antimalarial activity (615). Several of the structures approached the activity seen with artemisinin. Most analogs have kept an intact C ring because it provides a convenient scaffold for construction of the bicyclic AB system. There is some suggestion that the **6-methyl** contributes to the activity of artemisinin (575). Compounds with a simple dioxane ring instead of the trioxane such as (58) are active (601), again demonstrating that the trioxane is not a required component for antimalarial activity. Replacement of the methyl group at position 3 with other alkyl or **alkaryl** groups yields active compounds (610). In general, incorporation of substituents at position **3** leads



(58)

to compounds with less activity than compounds with substitution at position 9. The presence of an A ring provides compounds with greater activity; this may be because of the need for some degree of rigidity in the AB system.

There is no clear indication, either from the activity of racemic analogs or from the proposed mechanism of action, for chiral preferences in antimalarial behavior. For compounds derived from artemisinin itself, no question of absolute stereochemistry arises. Purely synthetic analogs have most often been prepared in racemic form; these compounds can have significant amounts of activity. Future studies may uncover stereochemical issues in the mechanism of action of the artemisinins with parasite proteins. Differences in relative stereochemistry can produce significant differences in antimalarial activity, however (612, 616). Much might be learned from examining the activity of the enantiomer of naturally occurring artemisinin. None has yet been prepared, as well as can be determined, either by asymmetric synthesis nor by separation of synthetic racemic material.

Acton et al., in the course of describing some artemisinin analogs with substituents at the 9 position, makes useful comments about artemisinin SAR (617). They observed that "lack of activity in a parent compound need not consign derivatives of that compound to the dustbin of chemistry since [structural changes] can convert compounds with little or no discernible activity into derivatives with quite respectable antimalarial behavior." An example of this is found in the work of Posner et al. (618), where a parent tricyclic 1,2,4-trioxane alcohol was relatively inactive while its derivatives showed good activity.

A number of quantitative structure-activity relationship (QSAR) studies have been carried out on artemisinin and on synthetic trioxanes using a number of different approaches and techniques (619–627). For the most part, these models gave satisfactory predictive results. It was suggested that the reactivity of this system is related to the rather dramatic region of negative potential that surrounds the molecule, including all three oxygens of the trioxane. The atoms of the trioxane ring along with the D ring oxygen, the lactol carbon, and the lactol oxygen were located as the important grouping for antimalarial activity. Cyclic voltammetry studies indicate that the irreversible reduction potentials of a number of artemisinins and analogs gave a good correlation with antimalarial activity reconfirming the essential nature of the peroxide moiety (628).

2.7.3 Mechanism of Action. The mechanism of action of the artemisinins against malaria has been the subject of fascinating recent work although the situation at present is far from settled. Several reviews are available that address this topic (534, 575, 629, 630). Early on it was determined that artemisinin had a direct parasiticidal action against P. *falciparum* in the erythrocytic stage both in vitro and in *vivo* (540,631,632). These compounds also show cytotoxicity at considerably higher concentrations than those necessary for antimalarial activity (633). The key morphologic changes observed on artemisinin administration to parasite-infected erythrocytes involves disruption of the food vacuole membranes (634). The breakdown of the vacuole membranes causes the release of the digestive enzymes resulting in harmful effects on the cytoplasm. Artemisinin enhanced the oxidation of lipid membranes in the presence of heme (635). Even so, the antimalarial action of artemisinin is not caused by a direct effect on the lipid structure of the membrane (636). A correlation in time between the morphological changes in the parasite and the biochemical suppression of protein synthesis by artemisinin was noted (637, 638). Tritiated dihydroartemisinin was reversibly and saturably accumulated from low concentrations into erythrocytes infected with P. falciparum (639). Uninfected erythrocytes concentrated the drug less than twofold, whereas infected

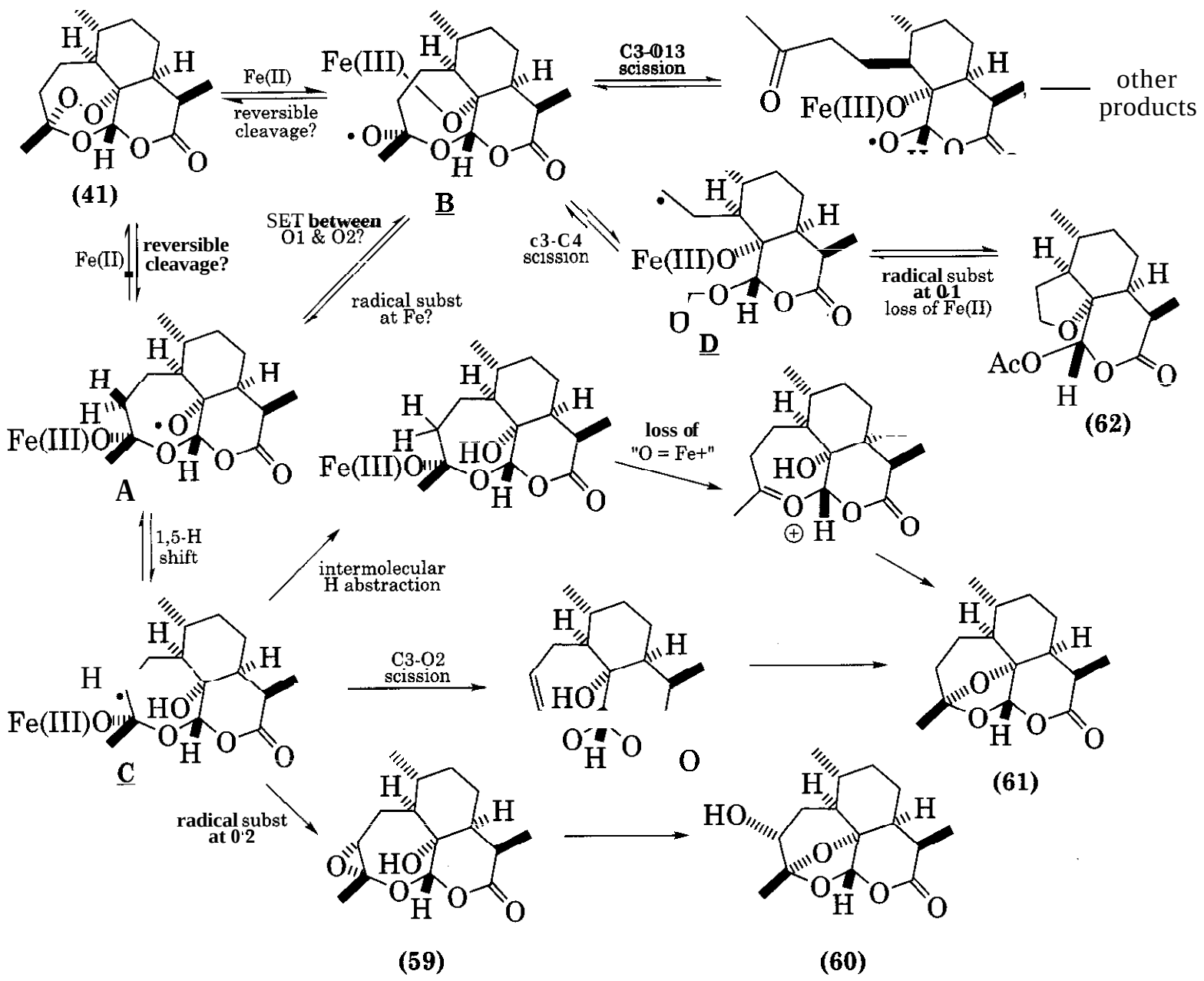
erythrocytes achieved more than 300 times the medium concentration. Artemisinin and its derivatives are transported by a vacuolar network induced by P. falciparum; interaction with this membrane network may be implicated in the mechanism of action (640). Interestingly, labeled dihydroartemisinin becomes bound to both proteins and lipids in isolated red cell membrane preparations but does not associate with the same components in intact red cells (641). One explanation is that infected erythrocytes have a altered distribution of membrane lipids which may promote drug binding. Interactions between artemisinin and DNA were not observed (642). Artemisinin has high permeability across biological membranes (643).

The requirement of iron for activity of artemisinin is well known. Whereas hemoglobin and hemozoin react poorly, free iron and heme-iron react readily under a number of experimental conditions (644). The binding affinity of artemisinin derivatives to heme seems to correlate well with antimalarial activity (645). Free radical scavengers and iron chelators are known to antagonize the drug's action (646–648). Interestingly, compounds in which an iron chelating group has been attached to the artemisinin central structure were as active as artemisinin but no more active (649). Iron is not the only metal that can promote the decomposition of artemisinin. When plasmodia infected mice were treated with a combination of artemisinin and a manganese porphyrin complex, which by itself was devoid of antimalarial activity, the effect of artemisinin was increased by 50%. The authors suggest that the activation of artemisinin by a metal porphyrin in vivo was confirmed by these results (650).

Although controversy remains about the details of artemisinin's mechanism of action (651,6521,a general scheme based on the radical decomposition of artemisinin by iron has been developed. A number of lines of evidence led Wu and colleagues to propose a "unified" mechanism (Scheme 18.2) (653), developed from the pioneering work of Meshnick (654), Posner (630, 655), and Jefford (656) and their co-workers. The pathway commences with cleavage of the artemisinin peroxy bond by single electron transfer (SET) from Fe(II) of heme. Two possible oxygen-centered radical anions could be generated by this process, with structures **A** and B. These intermediates may be interconvertible, explaining the different ratios of product obtained under various conditions. Further transformation of **A** by a 1,5-hydrogen shift yields a secondary carbon-centered radical **C** (655). Several routes lead from C to provide the known products of chemical and biological reactions (59), (60), (61), and (62). Alternatively, scission of the C3—C4 bond of B leads to primary carbon-centered radical D which is proposed to react intrarnolecularly to form a tetrahydrofuran product (62). Other scission pathways are also possible.

In support of this scheme, primary and secondary carbon radicals were detected in EPR experiments using artemisinin with iron in the presence of spin-traps in a ratio of 1:4, consistent with intermediates D and C (657). Subsequent transformations provided the products observed from a number of studies of the iron-mediated decomposition of artemisinin. Further evidence for the existence of a primary radical such as D was obtained from studies of various metalloporphyrins with artemisinin, artemether or Fenozan-50F (658–660). Covalent adducts between C4 of the drug and heme were fully characterized. Reaction of artemisinin with free iron in the presence of a sulfhydryl compound yielded a product that could only have been formed from D (661). Additional EPR data also exists in support of a secondary radical generated from arteflene (662).

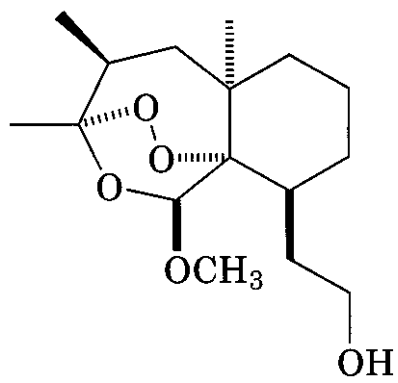
Structures and stabilities of 0-centered and C-centered radicals derived from a model trioxane (2,3,5-trioxabicyclo[2.2.3]nonane) using density functional theory found that the C-centered free radicals were predicted to be stable. The 1° C-radical (**D**) was more stable than the 2" C-radical (**C**) (663). Further calculational studies identified transition state structures for the radical transformations in the model compound and found a low activation energy for the 1,5-hydrogen shift process (664). From the calculated lifetimes of the O-centered radicals, the authors predict that these intermediates should be detectable at low temperature.



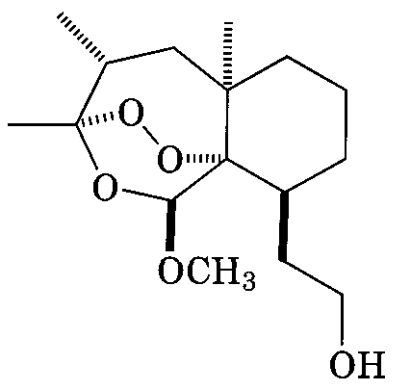
Scheme 18.2. Proposed radical decomposition of artemisinin.

Several workers have investigated the Fe(II)-mediated degradation of artemisinin derivatives and synthetic endoperoxides under biomimetic conditions (665–667). The major products obtained from these studies have been shown to be a tetrahydrofuran (**62**), supporting the intermediacy of a primary C-centered radical such as D and a hydroxy deoxo derivative (60) suggesting the involvement of a secondary C-centered radical intermediate such as **C.**

In additional studies, the possibility of the involvement of a carbon-centered radical **(A)** in the mechanism of action was explored (665). A series of analogs, some with abstractable 4α-hydrogen (63) and some without (**64**), were prepared and tested for antimalarial activity. Those compounds with an available 4α-hydrogen showed activity at 12–200 times that of the corresponding 4β-epimers (668). These results are interpreted to suggest the requirement of an abstractable H at the 4α-position for good antimalarial activity. As further proof of the proposed mechanism of action, Fe(IV)=O has been detected in reactions of artemisinin with ferrous ion (669, 670). In Fourier transform infrared and resonance Raman studies with artemisinin and



(63)



(64)

hemin dimer, researchers report identification of vibrational modes consistent with a heme ferryl-oxo (Fe=O) stretch (671). They believe this provides direct evidence for presence of this species in the interaction of artemisinin with heme. It is suggested that the high-valent iron-0x0 species and an electrophilic (and cytotoxic) epoxide (59) derived from artemisinin are formed concurrently.

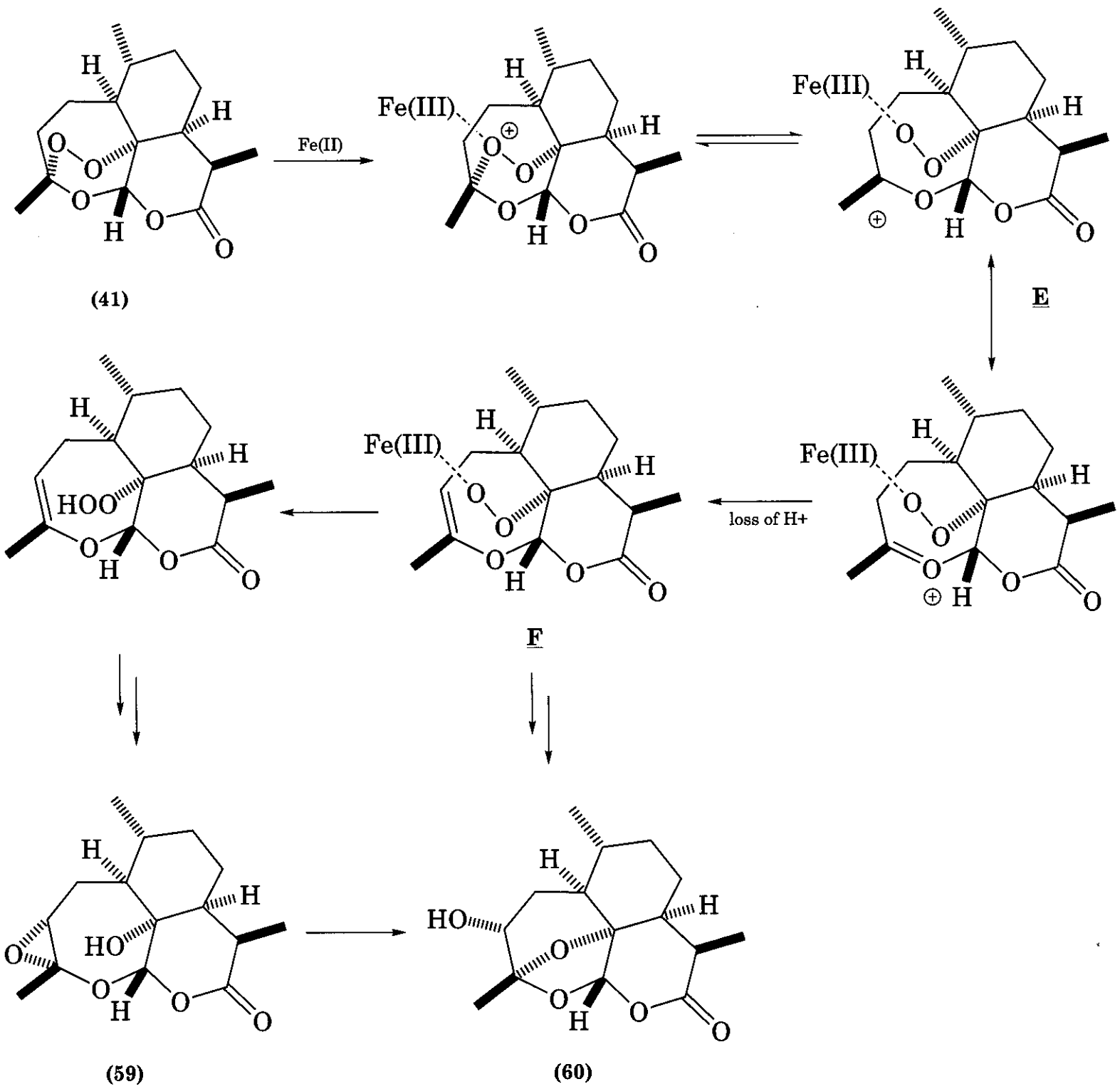
To test the requirements for the carbon-centered radical, Avery's group prepared an analog designed to stabilize such an intermediate (672). The analog lacked the non-peroxidic oxygen of the trioxane system and did not display significant antimalarial potency when compared with artemisinin. Some analogs with 4β-substituents that would be expected to provide stabilization for a radical were less active than the parent (unsubstituted) system (668). These results indicate that stabilization of a C-4 radical does not necessarily improve antimalarial activity. The exact role of a C-4 radical in the mechanism of action of artemisinin remains under discussion.

The selectivity of artemisinin's toxicity for infected erythrocytes, where the necessary heme-iron is available, is thereby accounted for as is the necessity for the peroxide functionality. Membrane damage is one result of the interaction between hemin and artemisinin (673). Molecular modeling studies of the interaction of artemisinin with heme suggest that a stable docking arrangement brings the endoperoxide bridge in close proximity to the heme iron (674).

It remains unclear how this pathway contributes to the overall destruction of the parasite. Several of the intermediates in the pathway are candidates for a reactive entity that confers cellular damage. The radical species themselves may act to alkylate proteins or heme. And the epoxide (**59**) has been suggested as a potential site for further reaction.

An alternative viewpoint suggests that whereas radicals may indeed be formed through interaction with iron sources *in vivo,* such radicals must be short-lived, evidenced by the trapping with porphyrins. The radical intermediates would not/do not have a sufficient lifetime to diffuse to other sites to accomplish parasite damage. Also, although formation of carbon-centered radicals is common in the reductive cleavage of endoperoxides, not all endoperoxides are active antimalarials. Rather, these authors envision a different pathway for artemisinin degradation and action (Scheme 18.3) (675–677). The peroxide may complex with Fe(II) or become protonated which then activates the ring-opening steps to generate an electrophilic cation E. Elimination of a proton yields an open hydroperoxide or a metal peroxide F. This intermediate then may be responsible for the remaining events. The biological significance of hydroperoxides in relation to hydroxylations and autoxidation is well established. It was observed that all active peroxidic antimalarials possessed groups that were capable of stabilizing the positive charge of E induced by heterolytic ring opening.

The next step in the proposed mechanism of action is the alkylation of proteins by artemisinin (678). A clear-cut correlation between antimalarial potency and the alkylative property of synthetic tricyclic trioxanes was reported (679). In the presence of hemin, artemisinin residues become covalently linked to human albumin, probably through thiol and amino groups (680). Artemisinin becomes bound to certain hemoproteins *in vitro* but not to heme-free globin and not to DNA (681). Further studies with artemisinin and artemisinin derivatives have demonstrated that the endoperoxides react with specific parasitic proteins to form covalently linked species (682). In P. *falciparum,* one of the main alkylation targets is the translationally controlled tumor protein (TCTP) homolog (683). In resistant parasites, expression of TCTP was increased over sensitive strains, although it is not clear whether this observation is connected with the mechanisms of resistance (684). Artemisinin also forms ad-



Scheme 18.3. Proposed heterolytic decomposition of artemisinin.

ducts with heme; a covalent alkylation product of heme with artemisinin was isolated and fully characterized (658). It is not clear, however, what role these drug-heme adducts may play in the mechanism of action of artemisinin. The heme-artemisinin adduct was not toxic to in *vitro* cultures of P. ***falciparum*** when added to the media (654).

Artemisinin also forms an adduct with glutathione, and it has been suggested that the reduction in the amount of available glutathione or the inhibition of glutathione reductase by the adduct may contribute to antimalarial activity (685). There are conflicting reports on the effect of artemisinin on hemozoin formation; one group states that they observed little effect from artemisinin (686) while others noted a great reduction in hemozoin production (687, 688) and potent inhibition of both hemoglobin breakdown and heme sequestration (689).

2.7.4 First Generation Artemisinins. More than 2 million patients have been treated with artemisinin agents. Clinical trials comparing various artemisinins in various routes of administration found no particular compound obviously superior to the others, nor was a particular route more efficacious; all treatments with all the compounds tested were

safe, well tolerated, and highly effective (690–692). Several formulations of artemisinin and its derivatives have been developed for clinical use. Artemisinin and artemether can be dissolved or suspended in oil for intramuscular injection. An aqueous suspension of artemisinin has been used intramuscularly. Artesunate solutions are available for either intramuscular or intravenous injection. Tablets of artemisinin or artesunate and capsules of artemether are available for oral dosing. Suppositories of artemisinin and of artesunate are increasingly used. When any of the artemisinin drugs are used in monotherapy for uncomplicated malaria, whether given orally, by intramuscular injection, by intravenous injection, or by suppository, the mean times to clearance of parasites and fever are several hours shorter than with other antimalarials (693, 694). In cases of cerebral malaria, shorter coma resolution is observed. The drugs are well tolerated and without evident toxicity, although questions have been raised concerning the neurotoxicity of artemether and arteether. In addition, the artemisinins have the broadest stage specificity of action of any antimalarial currently in use. Treatment with artemisinin derivatives was found to greatly reduce the subsequent carriage of gametocytes providing the potential for interrupting transmission and preventing the spread of multi-drug resistant parasite (695). Given the ease of use and the efficacy of these drugs, it is expected that they will make a strong positive impact in community-based treatment (696).

The percent of recrudescence is high with the artemisinins, with an average value of 24% for over 2000 patients in various trials. Because of the difficulty in separating the incidence of recrudescence from re-infection, this frequency may be overestimated in practice. To achieve good 28-day cure rates with artemisinin drugs alone, treatment over 5–7 days is required. Given the realities of human nature, when symptoms improve rapidly, patients are less likely to continue drug treatment for longer than 3–4 days. The necessity for a prolonged course of therapy has been attributed to the short half-lives of the parent drugs and their active metabolites in plasma. Combinations of artemisinin drugs with other antimalarials are particularly attractive in addressing the problem of recrudescence. Careful choice of the adjunct agent is important because of the observed antagonism of artemisinin with antifolates, chloroquine, and pyrimethamine (697). Synergy has been noted for the combinations of artemisinin with either mefloquine (698) or tetracycline (697). A shorter course of treatment can be adopted when the drugs are used in combination, and thus patients are more likely to complete the prescribed therapy. A wide variety of combination regimens have been studied and several are very effective. The efficacy, pharmacokinetics, pharmacodynamics, clinical properties, and tolerability of the artemisinins have been reviewed (137, 155, 262, 532, 533, 699, 700).

2.7.4.1 Artemisinin (42). Artemisinin has rapid action on all erythrocytic stages of the parasite (701). There is no sporontocidal action and no action against liver stages, but gametocytes are targets of the drug (702). Artemisinin kinetics demonstrated a decrease in plasma concentrations during the course of treatment, a time-dependent phenomenon observed in both healthy adults (703) and in malaria patients (704) that may partially explain the recrudescence commonly seen with these agents. A single dose was poorly absorbed and rapidly cleared with large inter-individual. variance (705), and the results do not differ between healthy volunteers and patients with uncomplicated falciparum malaria (706). Food intake had no effect on artemisinin pharmacokinetics (707). In a study of artemisinin suppositories, a formulation that has been demonstrated to be effective and well tolerated, therapeutic concentrations were reached (708). Artemisinin apparently induces its own elimination because it was shown in a study in rats that changes in transport associated with efflux by P-glycoprotein are not involved (709). Artemisinin metabolism is mediated primarily by CYP2B6 in human liver microsomes (710); in addition, artemisinin induces CYP2C19 as well as at least one other enzyme (711). This may have implications for drug metabolism in combination therapy. One of the elimination products of artemisinin is the glucuronide of dihydroartemisinin. The human metabolite has been unambiguously identified as the 12α-epimer (712).

The clinical efficacy and pharmacokinetics of artemisinin monotherapy and in combination with mefloquine have been studied (713). The combination produced a more rapid clearance of parasitemia than artemisinin alone and accomplished radical cures. The dose and duration of artemisinin treatment was reduced when used in conjunction with mefloquine. Single-dose combination treatments were equally effective in acute uncomplicated malaria whether the drugs were given together or sequentially (714). The high cost of mefloquine and its association with psychiatric disorders may limit the usefulness of this particular combination, however. A study in Vietnam showed that in uncomplicated falciparum malaria, a single dose of artemisinin along with 5 days of quinine was as effective as the standard 7-day quinine regimen (715). Combinations with tetracycline have also been examined (716).

As with all other known antimalarials, resistance to artemisinin can be induced (717), although this resistance is often unstable (684). In a study of P. berghei and P. yoelii strains, resistance to artemisinins and synthetic endoperoxides was induced by multiple passages of the parasite under drug pressure. When compared with resistance to amodiaquine, mefloquine, or atovaquone, resistance to the endoperoxides was at a low level and resistant parasites regained sensitivity once drug selection pressure was withdrawn (718). The possibility of cross-resistance with mefloquine, quinine, and halofantrine in vitro has been seen in some experiments (719). To provide baseline data for tracking the rise of resistance to the artemisinins, a study was carried out on drug susceptibility in Vietnam shortly after the introduction of artemisinins into nationwide use. All isolates were found to be sensitive to artemisinin, artesunate, dihydroartemisinin, and quinine. All were resistant to chloroquine and mefloquine (720).

WHO has issued guidelines for the use of artemisinin, suggesting that it be used as an alternative to quinine for the treatment of severe malaria in areas where sensitivity to quinine is reduced, that it be restricted to use in cases of multi-drug resistant infections, that it should always be used in combination with another effective drug or, in cases where combinations cannot be used, that a 7-day course of treatment is recommended with monitoring for compliance, and that it not be used as a prophylactic (721).

2.7.4.2 Dihydroartemisinin (47). Dihydroartemisinin is more active in vitro and in *vivo* than artemisinin or artesunate (722, 723) and more active in vitro than mefloquine or halofantrine (724). All currently available artemisinin derivatives are metabolized rapidly to dihydroartemisinin, the main human metabolite. Although clinical trials have been carried out in China (725), the use of dihydroartemisinin has been limited by its low solubility and supposed instability. Treatment is effective and well tolerated in acute uncomplicated falciparum malaria (726). As yet, no evidence of neurotoxicity has been observed. Sequential combinations of rectal dihydroartemisinin with mefloquine was found to be a useful alternative to parenteral drugs in severely ill patients (727). As with all the artemisinins, recrudescence is a problem. In a dosing study, researchers in China determined that cure rates of >97% were achieved with a 7-day regimen of dihydroartemisinin; a 5-day regimen provided cure rates of 80% (728). Pharmacokinetic studies in human volunteers have shown that dihydroartemisinin is more bioavailable than artemisinin (729, 730). The ^{1}H NMR spectrum, the X-ray crystal structure (731), and chemical stability studies (732) have been reported.

2.7.4.3 Arfesunate (49). Artesunic acid is most commonly available as the sodium salt, sodium artesunate. In viuo, artesunate has higher activity than artemisinin (723) and significantly lower parasite clearance and fever clearance times (733). Artesunate suffers from intrinsic instability. Aqueous solutions are unstable at neutral pH and must be prepared from the powder by dissolution immediately before either intramuscular or intravenous injection. This is the only artemisinin derivative that can be given intravenously, a route that might allow improved pharmacokinetic parameters over bolus administration (734). The pharmacokinetics and pharmacodynamics of oral, rectal, and intravenous artesunate in healthy volunteers (735) and in uncomplicated falciparum malaria patients has been studied (736–738). The drug is rapidly ab-

sorbed, distributed, and eliminated. Oral and intravenous routes were comparable, but the rectal route may offer some advantages. Pharmacokinetic studies of artesunate in P. *vivax* confirms that the drug is rapidly effective either orally or intravenously. The properties were comparable with those reported for uncomplicated falciparum malaria (739). Both artesunate and dihydroartemisinin, its major metabolite, are rapidly cleared leading to concerns that a once daily dosing regimen allows periods of nonsuppression (740). Recent studies have confirmed, however, that once-daily dosing gives results identical with those obtained from more frequent administration, suggesting that pharmacodynamic behavior may be more significant than pharmacokinetic properties in these drugs (741). Artesunate was found to inhibit cytoadherence, the pathological process that contributes to microvascular obstruction in severe falciparum malaria (742). Studies of artesunate with hemin using electrochemical methods indicated that artesunate possesses an identical mechanism of action as does artemisinin (743).

Clinical trials have been carried out in China (725), Vietnam (744), and Thailand (745). Both 5- and 7-day regimens of oral artesunate were effective and safe in treating uncomplicated falciparum malaria in Thailand (746). The completed clinical trials of rectal artesunate have been summarized (511). Short-term monotherapy with artesunate suppositories affected a rapid reduction in parasitemia, especially useful with patients who cannot take medication by mouth, who are unable to travel to health care facilities, or who live in areas where administration of parenteral drugs is problematic (747). In a small study, artesunate was administered to pregnant women and was well tolerated with no adverse drug effects noted; normal neurological development was observed in all the neonates that were able to be followed (748).

A goal in the design of therapeutic regimens with artesunate has been the development of a treatment of short duration that maintains efficacy. In comparison with a 7-day course of quinine/tetracycline, a 5-day course of artesunate monotherapy (749) or a 7-day combination of artesunate and tetracycline (750) produced lower rates of recrudescence, faster parasite and fever clearance times, fewer side effects, and equally high cure rates. Oral artesunate was superior to an intravenous quinine loading dose in patients at risk of developing life-threatening complications but who were able to take oral medications (751). Obviously, oral dosing is much more convenient especially in community treatment approaches and avoids risks of using needles.

A number of studies have shown the benefits of combining artesunate, administered either orally or as suppositories, with mefloquine in the treatment of multi-drug–resistant falciparum malaria (752–755). A sequential administration of artesunate followed by mefloquine was more effective than either drug alone, and it was well tolerated (756, 757). Artesunate rapidly reduces parasitemia, and mefloquine has a long-term effect in clearing residual parasites. This combination reduced the incidence of falciparum malaria and seemed to halt the progression of mefloquine resistance in a study in Thailand (758). Artesunate is synergistic with both quinine and mefloquine in vitro against P. *falciparum* (759).

The WHO has developed artesunate as an emergency treatment for malaria (760). The population most at risk of death are infants and children in Africa. In this group, symptoms can rapidly progress so that they are soon too ill to take an oral medication; the artesunate suppositories provide an effective alternative.

*2.7.4.4 **Artemether** (50).* Artemether (Paluther) is the methyl acetal derivative of dihydroartemisinin. The details of its structure have been elucidated by ^{1}H NMR and X-ray crystallography (731). This oil-soluble derivative has high blood schizonticidal activity comparable with that of arteether in vitro and in *vivo* (761). As with artesunate, artemether inhibits cytoadherence (742). The main metabolite of artemether is dihydroartemisinin, for which artemether may be considered a prodrug. A number of hydroxylated metabolites were formed by microbial transformation (762) and as biliary metabolites in rats (763). Pharmacokinetic studies have been carried out in healthy subjects using oral (764, 765) and intramuscular administration (765). Studies of the clinical pharmacokinetics,

safety, and tolerance of artemether in healthy volunteers and in patients with acute uncomplicated falciparum malaria have also been reported (339, 766, 767). Pharmacokinetics in patients from Thailand (768) and China (769) using multi-dose regimens were studied as well. The bioavailability of artemether in children with cerebral malaria was found to be highly variable, particularly when respiratory distress was also present (770).

Clinical trials have been carried out in China (725), Vietnam (744), Thailand (745), and India (771). There is a quick resolution of coma in children with cerebral malaria (772) and more rapid clearance of parasites than is seen with chloroquine (773) or quinine (774). In African children with severe falciparum malaria, artemether was highly effective especially against chloroquine and pyrimethamine resistant infections (775–777). Similar results were obtained in studies with Thai adults (778). Artemether compares favorably with, and may be superior to, quinine in treating severe falciparum malaria in children or adults (779–781). Of particular interest has been the identification of an agent to replace intravenous quinine for severe malaria in areas where quinine resistance is increasing. Intramuscular artemether was found to be as efficacious as intravenous quinine in Papua New Guinea (782) and in Nigerian children (783). Intramuscular artemether was completely effective in treating children with recrudescent P. *falciparum,* those having experienced treatment failures with other regimens (784). A meta-analysis of randomized clinical trials that compared artemether with quinine for severe malaria concluded that artemether was as effective as quinine (785). Substitution of artemether for quinine also avoids any cardiotoxic or hypoglycemic effects associated with quinine use.

A combination of artemether with mefloquine was found to be safe and effective in treating complicated falciparum malaria in Myanmar (786) and in treating drug-resistant malaria during the second and third trimesters of pregnancy (787). Very little recrudescence was observed with the combination. In a study of fresh isolates from Senegal (788) or Gabon (789) there were indications of *in vitro* cross-resistance of artemether with standard antimalarial agents. This is not necessarily an indication of *in vivo* cross-resistance but reinforces the need to use new drugs, particularly the artemisinins, in combination only.

2.7.4.5 ***Arteether*** **(51).** Arteether (Artemotil) is the ethyl acetal of dihydroartemisinin. The drug is approved for restricted use as alternative treatment for multi-drug resistant P. *falciparum* in India (790). Arteether was synthesized in 1988 (541) and chosen for development because of its greater lipophilicity than artemether and the advantage of less toxic metabolites relative to artemether (ethanol versus methanol). Other synthetic methods to the arteethers have been reported (791, 792), and a stereoselective synthesis of α-arteether from artemisinin has appeared (793). Synthesis of the specifically labeled arteethers 11-[^{3}H]-arteether (794) and 14-[^{2}H]-arteether (795) have been accomplished. The β-isomer is crystalline and therefore easily purified; the a-isomer is not and both arteethers are low melting solids. NMR data, both ^{1}H and ^{13}C, are available (796, 797) as is an X-ray structure determination (731). The acid decomposition reactions of arteether have been studied (798, 799); decomposition in simulated stomach acid yielded compounds which retained antimalarial activity (800). A number of metabolites of arteether have been identified: dihydroartemisinin, deoxydihydroartemisinin, 3α-hydroxydeoxydihydroartemisinin, 3α-hydroxyarteether, 9α-hydroxyarteether, 9α-hydroxydihydroartemisinin, 3α-hydroxydeoxyarteether, and tetrahydrofuran derivatives (801–804). A different metabolite, 1α-hydroxyarteether was isolated as a result of microbial metabolism (805). Glucuronides of these hydroxylated metabolites were synthesized and all were significantly less active than arteether (806). β-Arteether is metabolized to dihydroartemisinin primarily by CYP3A4 in human liver microsomes (807).

Arteether is comparable in activity with artemether *in vitro* and *in vivo* (761). A strain of P. *yoelii* that was highly resistant to chloroquine, mefloquine, and quinine was completely susceptible to arteether (808). Although the drug is not active against sporozoites, hypnozoites, nor exo-erythrocytic stages of P. *cynomolgi,* it is several times more active than artemisinin in curing P. *cynomolgi*

infections (809). Rings and young trophozoites are the most susceptible stages of the parasite life-cycle to arteether (810). The use of α,β-arteether in patients with severe falciparum malaria in India, given intramuscularly once daily for 3 days, gave good results with no evidence of toxicity. As with artemether, arteether may serve as a good alternative to intravenous quinine in severe cases far from primary health care (811). The pharmacology of α/β-arteether as a 30:70 mixture has been studied (812). In comparing the isomers, β and α/β (30:70) are equally effective against P. *cynomolgi* in monkeys; a-arteether is slightly less active (813). Pharmacokinetic studies in dog (814) and in humans (766) have shown the drug to be well tolerated with a long elimination half-life of 25–72 h. At higher doses, toxic effects in animals are seen on heart, brain, bone marrow, kidney, and liver (815). Arteether binds human plasma proteins in vitro (816), binding to α1-glycoprotein more tightly than to albumin; concentrations of α1-glycoprotein are markedly increased during malaria infection. Synergism has been observed between arteether and mefloquine or quinine in vitro (817).

Both arteether and artemether have been shown to cause neurotoxicity in vitro (818, 819) and in animal models (820–822), but the effects only occurred at high doses or after prolonged exposure (823). Arteether was demonstrated to produce irreversible neural injury in monkeys after 14 days of intramuscular administration. It remains unclear whether a 5-day course of treatment that would constitute a typical therapeutic regimen would produce similar damage (824). A single high dose of arteether produced brainstem neuropathology in rats, although behavioral indicators of toxicity were negative (825); this observation points out the difficulty in detecting the onset of arteether-induced toxicity. Arteether was shown to accumulate in plasma of rats after repeated intramuscular dosing of a oil solution. While this may contribute to efficacy, it also contributes to toxicity (826). There was a significant difference in toxicity depending on route of administration and length and amount or dose. Intramuscular administration or oral administration in the presence of peanut oil increased the toxic effects in mice (827) and dogs (828). On the other hand, once daily oral administration, especially under the usual 3- to 7-day course, was relatively safe.

Brainstem auditory pathways may be particularly vulnerable (829); in rats, there were significant decreases in auditory accuracy at high doses of arteether (830). An auditory evaluation of patients who had been treated with at least two courses of oral artemether or artesunate therapy were found not to differ significantly from controls. In this study, there was no evidence of significant neurotoxicity (831).

Since the original report of neurotoxicity in animals, clinicians have been looking for signs of clinical neurotoxicity. A study of adverse effects in thousands of falciparum malaria patients in Thailand found that artemether or artesunate treatment had fewer side effects than when therapy involved the combination of an artemisinin with mefloquine (832). One case was reported in which a patient suffered an extended period of tremors after two courses of artemether therapy (833).

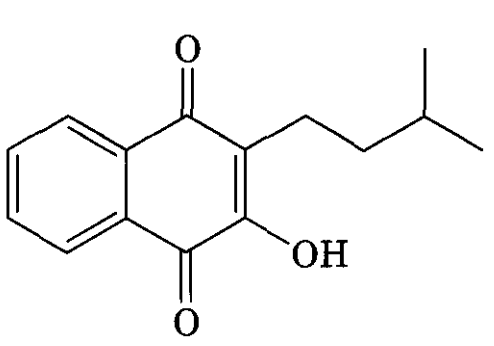
The results of the in vitro studies suggested a specific but as yet unidentified neuronal target. Compared with arteether and artemether, derivatives such as artesunate showed less injury, whereas dihydroartemisinin showed more by parenteral administration (834). In vitro studies have shown that the neurotoxic effects of artemisinins were enhanced by the presence of hemin (835). The presence of the endoperoxide is required for toxicity. Higher lipophilicity was associated with greater neurotoxicity in a study of the stereoelectronic properties of the artemisinins (836). Dihydroartemisinin produced changes in differentiating NB2a neuroblastoma cells that may be related to the neurotoxicity in animal models (837). Further, the neurotoxic effects seem to be mediated by drug binding to cellular proteins, binding that is increased in the presence of hemin (838). Antioxidants (ascorbic acid or glutathione) completely protected against the drug induced toxicity in vitro (839). Again, the interaction of artemether with hemin was suggested as a possible toxic mechanism of toxicity.

2.7.4.6 Artelinic Acid (52). Artelinic acid was developed as a water-soluble alternative to artesunate, and it has better stability in aqueous solution (840). A stereoselective syn-

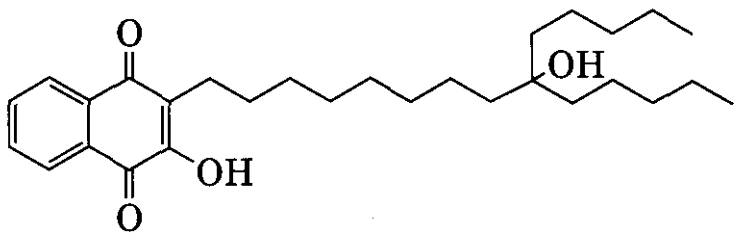
thesis of a-artelinic acid has been reported (841). The drug possesses superior activity against P. berghei in vitro compared with either artemisinin or artesunate (840). The pharmacokinetics and pharmacodynamics of artelinic acid in rodents have been studied (842) along with its clinical pharmacology (339). Sodium β-artelinate was demonstrated to be an active gametocytocide against P. *cynomolgi* by oral and intravenous administration (843). When compared with other artemisinin derivatives, artelinic acid had the highest plasma concentration, the highest oral bioavailability, the longest half-life, the lowest metabolism rate, and the lowest toxicity at equivalent doses (844), most likely because of its lower extent of conversion to dihydroartemisinin and slower elimination (845). The drug has not yet found wide use.

2.8 Other Agents

2.8.1 Atovaquone and Malarone. The potential for anti-protozoal activity of hydroxynaphthoquinones has been known for many years; hydrolapachol (**65**) was found to

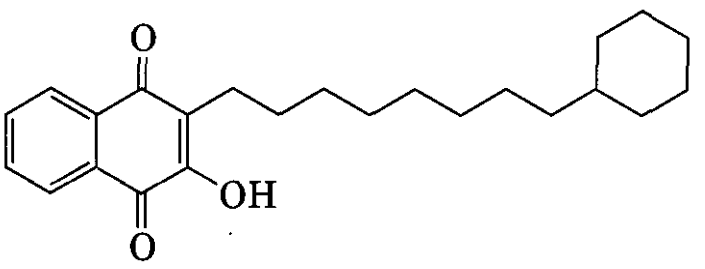


(65)
hydrolapachol

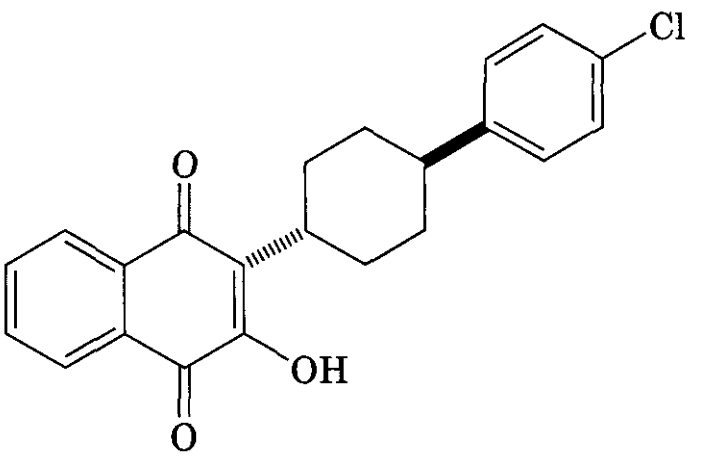


(66)
lapinone

be active against an avian malaria. Further studies identified lapinone (66) for clinical tests. Although effective against P. *vivax*, parenteral administration was required and interest lagged. Attempts to parlay these observations into clinically active compounds led to the development of, first, menoctone (**67**), and



(67)
menoctone



(68)
atovaquone

more recently, atovaquone (68) (846, 847). A short synthesis of atovaquone has been communicated (848). Menoctone, although active in mice, demonstrated no activity in humans infected with P. falciparum. Poor bioavailability was the cause of that disappointing result. Also known as BW 566C80, atovaquone was selected for development from a series of synthetic hydroxynaphthoquinones. Metabolic studies had shown that many of the compounds in the series were unsuitable for clinical use because of extensive human metabolism, even though good activity had been reported in animal models (849). Atovaquone, in contrast, was inert to human liver microsomes, displayed a plasma elimination half-life of 70 h, and was well tolerated. Absorption of the drug is slow and irregular, however. An increase in the plasma concentrations of atovaquone have been observed when dosing while giving with fatty foods (850).

Malaria parasites are unable to salvage pyrimidine bases and nucleotides; acquisition must be through *de novo* synthesis. Dihydro-

orotate dehydrogenase, a central enzyme in the de novo pyrimidine biosynthetic pathway, is moderately inhibited by atovaquone, which results in the accumulation of dihydroorotate and carbamoyl-aspartate, intermediates in pyrimidine biosynthesis (851, 852). Although growth of P. *falciparum* was inhibited by atovaquone, only moderate decreases in UTP, CTP, and dTTP levels were observed. Atovaquone at 15 μM decreased the activity of human dihydroorotate dehydrogenase by 50% (853). Kinetic data demonstrated that atovaquone was a competitive inhibitor with respect to the quinone co-substrate and an uncompetitive inhibitor with respect to the substrate dihydroorotate.

A second activity of this naphthoquinone is likely more directly related to antimalarial effect. Atovaquone is thought to act at the cytochrome *bc1* (Complex III) site of the mitochondrial electron transport chain by indirectly inhibiting several metabolic enzymes linked through ubiquinone (847, 854, 855). This results in the collapse of the plasmodia1 mitochondrial membrane potential. Parasite respiration was inhibited within minutes after drug treatment (856). The inhibitory effects were 1000 times higher in Plasmodia mitochondria than in isolated rat liver mitochondria. And purified ubiquinol-cytochrome c reductase from P. falciparum was more sensitive to atovaquone than was the mammalian enzyme (857). Intentionally developed resistance to atovaquone was accompanied by mutations in the cytochrome b gene of P. berghei (858) or P. yoelii (859). Recent evidence indicates that P. falciparum uses both a cytochrome chain and an alternative oxidase pathway for respiration (860). The action of atovaquone is potentiated by compounds that inhibit this alternative oxidase pathway, for example propyl gallate. Atovaquone inhibits up to 73% of P. falciparum oxygen consumption; this is consistent with known activity on the cytochrome bcl complex. Taken together, these findings strongly support the interaction of atovaquone with cytochrome bcl as a critical component of its mechanism of action.

Trials in vitro showed good response and no evidence of cross-resistance (861). Human trials with oral atovaquone indicated that initial responses were good, with prompt clinical and parasitologic response, but that rates of recrudescence were high regardless of length of treatment (862, 863). Re-treatment of the patients that suffered recrudescence with atovaquone were not successful, suggesting the development of drug resistance. Atovaquone demonstrated causal prophylactic activity in non-immune subjects who were challenged with mosquito transmitted P. falciparum (864). A number of agents were found to be synergistic with atovaquone including PS-15 (865), doxycycline (866), proguanil, tetracycline, or 5-fluoroorotate in vitro (867, 868). The elimination route for atovaquone in humans is almost exclusively in the feces, with no evidence of metabolites (869). Population pharmacokinetics in black, Oriental. and Malay groups have been reported (870).

Against P. berghei, atovaquone is highly potent against mosquito stages (871, 872), perhaps because of its ability to block the recruitment of asexual parasites and/or young (stage 1) gametocytes into the population of morphologically identifiable gametocytes (stages 2–5) of P. falciparum in vitro (873). Liver stage parasites are also inhibited when cultured in vitro (874).

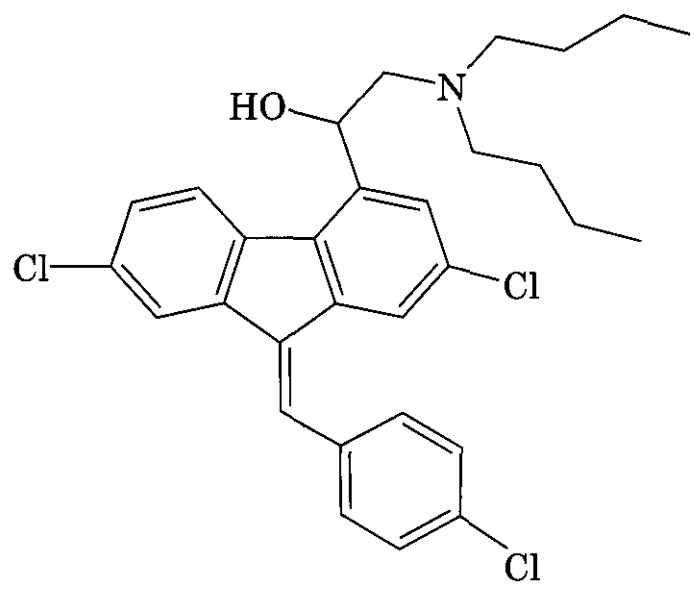
The combination of atovaquone and proguanil has been approved and is now marketed by Glaxo Wellcome as Malarone. The clinical development of Malarone has been reviewed (875). In randomized, controlled clinical trials, treatment with Malarone was significantly more effective than mefloquine (Thailand), amodiaquine (Gabon), chloroquine (Peru and Philippines), pyrimethamine/sulfadoxine (Zambia) (876), and chloroquine plus pyrimethamine/sulfadoxine (Philippines) (877). Overall cure rates exceeded 98% in more than 500 patients with falciparum malaria, even in strains that were resistant to proguanil or had been shown to be refractory to conventional therapy (878,879). The pharmacokinetics of atovaquone and proguanil and its metabolite cycloguanil were the same whether administered alone or in combination (880). The recommended regimen is 1000 mg atovaquone and 400 mg proguanil once a day for 3 days (863). It is effective in non-immune patients with uncomplicated falciparum malaria (881), safe and effective in children (882), effective when used as the suppressive agent

against P. *vivax* infections (883), and effective against P. *ovale* and P. *malariae* infections (884). In studies of Malarone for prophylaxis in various parts of Africa, the drug was found to provide excellent efficacy along with being well tolerated (885–888). The CDC includes Malarone among the agents recommended as prophylaxis for persons traveling to areas known to have chloroquine-resistance (92) and for presumptive self-treatment for travelers.

The two drugs are synergistic. Proguanil itself has no effect on electron transport nor mitochondrial membrane potentials (889). But it significantly enhanced the ability of atovaquone to collapse mitochondrial membrane potentials when used in combination.

Atovaquone is a potent, selective inhibitor of a variety of medically important protozoa as well as Plasmodium species. It is now used to treat a number of AIDS-related opportunistic infections including *Pneumocystis* carinii pneumonia, toxoplasmosis, and babesiosis. The drug is expensive to produce and will not be affordable in poorer areas, especially Africa. Glaxo Wellcome has announced its intention to provide the Malarone through a controlled donation program (890), part of which involves giving away 1,000,000 treatments each year to countries in Africa. The offer has met with skepticism by some governments, but pilot projects using Malarone are now underway (891). Extensive product information for Malarone is available on the web (892).

A triple combination of atovaquone with dapsone and either proguanil or PS-15 holds promise for treating multi-drug resistant parasites. It was more active than the combination of atovaquone with proguanil perhaps because of two pairs of synergistic interactions: proguanil with atovaquone and cycloguanil (the metabolite of proguanil) with dapsone (893,894).



(69)
lumefantrine

2.8.2 **Lumefantrine.** Lumefantrine (benflumetol, 69) was originally synthesized by the Academy of Military Medical Sciences in Beijing and underwent preliminary clinical trials in China. It belongs to the quinoline alcohol group that also includes quinine and mefloquine. A synthesis has been reported (895) as has a crystal structure (896). The compound is used in racemic form, and no substantial differences were found between the racemate or each enantiomer separately (897). A metabolite of lumefantrine, desbutyl-lumenfantrine is active in its own right (898). The accepted view of the mechanism of action involves the interaction of the drug with heme (899). Lumefantrine shares several of the pharmacokinetic properties of other antimalarials, notably mefloquine and halofantrine, in that it has a highly variable oral bioavailability between doses and between patients and is eliminated slowly. Pharmacokinetics and pharmacodynamics of lumefantrine in acute P. *falciparum* infections were reported (900).

The activity of lumefantrine did not differ between chloroquine-sensitive and chloroquine-resistant strains. Its in *vitro* activity against Cameroonian and Senegalese isolates was similar to that of mefloquine and slightly lower than that of artemisinin or pyronaridine (901, 902).

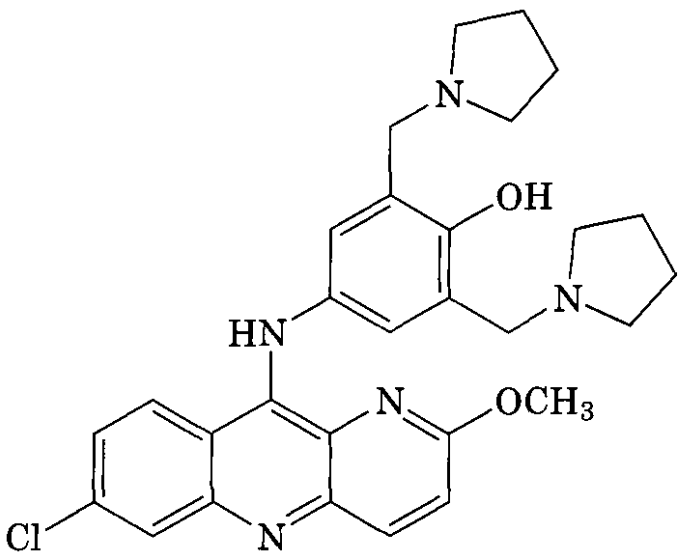
A fixed combination of lumefantrine with artemether (CGP 56697, Coartem, Riamet) has proven to be particularly promising. Phase II clinical trials of the combination were carried out in China. Parasite reduction at 24 h was 99.4%, the cure rate was 96%, and recrudescence was low (903). No adverse effects were encountered. Subsequent development has been carried out by Novartis Pharmaceuticals, and the combination was approved in Switzerland in 1999 (904). The clinical efficacy of the combination has been confirmed in studies carried out in Thailand

(905, 906), India (907), The Gambia (908), and Tanzania (909). In some cases, a relatively high level of recrudescence was noted (910). In others, the cure rates were lower than that seen with mefloquine (911). A higher dose regimen was suggested to improve these slight drawbacks. For a summary of completed clinical trials of lumefantrine/artemether, see Ref. 511.

Each tablet is 20 mg artemether and 120 mg lumefantrine, and a therapeutic regimen for adults is four doses of four tablets each at 0, 8, 24, and 48 h. In cases of multi-drug resistant infections, six doses of four tablets would be extended over 3 days. The fixed combination achieves its effect through an initial large reduction in parasitemia by the rapidly absorbed artemether and the subsequent removal of any remaining viable parasites by the intrinsically less active but more slowly eliminated lumefantrine (904). The synergism between the two drugs was documented even in a multi-drug resistant strain (912), and reviews of clinical pharmacokinetics (904, 913) and pharmacodynamics (913) of the combination are available.

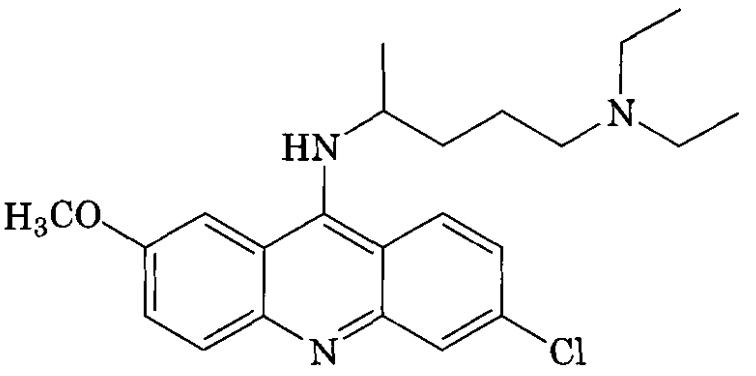
WHO and Novartis are providing developing countries with Coartem at a cost of approximately US$0.10 per tablet, amounting to less than US$2.50 per treatment for adults and even less for children.

2.8.3 Pyronaridine. The drug pyronaridine (70) (or malaridine), first synthesized in 1970,

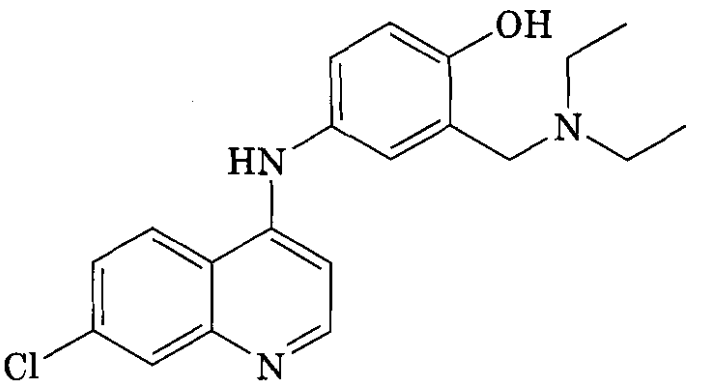


(70)
pyronaridine

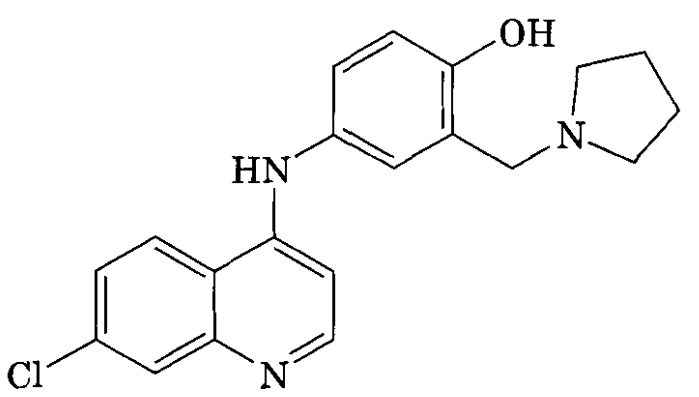
is the product of many years of research that began with the quinacrine (mepacrine) nucleus (71), work that has been carried out primarily in China. Quinacrine was selected as the lead compound because of the activity of its derivatives against chloroquine-resistant strains of Plasmodium but was not pursued as an antimalarial agent itself because of toxicity. The side-chain of pyronaridine is similar to those of amodiaquine (72) and amopyroquine (73). Issues leading to the design of pyronaridine and its synthesis have been reviewed (914). Reviews has also covered the actiyity in vitro and in vivo, toxicity, pharmacokinetics, and clinical studies (914–917). Pyronaridine is active against the erythrocytic



(71)
quinacrine



(72)
amodiaquine



(73)
amopyroquine

stages of malaria, although its mode of action is not well understood. It is not active against gametocytes (918). Early reports suggested that inhibition of the P. *falciparum* topoisomerase II enzyme was associated with the mechanism of action of the drug (919). Pyronaridine inhibited parasite growth in the low nanomolar range but inhibited topoisomerase II only in the micromolar range, with no evidence of drug concentration in the parasite nucleus. In more recent studies, pyronaridine failed to show topoisomerase II activity against P. *falciparum* enzyme *in situ* (920). It has been observed that similar morphologic changes are seen in both pyronaridine- and chloroquine-treated parasites (921) and that the parasitic digestive system is affected (922). Pyronaridine is converted into reactive intermediates by oxidation of its *p*-aminophenol moiety (923).

Preliminary clinical studies in China demonstrated the efficacy of pyronaridine against P. *falciparum* and *P. vivax* (916,917). Similar results were obtained when the drug was used to treat P. *ovale* and *P. malariae* infections (924). Field testing in China has included several thousand patients, and the drug is well tolerated, with few major adverse effects being reported. Testing in other areas (Thailand, Cameroon) has confirmed the Chinese results (925–927). For a summary of completed clinical trials, see Ref. 511.

Pyronaridine is available as its phosphate salt and can be given orally, intramuscularly, and intravenously. Recrudescence rates with pyronaridine alone are ~12%, which is good but not exceptional (925). Given the propensity for resistance to pyronaridine to develop rapidly in *in vitro* studies (928, 929), most researchers are advocating the development of combinations with pyronaridine rather using the drug in monotherapy. In particular, a triple combination of pyronaridine, sulfadoxine, and pyrimethamine has been studied with promising results; a single optimized dose gave a 100% cure rate in patients with acute falciparum malaria (930). In addition, a 5-year study of the triple combination, during which it was given as the only antimalarial drug at the study site in the Hainan Province, China, demonstrated that its effectiveness remained at the 100% level (931). No evidence of resistance was obtained. In an effort to evaluate alternatives for radical cure of vivax malaria, a combination of pyronaridine with primaquine was shown to have lower toxicity than chloroquinelprimaquine in mice and rats (932). The effects of pyronaridine with primaquine were reported to be synergistic (933). Potentiation of antimalarial action was observed when pyronaridine and artemisinin were used in combination with either an artemisinin-resistant or a pyronaridine-resistant strain of P. *yoelii* (929).

Although cross-resistance with chloroquine might be expected based on their structural similarities, pyronaridine is effective against chloroquine-sensitive, chloroquine-resistant, and multi-drug–resistant parasite strains, comparing well with other common antimalarials *in vitro* and in rodent models (934–939). Paradoxically, a pyronaridine-resistant strain was shown to be resistant to chloroquine. Interesting patterns of cross-resistance among pyronaridine and several structural relatives in rodent malarias were noted; resistance to one does not necessarily imply resistance to the whole group (940,941).

Estimates of the economics of pyronaridine use suggested that in Cameroon, pyronaridine treatment costs about three times as much as chloroquine and about twice as much as amodiaquine or sulfadoxine/pyrimethamine. It is less than one-half as expensive as a full course of oral quinine, halofantrine, or sulfadoxine/pyrimethamine/mefloquine. Given the increasing resistance to chloroquine in Africa and the toxicity of amodiaquine, pyronaridine has been suggested as a cost-effective alternative treatment (926). A note of caution has been sounded, however, about the rapid deployment of this agent in endemic areas such as West Africa (942). Additional detailed pharmacokinetic data are required as well as long-term toxicity studies, especially given the structural similarities between the side-chains of pyronaridine and amodiaquine.

A review of the development of pyronaridine is available (943). The drug has been developed and commercialized in China but is not routinely available elsewhere. At present no commercial partner has seemed interested in working toward international registration.

2.8.4 **Amodiaquine.** Amodiaquine (72, camoquine) is a 4-aminoquinoline and is structurally related to chloroquine (944). The crystal structure has been reported (945). The use of amodiaquine in the treatment of uncomplicated malaria has been reviewed (946). It was found that amodiaquine was decidedly more effective than chloroquine, despite the similarities in chemical structure, with times to parasite clearance being significantly shorter and fever clearance marginally faster. As might be expected, amodiaquine was less potent against chloroquine-resistant than against chloroquine-sensitive strains, but more potent than chloroquine against the resistant strains in *vitro* (947). Its toxicity seemed comparable with that of chloroquine when administered at doses up to 35 mg/kg over 3 days. Even though amodiaquine has not been as widely used in the treatment of malaria as chloroquine, good results have been obtained in comparison with other agents (948–950).

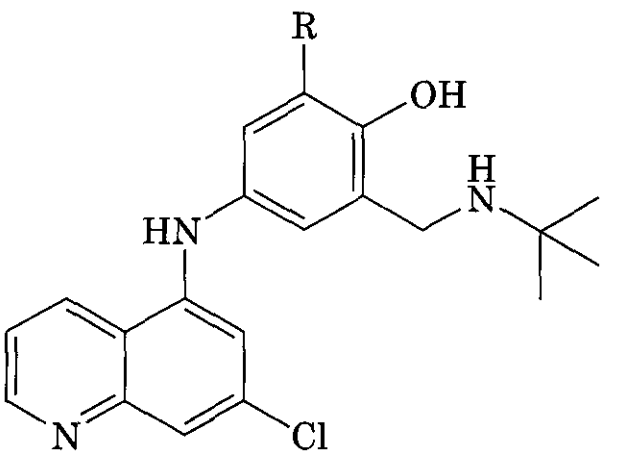
A combination of amodiaquine with pyrimethamine/sulfadoxine gave better control of clinical symptoms than did the antifolates alone with no evidence of serious side-effects (951). Resistance to amodiaquine is not prevalent (879) but has been observed (950, 952, 953). An association between resistance to amodiaquine and resistance to chloroquine has been demonstrated in P. *falciparum* isolates, although the resistance mechanism is much less effective for amodiaquine than for chloroquine (954).

Amodiaquine was thought to be well tolerated until it began to be used widely for prophylaxis. At that point, evidence for its long-term toxicity emerged; consequently, amodiaquine is no longer used prophylactically. Severe adverse effects associated with amodiaquine are agranulocytosis (955–957) and liver toxicity (957, 958). It has been suggested that the toxicity of amodiaquine is related to reactive derivatives formed by oxidation of its phenolic side-chain, especially to the formation of a quinone-imine metabolite by biological oxidation (923,959–962). Much like chloroquine, amodiaquine has been shown to interact with ferriprotoporphyrin IX (963).

The pharmacokinetics of amodiaquine have been studied (121, 964–966). The drug disappears rapidly from the blood with a terminal half-life of about 2 h after intravenous injection. An increased level of amodiaquine accumulation in comparison with chloroquine may be caused by enhanced affinity for a parasite binding site and may also explain the greater inherent activity against P. *falciparum* (967). Amodiaquine could be considered a prodrug; its main metabolite, monodesethylamodiaquine, is found in high concentrations in the blood after dosing and is highly active (966, 968, 969). Other metabolites that have been identified are bi-desethylamodiaquine and hydroxydesethylamodiaquine, both of which have negligible activity (970, 971). Amodiaquine was shown to be very weakly mutagenic in an Ames mutagenicity assay (972).

SAR work on amodiaquine had previously shown that wide variations in the side-chain are accommodated with retention of antimalarial activity.

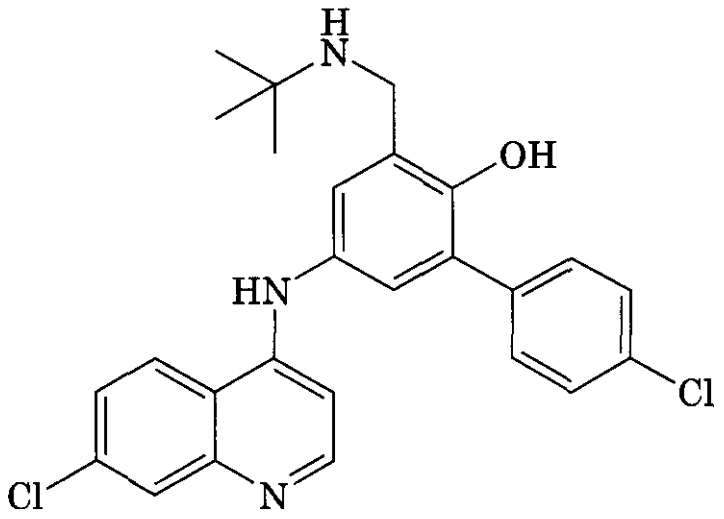
Blocking of bioactivation pathways either through removal of the 5' phenol or introducing a non-reactive substituents has been the main strategy (973, 974). Replacement of the 3-diethylamino group with cyclic amines or with a tert-butyl group provided analogs (74) that were more active than amodiaquine (975–977). Reducing bioactivation also seems to result in compounds with slower elimination and increased tissue accumulation (978). The suggestion is made that a specific binding site for amodiaquine-like compounds may exist and that characterization of such a site would facilitate further drug design. A conformational analysis on structures related to amodi-



(74)
R = propyl, isopropyl

aquine revealed that the interatomic distance between the ring and side-chain nitrogen atoms is an important determinant of antimalarial activity (979). Although amodiaquine does not form a complex with heme, it does inhibit hemozoin formation (IC_{50}, 250 m*M*) (32).

One analog of amodiaquine in particular was considered quite promising at one time. Tebuquine (75) was found to be more potent

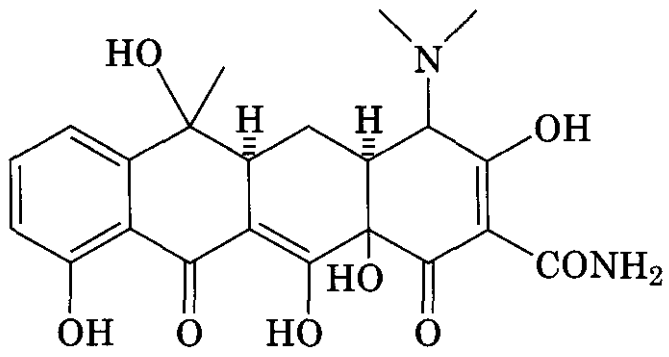


(75)
tebuquine

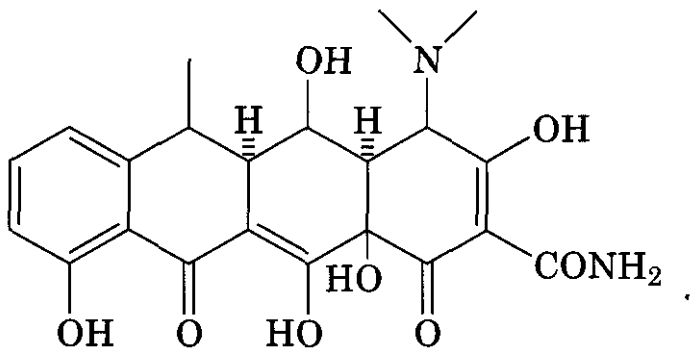
than amodiaquine or chloroquine, have excellent activity against resistant parasite strains, was curative in primate models, and had an extended duration of action (980). Additional tebuquine analogs were prepared and studied, although none was superior to the original (981).

A difference of opinion has arisen about the future of amodiaquine in malaria management. The drug is clearly not appropriate for suppressive prophylaxis. However, there may be more leeway in regard to therapeutic use. At present, amodiaquine is not recommended for treatment or prophylaxis by the World Health Organization because of hepatic and hematological toxicity. Even so, some researchers are advocating an increased use of amodiaquine in treatment because it has higher activity than chloroquine, it has efficacy in areas where chloroquine resistance is reported, and it is often produced locally, making it an inexpensive drug (948,982). At a time when many countries in Africa are faced with identifying a first-line drug to replace chloroquine, amodiaquine may be a viable alternative despite its adverse effects (983–985), although caution is certainly warranted (986).

2.8.5 **Antibiotics.** Early studies revealed that chlortetracycline was active against avian malarias, although as a group the antibiotics were slow-acting, not truly curative, and not causally prophylactic. As such, no benefit from antibiotic use was envisioned. With the increase in drug-resistant strains of Plasmodia, the practicality and use of antibiotics has been re-examined. The most common antibiotics used against malaria are tetracycline (76) and doxycycline



(76)
tetracycline



(77)
doxycycline

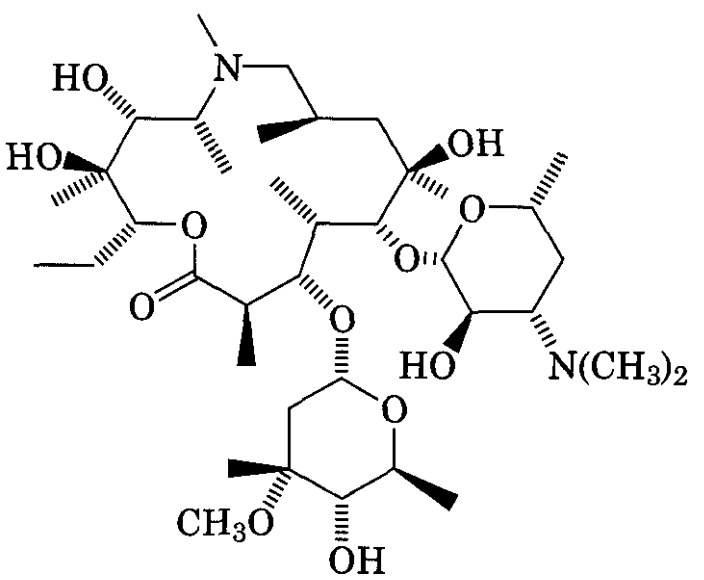
(77). Doxycycline is a structural isomer of tetracycline with improved oral absorption; thus, a smaller therapeutic dose is required.

Because of the slow-acting nature of these drugs, they are almost always used in combination with a fast-acting agent such as chloroquine or quinine in treating acute malarial attacks. In particular, a 7-day course of a tetracycline-quinine combination has been found to be useful in treating chloroquine- and multi-drug–resistant strains (987, 988). Tetracycline serves to increase the plasma quinine levels above those seen with equivalent dosing of quinine alone (131).

Although tetracycline displays activity against primary tissue schizonts of chloroquine-resistant strains of P. falciparum, its

long-term use as a prophylactic agent is not advised. On the other hand, doxycycline has been suggested for short-term prophylaxis by non-immune travelers (505, 989). It was highly efficacious and well tolerated as a prophylactic agent in Indonesian soldiers (990) and in semi-immune volunteers in Kenya (991). Another study with soldiers in hyperendemic areas demonstrated that doxycycline could not be relied on for causal prophylaxis, however, even when combined with primaquine (69). Because of their adverse effects on bones and teeth, tetracyclines should not be given to pregnant women or children less than 8 years old (155), precisely the populations most vulnerable to malaria in endemic areas. The tetracyclines act against the blood stages of Plasmodia. In contrast with what is known about the action of tetracycline on prokaryotes, its mode of action on Plasmodia is not completely understood. The site of action of tetracyclines is the parasite mitochondrion, with inhibition of mitochondrial protein synthesis the result (992). This would account for slow onset of action of the tetracyclines, because mitochondrial replication may be restricted to a limited part of the cell cycle.

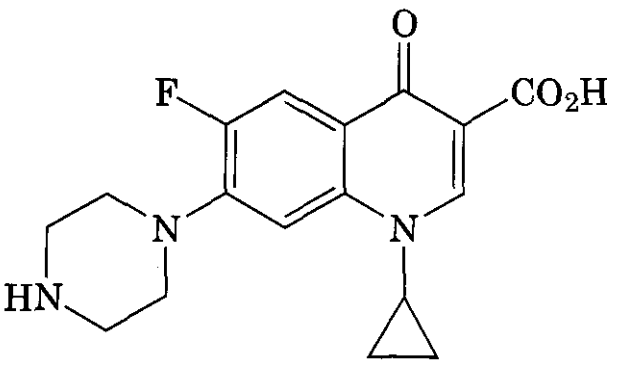
Azithromycin (78), an erythromycin analog, is used to treat bacterial and chlamydia1



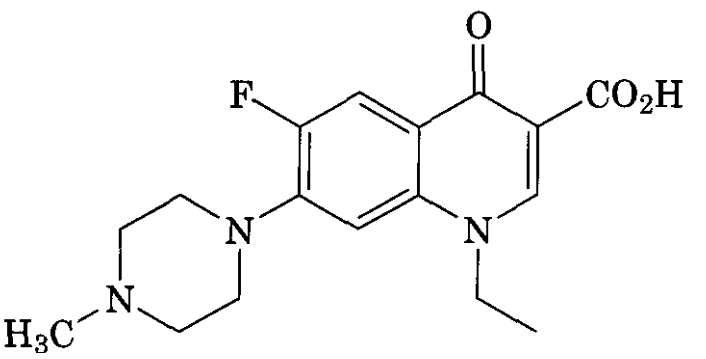
(78)
azithromycin

infections. When combined with fast-acting agents such as quinine, halofantrine, chloroquine, and artemisinin, the effects were additive (993). Azithromycin may also have promise as a prophylactic agent, given its increased half-life over doxycycline (994). Daily azithromycin provided excellent prophylactic results against P. *vivax*, comparable to those seen with doxycycline, but was less effective against P. ***falciparum*** in a study in Indonesia (995). A further advantage in substituting azithromycin for tetracycline in combination therapies is that there is no contraindication to administration of azithromycin to pregnant women or young children (996).

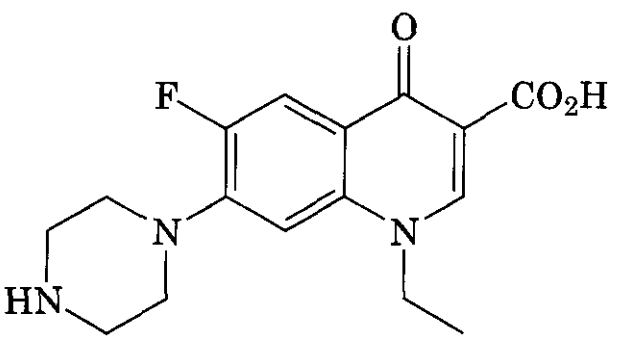
Fluoroquinolone antibiotics such as ciprofloxacin (79), pefloxacin (80), norfloxacin (81),



(79)
ciprofloxacin

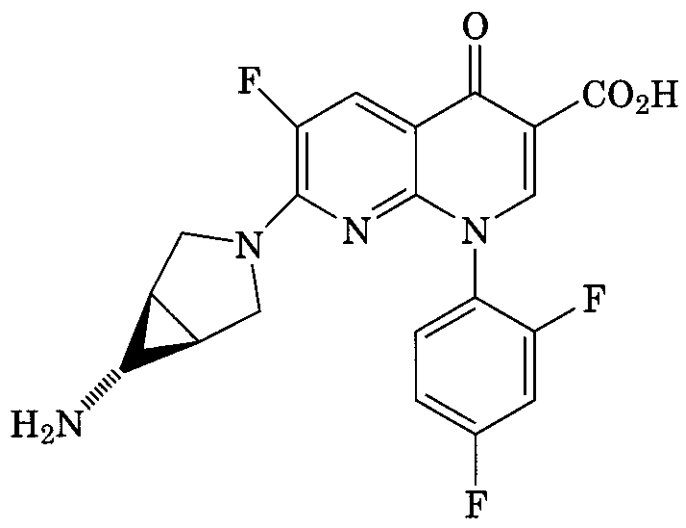


(80)
pefloxacin



(81)
norfloxacin

trovafloxacin (82) have been shown to be active against P. *falciparum* ***in vitro*** (997, 998) and

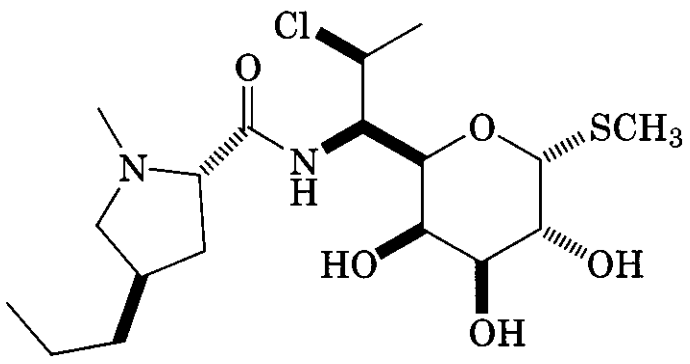


(82)
trovafloxacin

against chloroquine-resistant P. yoelii in mice in vivo (999). Preliminary studies with drugs of this class suggested clinical antimalarial efficacy but more detailed investigations failed to confirm the earlier observations (1000). In combination with quinine, pefloxacin did not potentiate the activity of quinine in terms of speed of parasite or fever clearance (1001). Norfloxacin was less effective than chloroquine in clinical studies (1002). The future role of the fluoroquinolines is not likely to include first-line therapy nor single-agent regimens.

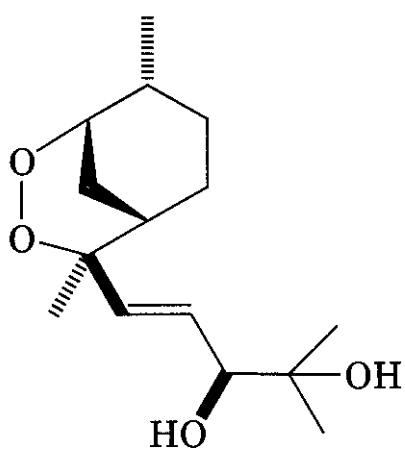
An unusual organelle, the apicomplexan plastid or apicoplast, found in certain parasites including Plasmodium, may be the site of action of ciprofloxacin. This antibiotic inhibits replication of the apicoplast, blocking parasite replication (50). The specific action may involve inhibition of a topoisomerase II activity associated with the apicoplast (1003).

Clindamycin (**83**), a lincosamide antibiotic that can be given to children, has activity against Plasmodia. The addition of clindamycin to a standard quinine treatment significantly improved and shortened chemotherapy in children and adults in Africa and Thailand (1004–1006). A 3-day clindamycin-quinine regimen was well tolerated by children and had an efficacy rate of 97% by day 20 (1007). The mode of action of clindamycin and related compounds against malaria parasites may involve effects on apicoplast replication (50). It was noted that the antibiotic effect of clindamycin may be beneficial in treating the concomitant bacterial infections that are often found in cases of severe malaria.



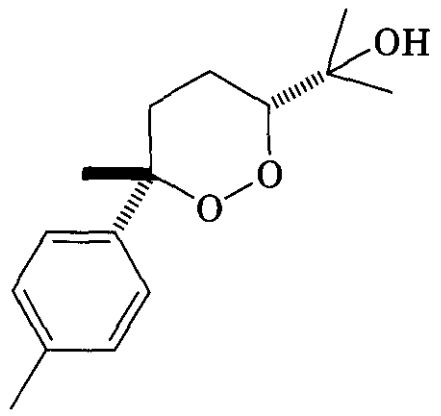
(83)
clindamycin

2.8.6 Yingzhaosu A, Yingzhaosu C, and Arteflene (Ro 42-1611). Given the success of the peroxide natural product artemisinin in drug development efforts, it was obvious for researchers to explore the antimalarial properties of other naturally occurring peroxides. Two cyclic compounds containing a bisabolene skeleton have been isolated from the roots of the plant yingzhao (*Artabotrys uncinatus*), which has been used as a folk treatment for malaria. Yingzhaosu A (84) was first isolated,

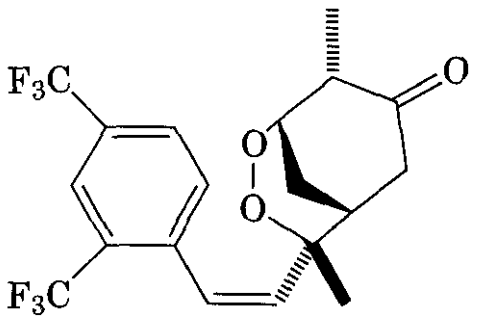


(84)
yingzhaosu A

and the structure was proposed in 1979 (1008). The structure and stereochemistry of yingzhaosu C (85) were determined by spectroscopic methods, derivatization, and by conversion to a known compound (1009). Both compounds have been synthesized (565, 1010–1012) and both are active antimalarials (592, 1013, 1014). Analogs have been prepared, and some demonstrate significant antimalarial activity in vitro (667, 1015).



(85)
yingzhaosu C



(86)
arteflene

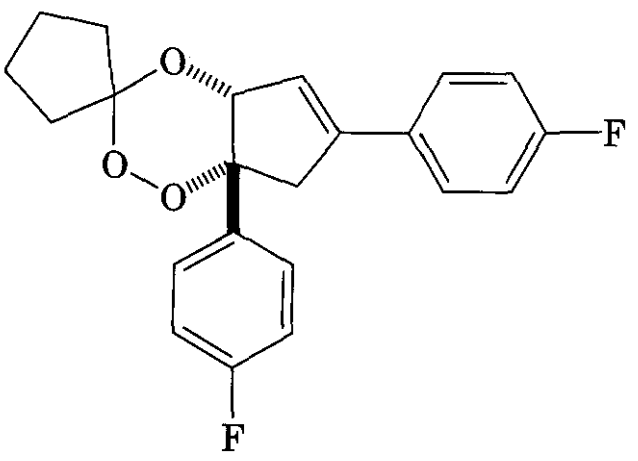
A particular example is arteflene (86), a synthetic antimalarial agent developed as an analog of yingzhaosu **A** (1016). The central core ring structure (2,3-dioxabicyclo[3.3.1] nonane) was shown to have inherent antimalarial activity. A flexible synthetic strategy was devised that afforded a variety of analogs, with the more lipophilic compounds displaying higher activity. The most active of these was arteflene. It is as active as artemisinin, and has better chemical stability. In a mouse model, arteflene was fast-acting and a single dose lasts significantly longer and results in much lower recrudescence than with artemisinin (1017). The pharmacokinetics and metabolism of arteflene have been studied in animal models (1018) and in humans (1019). Like artemisinin, the compound has low human toxicity and seems to interact with specific parasite proteins (682). In preliminary clinical trials, arteflene is effective in reducing clinical symptoms and is well tolerated (1020–1022). Arteflene may find use both in treatment and as a prophylactic agent, although interest in the drug seems to have waned recently.

3 ANTIMALARIAL AGENTS FOR CHEMOTHERAPY AND PROPHYLAXIS: EXPERIMENTAL AGENTS

3.1 Synthetic Experimental Antimalarial Agents

A number of new structural lead compounds have been discovered or prepared in the quest for new antimalarial agents. In addition, the analysis of the biochemistry of the parasite has led to the identification of novel molecular targets. The more active and promising frontier areas are discussed below.

Although many trioxane compounds related to artemisinin have been prepared and studied, Fenozan-50F (87) is the first trioxane

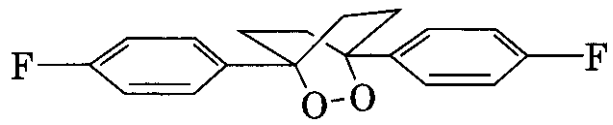


(87)
Fenozan-50F

structurally unlike artemisinin to possess the possibility of therapeutic use. Stemming from general studies on the trioxane system, a large number of cis-fused cyclopentenotrioxanes were synthesized (1023). From these, Fenozan-50F was identified as the most active agent (1024, 1025). The compound is active against blood stages of P. ***berghei*** and ***P. yoelii***, even against a wide spectrum of drug-resistant parasite strains, and is a potent gametocytocide (1026). There are significant differences between Fenozan-50F and the artemisinins in their blood schizontocidal actions (1026). Fenozan-50F is about one-half as active as arteether in P. ***berghei*** infected mice but three times as active as artesunate, and its toxicity seems to be low. The enantiomers of Fenozan-50F are equally active with the racemic mixture (1027). Fenozan-50F exerts complex effects when combined with other antimalarial drugs

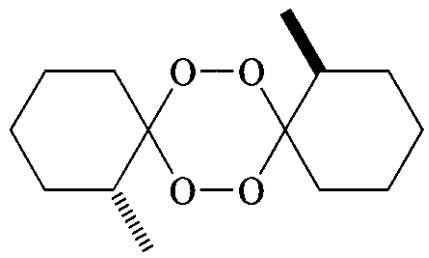
against mouse parasites **(1028).** With mefloquine, for example, observed effects were additive, potentiating, or antagonistic depending on the strain of *Plasmodium* used. Studies on its mechanism of action demonstrated that the trioxane reacted with ferrous ion to produce a number of product esters; oxygen transfer from the peroxide did not occur **(1029).** Resistance to Fenozan-50F could be established in *P. yoelii,* but the resistance was not stable once drug pressure was removed (718).

Even very simple bicyclic endoperoxides such as **(88)** were found to have approximately



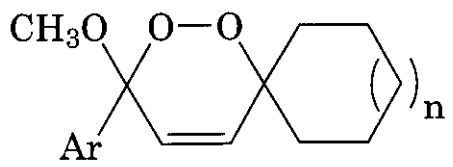
(88)

15% of the activity of artemisinin **(1030).** The mechanism of action of these compounds may in fact differ from that of the artemisinins; alkylating agents such as epoxy ketones and/or ethylene oxide may be generated *in situ* and may account for the observed parasiticidal activity. Other peroxide systems such as the readily available tetraoxane **(89,** WR148999)



(89)
WR148999

possess IC_{50} values in the nanomolar range (1031), although the oral activity was poor. Attempts to design compounds with improved oral activity led to the identification of a tetramethylated compound with *in vivo* activity better than that of artemisinin but not as good as arteether **(1032).** A number of other structural types of tetraoxacycloalkanes have been produced that have antimalarial activity **(1033–1036).** Cyclic peroxy ketals, reminiscent of certain marine natural products, have been prepared and tested as antimalarial agents **(90) (1037).** Some of the compounds

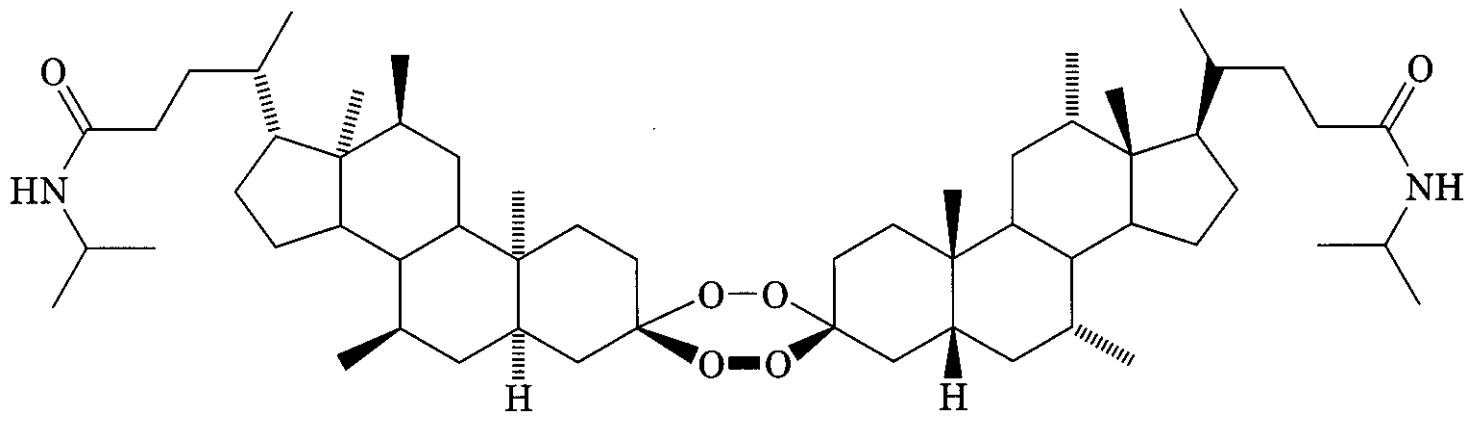


(90)
n = 1. 2

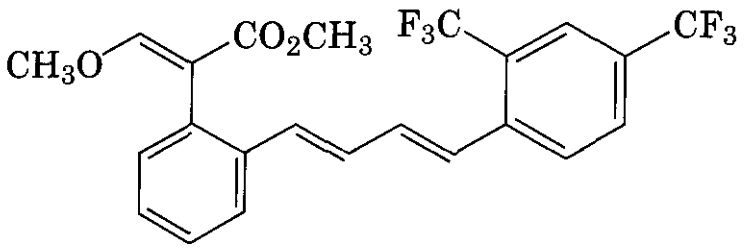
had IC_{50} values in the range of **30–80** n*M* against P. *falciparum in vitro.* Generalizations about SAR in this series were presented. More complex tetraoxanes could be prepared from steroidal ketones; one compound derived from a cholic acid amide **(91)** was almost as active as artemisinin and displayed low cytotoxicity **(1038).** A hydroperoxide, 15-hydroperoxyeicosatetraeneoicacid (HPETE), induced a **96%** inhibition of *P. falciparum* growth at **40** μM **(1039).**

Compounds incorporating a new antimalarial pharmacophore, a phenyl β-methoxyacrylate exemplified by (**92**), have been reported to interfere with mitochondrial electron transport **(1040).** These compounds were superior to chloroquine both *in vitro* and *in vivo* and may provide a lower cost alternative to atovaquone.

Given the promising introduction of the hydroxynaphthoquinone atovaquone, a series of

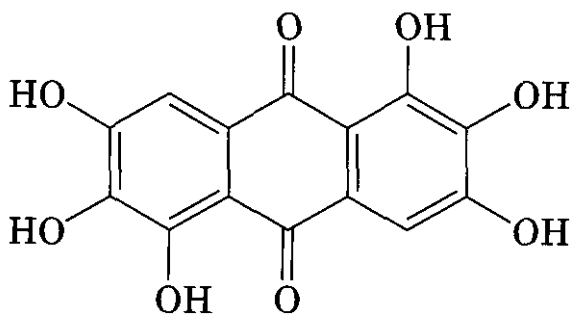


(91)



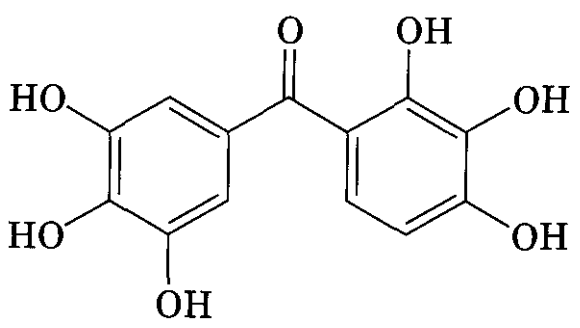
(92)

hydroxy- and polyhydroxyanthraquinones were synthesized and screened for antimalarial activity (1041). Rufigallol (**93**), easily pre-



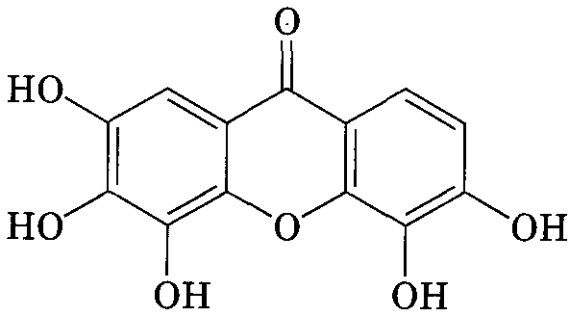
(93)
rufigallol

pared by dehydration of gallic acid, displayed an IC_{50} of 35 n*M* against chloroquine-sensitive or resistant P. falciparum in *vitro* and was the most active compound in the group. When combined with exifone (**94**), a strong potenti-



(94)
exifone

ation of antimalarial effect was noted (1042). Exifone is also synergistic with other oxidants such as ascorbic acid; the authors suggested that exifone may undergoes oxidation to a toxic xanthone in *vivo* in presence of rufigallol or ascorbic acid (1043). Hydroxyxanthones such as X5 (**95**) inhibit P. falciparum growth

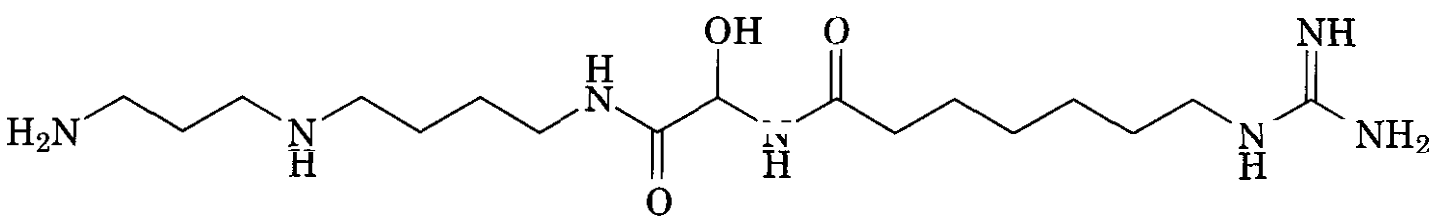


(95)
X5

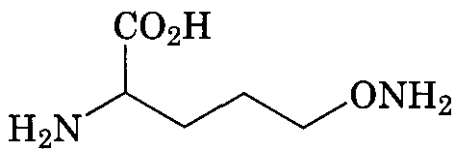
in *vitro*, and they seem to act at the point of heme sequestration (1044). The antimalarial effect occurred during the second half of erythrocytic cycle, when free heme production peaks (1045).

dl-α-Difluoromethylornithine and *dl*-α-monomethyldehydroornithine methyl ester, inhibitors of ornithine decarboxylase, part of the polyamine biosynthesis pathway, block exoerythrocytic schizogony (1046). The immunosuppressive agent deoxyspergualin (96) has shown activity in *vitro* and in *vivo*, most likely through inhibition of polyamine biosynthesis. Although the drug acted slowly, complete eradication of parasitemia was accomplished in mice (1047). Clinical studies are underway. Another group of ornithine decarboxylase inhibitors includes hydroxylamino compounds. A number of structures including canaline (97) and CGP51905A (98) were prepared and found to have sub-micromolar inhibition of P. falciparum in *vitro* (1048).

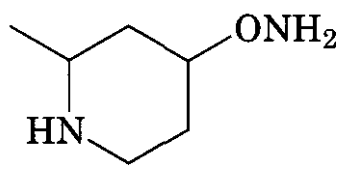
5-Fluoroorotate, a pyrimidine analog, inhibits parasite proliferation at low concentrations with little toxicity to mammalian cells (868, 1049). Inhibition of the plasmodial thymidylate synthase, a key enzyme in the path-



(96)
deoxyspergualin



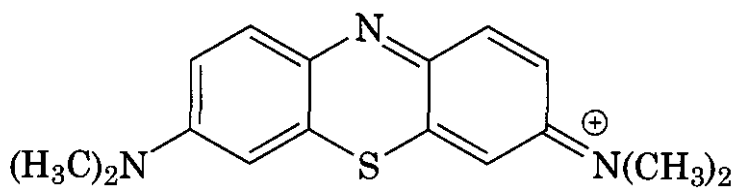
(97)
canaline



(98)
CGP1905A

way of de novo pyrimidine synthesis, seems to be the mode of action (1050).

One of the earliest known antimalarial agents after quinine was the synthetic thiazine dye, methylene blue (**99**). The in vitro

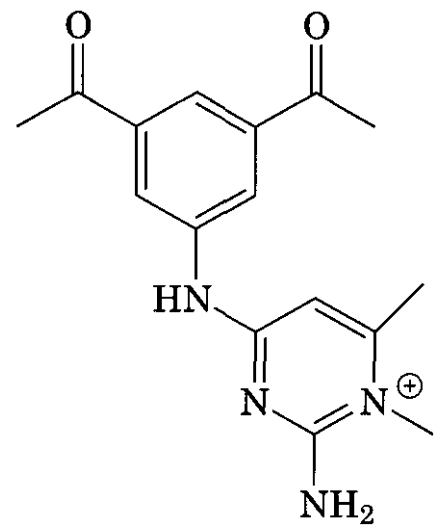


(99)
methylene blue

activities of methylene blue and several other dye compounds were reexamined recently (1051). IC_{50} values in the nanomolar range were obtained for the thiazine dyes, and some were more active than chloroquine in sensitive strains. No cross-resistance with chloroquine was observed. The mechanism of action of methylene blue has been studied (1052) and may involve inhibition of plasmodial glutathione reductase (1053). Although the potential toxicity of these compounds will likely prohibit their clinical use as antimalarial agents, the phenothiazine structure may provide a useful lead for further drug design efforts.

Because the end product of hemoglobin digestion is hemozoin, a sequestration product of hematin Fe(III), Fe(II) in hemoglobin is oxidized to Fe(III). It seems that this oxidation occurs at the level of hemoglobin to methemoglobin, because it has been demonstrated that P. falciparum increases the levels of methemoglobin in infected erythrocytes and the increase is restricted to ingested hemoglobin (1054). Riboflavin was active in vitro against P. falciparum at levels commonly used clinically to treat methemoglobinemia without adverse effects. Both riboflavin and methylene blue may act by reduction of methemoglobin to hemoglobin in *vivo*.

A novel arylene bis(methylketone), compound CNI-H0294 (100) was shown to have

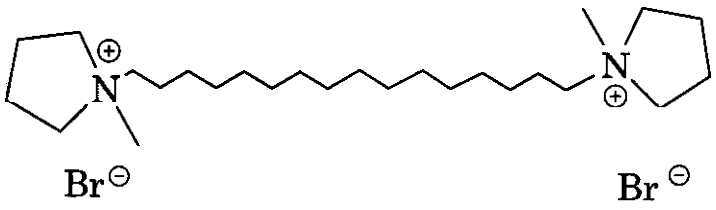


(100)
CNI-H0294

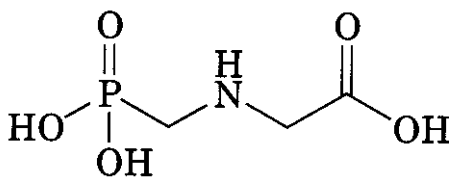
good activity in vitro against chloroquine and pyrimethamine resistant P. falciparum in vitro and against P. berghei in *vivo*. The drug displayed activity against the plasmodial dihydrofolate reductase at achievable concentrations, and this represents a possible mechanism of action for the drug (1055, 1056).

Another target for the design of new antimalarial agents has been the phospholipid metabolism of P. falciparum. Phosphatidylcholine, the major phospholipid of infected erythrocytes, is synthesized from choline, drawn mainly from plasma using parasitic enzymes. Interference with the de novo biosynthesis of phosphatidylcholine is lethal to the parasite. The transport of choline is impaired by quaternary ammonium compounds (1057, 1058). Several inhibitors showed excellent in vitro action against drug-resistant strains of P. falciparum. One compound in particular (101) had an IC„ value in the sub-nanomolar range (1059). From the data obtained for this series, the authors propose a model for binding of these inhibitors to the choline carrier.

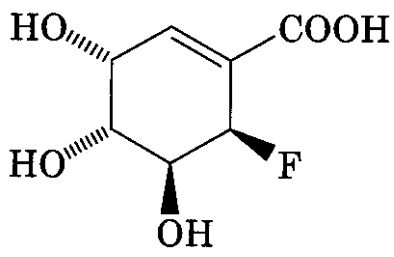
Specific inhibitors of the shikimate pathway include the herbicide glyphosate (102) (46) and fluorinated derivatives of shikimic



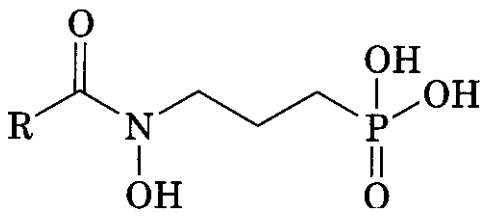
(101)



(102)
glyphosate



(103)

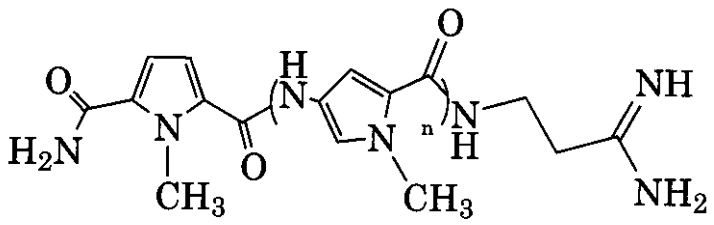


(104)
fosmidomyin, R = H
(105)
FR-900098, R = CH_3

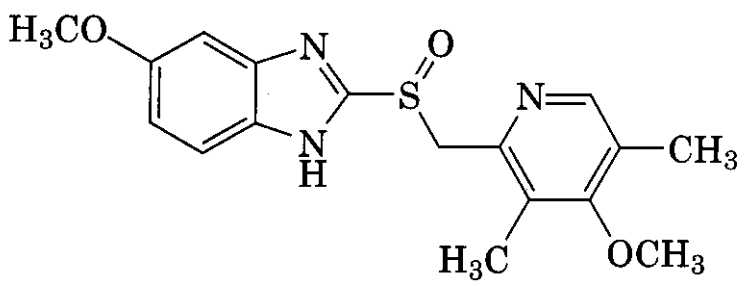
acid such as (**103**) **(1060).** Inhibitors of DOXP reductoisomerase were already known, including fosmidomycin **(104)** and FR900098 **(105).** These two agents were shown to inhibit P. *falciparum* in culture (~300 and **—140 n*M***, respectively) and active *in vivo* against *P. vinckei* in mice. Both drugs have very low toxicity but also had very short half-lives and may provide leads for new drug development. Indeed, modification of the structure of FR900098 as phosphodiaryl ester resulted in compounds with improved oral activity **(1061).**

Analogs of distamycin, a compound that binds in the minor groove of duplex DNA, were shown to have antimalarial activity in the sub-micromolar range. Distamycins bind dA-dT-rich DNA sequences that make up a large percentage of the P. *falciparum* genome **(82%).** In contrast with distamycin itself, active analogs such as (**106**), were much less toxic **(1062).**

In a study of the antimalarial action of benzimidazole compounds, omeprazole (**107**),



(106)
n = 4

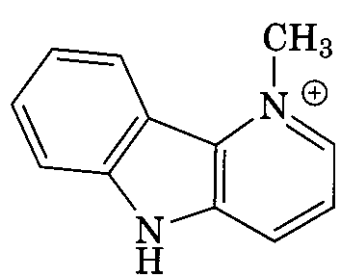


(107)
omeprazole

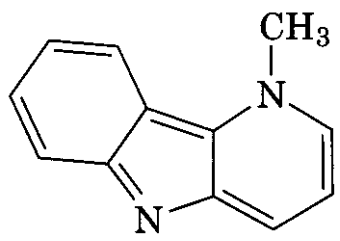
a proton pump inhibitor, was found to be the most active *in vitro*, primarily against the later stages of parasite development **(1063).** Its action is synergistic with that of quinine but antagonistic with chloroquine **(1064).**

Vitamin A (**retinol**) is central to normal immune function and has been shown to possess antimalarial activity on its own. Supplementation therapy with high dose vitamin A was beneficial in young children in Papua New Guinea (1065), and *in vitro* studies showed that retinol potentiated the actions of quinine and omeprazole **(1066).**

δ-Carbolines were prepared and tested for antimalarial activity **(1067).** These compounds **(108, 109)** were active *in vitro* against



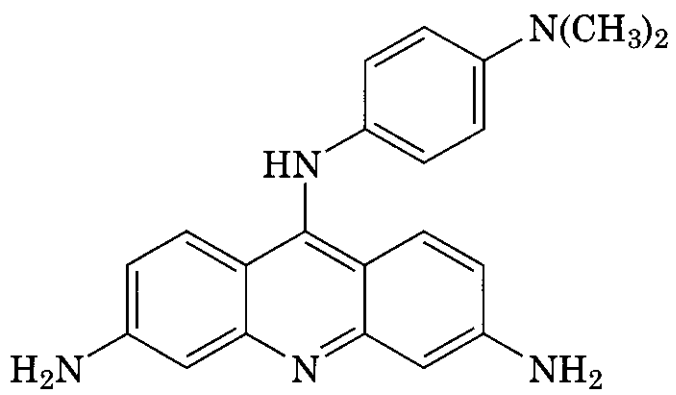
(108)



(109)

P. falciparum in the low micromolar range and were weakly cytotoxic. These agents specifically accumulate in the intracellular parasite and may interact with parasite DNA.

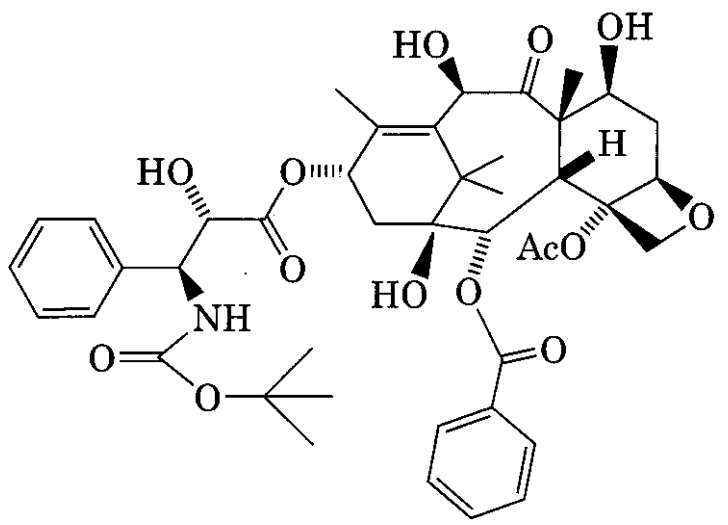
A series of 9-anilinoacridines (110) has been synthesized and evaluated for their antimalarial activity as topoisomerase inhibitors (919, 1068). It was found that a 3,6-diamino substitution pattern on the acridine conferred high potency against P. *falciparum* in erythrocyte culture tests. Inhibitors that acted against the parasite topoisomerase II enzyme both *in vitro* and *in vivo* were identified based on the ability of the candidate compounds to generate cleavable complexes *in situ* (920).



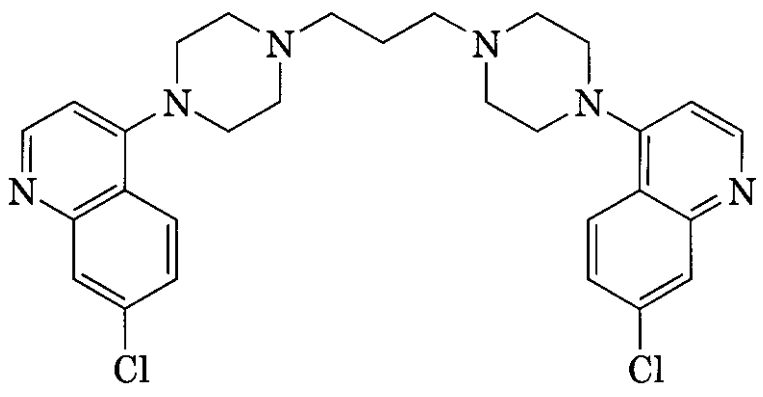
(110)

Docetaxel (taxotere, **111**), a taxol-type drug, was found to inhibit P. *falciparum* development *in vitro* with an IC_{50} value in the nanomolar range (1069), although the inherent cytotoxicity of microtubule inhibitors currently available limits the likelihood of their being used in malaria therapy (1070).

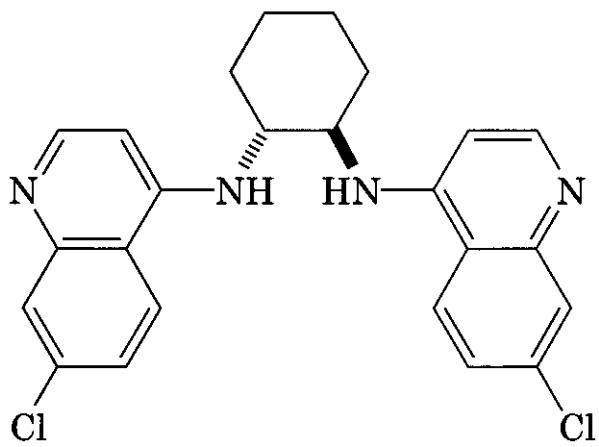
Earlier efforts in improving the chloroquine structure involved the development of bisquinoline compounds linked by a covalent bridge, including compounds such as piperaquine (**112**) (1071). Several of the compounds with simple alkyl bridges exemplified by 113 displayed good activity and were in fact more active against a chloroquine-resistant strain than against a sensitive one (1071).



(111)



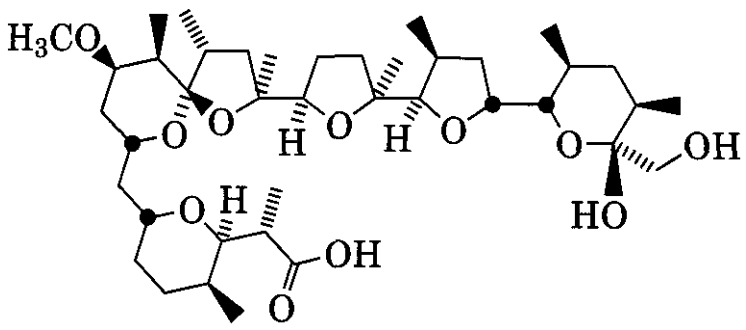
(112)
piperaquine



(113)

During parasite development in the host red blood cell, Na^+ and K^+ levels within the cell are disrupted, and the parasite creates a gradient between its own cytosol and the erythrocytic cytosol. Because ionophores are known to disturb ionic gradients, derivatives of the ionophore monensin were synthesized and evaluated for antimalarial activity (1072). The activity of the ionophores correlated with the transport efficiency for total Na^+ and K^+ rather than with ion selectivity. Several of the

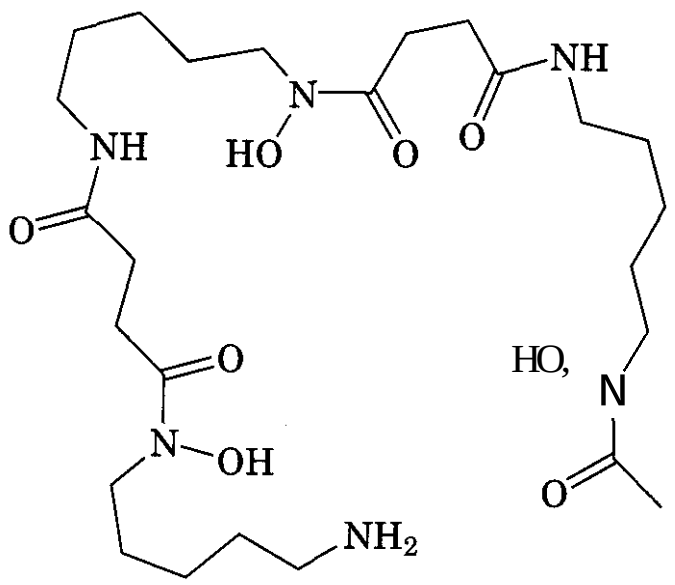
compounds exhibited IC_{50} values in the nanomolar range against P. ***falciparum in vitro.*** Nigericin (**114**), an ionophore selective for



(114)
nigericin

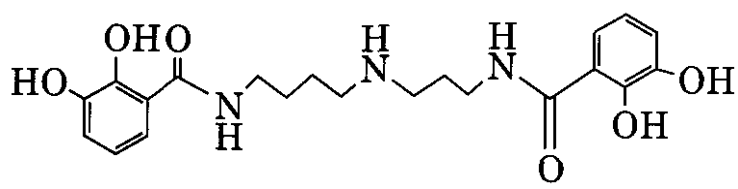
K^+/H^+ exchange also, had good ***in vitro*** activity, and its action was synergistic with that of monensin (1073).

Malaria parasites are very susceptible to the action of iron chelators and a number of studies have been carried out to evaluate the clinical potential of agents of this type (1074, 1075). Iron chelators may act by one of two possible mechanisms against malaria parasites. They may act either by depriving the rapidly multiplying parasites of essential iron, or they may form complexes with iron that are ultimately toxic to the parasite. Desferrioxamine (DFO, **115**), a known iron chelator, has



(115)
desferrioxamine

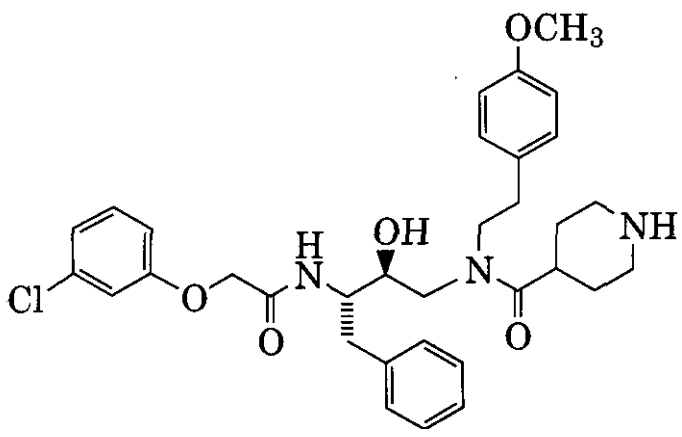
been examined as a therapeutic agent with mixed results (1076). DFO inhibits ***in vitro*** growth of P. ***falciparum*** in humans. Clinical cases are resolved faster with co-administration of DFO and a traditional antimalarial (339). For example, parasite clearance and recovery from coma were faster when DFO was used in combination with quinine than when quinine was used alone (1077). Recrudescence occurred in most subjects, however. Clinical efficacy of DFO required continuous parenteral administration, and its use in therapy must therefore be somewhat limited. In addition to effects on iron levels, DFO may protect against iron-mediated oxidant damage (1077). DFO antagonizes the action of chloroquine (1078), apparently by increasing the concentration of soluble forms of hematin and enhancing hematin sequestration (1079). DFO does not seem to extract iron from intact enzyme but may prohibit the initial iron-enzyme interaction necessary to form the active species. Derivatives of DFO demonstrated significant differences in activity against parasites vs. mammalian cells, the parasites being more susceptible to interference from the chelator (1080).Other iron chelators have been studied as well, especially a group of compounds known as reversed siderophores, synthetic analogs of the natural siderophore ferrichrome (1076, 1081, 1082). The most lipophilic of a series was the most potent antimalarial agent and the most efficient in extracting iron from the infected cells (1082). A novel siderophore (**116**) was shown to have sub-micromolar ac-



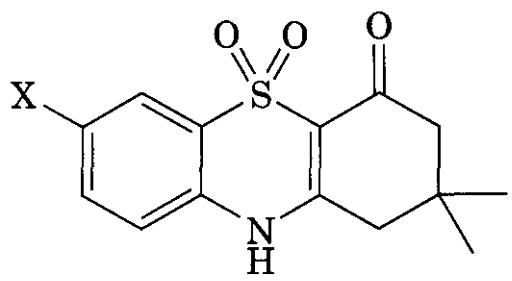
(116)

tivity against P. ***falciparum*** clones, presumably because of iron deprivation (1083).

Compounds that act on the plasmodial proteases that degrade hemoglobin are receiving attention as potential antimalarial agents. Non-peptide inhibitors of plasmepsin II, an aspartyl protease, were identified using combinatorial chemistry and structure-based design. One inhibitor (**117**) had a K_i of 4.3 nM and had good selectivity for the plasmodial enzyme over cathepsin D (1084). Phenothiazines were studied as inhibitors of falcipain, a cysteine protease of *P. falciparum* (1085). Certain

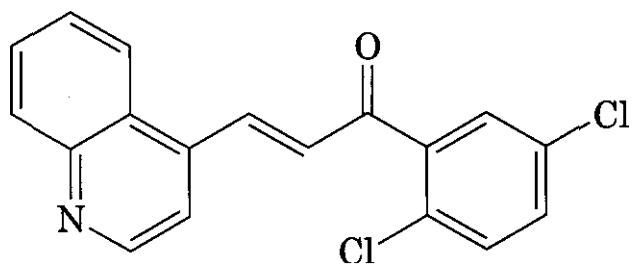


(117)



(118)
X = Cl, F

structures (**118**) were shown to inhibit parasite development, but the effect required relatively high concentration. Some chalcones (119) have been synthesized that also act on

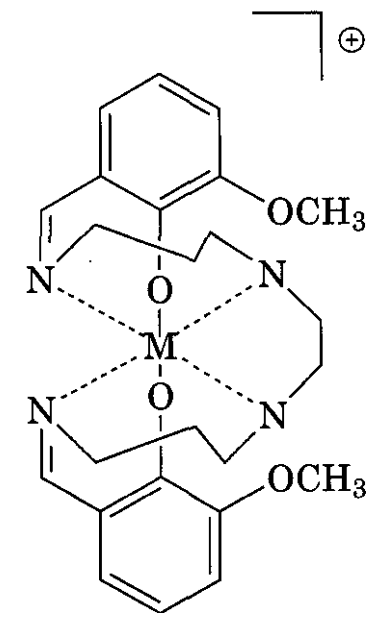


(119)

falcipain (1086–1088). The compound has an IC_{50} of 200 n*M* against either chloroquine-sensitive or chloroquine-resistant strains of P. ***falciparum.*** *A* combination of agents that inhibit both falcipain and plasmepsin were shown to behave synergistically (1089).

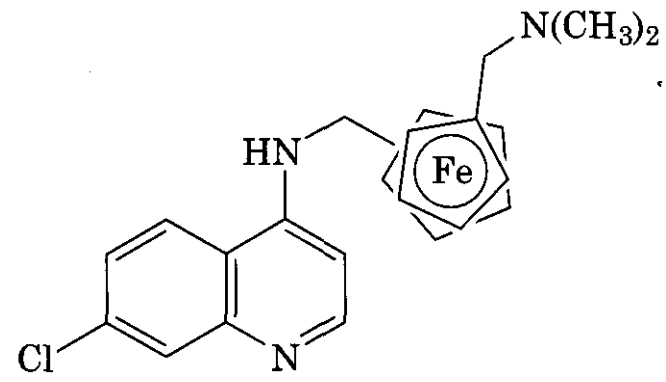
The hemolytic peptide dermaseptin S4 was shown to exert antimalarial activity through lysis of infected cells (1090). Further inquiry into the basis for this action may lead to the design of a new class of antimalarial agents.

A new class of antimalarial agents, metal complexes such as (**120**), are active in the low



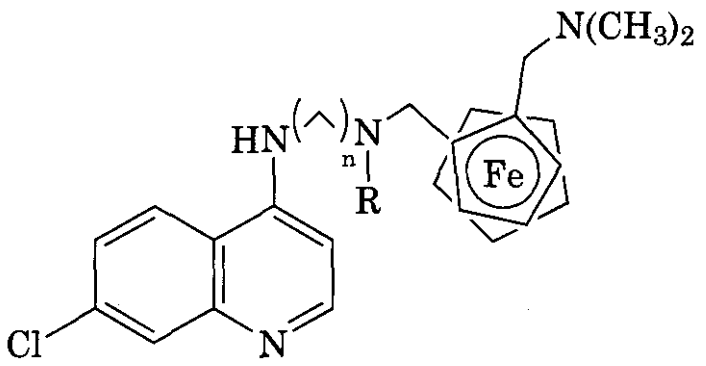
(120)
M = Fe(III), Ga(III)

micromolar range (1091, 1092). Both the Fe(III) and Ga(III) complexes seem to inhibit hemozoin aggregation. It has been suggested that the inhibition occurs through formation of a salt complex between the propionate of the heme and the cationic complex (1093). Compounds linking a 4-aminoquinoline to a ferrocenyl unit such as (121) were active



(121)
ferrochloroquine

against a chloroquine-resistant strain of P. ***falciparum in uitro*** and ***in vivo*** against ***P. berghei*** and ***P. yoelii*** (257). Using the metabolites of chloroquine as the model, possible metabolites of ferrochloroquine were synthesized and found to have activity ***in uitro*** (1094). Amine analogs of ferrochloroquine (**122**) were more active than chloroquine against both sensitive and resistant strains (1095). Ferrocene-chloroquine analogs were active in clinical isolates, 95% of which were resistant strains (1096). IC_{50} values were in the low



(122)

nanomolar range. The use of metal complexes in malaria therapy has been reviewed (1097).

3.2 New Targets for Antimalarial Chemotherapy

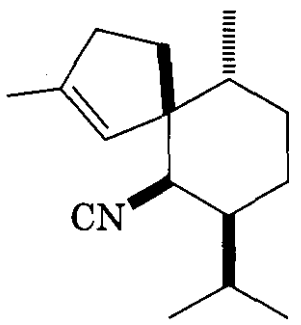
Many initial efforts towards new and experimental targets have been presented above. A number of writers have compiled lists of plasmodial targets with potential in antimalarial chemotherapy (1098–1100). Of particular interest is the inhibition of the proteases that specifically degrade hemoglobin in the parasite food vacuole, the plasmepsins, falcipain, and perhaps falcilysin (1101). Also very promising are targets associated with pathways specific to the apicoplast, such as the shikimate pathway and the 2-deoxy-D-xylulose-5-phosphate (DOXP) pathway (50,1102). Cellular and biochemical targets in the mosquito vector are worthy of consideration as well (1103). One group of workers has demonstrated the viability of using a molecular connectivity QSAR approach to identifying new active antimalarial agents (1104).

3.3 Antimalarial Natural Products

Given the increasing problem of drug resistance, researchers are turning with renewed interest to natural sources for the discovery of novel structural types for antimalarial investigations. Many natural products have been shown to have antimalarial activity in a variety of screens. Most, however, are only mildly active or have toxicity that precludes any serious interest in pursuing the new structures as lead compounds for drug studies. A very few examples are provided below of compounds providing interesting possibilities or structures for continuing research.

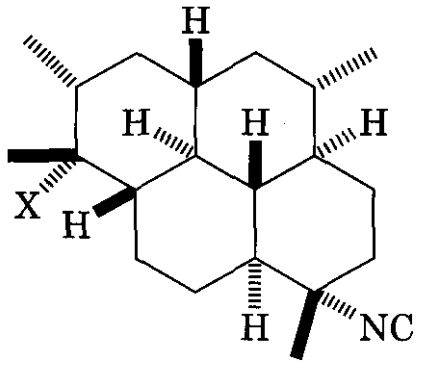
Reviews in the area of antimalarial natural products include a review of traditional medicinal plants as sources of antimalarial agents (1105), a review of plants more generally as sources of antimalarial. compounds (1106, 1107), and the selective screening and testing of natural products for antimalarial activity (1108). A number of marine organisms have been screened for natural products with selective antimalarial activity, and the structures of active components have been reported (1109, 1110).

The initial report of isonitrile compounds, isolated from marine organisms, that had significant antimalarial activity was that of axisonitrile-3 (123) from the sponge Acanthella



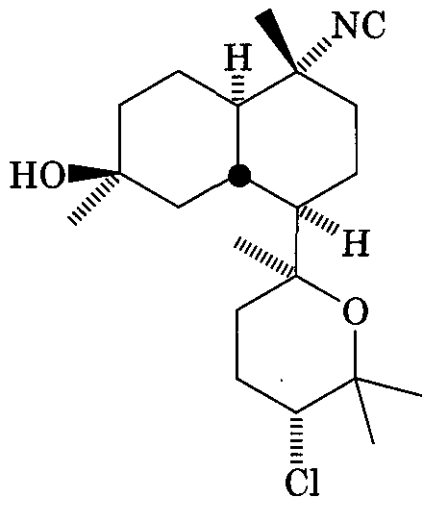
(123)
axisonitrile

klethra (1111). Further examination of marine sources led to the isolation of a series of diterpene isonitriles and isocyanates from the sponge Cymbastela hopperi (1112, 1113). Two of the compounds, (124), have IC_{50} values (ng/



(124)
X = NC, NCO

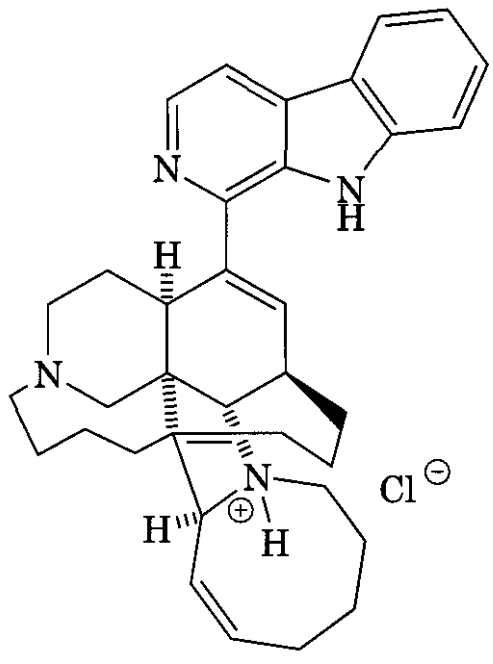
mL) in the 2.5–4.7 range for both chloroquine-sensitive and chloroquine-resistant clones of P. falciparum. A marine sponge Acanthella sp. provided kalihinol A (125) (1114). As with the other isonitrile natural products, this one has activity in the nanomolar range in uitro and



(125)
kalihinol **A**

was quite selective. Molecular modeling studies employing 3D-QSAR were able to derive a pharmacophore schema consistent with the biological activity (1115). The isonitrile compounds seem to act on heme detoxification processes.

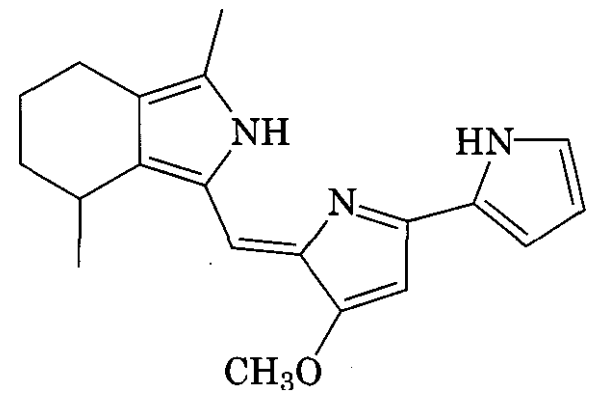
Manzamine A (**126**), an unusual β-carboline isolated from a number of marine



(126)
manzamine A

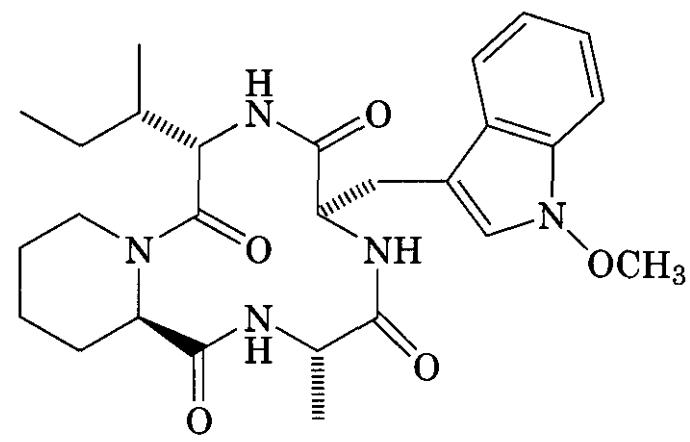
sponges, was an active antimalarial ***in vitro*** and ***in vivo*** (1116). Despite a narrow therapeutic index, a recent total synthesis should enable SAR studies to be carried out (1117).

A marine bacterium, ***Pseudoalteromonas denitrificans,*** was the source of cycloprodigiosin (127) (1118). The ***in vitro*** activity of 11 n*M* was particularly promising given the low toxicity. Cycloprodigiosin has been studied for a wide range of biological activities beyond antiprotozoal effects.



(127)
cycloprodigiosin

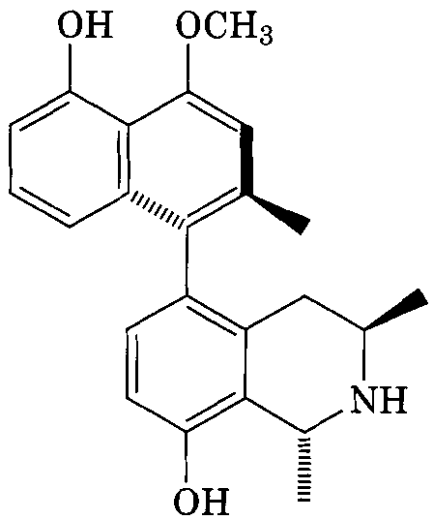
Apicidin (128) is a tetrapeptide produced by ***Fusarium pallidoroseum.*** The compound is



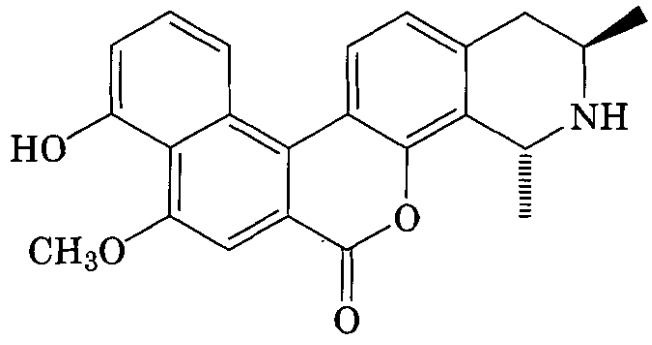
(128)
apicidin

orally active against ***P. berghei*** in mice at less than 10 mg/kg and seems to act by inhibition of the apicomplexan histone deacetylase (1119).

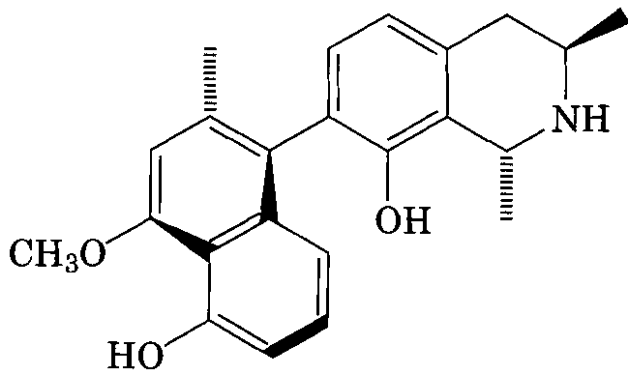
The naphthylisoquinoline alkaloids of ***Triphyophyllum peltatum,*** the most widespread species of Dioncophyllaceae, have been reviewed (1120). This group includes dioncophylline C (**129**), dioncophylline B (**130**), dioncopeltine A (**131**), dioncolactone A (**132**), and 5′-*O*-demethyldioncophylline A (**133**) (1121). A related compound is ancistrocladine (134) from ***Ancistrocladus abbreviatus.*** All of these display good activity ***in vitro,*** and some SAR studies of dioncophylline have been carried out (1122). Dioncophylline C and dioncopeltine A were also effective as oral agents against chloroquine-resistant ***P. berghei*** in mice (1123). Dioncophyllines A and C and an-



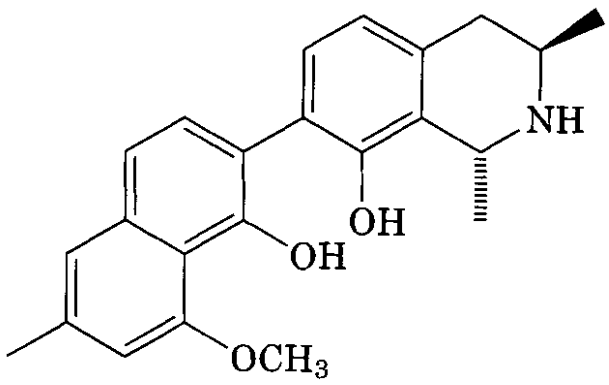
(129)
dionophylline C



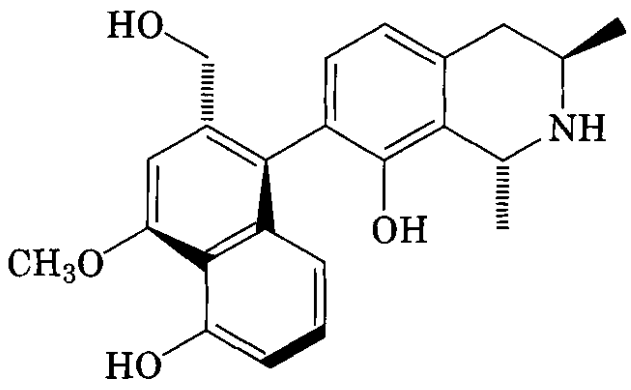
(132)
dioncolactone **A**



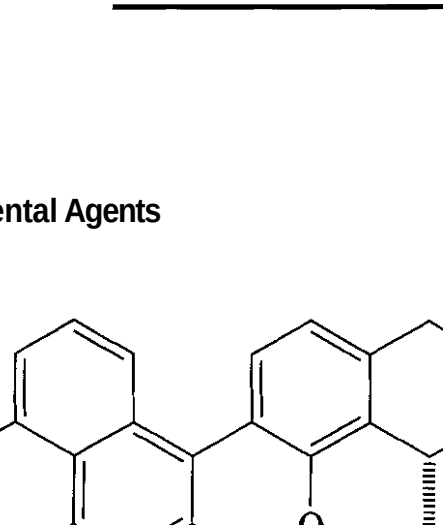
(133)
5'-O-demethyldionophylline A



(130)
dioncophylline **B**



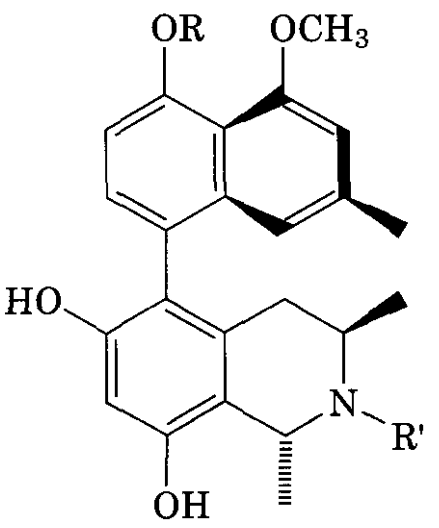
(131)
dioncopeltine **A**



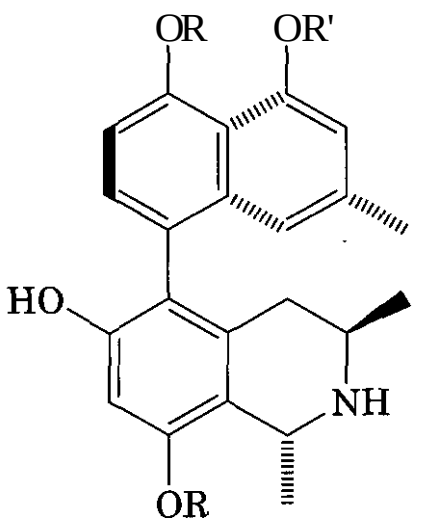
(134)
ancistrocladine

cistrocladine were shown to be active not only against erythrocytic forms of the parasite but also against exoerythrocytic forms **(1124).** ***Ancistrocladus korupensis,*** a tropical liana, has been another rich source of naphthylquinoline alkaloids. Of particular interest are the korupensamines A-E (**135–139**) **(1125,1126).** The compounds, being very similar in structure to the alkaloids from T. ***peltatum,*** are active both ***in vitro*** and ***in vivo*** **(1127).** The naphthylisoquinoline alkaloids have inspired a number of total syntheses directed at T. ***peltatum*** compounds **(1128–1130)** and those from ***A. korupensis*** **(1131–1133).** ***A. korupensis*** has provided, in addition to the agents mentioned above, a heterodimeric naphthylisoquinoline, korundamine **A (140) (1134).** This is the most potent ***in vitro*** of the members of this class yet identified.

Bisbenzylisoquinolines make up a large group of natural products, several of which



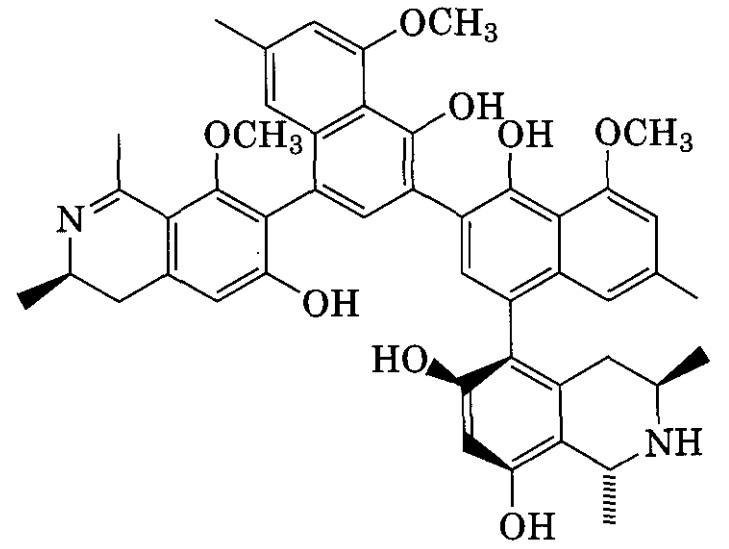
(135)
korupensamine A, R = R′ = H
(137)
korupensamine C, R = CH_3, R′ = H
(138)
korupensamine D, R = H, R' = CH_3



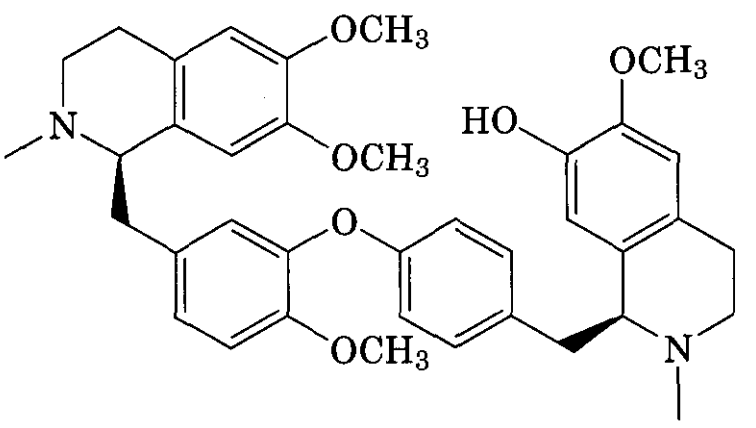
(136)
korupensamine **B,** R = H, R' = CH_3
(139)
korupensamine E, R = CH_3, R′ = H

have been shown to possess antimalarial activity ***(1135).An*** evaluation of over ***50*** structures led to the identification of six compounds that not only had appreciable activity against chloroquine-sensitive and resistant strains of P. ***falciparum,*** but were also somewhat selective.

The roots of ***Dichroa febrifuga*** have been used for centuries in China to treat malarial fevers. Febrifugine ***(147)*** and isofebrifugine ***(148)*** were isolated from D. *febrifuga* (1136–***1138)*** and have attracted considerable attention as antimalarial agents. In studies in mice, febrifugine significantly reduced mortality. The drug seems to potentiate the production of nitric oxide in acute immune responses



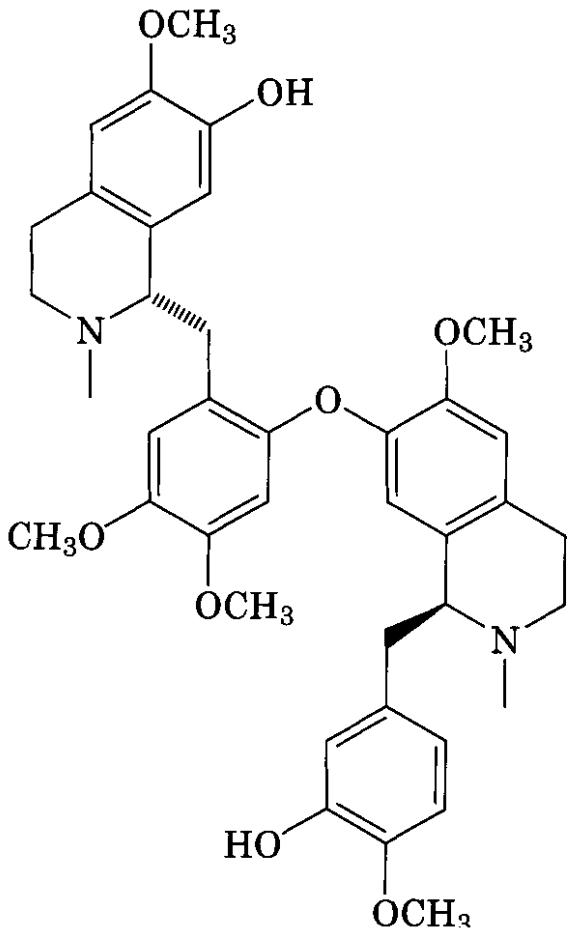
(140)
korundamine **A**



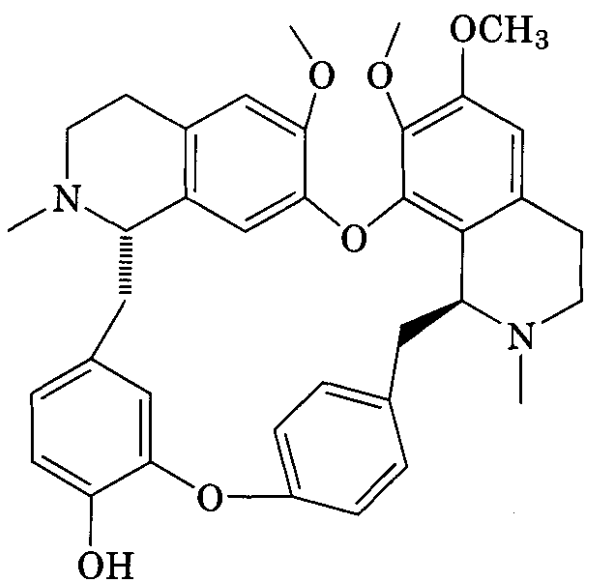
(141)
(+)-temuconine

(1139).Both compounds have been prepared by asymmetric synthesis ***(1140).***

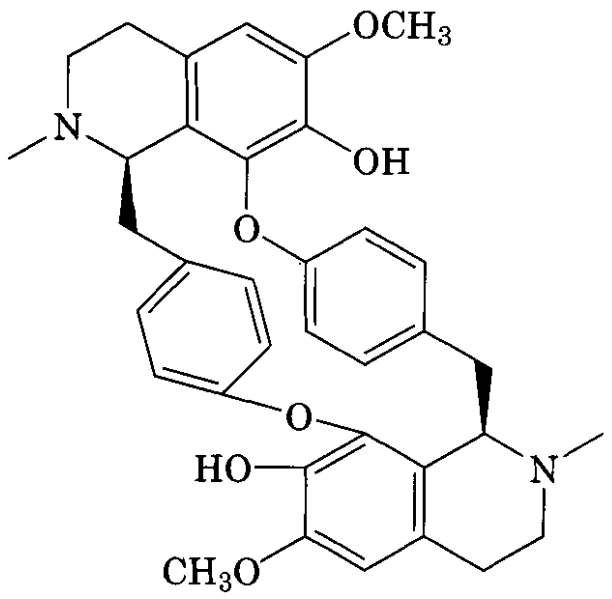
Folk medicine has often offered leads in locating biologically active compounds. Neem (*Azadirachta* ***indica)*** is a popular source of traditional remedies in Africa. Gedunin ***(149)*** is the main antimalarial component with good activity against both chloroquine sensitive and resistant strains of P. ***falciparum (1141, 1142).*** The lack of ***in vivo*** activity against ***P. berghei*** was surprising, however, given the widespread use of ***A. indica*** in herbal preparations. Other limonids from neem were isolated and found to have some antimalarial activity ***(1143).*** The stem bark of ***Tabebuia ochracea*** spp. ***neochrysantha*** has been used by Indians of the Colombian Amazon as an antimalarial. Extracts provided an inseparable mixture of furanonaphthoquinones ***(150) (1144).*** The mixture demonstrated significant activity ***in vitro*** against ***P. falciparum. Vismia guineësis***



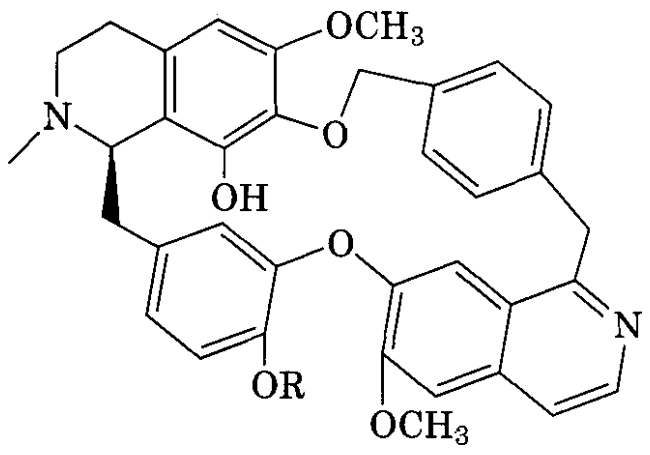
(142)
(+)-malekulatine



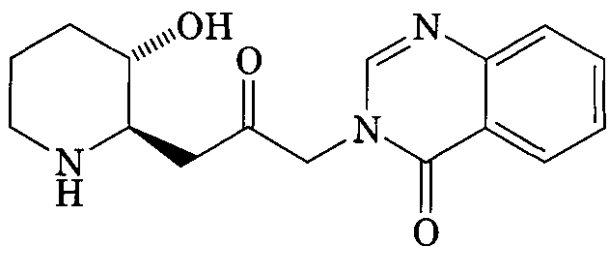
(143)
(-)-repandine



(144)
(-)-cycleanine

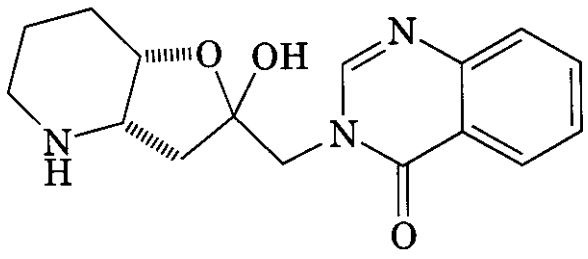


(145)
R = CH_3, (+)-cyclatjehenine
(146)
R = H, (+)-cycleatjehine

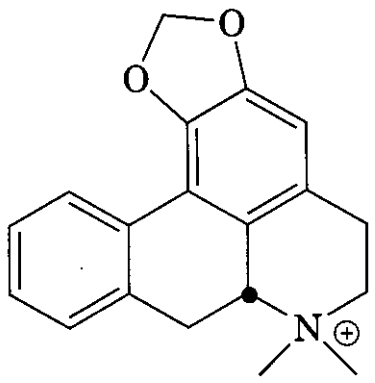


(147)
febrifugine

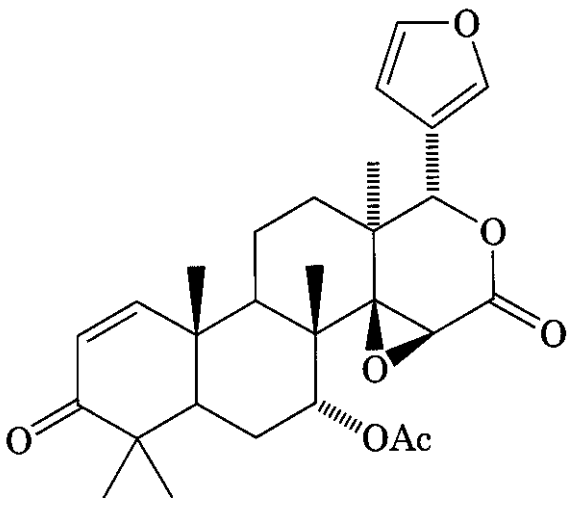
has been used to treat malaria in West Africa; it is the source of **vismione** H (**151**) (1145). This prenylated preanthraquinone was the active component, and several analogs were studied and found to be active as well. An aporphine, (−)-**roemrefidine** (**152**), was isolated from *Sparattanthelium amazonum*, a vine of the subtropical rain forests of South American, where it is used by the native **com**-munity (1146). *In vivo* against *P. berghei* in mice, the compound was not only effective but displayed little toxicity. Tubulosine (**153**) from *Pogonopus tubulosus*, another source of traditional medicines from the South American subtropical rain forest, was more active than chloroquine in sensitive strains of *P. falciparum* and significantly more active in **resis**-



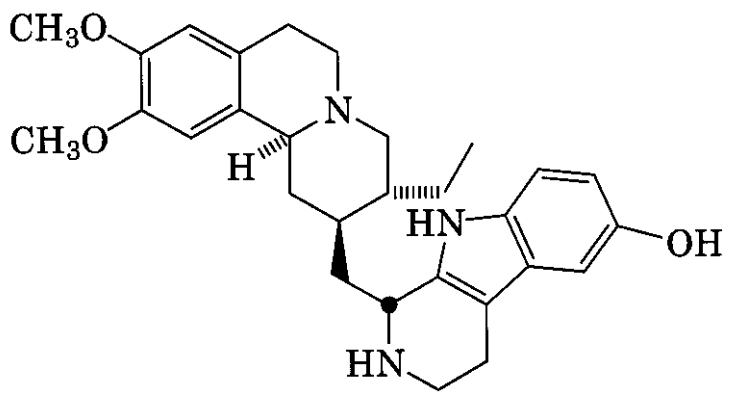
(148)
isofebrifugine



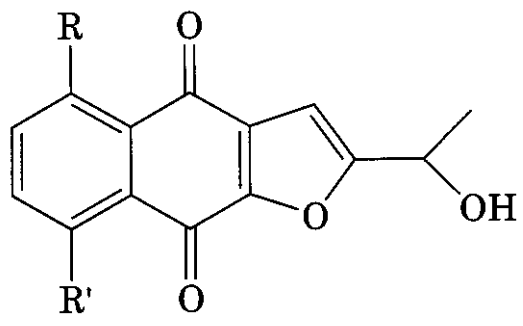
(152)
(–)-roemrefidine



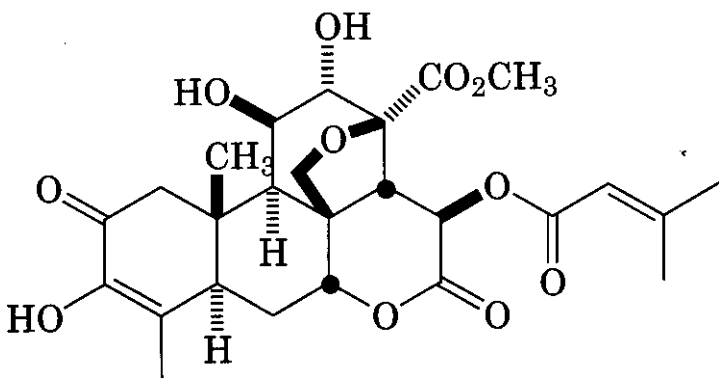
(149)
gedunin



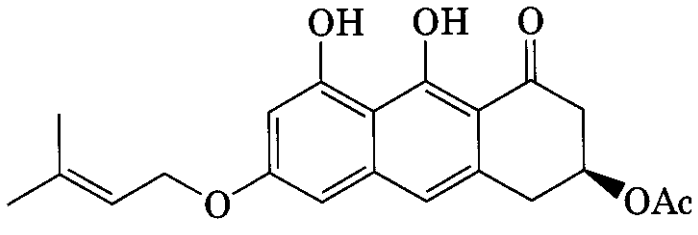
(153)
tubulosine



(150)
R = H, R' = OH
R = OH, R' = H

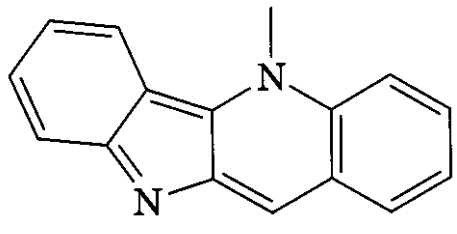


(154)
brusatol



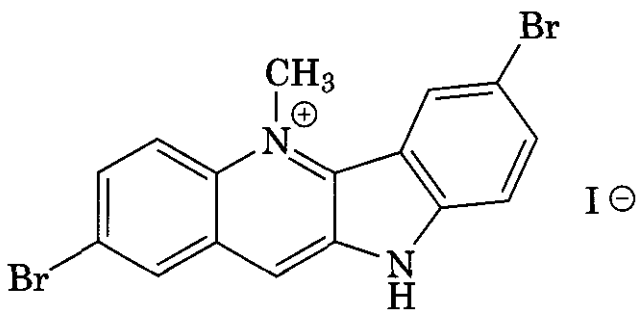
(151)
vismione H

tant ones in vitro (1147). **Brusatol (154)**, from Brucea javanica, a plant used in Chinese herbal remedies for malaria, was shown to be active against a chloroquine-resistant strain of P. falciparum (1148). Investigation of acetylated derivatives led to the discovery of compounds active in *vivo* with selective toxicities (1149, 1150). Cryptolepine (**155**) was isolated from Cryptolepis sanguinolenta, an African climbing liana (1151). Analogs of the natural

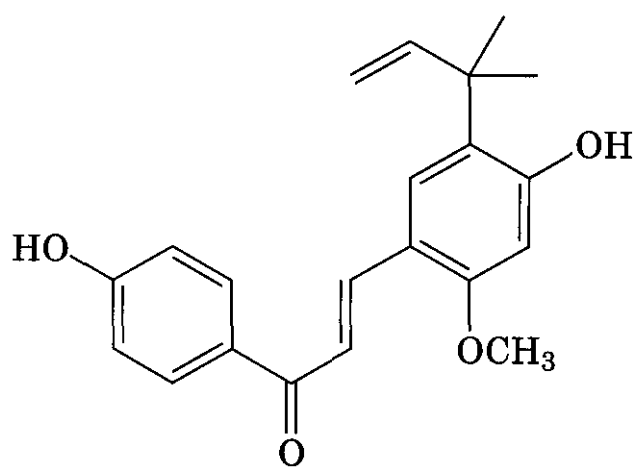


(155)
cryptolepine

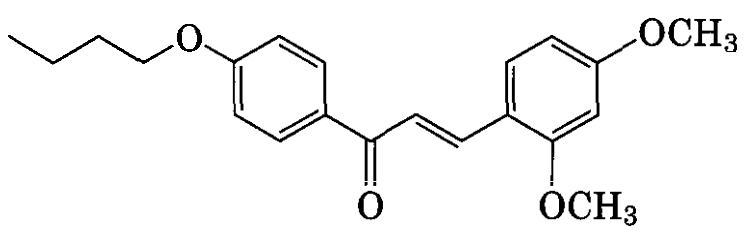
product were shown to be effective against P. berghei in infected mice (1152). The synthetic compounds (**156**) were active in both chloroquine-sensitive and chloroquine-resistant strains and showed no evidence of cross-resistance with chloroquine. Licochalcone A (157) was isolated from Chinese licorice root, a traditional treatment for a number of disorders. It acts against chloroquine-sensitive and -resistant strains of P. falciparum (1153) and in *vivo* against P. yoelii in infected mice (1154). An analog of licochalcone A, 2,4-dimethoxy-4'-butoxychalcone (**158**), was active in the micromolar range in rodent models, was orally active, and was much less toxic than the natural product (1155). An aminosteroid, sarachine (**159**), was isolated from Saracha punctata, a Bolivian shrub (1156). The compound had good activity both in vitro and in *vivo* (*P.* vinckei), although there was some cytotoxicity.



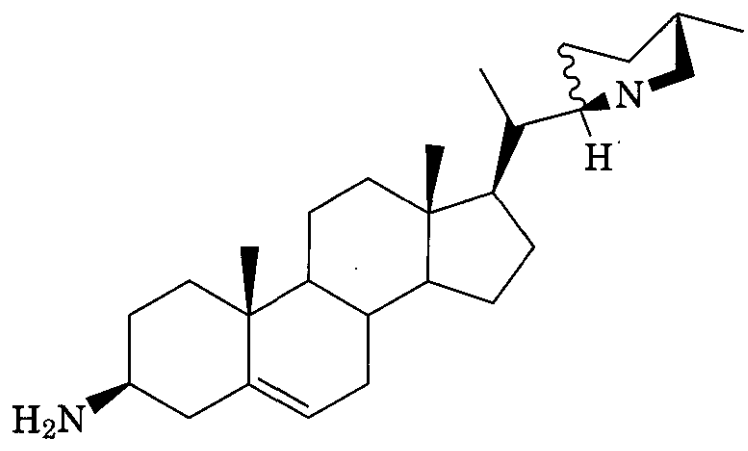
(156)



(157)
licochalcone



(158)



(159)
sarachine

4 RESOURCES

General information about malaria can be found on the World Wide Web at sites maintained by the United States Centers for Disease Control (1157) and by the World Health Organization (1158).

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CHAPTER NINETEEN

Antiprotozoal Agents

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1 INTRODUCTION

The topics covered in this chapter are the kinetoplastid protozoan infections, the African and American trypanosomiases, and the leishmaniases. Malaria, the remaining major parasitic protozoan infection, is covered in chapter 18 of this volume. These diseases share the common characteristic of predominately affecting people living in tropical regions of the world. The countries where the diseases are endemic are, for the most part, developing or undeveloped, and they lack the economic resources to combat the diseases successfully. Companies are unwilling to target these diseases for drug development because of the lack of economic incentive. Of the 1393 new drugs introduced between 1975 and 1999, only 16 were indicated for the treatment of tropical diseases that the World Health Organization, Tropical Drug Research (TDR) division, has placed on its list of targeted diseases (Table 19.1) (1, 2). Yet, this group of diseases accounts for an estimated 772 million cases and 2.9 million deaths per year. The statistics accentuate the need for the development of new drugs for these diseases.

Most of the drugs used to treat the diseases reviewed in this chapter were developed more than 50 years ago and their origins can be traced back to the pioneering research of Paul Ehrlich and others on dyes (e.g., suramin), organic arsenicals (e.g., melarsoprol), and antimonials (e.g., Pentostam). Resistance has developed, to varying degrees, to most of the drugs. Many of the existing agents are either too expensive to manufacture, too toxic, or too difficult to administer for use in broad-scale disease treatment or prevention programs in Third World countries.

Because of similarities in kinetoplastid biochemistry and molecular biology, some drug families display selective toxicity across all of the organisms. Conversely, species that are very closely related [e.g., *Trypanosoma* brucei gambiense (T. b. gambiense) and T. brucei *rho-*

Table 19.1 WHO Tropical Disease Research (TDR) List of Target Diseases

Disease	Infective Organism	Disease Burden[a] (DALYs)	Cases[b] (worldwide)	Deaths/Year[b] (worldwide)
African sleeping sickness	***Trypanosoma brucei***	1.8 million	300,000–500,000	41,000
Chagas' disease	***Trypanosoma cruzi***	680,000	16–18 million	21,000
Dengue fever	***Flaviviridae*** viruses	433,000	50 million	12,000
Leishmaniasis	***Leishmania donovani***	1.7 million	500,000	60,000
Leprosy	***Mycobacterium leprae***	141,000	740,000	2,000
Lymphatic filariasis	***Wuchereria bancrofti*** ***Brugia timori,*** and others	5.5 million	120 million	0
Malaria	***Plasmodium*** spp.	40.2 million	300 million	1,080,000
Onchocerciasis	***Onchocerca volvulus***	951,000	20 million	0
Schistosomiasis	***Schistosoma mansoni,*** and additional ***S.*** spp.	1.6 million	200 million	50,000
Tuberculosis	***Mycobacterium tuberculosis***	35.8 million	62 million	1,660,000

[a]DALYs, disability adjusted life years = the number of healthy years of life lost because of premature death and disability.
[b]Estimates extracted from WHO Fact Sheets.

desiense (*T.* b. rhodesiense)] may not always respond to the same drug treatments. Nonetheless, the parasites have a number of biochemical pathways that differ significantly from their mammalian hosts and therefore offer opportunities for drug development. However, the potential for the development of selective new drugs has not been aggressively pursued and provides the medicinal chemist with numerous and often validated targets for the design and synthesis of new drug candidates.

2 KINETOPLASTID PROTOZOAN INFECTIONS

Trypanosomes and leishmania parasites are single-cell eukaryotes belonging to the order Kinetoplastida **(3,** 4). This order takes its name from the kinetoplast, a round or oval body situated near the base of the flagellum. **As** the name suggests, its original function was ascribed to motility of the flagellum. Subsequently, the kinetoplast was identified as a specializedregion of the single mitochondrion, constituting a mass of catenated small (minicircles) and large (maxicircles) circular DNA molecules that form the mitochondrial genome of these parasites **(5).** Unlike other organisms, maxicircle RNA transcripts undergo a remarkable process of RNA editing in which small "guide" RNAs direct the insertion and deletion of uridine residues to produce functional mRNA (6, 7). The genera *Trypanosoma* and Leishmania belong to the suborder Trypanosomatina, family Trypanosomatidae, and are consequently frequently referred to as "trypanosomatids."

2.1 Human African Trypanosomiasis (African Sleeping Sickness)

Human African trypanosomiasis (HAT) is also known as sleeping sickness. In cattle, the disease is known as nagana. The parasite is transmitted between vertebrate hosts by the tsetse fly of the genus Glossina. The infection can also be spread transplacentally and by accidental contact with the blood of an infected person or animal. There are three subspecies of *Trypanosoma* brucei, two of which cause disease in humans. T. b. gambiense, found in West and Central Africa, causes Western African sleeping sickness. T. b. rhodesiense occurs in Eastern and Southern Africa, where it causes Eastern African sleeping sickness. The third subspecies, *Trypanosoma* brucei brucei, which is morphologically and biochemically indistinguishable from T. b. rhodesiense, does not infect humans because of a lytic factor in the high density lipoprotein fraction of human serum (8, 9). Along with the two other major species T. congolense and T. *vivax*, T. b. brucei causes nagana in cattle, sheep, and goats. To-

gether, these species constitute the main obstacle to the cattle industry in Africa (6, 10).

T. ***b. rhodesiense*** is more virulent, but much less common in occurrence than is T. ***b. gambiense. T. b. rhodesiense*** causes acute infection that emerges within a few weeks of the fly's bite. As such, it is much more easy to detect than T. ***b. gambiense,*** which may not show symptoms for months or years. Once T. ***b. gambiense*** does emerge, it is already in an advanced stage and difficult to treat. HAT threatens over 60 million people in 36 countries of sub-Saharan Africa. Almost 45,000 cases of HAT were reported in 1999 and the World Health Organization (WHO) estimates that the actual number of cases is between 300,000 and 500,000 (11).T. ***b. gambiense*** is epidemic (20–50% infection rate in some villages) in Angola, the Democratic Republic of Congo, and southern Sudan and it is highly endemic in Cameroon, Central African Republic, Chad, Congo, Côte d'Ivoire, Guinea, Mozambique, Uganda, and the United Republic of Tanzania.

When an infected (female) tsetse fly bites an uninfected human to obtain a blood meal, trypanosomes are injected with the salivary secretions. The metacyclic trypanosomes multiply at the site of the bite, which can develop into a painful indurated swelling (trypanosomal chancre). The slender trypomastigote forms then migrate through the lymphatic system to reach the bloodstream. Here they multiply by asexual binary fission once every 5 to 10 h (12) and spread to the intercellular spaces of other tissues. In contrast to American trypanosomiasis and leishmaniasis, there are no intracellular stages. In the advanced stage of infection, some trypanosomes pass through the choroid plexus to invade the central nervous system (CNS), causing lesions that lead to the classical symptoms of sleeping sickness. In a chronic relapsing infection, the bloodstream and tissue forms display considerable variation in length and shape (pleomorphism), ranging from "long-slender," through "intermediate" to "short-stumpy." The latter forms are nondividing and are thought to be preadapted for survival when taken up into the tsetse fly midgut, where they transform into procyclic forms to perpetuate the life cycle. The parasites are remarkably well adapted to the major circulating energy source of their hosts (glucose in mammalian blood and proline in the tsetse hemolymph). In the mammalian stages, they completely lack cytochromes and a citric acid cycle and use glucose as the sole source of energy, whereas in the insect midgut they switch on a functional cytochrome-dependent electron transport system and citric acid cycle to metabolize proline and other citric acid cycle intermediates (13, 14).

The development of host immunity is compromised by the ability of trypanosomes to vary their outer glycoprotein coat (15). Each metacyclic and bloodstream form trypanosome is coated with a monomolecular layer of glycoprotein, known as the variant surface glycoprotein (VSG). Trypanosomes possess a virtually limitless repertoire of immunological distinct forms of VSG. By periodic switching between different antigenic types, the parasite effectively evades the host's antibody response. The ability of the trypanosomes to undergo antigenic variation keeps them one step ahead of the host's immune defense system and renders development of a vaccine unlikely. Thus, chemotherapy remains the best available treatment for HAT.

HAT has a high chance of cure, if it is diagnosed early (16). Early phase symptoms are fever, headaches, joint pains, and pruritus. The second phase is the neurological phase that occurs after the parasite has invaded the CNS. The signs and symptoms of the neurological phase are confusion, sensory disturbances, loss of coordination, disturbance of the sleep cycle (from which the name of the disease originates), coma, and death. Neurological damage can be irreversible, even if late-stage treatment is successful in clearing the parasites from the patients. Left untreated, HAT is always fatal.

Drugs used to treat HAT are listed in Table 19.2 and structures are given in Fig. 19.1. Suramin (1) and pentamidine (2) are only useful for early-phase disease, whereas melarsoprol (3) and eflornithine (4) are effective against both phases. The drugs are difficult to administer and all must be given by injection over a relatively long period. In many parts of Africa, medical facilities and staff for long-term treatment and follow-up of patients do not exist. The success rate of the drugs in

Table 19.2 Drugs Used to Treat Human African Trypanosomiasis

Drug	Use	Adult Dose	Pediatric Dose
Suramin sodium	Initial phase, *T. b. rhodesiense*	100–200 mg (test dose) i.v.; then up to 1 g i.v. on days 1, 3, 7, 14, 21	20 mg/kg, days 1, 3, 7, 14, 21
Pentamidine	Initial phase, *T. b. gambiense*	4 mg kg^{-1} day^{-1}; i.m.; 10 days	Same as for adults
Melarsoprol	Advanced phase, both subspecies	2.0–3.6 mg kg^{-1} day^{-1}; i.v.; 3 days–week 1	18–25 mg/kg total in 1 month
		3.6 mg kg^{-1} day^{-1}; i.v.; 3 day,–week 2	0.36 mg/kg; i.v.; every 1–5 days; total 9–10 doses
		Repeat after 10–21 days	
Eflornithine	Advanced phase, *T. b. gambiense*	400 mg kg^{-1} day^{-1}, i.v.; in four doses × 14 days	

treating patients with neurological phase HAT is marginal, under the best circumstances. Side effects of the drugs are severe. Supplies of the existing drugs cannot always be guaranteed, and drug companies periodically abandon production because of lack of profitability and environmental issues.

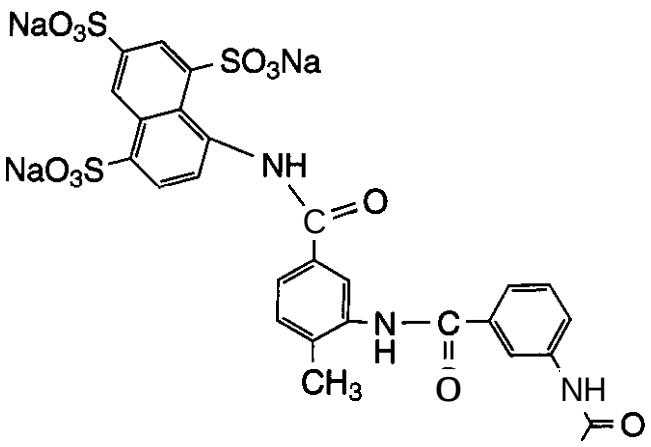
Homidium (ethidium bromide; **5**), diminazene (**6**), and isometamidium (**7**) are available to treat T. brucei infections in animals (17) (Fig. 19.1B). These same drugs are used to treat Trypanosoma evansi in camels and the Trypanosoma equiperdum infection in horses (17). In addition, quinapyramine (8) and melarsenoxide cysteamine (9; Mel Cy, Cymelarsan) are available for treatment of T. evansi infections (18).

2.1.1 Mechanisms of Action of Drugs Used to Treat HAT

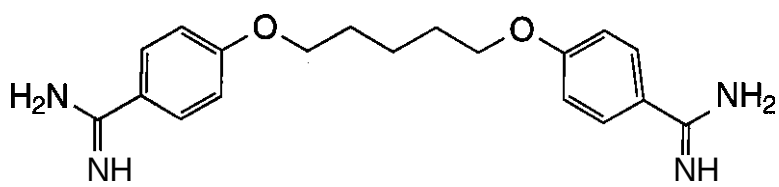
2.1.1.1 Suramin Sodium. Suramin sodium ***(1)*** is also known as Bayer 205 and Germanin. It is a sulfonated naphthylamine polyanionic dyestuff (MW 1429), chemically related to Trypan Red and Trypan Blue, which, as their names suggest, also possess antitrypanosomal activity. Suramin was introduced in the early 1920s and it remains the drug of choice for treating the early stages of T. b. rhodesiense infections. The drug is highly water soluble and must be given by intravenous injection. Suramin is unstable in solution exposed to air and must be dissolved for injection at the time of use. The ineffectiveness of suramin against the neurological stages of the disease is predictable because the highly polar drug does not cross the blood-brain barrier to any significant extent. The selective toxicity of suramin is explained by the ability of trypanosomes to accumulate the drug. Structure modifications of suramin that result in decreased uptake generally cause loss of antitrypanosomal activity. Fairlamb and Bowman (19, 20) showed that in the presence of serum proteins, trypanosomes take up suramin by a receptor-mediated endocytosis at a rate that is 18-fold higher than could be explained by fluid endocytosis alone. There is evidence that low density lipoproteins are the most important binding protein for suramin endocytosis (21). However, a recent study suggests that uptake of suramin is not mediated through an LDL receptor, but by another as yet unidentified receptor mechanism (22). Fairlamb and Bowman (23) found that in the suramin-treated infected rats, where the plasma suramin reaches a level of 100 μM, the suramin taken up by T. brucei amounts to about 0.5 nmol/mg protein. Assuming a cellular protein concentration of about 200 mg/mL in T. brucei (24), the average intracellular suramin concentration is calculated to be about 100 μM, which is equivalent to the exogenous concentration. If suramin were to be retained within the endocytic compartments, then the local concentration would be much higher.

Once suramin is "inside" trypanosomes (it is debatable whether such a highly charged polysulfonated molecule could exit from the endocytic system and enter the cytosol), the mechanism of its trypanocidal action remains uncertain. Suramin has an inhibitory activity against a number of trypanosomal enzymes and multiple mechanisms are probably in-

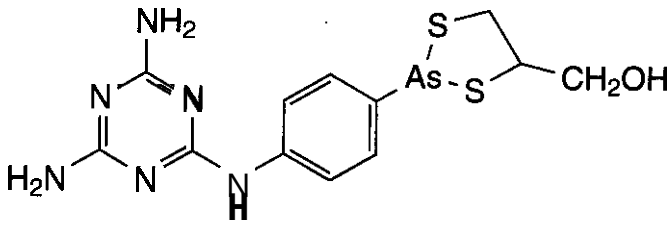
Part A.



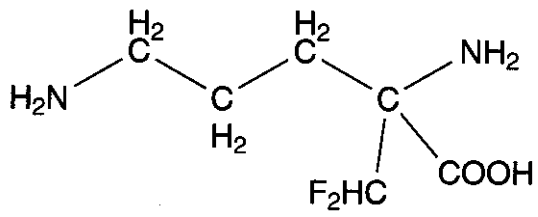
Suramin Sodium **(1)**



Pentamidine **(2)**

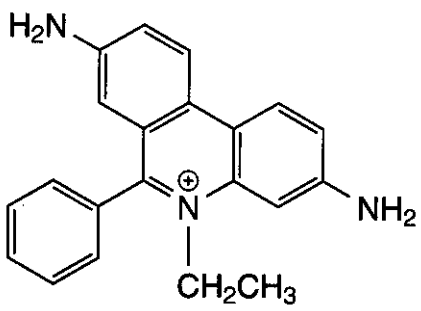


Melarsoprol (**3**)

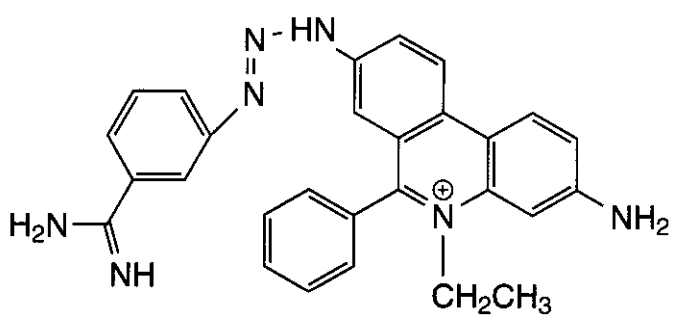


Eflornithine **(4)**

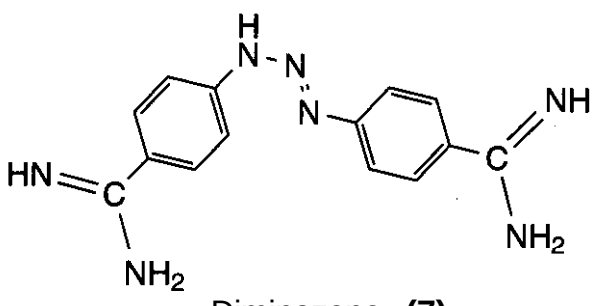
Part **B.**



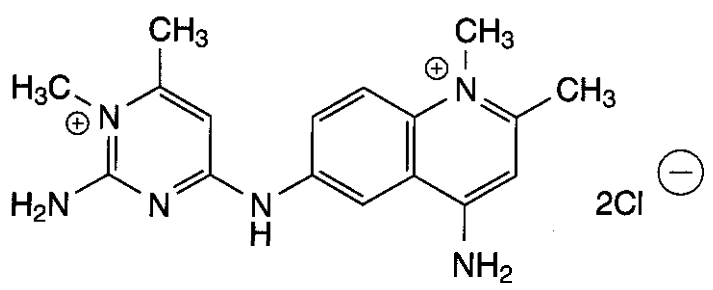
Homidium **(5)**



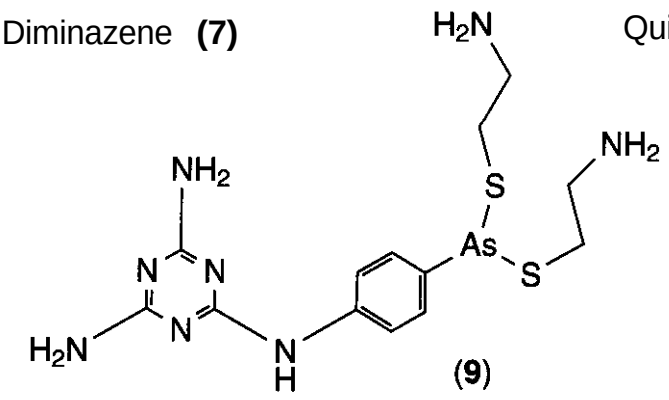
Isometamidium **(6)**



Diminazene **(7)**



Quinapyramine **(8)**



(**9**)

Melarsenoxide cystemine,
Cymelarsen

Figure 19.1. Agents used to treat African trypanosomiasis. **(Part** A) Agents for **human** disease. **(Part** B) Agents for livestock.

volved in its therapeutic effect. In T. ***brucei,*** suramin has been found to inhibit dihydrofolate reductase (25) and thymidine kinase (24). Morty et al. (26) implicated the inhibition of trypanosomal cytosolic serine oligopeptidase in the activity of suramin. It is also a potent inhibitor of the glycolytic enzymes in T. ***brucei*** (27). However, the glycolytic enzymes are contained within a membrane-bound organelle called the glycosome and it is unlikely that the highly polar suramin can penetrate the organelle's membrane to reach these enzymes (28). Inhibition of the glycolytic enzymes would result in a rapid death and lysis of the trypanosomes because they are totally dependent on glycolysis for energy production; however, it is found that trypanosomes exposed to suramin die slowly over a period of several days (20, 29). The pronounced synergism between difluoromethylornithine (eflornithine) and suramin might implicate polyamine metabolism in its mode of action (30, 31). However, no satisfactory biochemical explanation has been advanced to explain this effect. The fact that no significant resistance to suramin has been reported after 80 years of use is consistent with the drug having multiple sites and mechanisms of action.

Suramin has been found to be a potent inhibitor of HIV reverse transcriptase ***in vitro***, but it lacks antiviral activity ***in vivo***. It has also shown antiproliferative activity at high doses and has been tested against a number of metastatic tumors (32). Suramin's only other proven clinical use is as an antifilarial agent in treating the disease onchocerciasis. Its action against the adult worm is not understood.

Suramin is 99.7% protein bound after a 1 g intravenous dose. Tight protein binding is believed to be the cause of its long terminal half-life of 90 days (33). Suramin undergoes very little metabolism and about 80% of the drug is eliminated by slow renal clearance. Suramin's long half-life makes it an excellent prophylactic agent for trypanosomiasis. Major side effects frequently induced by suramin include vomiting, pruritus, urticaria, paresthesias, hyperesthesia of hands and feet, photophobia, and peripheral neuropathy. Occasional side effects are kidney damage, blood dyscrasias, shock, and optic atrophy.

2.1.1.2 ***Pentamidine.*** Pentamidine (2), an aromatic diamidine, was first synthesized and tested as a hypoglycemic agent in 1937. It was soon found to have antiprotozoal activity and was introduced to treat trypanosomiasis in 1941. Pentamidine is currently marketed as the di-isethionate (Pentam 300, Nebupent) and dimesylate (Lomidine) salts. It is effective against early-phase T. ***b. gambiense*** and is used as well against both antimony-resistant ***Leishmania donovani*** (34) and ***Pneumocystis carinii*** in patients intolerant to sulfamethoxazole-trimethoprim (35). Pentamidine as the di-isethionate salt is highly water soluble and relatively unstable in solution. It must be given by intravenous or intramuscular dosage and it should not be dissolved for injection until just before use. Diminazene (Berenil, 7) is a related and more toxic diamidine that is approved for veterinary use (36). Although diminazene has been used for the treatment of HAT, its safety and efficacy for human use has not been properly evaluated (36). The diamidines are nearly fully protonated at both amidine groups at physiological pH and are thus not well absorbed after oral dosing, nor do they readily cross the blood-brain barrier. Their extremely poor penetration into the CNS makes them useless against the neurological phase of trypanosomiasis.

The activity of pentamidine and other diamidines is dependent on active uptake by the parasites (37, 38). One route of entry for pentamidine has been identified to be the high affinity purine 2 (P2) transporter that carries adenine and adenosine into cells (38). Melarsoprol (3) and the investigational trypanocide megazol use this same transporter. de Koning and Jarvis (39) identified at least one additional transporter for pentamidine in T. ***b. brucei.*** Molecules imported by the P2 transporter have a common structural unit (Fig. 19.2) that is recognized by the transporter. Cross-resistance between these agents is known to occur through downregulation of the P2 transporter (40, 41). Some T. ***brucei*** strains resistant to pentarnidine have decreased ability to import diamidines (42), whereas others show no such defect (43), indicating that multiple resistance mechanisms may be involved.

The mechanism by which pentamidine causes a trypanocidal action remains undeter-

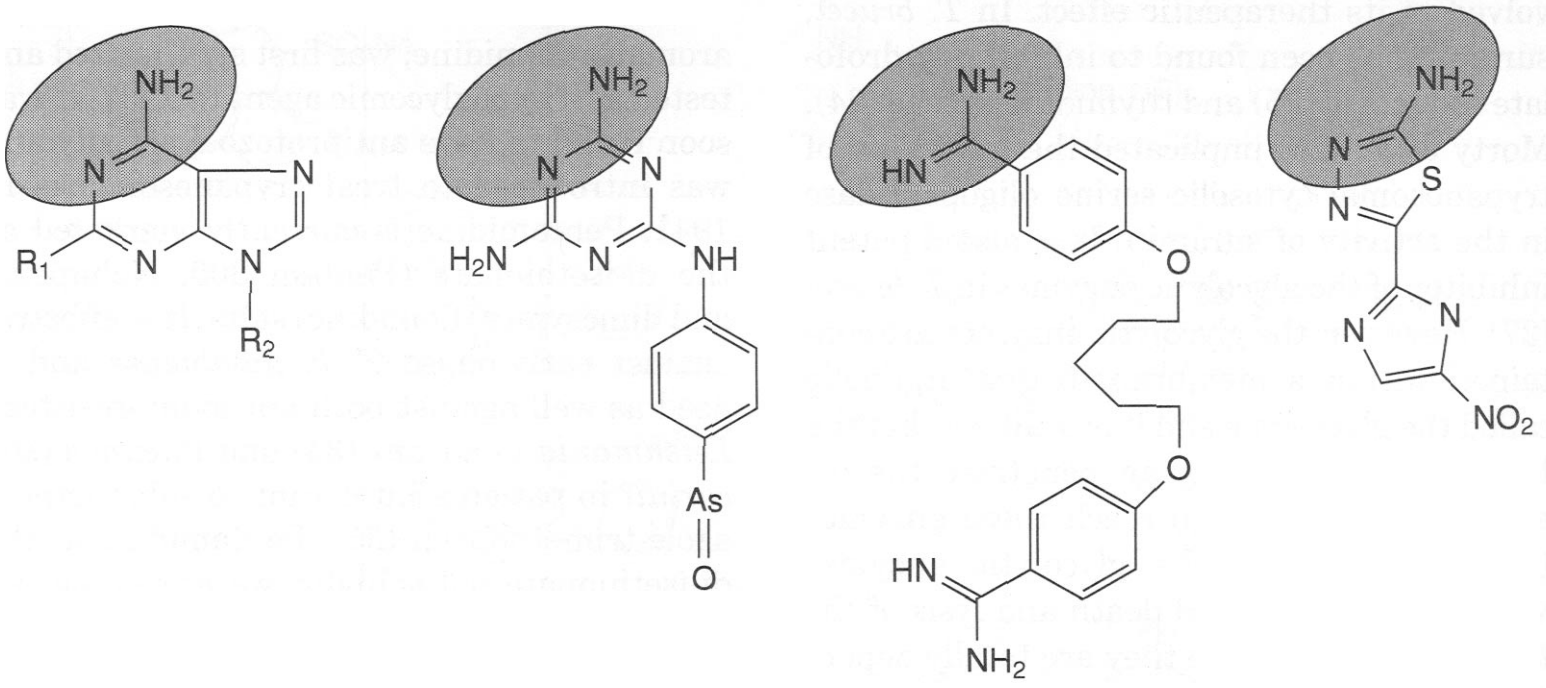


Figure 19.2. Substrates for active uptake of drugs into trypanosomes by the P2 transporter. The shaded area of each structure represents the common structural feature thought to be important for transporter recognition.

mined. A number of effects on trypanosome biochemistry have been shown *in vitro*, but none has been confirmed as the major cause of parasite death. Pentamidine reaches millimolar concentrations in cells and has been shown to bind to a number of negatively charged cellular components, including DNA, RNA, phospholipids, and a number of enzymes. Kapusnik and Mills (44) and Bailly et al. (45) reported the binding of pentamidine to nucleic acids and Edwards et al. (46) cocrystallized pentamidine bound to the dodecanucleotide d(CGCGAATFCGCG)2. Pentamidine is found bound, in a crosslinking manner, to the N3 positions of adenines in the 5'-AATT minor groove region of the duplex. Pentamidine is known to bind preferentially to the minor grooves of the kinetoplast DNA in T. *brucei*. It disrupts the kinetoplast DNA (47) and generates dyskinetoplastic cells that retain mitochondrial membranes but lack detectable kinetoplast DNA (48). Shapiro and Englund (49) reported that pentamidine, at 5 μM, promotes cleavage of the kinetoplast circular DNA to generate linearized DNA in a manner similar to that of a topoisomerase II inhibitor. However, the fact that trypanosomes lacking functional kinetoplasts can survive in the vertebrate host makes the importance of this mechanism uncertain.

Pentamidine has additional actions on trypanosomes. Berger et al. treated rats with pentamidine then infected the rats with T. b. *brucei* (50). After 4 h, they found a 13-fold increase in lysine content and a 2.5-fold increase in arginine in the trypanosomes. The reason for this drug effect was not determined. Benaim et al. (51) reported that pentamidine inhibits a high affinity (Ca^{2+}, Mg^{2+})-ATPase. Although Bitonti et al. (52) reported the *in vitro* inhibition by pentamidine of S-adenosyl-L-methionine decarboxylase (AdoMetDC), a key enzyme in the biosynthesis of polyamines, no perturbation of polyamine metabolism in intact T. *b. brucei* was noted by Berger et al. **(50).** Moreover, null mutants and overproducers of AdoMetDC in L. *donovani* showed no alterations in sensitivity to pentamidine, berenil, or methylglyoxal bis(guanylhydrazone), eliminating AdoMetDC as the major target for these drugs (53). Pentamidine was also found to inhibit the *in vitro* splicing of a group I intron in the transcripts of ribosomal RNA genes from P. *carinii* (54). It is possible that RNA editing in trypanosomes could also be affected by this drug.

Frequently observed side effects of pentamidine include hypotension, hypoglycemia that may lead to diabetes mellitus, vomiting, blood dyscrasias, renal damage, pain at the in-

jection site, and gastrointestinal disturbances. Occasional side effects are shock, hypocalcemia, hepatotoxicity, cardiotoxicity, delirium, and rash. Rare side effects from pentamidine are Herxheimer-type reactions that include anaphylaxis, acute pancreatitis, hyperkalemia, and ventricular arrhythmias.

2.1.1.3 Melarsoprol. Melarsoprol (Mel B; Arsobal; 3) is the 2,3-dimercaptopropanol adduct of melarsen oxide. It is a 3:1 mixture of two diastereomers (55). Melarsoprol was introduced as an antitrypanosomal agent in 1949. It is water insoluble and is formulated as a 3.6% (w/v) solution in propylene glycol for intravenous dosage through use of a glass syringe. When introduced, it was the only drug effective against late-stage HAT caused by either T. *b. gambiense* or *T. b. rhodesiense.* Resistance to melarsoprol in both subspecies has developed and eflornithine (difluoromethylornithine) is now used for late-stage T. *b. gambiense* infection. The drug crosses the blood-brain barrier in sufficient amounts to kill parasites in the CNS.

Melarsoprol is a prodrug. It is converted in patients to melarsen oxide with a half-life of 30 min. Melarsen oxide disappears relatively rapidly from the serum ($t_{1/2} = 3.8$ h) and no other free arsenic or organoarsenic compound can be detected by atomic absorption spectroscopy or high pressure liquid chromatography analysis after 24 h (56). In rats, significant biliary excretion of melarsoprol as the glucuronide or as the metabolite melarsen-diglutathione conjugate has been reported (57). Melarsen oxide binds rapidly and reversibly to serum proteins (apparently as unidentified protein-S-As or protein-N-As complexes), which serve as a reservoir from which the melaminophenylarsenical is released with a half-life of 35 h in serum and 120 h in cerebrospinal fluid. Trypanocidal levels of the melaminophenylarsenical do remain in the serum and other tissues and are detectable by bioassay. Selective concentration of trace amounts of free melarsoprol or melarsen oxide by the trypanosome P2-purine transporter may be important in selective toxicity (58). Figure 19.3 summarizes the mechanism of action of melarsoprol.

The controversy about melarsoprol's mechanism of action concerns the identification of the protein-arsenical complex in trypanosomes that is the most important for trypanocidal activity. Historically, the dominant hypothesis for melarsoprol's mechanism of action has been the blockage of enzymes essential for glycolysis in bloodstream forms of the African trypanosome (59). This was inferred to result from inhibition of pyruvate kinase, given that phosphoenolpyruvate accumulated in treated cell suspensions. In fact, this inhibition occurs indirectly because of depletion of fructose-2,6-bisphosphate [Fru(2,6)P_2], a potent activator of pyruvate kinase (60). Melarsen oxide was found to inhibit trypanosomal 6-phosphofructo-2-kinase (PFK 2) ($K_i < 1$ μM) more than fructose-2,6-bisphosphatase ($K_i = 2$ μM), leading to a depletion of the activator. However, the observed lytic effects of the drug preceded depletion of Fru(2,6)P_2, and the authors concluded that inhibition of glycolysis is not the cause, but rather the consequence, of lysis (60).

More recently, Fairlamb et al. (61) published that melarsen oxide or melarsoprol can form a stable adduct with trypanothione (11; Mel T), a bis(glutathionyl)spermidine adduct essential for redox homeostasis in trypanosomes (Fig. 19.3). Mel T inhibits T. *b. brucei* trypanothione reductase ($K_i = 17.2$ μM), a key enzyme in regulating the thiol-disulfide state of trypanothione (62, 63). The combination of depletion of trypanothione and inhibition of trypanothione reductase may be sufficient to kill trypanosomes.

Melarsen oxide readily forms coordination complexes with a variety of dithiol-containing enzymes and lipoic acid (64). In addition to the arsenic-protein complexes described earlier, melarsen forms stable complexes with dihydrolipoamide dehydrogenase and a number of other proteins in which cysteine residues are positioned close together. The drug may be a nonspecific inhibitor of many different enzymes, which may explain the many toxic side effects.

Melarsoprol causes reactive encephalopathy in 5 to 10% of the patients treated and has a fatal outcome in 10 to 50% of those patients (65). There is some controversy about the cause of the encephalopathy. One theory places the cause on covalent binding of the drug or its metabolites to proteins that then trigger immune reactions (66, 67). Another

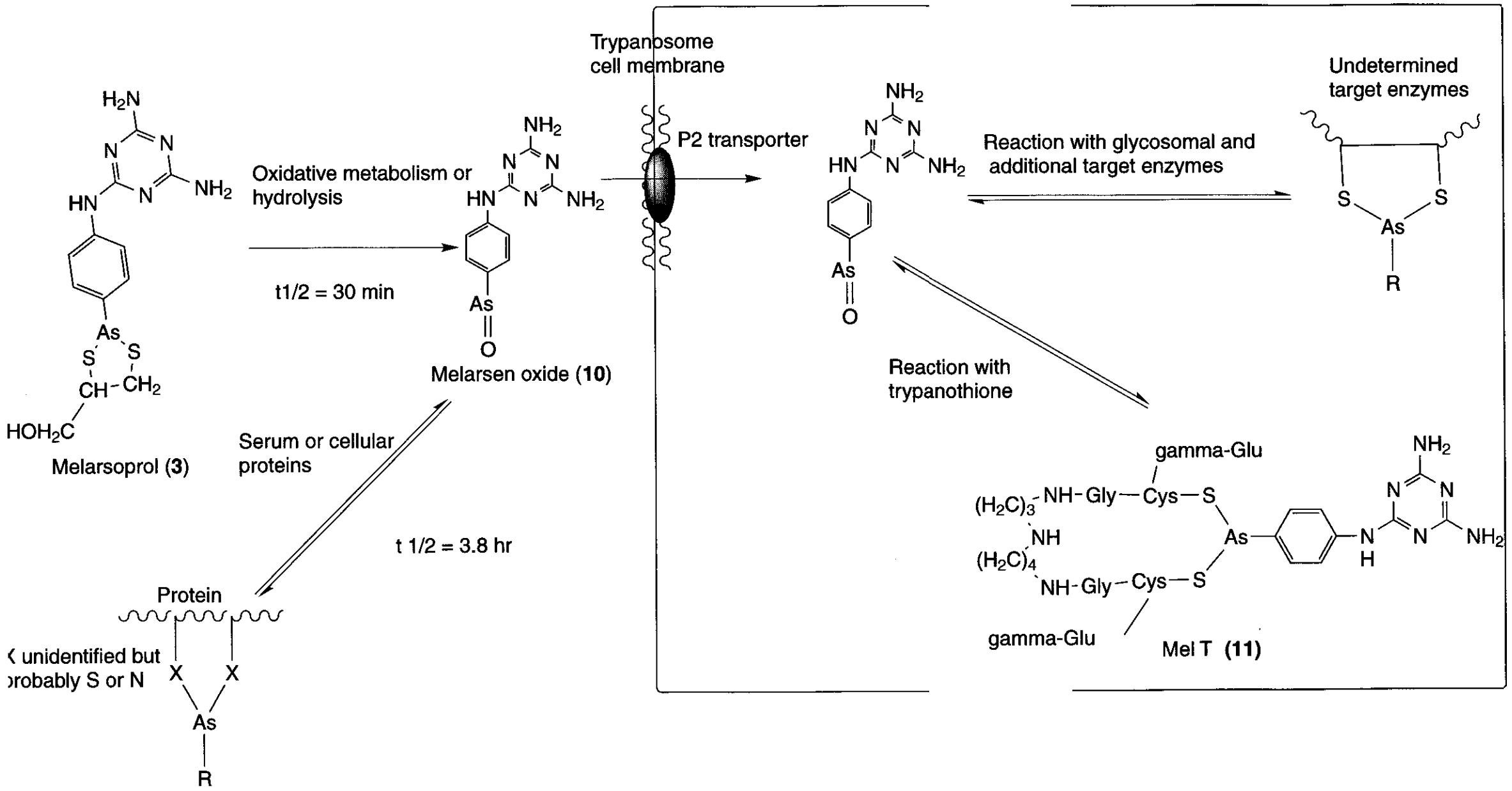


Figure 19.3. Mechanism of action of melarsoprol.

theory implicates the release of large concentrations of antigens in the CNS from trypanosomes dying from melarsoprol therapy as the cause of the encephalopathy (68, 69). Interestingly, the occurrence of melarsoprol-induced encephalopathy has been shown to be unrelated to the stage of the disease (peripheral or neurological) or the dosing regimen of the drug (70). Encephalopathy is most likely to occur on the second injection of the drug. These observations support a covalent binding mechanism for induction of encephalopathy. Attempts to reduce the incidence of encephalopathy with a new therapeutic dosing schedule proved unsuccessful (71). Other frequent side effects reported with melarsoprol therapy are peripheral neuropathy, hypertension, myocardial damage, albuminuria, hepatotoxicity, vomiting, and abdominal colic (72).

2.1.1.4 Eflornithine. Eflornithine [Ornidyl; DFMO; (*R,S*)-α-difluoromethylornithine, **4**] was reported by Metcalf et al. (73) as a potential mechanism-based inhibitor of ornithine decarboxylase (ODC). ODC is an essential and rate-limiting enzyme in the pathway leading to the biosynthesis of polyamines. Initial studies with eflornithine in cell culture showed extensive depletion of the polyamines putrescine and spermidine and eflornithine exhibited a strong cytostatic action. Eflornithine failed in early *in vivo* anticancer screens, probably because of the ability of mammalian cells to induce rapid ODC biosynthesis and to induce transporters for the uptake of polyamines from extracellular fluids (74). Interestingly, eflornithine has been found to be effective in the treatment and chemoprevention of colorectal cancers (75). Bacchi et al. (76) reported eflornithine to have antitrypanosomal activity in 1980, and it was approved in the United States in 1990 and Europe in 1991 for treatment of early- and late-stage ***T. b. gambiense*** infections (77). Because of its expense and the need for continuous intravenous infusion, it is used only in late-stage disease. Eflornithine lacks effectiveness against T. ***b. rhodesiense*** and all other ***Trypanosoma*** spp. on which it has been tested.

Poulin et al. (78) confirmed the proposed mechanism-based enzyme inhibitor action of eflornithine by isolating a covalent adduct between the drug and residue cysteine 360 in mouse ODC (Fig. 19.4). The adduct is consistent with the predicted mechanism of eflornithine as a suicide inhibitor, where the cysteine 360 acts as an attacking neucleophile in the active pocket of mouse ODC. Human ODC shares 99% identity with mouse ODC (79), and it is presumed to be inhibited by eflornithine in the same manner.

The gene encoding T. ***b. brucei*** ornithine decarboxylase has been cloned and sequenced, and the recombinant protein expressed in transformed ***Escherichia coli*** (80, 81). The protein is a homodimer, with an estimated subunit molecular mass of 45 kDa compared to the 53-kDa subunits in the mouse ODC homodimer (82). There is 61.5% sequence identity and 90% similarity between T. ***b. brucei*** and mouse ODC. The crystal structure of ***T. b. brucei*** ODC in complex with eflornithine indicates that the drug forms a Schiff base with the pyridoxal phosphate cofactor and is covalently attached to cysteine 360 (83), as predicted for the mammalian enzyme (78).

The reason for the selective toxicity of eflornithine is not fully understood. The susceptibility of ODC from T. ***b. brucei*** and mouse to inhibition by eflornithine is remarkably similar (K_i values are 220 and 39 μM and k_{inact} values are $4.3 \times 10^{-3}\ s^{-1}$ and $3.7 \times 10^{-3}\ s^{-1}$ for trypanosome and mouse ODC, respectively) (73, 80). The major difference between the two enzymes is an extra 36 amino acid peptide at the C-terminus of the mouse enzyme (81). This extension contains a PEST sequence, which is found in many eukaryotic proteins that are known to turn over rapidly ***in vivo*** (84, 85). Because the T. ***b. brucei*** ODC is highly stable and does not turn over at a detectable rate, it has been proposed that differential toxicity is attributable to the differences in turnover between host and parasite ODCs (81). Expression of ***T. b. brucei*** ODC in CHO cells demonstrated that the intracellular stability is an intrinsic feature of the trypanosomal enzyme rather than the cellular environment (86). In addition, when full-length mouse ODC was expressed in T. ***b. brucei,*** it was found to be stable, suggesting that the pathway for degradation was lacking or inactive in T. ***b. brucei*** (87, 88). Another possibility, which would account for the failure of eflornithine as an anticancer agent, is that mammalian cells are able to bypass inhibition of ODC by taking up putrescine and spermidine from the extracellular

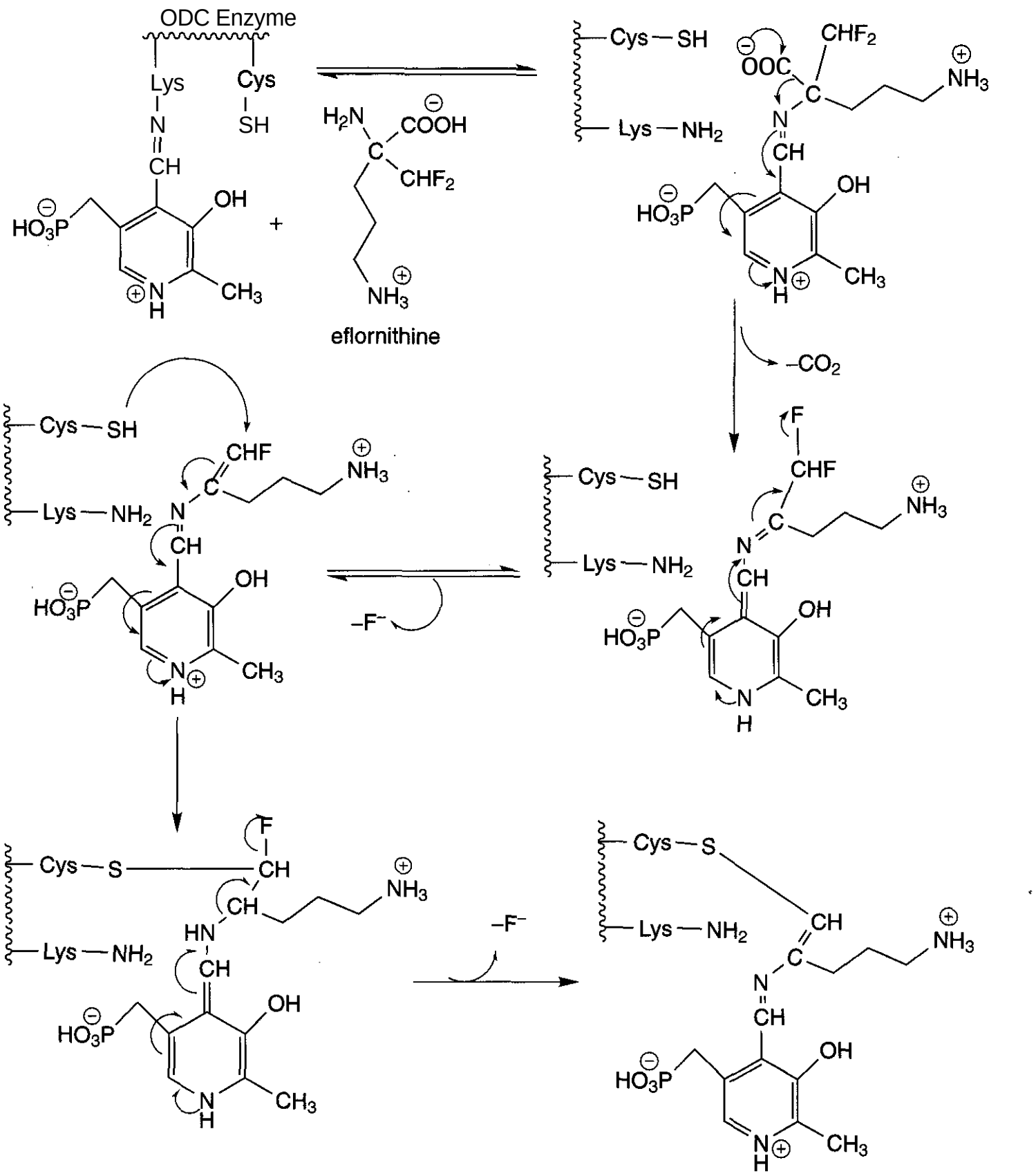


Figure 19.4. Proposed mechanism of action for eflornithine: mechanism-based inhibition of ornithine decarboxylase.

medium. Serum concentrations of polyamines are low (89) and T. ***b. brucei*** has ***an*** extremely low capacity for polyamine transport compared to that of T. *cruzi*. Thus, failure to import polyamines could contribute to the selective toxicity of eflornithine toward T. ***gambiense.***

The reason for the clinical ineffectiveness of eflornithine against T. ***b. rhodesiense*** is not fully understood. Drug-susceptible T. ***b. gambiense*** and refractory ***T. b. rhodesiense*** strains showed no difference in uptake of eflornithine or in uptake of physiologically relevant concentrations of putrescine (90). ODC inhibition by eflornithine was not significantly different between the two species. However, ODC activity was threefold higher in the eflornithine-refractory T. ***b. rhodesiense and*** appeared to have a higher turnover rate than that of T. ***b.***

gambiense (90). Because ODC from T. b. *brucei* and T. b. gambiense are identical at the amino acid level, other cellular factors must be involved in the difference in turnover rate. Bacchi and coworkers also compared drug-susceptible and refractory clinical isolates of T. b. rhodesiense (91). No clear consensus emerged from their studies; however, the data suggested that alterations in AdoMet metabolism may be responsible for resistance.

Eflornithine depletes putrescine and spermidine from T. b. brucei both in *vitro* and in *vivo* (92). The trypanosomes stop growing and transform to a short, stumpy form that apparently lacks the ability to alter its VSG and is eventually killed by the host's immune system (93). The drug effects can be reversed by putrescine in *vivo* (92, 94) and in vitro (92, 95) but not by L-ornithine in vitro (95). Depletion of polyamines in eflornithine-treated trypanosomes causes a 40 to 60% decrease in the content of trypanothione and monoglutathionylspermidine, respectively (96). It is possible that reduction in trypanothione contributes to eflornithine's mechanism of action as well as accounting for the synergistic effect with arsenical drugs in *vivo*.

2.2 American Trypanosomiasis (Chagas' Disease)

American trypanosomiasis, also known as Chagas' disease, is caused by the hemoflagellate Trypanosoma cruzi. Carlos Chagas, a Brazilian physician, first described the disease in 1909 (97). The vectors are the bloodsucking triatomine (cone-nosed) bugs of several genera (Triatoma, Rhodnius, Panstrongylus), commonly known as reduviid bugs, kissing bugs, and assassin bugs. The type of vector in any particular case will depend on the living conditions (i.e., mud-walled vs. thatched vs. wood huts, etc.) and the alternate animal host involved. Wild and domestic animals of all sorts (cats, dogs, opossums, armadillos, rodents, etc.) are reservoirs for the parasite, which can be transmitted to humans through contaminative inoculation following an insect bite. The disease can also be transmitted by blood transfusions, and this has been a major cause of the spread of the disease in some countries. Transplacental infection (98) and posttransplantation infection (99) have also been documented.

Chagas' is a disease of the Americas. It has a geographic range from Argentina in South America to parts of southern and western United States. It is considered a tropical disease because most of the persons infected with the disease live in poor areas of tropical South and Central America. It is estimated that 24 million people within the geographical area are infected with the parasite and five to six million have developed incurable chronic disease. In the United States between 50,000 to 300,000 people (mostly immigrants) are estimated to be carriers of the disease (98). Most of the persons were infected with the disease before they immigrated to the United States from Central or South America. Spread of the disease in the United States is minimal at this time because of the paucity of vectors and generally higher public health standards than are found in endemic countries (98).

Excellent reviews on the life cycle of T. cruzi can be found in Kirchhoff (98) and Tyler and Engman (100). In brief, the parasite, in its metacyclic trypomastigote life form, is transferred to the human, or alternate host animal, from the excreta of the triatomine bug through infection of the bug's bite wound or through the mucous membrane of the host. In humans, a local swelling or skin nodule called a chagoma forms at the site of the insect bite. Here the metacyclic trypomastigotes invade tissue cells and transform into amastigotes. The arnastigote stage resides in the host cytoplasm, where it rapidly multiplies to fill the host cell to the bursting point. Amastigotes then transform into actively motile trypomastigotes, which rupture the host cell and escape into the bloodstream. The nondividing extracellular trypomastigotes are then spread throughout the body, after which they invade smooth muscle tissue and ganglia of the heart, esophagus, and colon where they transform back into the intracellular proliferative amastigote stage. Infected tissue can lead to chronic disease manifested as dysrhythmias, cardiomyopathy, megaesophagus, megacolon, and occasionally meningoencephalitis. The initial infective phase of the disease lasts for weeks to months and causes fever, local swelling, skin

Table 19.3 Drugs Used to Treat American **Trypanosomiasis** (Chagas' Disease)

Drug	Adult Dose	Pediatric Dose
Nifurtimox	8–10 mg kg^{-1} day^{-1}; p.o.	1–10 years old; 15–20 mg kg^{-1} day^{-1}; p.o. 11–16 years old; 12.5–15 mg kg^{-1} day^{-1} p.o. In four divided doses for 90–120 days for all
Benznidazole	5–7 mg kg^{-1} day^{-1}; p.o.	1–12 years old; 10 mg kg^{-1} day^{-1}; p.o. In two divided doses for 30–90 days for all

rashes, myocarditis, and hepatosplenomegaly. About 10% die in the acute phase of infection, and those who survive enter an asymptomatic phase of chronic infection that can last for more than a decade. Up to 70% of infected individuals never show signs of chronic disease. The remainder develop cardiac and/or gastrointestinal symptoms that often end in death. Sudden death of young adults (age 35–45 years), caused by cardiac arrhythmias induced by T. cruzi infection, is a common occurrence in many parts of South America. Chagas' disease is extremely virulent and difficult to treat in patients who also have HIV/AIDS or are immunosuppressed for other reasons.

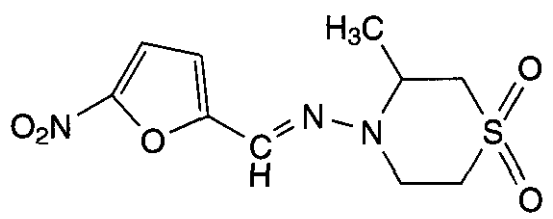
Vector eradication has proved extremely effective in controlling Chagas' disease in specific geographic areas. Programs that use insecticides to eradicate vectors in Argentina, Brazil, and other "Southern Cone" countries are currently in progress. These programs, coupled with blood bank screening, have greatly reduced the incidence of Chagas' disease in specific geographic areas of South America and provide hope for elimination of the disease (101).

Dosage regimens for nifurtimox (12) and benznidazole (**13**), the only two drugs approved to treat T. cruzi (American trypanosomiasis), are given in Table 19.3 and structures are given in Fig. 19.5. Both of these drugs give modest cure rates of 50% or less in patients with chronic disease and 70% in the acute stage of infection. Both have a number of toxic side effects. Another disadvantage of the drugs is the requirement for prolonged treatment periods. Improved drugs to treat American trypanosomiasis are desperately needed.

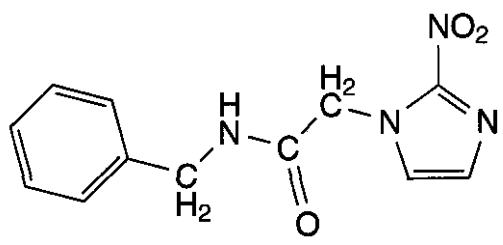
2.2.1 Mechanisms of Action of Drugs Used to Treat American Trypanosomiasis

2.2.1.1 Nifurtimox. Nifurtimox (Lampit; Bayer 2502, 12) is a nitrofuran derivative introduced to treat American trypanosomiasis in 1976 (102). The drug is no longer marketed and it appears unlikely that it will continue to be used. It remains the only drug approved in the United States for the treatment of American trypanosomiasis and is available from the Center for Disease Control in Atlanta. In addition to its action against T. cruzi, nifurtimox has activity against T. brucei and is being tested in combination with other agents in the treatment of drug-resistant strains of T. b. rhodesiense.

The exact mechanism of action for nifurtimox remains uncertain. It is clear that nifurtimox requires one electron reduction to form the nitro ion radical (Fig. 19.6). Both NADH and NADPH can serve as electron donors, but the enzyme(s) catalyzing this reaction are not known. The nitro ion radical is thought to reduce molecular oxygen to form superoxide anion and regenerate the parent nitro compound through redox cycling. Overproduction of superoxide anion swamps the cell's capacity to remove it and other reactive oxygen species



Nifurtimox (12)



Benznidazole (13)

Figure 19.5. Agents used to treat American trypanosomiasis (Chagas' disease).

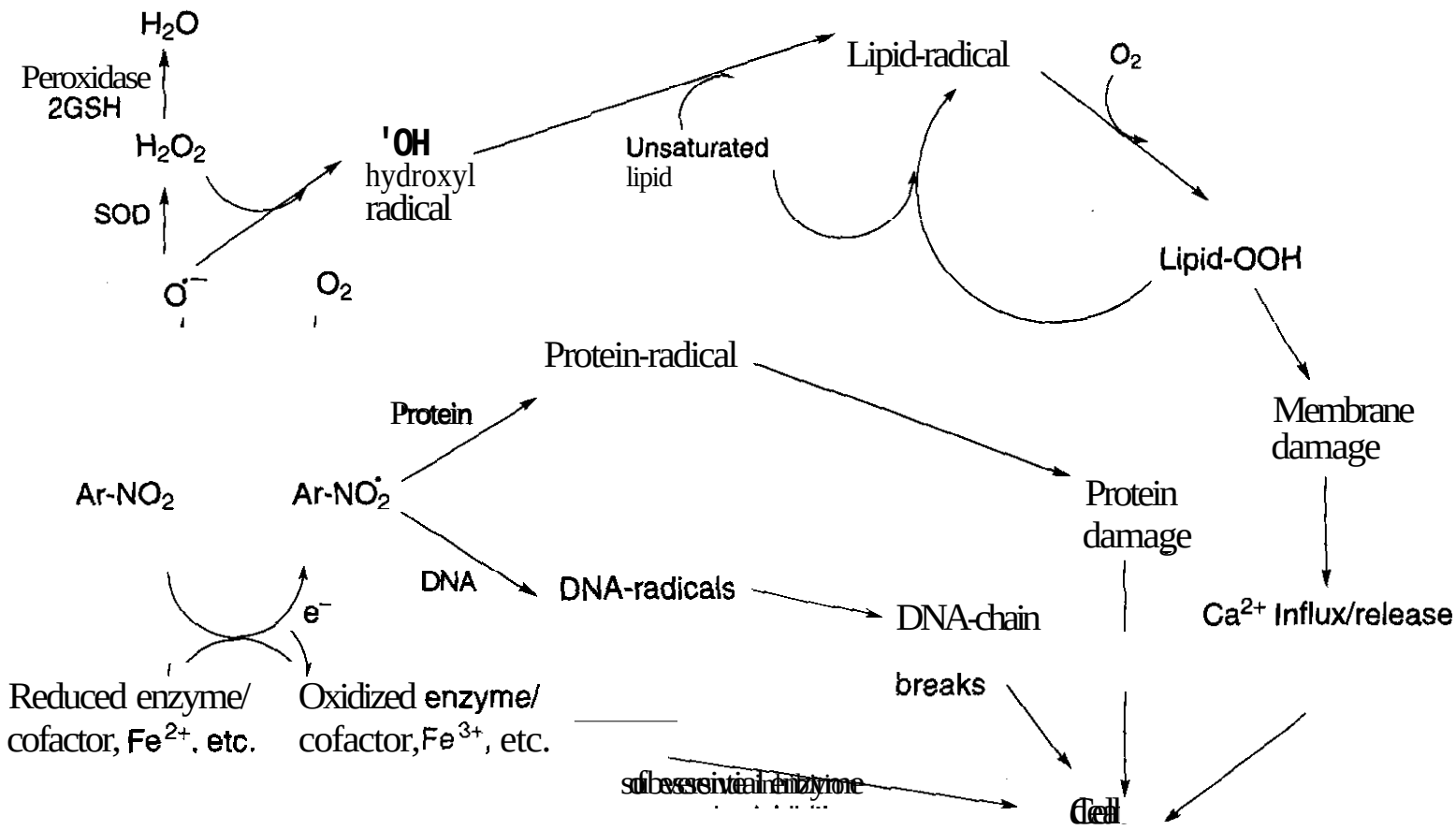


Figure 19.6. Mechanism of action for nitroimidazoles and nitrofurans (As-NO,): multiple pathways to cellular toxicity through free-radical generation and induction of oxidative stress. SOD, superoxide dismutase.

(e.g., H_2O_2 and OH') are formed, resulting in lipid peroxidation and damage to membranes, proteins, and DNA. Involvement of reactive oxygen in the mechanism of action of this organism is consistent with the aerobic environment in which they live. *T. cruzi* is reported to be relatively susceptible to oxidative damage (103). Nifurtimox weakly inhibits trypanothione reductase, an essential enzyme for protection of trypanosomes from oxygen free-radical damage (104).

Nifurtimox undergoes extensive metabolism; however. neither the structures of the metabolites nor their antitrypanosomal activity have been determined. The drug has a 3-h elimination half-life with only 0.5% of the drug being excreted unchanged in the urine. Gastrointestinal side effects include nausea, vomiting, abdominal pain, anorexia, and weight loss. Neurological side effects are restlessness, insomnia, paresthesias, polyneuritis, and seizures. Allergic reactions, including dermatitis, fever, icterus, pulmonary infiltrates, and anaphylaxis, may occur. The drug is generally better tolerated in children than in adults.

2.2.1.2 Benznidazole. Benznidazole (Rochagan, Roche 7–1051, 13) is a nitroimidazole analog introduced in 1979 (105). Benznidazole is recognized as the drug of choice for treatment of American trypanosomiasis. Cure rates with benznidazole treatment are nearly identical to those obtained with nifurtimox. Toxic side effects from benznidazole, although still severe, are slightly less than with nifurtimox.

The mechanism of action of benznidazole is similar to that of nifurtimox. Activation of benznidazole, by one-electron transfer from cellular components, is required. The resultant nitro anion radicals cause undetermined cellular damage that leads to, or facilitates, trypanosome eradication. Many isolates of T. *cruzi* are inherently resistant to both nifurtimox and benznidazole. The mechanism of resistance is not known.

2.3 Leishmaniasis

More than 20 species of *Leishmania,* distributed worldwide in tropical and subtropical regions, are known to be pathogenic to humans. The World Health Organization estimates that 350 million people are at risk of the dis-

ease (106). The severity of the disease depends on the *Leishmania* species causing the disease and the immune response that can be mounted by the host. The most severe infection, known as visceral leishmaniasis or kala-azar disease in India, is caused by *Leishmania donovani* (East India, Bangladesh, Sudan, Northeast Africa). Kala-azar is Hindi for black fever, the name coming from the fever and hyperpigmentation of the skin that often occur as symptoms of the disease. *Leishmania chagasi* (South America) and *Leishmania infantum* (Mediterranean and Middle East) can also develop into visceral infection. Worldwide cases of visceral leishmaniasis are estimated to be 500,000 per year. Cutaneous and mucosal forms of leishmaniasis are more common, but cause less severe pathology than that of visceral forms, but can still be highly disfiguring. Important genera responsible for cutaneous and mucosal leishmaniasis are *Leishmania major* and *Leishmania mexicana*. *Leishmania braziliensis* and *Leishmania panamensis* are known to have a high risk for development into mucocutaneous disease. Cutaneous leishmaniasis will most often heal without treatment over a period of months to years. The disease is usually treated to reduce scarring. Mucocutaneous leishmaniasis will seldom heal spontaneously and requires treatment (107).

The vectors for leishmaniasis are female sandflies of over 30 species from the genus *Lutzomyia* (Americas) or *Phlebotomus* (Europe, Asia, and Africa). The sandfly ingests the parasite in the amastigote form during a blood meal from an infected animal. Alternate animal hosts to humans are most often rodents, opossums, canines, sloths, and other small animals. Amastigotes transform to divide as promastigotes in the fly's gut, before migrating to the mouthparts. Promastigotes are spread to other animals, including humans, by subsequent bites of the fly. In the host, the promastigotes are ingested by macrophages at the bite site. Promastigotes transform to amastigotes that multiply in the phagolysosomes of the mononuclear cell until it fills and bursts. Amastigotes can live and reproduce only in macrophage. Released amastigotes infect new cells and spread the infection. Sandflies then become infected with the amastigotes on taking a blood meal from the host, thus completing the infectious cycle of the parasite. Cutaneous leishmaniasis will present itself as a skin ulcer with raised borders. Mucosal forms of the disease can cause disfiguring erosions of the skin around areas of the mouth, nose, palate, cheeks, and pharynx. Visceral leishmaniasis causes hepatosplenomegaly, fever, weight loss, thrombocytopenia, and hypergammaglobulinemia. Visceral leishmaniasis is fatal if left untreated (108).

Leishmania1 infections are most often resolved by the host's immune system. It is not clear why some individuals develop advanced forms of the disease. Certainly, the immunocompetence of the host and the many species of *Leishmania* that can cause disease are important factors. Leishmaniasis is becoming established as a major opportunistic disease in immunocompromised persons. Visceral leishmaniasis in particular is exacerbated in patients coinfected with HIV-associated immunodeficiency (109).

Vector control can sometimes be achieved by the use of insecticides in or near homes and in populated areas. However, much of the disease is sylvan in nature and is difficult to eradicate the vectors in these settings. Use of insect repellents can reduce incidence of infection. To date, no effective vaccine has been developed.

Dosing schedules for the major drugs used to treat leishmaniasis are given in Table 19.4 and structures of the drugs are in Fig. 19.7. Pentavalent antimony has been the agent of choice for treating leishmaniasis. Considerable resistance to the antimony-based drugs has developed and amphotericin B has become the main replacement drug. All of the major drugs used until this time have been relatively toxic, have required parenteral administration, and have relatively long durations of treatment. Amphotericin B-lipid has limited availability and is very expensive. Nonlipid dosage forms of amphotericin B can be effective, but cause a greater incidence of side effects. Interferon-gamma is sometimes used in combination with the antimonial agents and does increase cure rates, but this agent is too expensive for use in undeveloped nations. Miltefosine is potentially a major advancement in the therapy of leishmaniasis. Clinical

Table 19.4 Drugs Used to Treat Leishmaniasis

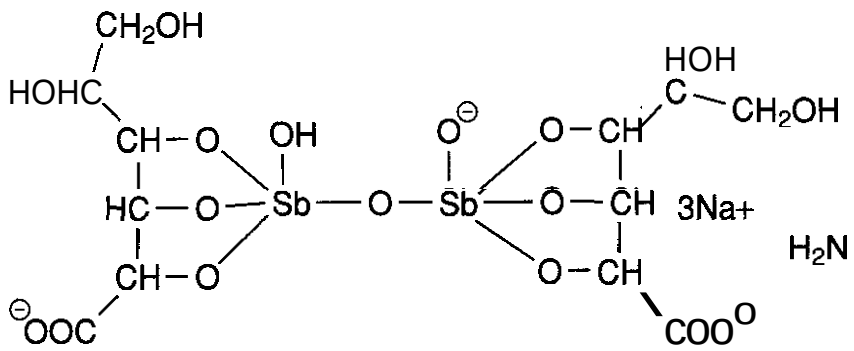
Drug	Adult Dose	Pediatric Dose
Amphotericin B-Lipid	3 mg kg^{-1} day^{-1}; i.v.; days 1–5, 14, 21 Immunosuppressed patients: 4 mg kg^{-1} day^{-1}; i.v.; days 1–5, 10, 17, 24, 31, 38	3 mg kg^{-1} day^{-1}; i.v.; days 1–5, 14, 21
Arnphotericin B deoxycholate	0.5–1.0 mg kg^{-1} day^{-1} or alternate day; i.v.; for 8 weeks	0.5–1.0 mg kg^{-1} day^{-1} or alternate day; i.v.; for 8 weeks
Sodium stibogluconate	20 mg Sb kg^{-1} day^{-1}; i.v. or i.m.; for 20–28 days	20 mg Sb kg^{-1} day^{-1}; i.v. or i.m.; for 20–28 days
Meglumine antimonate	20 mg Sb kg^{-1} day^{-1}; i.v. or i.m.; for 20–28 days	20 mg Sb kg^{-1} day^{-1}; i.v. or i.m.; for 20–28 days
Paromomycin	25–35 mg kg^{-1} bid^{-1}; i.v.; × 15 days	25–35 mg k g ^{1} bid^{-1}; i.v.; × 15 days
Pentamidine	2–4 mg kg^{-1} day^{-1} or alternate day; i.v.; × 15 days	2–4 mg kg^{-1} day^{-1} or alternate day; i.v.; × 15 days
Miltefosine	100–150 mg/day; p.o.; × 28 days	Undetermined

trials with the agent have shown that the drug can be dosed orally and gives 95% cure rates in visceral leishmaniasis.

2.3.1 Mechanisms of Action of Drugs Used to Treat Leishmaniasis

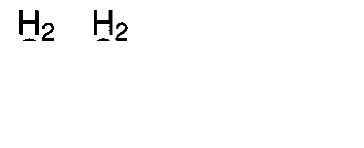
2.3.1.1 **Sodium Stibogluconate and *Meglumine* Antimonate.** These two pentavalent derivatives of antimony have been the agents of choice for the treatment of leishmaniasis for the past 50 years. Sodium stibogluconate (14; Pentostam) is used in most of the world, whereas meglumine antimonate (15; Glucantime) is used predominately in French-speaking countries. There is no discernable difference in treatment outcomes with the two drugs. The drugs are prepared by reacting gluconic acid (sodium stibogluconate) or meglumine (*N*-methyl-D-glucamine; meglumine antimonate) with pentavalent antimony. The reaction mixture is allowed to age and a complex mixture of antimony-sugar polymeric compounds is isolated. It is important to note that structures (14) and (15) represent the main components of the polymeric mixtures and not the actual chemical composition of the formulated drug. The drugs are assayed for antimony content and aqueous solutions prepared containing 100 mg Sb^{5+}/mL. The polymeric composition of meglumine antimonate in solution has been shown to change in time, as is evidenced by a steady increase in osmolarity of solution over 8 days. There is some concern that different batches of these agents give different therapeutic outcomes because of different polymeric mixtures in the formulations. However, Roberts and Rainey (110) separated sodium stibogluconate into 10 fractions by ion-exchange chromatography and found all fractions to have the same efficacy (based on Sb^{5+} content) against Leishmania amastigotes in vitro. Some of the confusion concerning the antileishmanial action of Pentostam against the promastigote stage can be attributed to m-chlorocresol added as a stabilizer to the drug preparation (110). Perhaps the variability in treatment outcome may result from differential degrees of resistance of the treated organisms.

Sodium stibogluconate and meglumine antimonate are prodrugs, requiring reduction of Sb^{5+} to Sb^{3+} for activity on leishmanial enzymes (111–113). Activation occurs selectively in the amastigote stage of parasite life cycle (114), indicating that there may be some enzyme or factor expressed only in the amastigote that is responsible for the activation (115). It has been suggested that nonenzymatic reduction by thiols such as glutathione could contribute to activation (116), but this is favored only at low pH. The mode of action of the antimonial drugs is not understood. High concentrations of trivalent antimony have been reported to inhibit glucose catabolism and fatty acid oxidation in amastigotes (117). In schistosomes, trivalent antimony is thought to inhibit glycolysis by selective inhibition of phosphofructokinase (118). However, other studies on phosphofructokinase and other glycolytic enzymes from L. mexicana revealed no



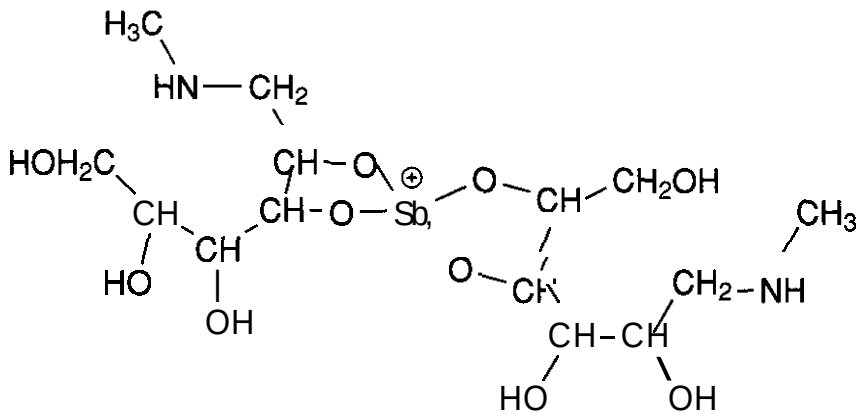
Sodium Stibogluconate

(14)



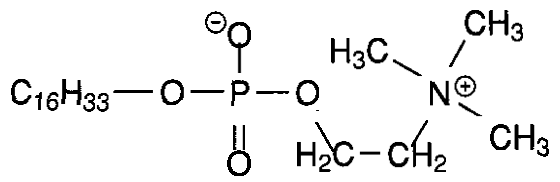
Pentamidine

(2)



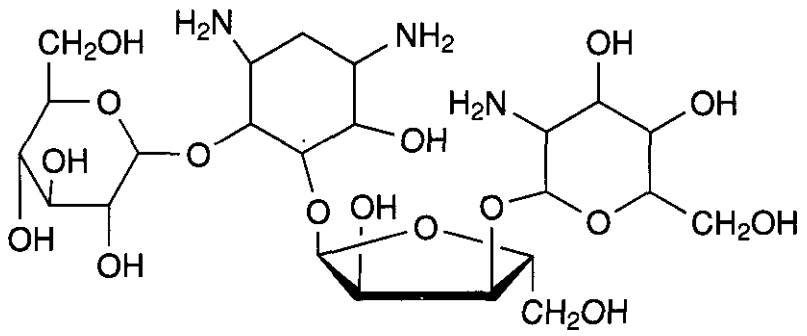
Meglumine Antimonate

(15)



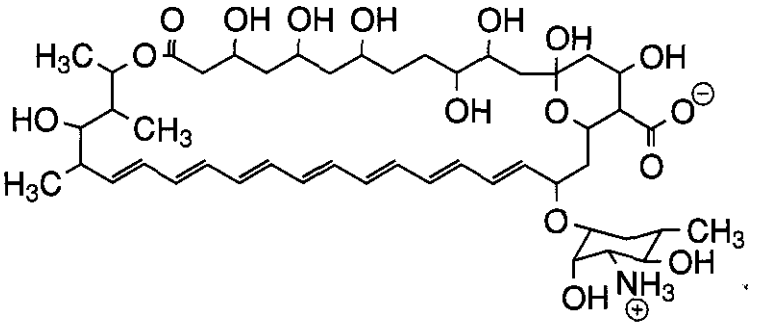
Miltefosine

(17)



Paromomycin

(16)



Amphotericin B

(18)

Figure 19.7. Agents used to treat leishmaniasis.

inhibitory activity and it was concluded that glycolysis is not the target for antimonial drugs (119). Trivalent antimony might be expected to bind to and inhibit a number of sulfhydryl-containing enzymes in the parasite. To date, only trypanothione reductase has been reported to be sensitive to inhibition by trivalent antimony (120). However, the significance of this finding is not clear because glutathione reductase is inhibited to a similar extent (120). Antimonials also induce DNA fragmentation in L. *infanturn* (121), but this may be a late consequence of cell killing by another mechanism.

Resistance to the antimonial agents is known and represents an increasing clinical problem, especially in Bihar, India and in parts of Bangladesh. Cure rates in these areas have dropped to 50% or less compared to the normal 90% cure rates of the antimonial agents. Various mechanisms for resistance have been postulated (122), including decreased activation, increased trypanothione levels (123), and increased export of the drug (124,125) through a pump that actively transports thiol-metal conjugates (126).

Frequent and often severe side effects associated with therapy with the pentavalent

antimonials include T-wave flattening or inversions (sometimes leading to fatal arrhythmias), transaminase elevations, muscle and joint pain, fatigue, nausea, and pancreatitis.

2.3.1.2 Paromomycin. Paromomycin sulfate (Humatin; aminosidine, 16) is an effective alternate to the trivalent antimonials for the treatment of all forms of leishmaniasis (127). Paromomycin is an aminoglycoside antibiotic closely related to neomycin. The agent is also effective in combination with the antimonials (128). Aminoglycosides exert a cytocidal action by binding to polysomes, inhibiting protein synthesis, and causing misreading and premature termination of translation of mRNA (129). Paromomycin is effective against antimony-resistant leishmaniasis, but offers no advantage to amphotericin B therapy. Paromomycin is licensed in Europe for parenteral use; however, it is not commercially available at this time. Paromomycin is also useful as an ointment preparation for the topical treatment of cutaneous leishmaniasis (130).

The major toxic effect of paromomycin is nephrotoxicity and damage to the eighth cranial nerve, resulting in hearing loss. To minimize these toxicities care must be taken to avoid exceeding the recommended dosage and the concomitant use of other agents with similar toxicities (130).

2.3.1.3 Amphotericin B-Lipid or Deoxycholate. Amphotericin B ***(18)*** is a polyene antibiotic used extensively against fungal infections. Amphotericin B-lipid complex (Am-Bisome, Amphocet, Abelcet, and others) and the older Amphotericin B deoxycholate (Fungizone) are highly effective against antimony-resistant strains of *Leishmania* (131, 132). The advantage of the lipid complex dosage forms is the ability to give higher doses with lower side effects. Amphotericin B gives cure rates, on all forms of leishmaniasis, that approach 100%. The problems with the drug are its high cost (especially the lipid dosage form), the necessity for parenteral administration in a hospital setting, and the long treatment periods required.

Amphotericin B works by complexing with ergosterol in the cell membrane of *Leishmania*. Pores are formed in the membrane and ions are allowed to pass into the cell, which leads to cell death (133). Resistance to amphotericin B is rare. When resistance does occur, it is generally the result of decreased ergosterol concentrations in the membrane or the substitution of a structurally altered steroid for ergosterol to which amphotericin B does not bind.

The major adverse side effect of amphotericin B is nephrotoxicity. Acute side effects of fever, chills, muscle spasms, vomiting, headache, hypotension, and anaphylaxis occur more often with amphotericin B deoxycholate preparations than with amphotericin B-lipid complex preparations.

2.3.1.4 Pentamidine. Pentamidine ***(2)*** has activity against leishmaniasis. However, pentamidine has greater overall toxicity, and resistance to it has developed in parts of India; thus, it is considered back-up therapy to the pentavalent antimonials and to amphotericin B-lipid complex. The mechanism of pentamidine is covered earlier in this chapter.

2.3.1.5 Miltefosine. Miltefosine (Impavido, 17) promises to be a breakthrough drug for the treatment of leishmaniasis. The drug has produced impressive results in phase I (134) and Phase II (135) clinical trials against Indian leishmaniasis and the drug was licensed in India in June 2002. A significant advantage of miltefosine is that it is active by oral (p.o.) administration, unlike the other antileishmanial drugs. The effective dose range of oral rniltefosine is 50 to 200 mg/day, but the optimal dose is 100–125 mg/day. In combined studies (134, 135), 50 of 51 patients with mild visceral leishmaniasis treated with 100 mglday miltefosine, p.o., for 28 days, were parasite free *6* months after the end of therapy. Some of the patients had received therapy with antimony agents before the drug trial. The 6-month cure rate with this drug regimen in India was 98%. Trials of miltefosine have been extended to American leishmaniasis. Soto et al. (136) reported a 94% cure rate of American cutaneous leishmaniasis with oral miltefosine after 3- to 4-week treatment with 133–150 mglday.

Miltefosine (17) is the simple ether for ester bioisostere of phosphatidylcholine (lecithin). The agent is a member of the lysophospholipid or ether-lipid class of drugs that were synthesized and developed as potential anticancer agents. Early clinical trials as systemic antitumor agents failed, but the compounds

have topical antitumor activity. The compound subsequently was found to have ***in vitro*** activity against leishmaniasis. These findings led to the clinical trials of oral miltefosine described earlier.

The exact mechanism of action of miltefosine is not known. In mammalian cell culture, miltefosine has been shown to have effects on signal transduction (137, 138), lipid metabolism (139, 140), and calcium homeostasis (141). Miltefosine inhibits phosphatidylcholine biosynthesis, resulting from the inhibition of phosphatidylethanolamine-phosphatidylcholine-N-methyltransferase in T. *cruzi* (142). Perturbation of ether-lipid remodeling is implicated as a target in ***Leishmania*** (143).

Side effects from oral miltefosine are significant but generally tolerable. Acute effects are primarily gastrointestinal distress, consisting mainly of vomiting and diarrhea. Gastrointestinal episodes occurred frequently in 62% of the patients and did not dissipate with continuing therapy; however, no patients left the study because of the gastrointestinal side effects, and overall, the patients experienced a slight weight gain over the 4-week treatment period. A more serious side effect was elevated serum aspartate aminotransferase levels. Twelve of 80 patients displayed elevated serum aspartate aminotransferase levels in the initial days of therapy, but these decreased during subsequent days of treatment. One patient had to discontinue therapy because of liver toxicity. Creatinine levels rose in several patients. All abnormal laboratory values returned to normal shortly after discontinuation of therapy.

3 PERSPECTIVES FOR NEW DRUG THERAPIES FOR KINETOPLASTID PARASITIC DISEASES

The kinetoplastid parasitic diseases are endemic, if not epidemic, in most of the tropical and subtropical regions of the world. Agents available to treat these diseases are, for the most part, inadequate because of poor efficacy, toxicity, high cost, and route of administration. Many of the older drugs do not meet current standards for regulatory approval for clinical use. Leading medical journals publish editorials on a regular basis calling for the development of new drugs for tropical diseases (144–147). Despite knowledge of a number of enzymes or enzyme systems that could be targeted for development of selectively toxic chemotherapeutic agents, little development of new drugs for these diseases has occurred. The following section explores some of the opportunities for the development of drugs to treat kinetoplastid diseases.

3.1 Glycosomal and Other Carbohydrate Metabolism

Bloodstream forms of T. ***brucei*** lack energy stores and essentially make all of their ATP from glycolysis. Two features of this pathway have attracted considerable interest for drug design. First, these parasites lack cytochromes and respiration is through a plantlike, cyanide-insensitive, glycerophosphate oxidase mitochondrial process. Second, as first described Opperdoes and Borst (148), the first nine enzymes of glucose and glycerol catabolism are contained within a peroxisome-like organelle known as the glycosome. Compartmentalization of the enzymes in the glycosome may make it possible for T. ***brucei*** to carry out glycolysis at a rate approximately 50 times higher than that of mammalian cells (149), although this has been disputed (150). The high rate of aerobic glycolysis is necessary to compensate for the poor yield of only two ATP molecules per glucose molecule that is processed (151) (Fig. 19.8). Trypanosomes require a large amount of energy to replicate every 6 to 8 h in mammalian blood (152), and to change their surface glycoproteins (VSGs)

Figure 19.8. Glycolysis and glycolytic enzymes associated with the glycosomes of bloodstream *Trypanosoma brucei*. The enzymes that catalyze the reactions are: (1) hexose kinase; (2) glucose-6-phospate isomerase; (3) phosphofructokinase; (4) fructose-1,6-bisphosphate aldolase; (5) triose-phosphate isomerase; (6) glycerol-3-phosphate dehydrogenase; (7) glyceraldehyde-3-phosphatedehydrogenase; (8) phosphoglycerate kinase; (9) phosphoglycerate mutase; (10) enolase; (11) pyruvate kinase; (12) glycerol kinase; (13) mitochondrial glycerol-3-phosphate dehydrogenase; (14) ubiquinol oxidase, (13 + 14 = glycerophosphate oxidase); (15) fructose-6-phosphate 2-phosphokinase; (16) fructose-2,6-bisphosphate 2-phosphatase; ⊕ = activation of pyruvate kinase.

Energy yield from glucose metabolism

Aerobic conditions:

$$\text{Glucose} + 2\ \text{ADP} + 2\ P_i + O_2 \longrightarrow 2\ \text{pyruvate} + 2\ \text{ATP} + 4\ H_2O$$

Anaerobic conditions:

$$\text{Glucose} + \text{ADP} + P_i \longrightarrow \text{pyruvate} + \text{ATP} + H_2O + \text{glycerol}$$

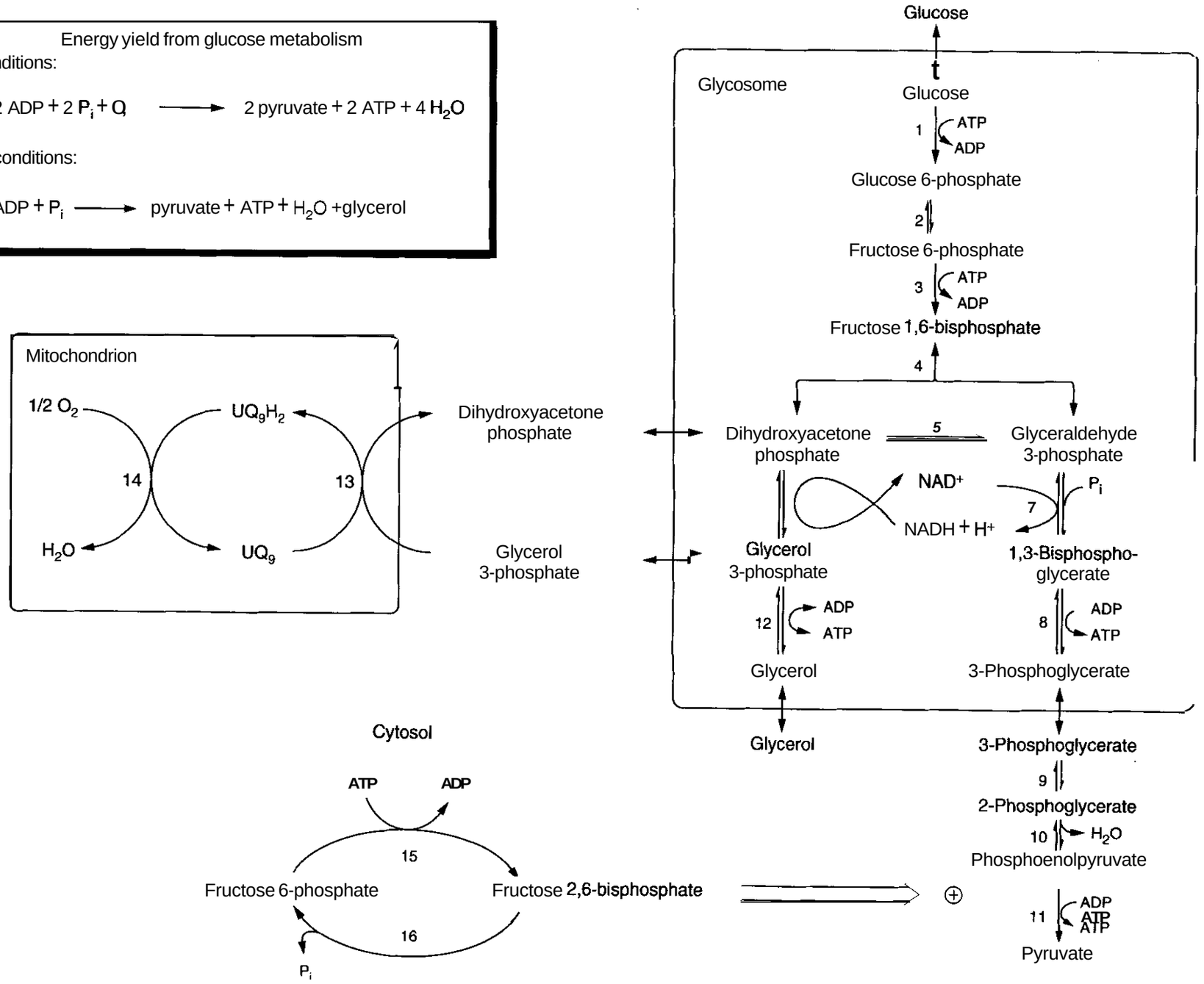
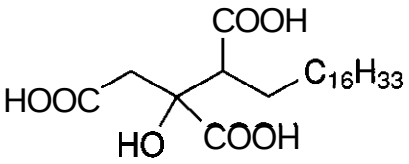
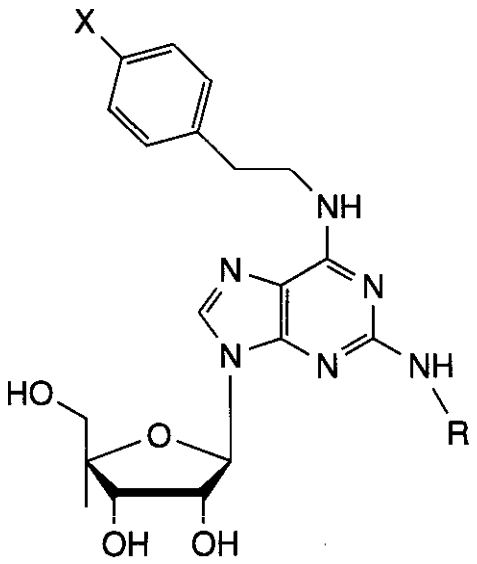


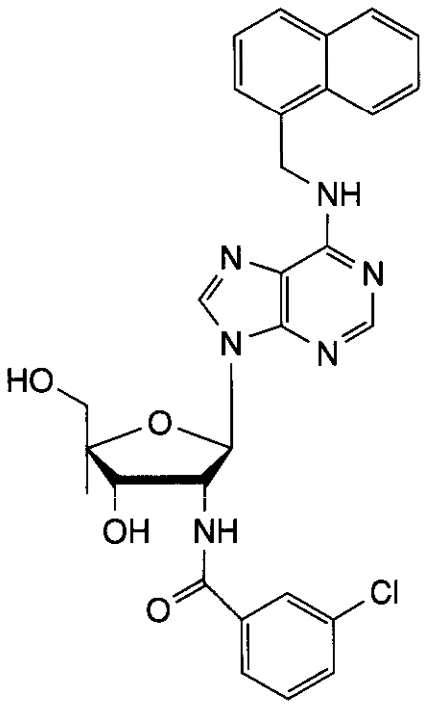
Figure 19.8.



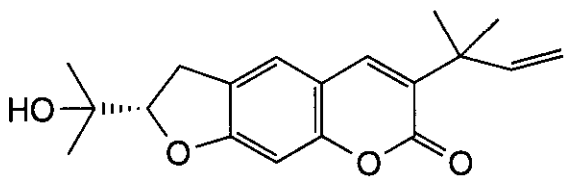
Agaricic acid
(**19**)



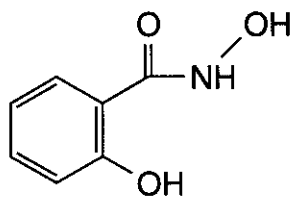
(**20a**): X = OH, R = H
(**20b**): X = H, R = $CH_2CH_2C_6H_5$



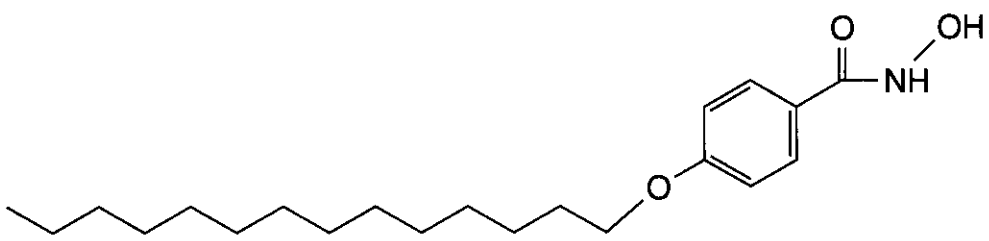
(**21**)



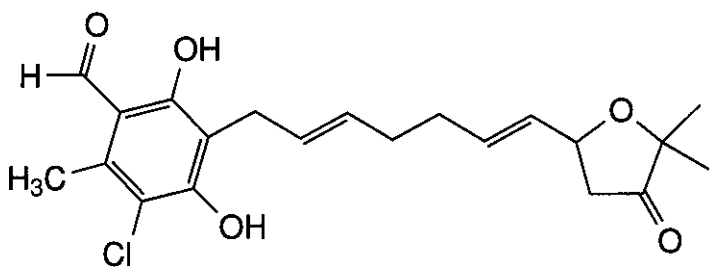
Chalepin
(**22**)



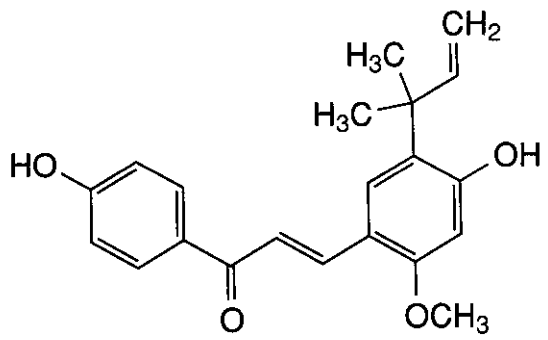
Sham
(**23**)



(**24**)



Ascofuranone
(**25**)



Licochalcone A
(**26**)

Figure 19.9. Inhibitors of glycosomal enzymes and additional enzymes associated with glycolysis in the trypanosome.

at a rate high enough to evade host immune response (15). Other species of the order Kinetoplastida are not as heavily dependent on glycolysis for energy supply as are the African trypanosomes (T. *brucei* spp.); however, many of the enzymes from kinetoplastids have similar structure and function, thus potent inhibitors to key enzymes may have selective toxicity toward many or all of the species (153). Excellent reviews on glycosomes and the enzymes they contain are given by Clayton and Michels (154), Wang (155), and Opperdoes and Michels (156). Structures of compounds known to inhibit glycolysis and respiration are shown in Fig. 19.9.

Glycosomes and the glycosomal enzymes are essential for trypanosome survival and growth. Although the glycosomal enzymes differ significantly in amino acid sequence and structure from the corresponding mammalian enzymes catalyzing glycolysis, this is less evident at their active sites and may present a

significant challenge for drug design. Many of the glycosomal enzymes have been sequenced, cloned, and the three-dimensional structures determined. As discussed earlier, the mechanisms of action of the arsenic- and antimony-containing trypanocides and leishmanicides have been attributed to inhibitory activity on enzymes of the glycolysis pathway, but this is far from certain. Because of the uniqueness of these enzymes to trypanosomes and kinetoplastids in general, they are considered by many authorities as excellent targets for structure-based drug design.

Bakker et al. (150, 157, 158) performed mathematical modeling experiments on the enzymes of the glycosomal pathway to determine the enzymes most important for control of "glycolytic flux." The modeling should identify the enzymes most susceptible to control by enzyme inhibitors. Interestingly, transport of glucose into the glycosome was calculated to be rate controlling on the glycolytic flux. Other highly important enzymes are calculated to be aldolase, glyceraldehyde-3-phosphate dehydrogenase, glycerol-3-phosphate dehydrogenase, and phosphoglycerate kinase (158). However, it should be noted that other modeling studies have concluded that there is little prospect of killing trypanosomes by depressing glycolytic flux except by the use of specific irreversible inhibitors (159) or by inhibition of pyruvate export.

3.1.1 Hexose Transport as a Drug Target. Bloodstream T. brucei import glucose by facilitated diffusion (160), and the uptake of glucose accounts for greater than 50% control of the glycolytic flux (158, 161). The glucose transporter will also accumulate fructose and mannose. Trypanosomes contain two genes encoding glucose transporters, THTl and THT2 (162). THTl glucose transporters are predominantly expressed in the bloodstream form and have low affinity for glucose. Cytochalasin B is a moderately potent inhibitor of THT1 (163). THT2 has high affinity for glucose and is expressed in the procyclic life form of the trypanosomes (164). Both THTl and THT2 could be considered as potential targets for antitrypanosomal chemotherapy (165), although an opposing view has been published (159). It is also theoretically possible that the glucose transporter could be used as a carrier to target chemotherapeutic drugs in trypanosomes (166).

3.1.2 Inhibition of Hexose Kinase. Hexose kinase (HK) is the first enzyme in the glycosomal glycolysis pathway (Fig. 19.8, enzyme 1). The enzyme has been sequenced (156) and found to have 36% identity to human HK. The enzyme has low substrate selectivity for glucose, phosphorylating a number of hexoses (167). The enzyme also has low selectivity for ATP, and can even use UTP or CTP in its place (156). The enzyme is not regulated by glucose-6-phosphate or glucose-1,6-bisphosphate as is the mammalian enzyme. Analogs of glucose have been found to inhibit the enzyme in *vitro* (168). Because HK is predicted to have a low degree of control over the glycolytic flux in the glycosome, it is not the best potential target for drug design.

3.1.3 Inhibition of Glucose-6-Phosphate Isomerase. Glucose-6-phosphate isomerase (PGI), the second enzyme in the glycosomal pathway (Fig. 19.8), has been sequenced, cloned, and characterized (169). PGI has 54–56% sequence homology with the equivalent enzymes from yeast and mammalian sources. The enzyme from trypanosomes, but not the enzyme from rabbit muscle, is reversibly inhibited by suramin (K_i = 0.29 mM) and irreversibly by agaricic acid (19)(169). Hardre et al. (170) synthesized a transition-state analog inhibitor of PGI (K_i = 50 nM); however, the compound has poor selectivity for the trypanosomal enzyme and, because of its ionic character, it is not likely to have in *vivo* activity. PGI is predicted to have little control on the glycolytic pathway (158); thus its potential as a drug target remains to be validated.

3.1.4 Inhibition of Phosphofructokinase. The next enzyme in the glycolytic pathway of T. brucei, phosphofructokinase (PFK; Fig. 19.8, enzyme 3), has been sequenced and cloned (171). PFK from T. brucei has the structure of a typical pyrophosphate-dependent kinase, but it will not function with pyrophosphate as a substrate. Rather, it requires ATP for activity. The differences in structure of T. brucei PFK, compared to that of the

mammalian enzymes having the equivalent function, make the enzyme attractive as a drug design target. However, the enzyme is not predicted to be important in controlling the glycolytic flux (158) and no inhibitors to the enzyme have been reported.

3.1.5 Inhibition of Fructose-1,6-Bisphosphate Aldolase. This enzyme (Fig. 19.8, enzyme 4) has been cloned (172) and expressed (173), and the structure of the recombinant protein was determined to 1.9-Å resolution (174). Because of the structural similarities in the active sites of the T. ***brucei,*** L. ***mexicana,*** and the three human aldolase isoenzymes, selective inhibition of the parasite enzymes is a remote possibility (174). However, the parasite enzymes both contain a type II peroxisomal targeting sequence at their N-termini, which might be exploited to inhibit aldolase import into the glycosome (see section 3.2).

3.1.6 Inhibition of Triose-Phosphate Isomerase. Triose-phosphate isomerase (TIM) is an essential enzyme for the conversion of dihydroxyacetone phosphate to glyceraldehyde-3-phosphate. TIM from T. ***brucei*** has been cloned (175) and the three-dimensional structure determined (176,177). Helfert et al. (178) used knockout constructs to demonstrate that TIM activity is essential for trypanosomes to survive. Surprisingly, neither the glycolytic scheme in Figure 19.8 (reaction 5) nor the computer model of Bakker et al. (158) predicts this enzyme would be essential for ATP synthesis through glycolysis, even though the gene knockout experiments validate the enzyme as essential. However, although essential, the structural similarities with mammalian TIM do not make it a good target for inhibition.

3.1.7 Inhibition of Glyceraldehyde-3-Phosphate Dehydrogenase. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is the most studied enzyme of the glycosomal enzymes (Fig. 19.8, enzyme 7). Crystal structures of GAPDH from T. ***brucei*** (179), ***T. cruzi*** (180), and ***L. mexicana*** (181) are all known and have been compared to the human enzyme. A major difference between the parasitic and mammalian enzymes lies in the region of the enzyme that binds NAD^+. Aronov et al. (182,183) performed molecular modeling on the NAD^+ binding site of GAPDH from L. ***mexicana*** for the design of adenosine analogs as tight binding inhibitors. Adenosine analogs (**20a**), (**20b**), and (21) were among the most active compounds reported by this group. Interestingly, compound (21) had good activity against cultured L. ***mexicana,*** T. ***brucei,*** and ***T. cruzi*** with IC_{50} values of 10, 30, and 14 μM, respectively, and showed three- to 15-fold selectivity (based on ED_{50} values on cultured cells) for parasites over mammalian cells. Increasing the potency, selectivity, and bioavailability of the lead compounds represents a significant challenge. Nonetheless, the lethal activity of (21) on all three species demonstrates the potential for antiparasitic activity against all kinetoplastid species. Ladame et al. (184) reported the inhibition of GAPDH by phosphorylated epoxides and alpha-enones. These agents are apparently affinity labels for GAPDH and may show some ***in vivo*** selectivity for the trypanosomal enzyme. Pavao et al. (185) identified chalepin (22), a natural product coumarin-related analog, as an inhibitor (IC_{50} = 64 μM) of GAPDH from T. ***cruzi*** and have obtained a crystal structure with (22) bound to the enzyme. Chalepin binds in the NAD^+ binding pocket of the enzyme by a unique mode. The X-ray structure provides a basis for structure-based drug design to maximize activity of (22).

3.1.8 Inhibition of Glycerol-3-Phosphate Dehydrogenase. NAD^+-dependent glycerol-3-phosphate dehydrogenase (G3PDH) is predicted by mathematical modeling of the glycosomal enzymes to be highly important for the control of the glycolytic flux (Fig. 19.8, enzyme 6). G3PDH has been cloned from several species (186, 187) and three-dimensional structure from L. ***mexicana*** was reported by Suresh et al. (188). G3PDH from L. ***mexicana*** is very similar to G3PDH from T. ***brucei*** (63% sequence homology), but has only 29% amino acid sequence homology with the mammalian enzyme. Agaricic acid, suramin, melarsen oxide, and cymelarsan inhibit trypanosomal G3PDH (188–190).

Denise et al. (191) applied glycosomal protein extracts to affinity chromatography col-

umns containing bound cymelarsan (9). Two proteins of molecular weight 36 and 40 kDa bound to the column and could be eluted by high concentrations of cymelarsan. The 36-kDa protein was identified as G3PDH and the 40-kDa protein as fructose-1,6-bisphosphate aldolase. These experiments implicate these two essential enzymes as targets for drug development.

3.1.9 Inhibition of the Dihydroxyacetone Phosphate/Glycero-3-phosphate Shuttle. In T. brucei, the glycosomal NAD^+ used in glycosomal metabolism must be regenerated from NADH through a dihydroxyacetone phosphate (DHAP) for glycerol-3-phosphate shuttle. The shuttle consists of the glycosomal NAD^+-dependent glycerol-3-phosphate dehydrogenase and a mitochondrial glycerol-3-phosphate oxidase system (192). The system constitutes a mitochondrial FAD-dependent glycerol-3-phosphate dehydrogenase (Fig. 19.8, enzyme 13) (193) that transfers electrons from glycerol-&phosphate to ubiquinone, which is then oxidized through a cytochrome-dependent ubiquinol oxidase (Fig. 19.8, enzyme 14), also known as the trypanosome alternative oxidase (194, 195). During anaerobic conditions the oxidase cannot function and glycerol-3-phosphate accumulates in the cell to millimolar concentrations (192). However, net ATP synthesis occurs because of the mass action effect of glycerol-3-phosphate driving the glycerol kinase reaction (Fig. 19.8, enzyme 12) toward the formation of glycerol and ATP (196). As long as glycerol concentrations remain low, the cells can survive considerable periods of anaerobiosis in vitro. A similar effect can be demonstrated both in vivo and in vitro by use of salicylhydroxamic acid (SHAM, **23**), an inhibitor of the glycerol-3-phosphate oxidase. However, addition of glycerol neutralizes this mass action effect and trypanosomes rapidly lose motility and lyse within minutes in vitro (197) and in vivo (259). Indeed, trypanosome-infected mice can be cured by a combined treatment with SHAM and glycerol (198), but the curative dose of SHAM in the combination caused unacceptable animal mortality rates (199).

Additional attempts have been made to find less toxic agents to apply to this chemotherapeutic approach. Grady et al. (200) made *p-n*-tetradecyloxybenzhydroxamic acid (24) and found it to be 70 times more potent as an inhibitor of glycerol-3-phosphate oxidase and 10- to 20-fold more effective as an antitrypanosomal agent in vitro than SHAM. However, no therapeutic improvement was found in vivo. Ascofuranone (**25**), an antibiotic isolated from Ascochyta visiae (201), has been found to potently inhibit the glycerol-3-phosphate-dependent mitochondrial O_2 consumption by bloodstream forms of T. b. brucei in vitro (202). The mechanism of action of (25) is attributed to its binding at the coenzyme Q site of the ubiquinol oxidase, an enzyme that has no counterpart in mammalian metabolism. This finding led to a trial of orally and intraperitoneally administered (25) combined with glycerol for the treatment of T. b. brucei-infected mice. Either agent alone was ineffective in the dose range tested; however, (25) at 100 mglkg combined with glycerol at 3 glkg given orally, or (25) at 25 mglkg combined with glycerol at 3 g/kg given intraperitoneally gave a 100% cure rate in five of five mice in each test group. In each experiment, (25) was given as a single dose and the glycerol was dosed at 30-min intervals at 1 g/kg dose. Ascofuranone was found to be highly nontoxic to mice, having an LD_{50} value of over 5 g/kg. If the combination of ascofuranone/glycerol is able to cure T. brucei in humans and/or livestock by single oral treatment, it would afford a major advancement in the management of African trypanosomiasis. However, glycerol does not readily cross the blood-brain barrier, so the infection would not be eliminated from the CNS. An alternative strategy, therefore, would be to simultaneously inhibit glycerol kinase and the alternative oxidase.

3.1.10 Inhibition of Glycerol Kinase. Kralova et al. (203) and Steinborn et al. (204) independently reported the cloning and molecular characterization of glycosomal glycerol kinase (GK) from T. brucei. A unique feature, compared to the mammalian enzyme, is the replacement of Ser137 with Ala. The Ala137 substitution results in lower affinity of the substrates for the enzyme compared to those of other GKs. The Ala137 substitution is found in other trypanosome species that can

convert glucose directly to glycerol and pyruvate under anaerobic conditions. This unique feature of T. brucei GK offers a potential for the design of selectively toxic drugs for use in combination with inhibitors of the alternative oxidase.

3.1.11 Inhibition of Phosphoglycerate Kinase. Phosphoglycerate kinase (PGK, Fig. 19.8, enzyme 8) exists as three isoenzymes in most *Trypanosomatidae*. Mathematical modeling of the glycosomal pathway places PGK as highly important to the overall glycolytic flux in the trypanosome (157, 158). Two of the forms are glycosomal (PGKA and PGKC) and the third (PGKB) is cytosolic in both T. brucei and T. cruzi. PGKA is expressed at low levels throughout the life cycle, whereas cytosolic PGKB is strongly expressed in the insect procyclic stage, but hardly at all in the mammalian bloodstream form, whereas the reverse is true for glycosomal PGKC. Overexpression of cytosolic PGKB in bloodstream forms is lethal (205), implying that compartmentation plays a vital role in maintaining their energy supply. Cloning and characterization of PGK from multiple species has been accomplished (206) and the structure has been determined (207). PGK from T. brucei has a high preference for ATP binding, whereas enzymes from yeast and rabbit muscle will readily accept GTP and ITP as cosubstrates. Suramin (1) selectively inhibits the glycosomal form of PGK from T. brucei (208).

3.1.12 Inhibition of Phosphoglycerate Mutase and Pyruvate Kinase. Phosphoglycerate mutase (Fig. 19.8, enzyme 9) in T. brucei has been found to be a cofactor-independent enzyme, unlike its mammalian counterpart enzymes that are all cofactor dependent. This major difference in structure of trypanosomal phosphoglycerate mutase is proposed to be an excellent target for drug development (209).

Pyruvate kinases from trypanosomal species possess unique properties that may be exploitable for the purpose of selective drug development. The most notable difference from the host is that the parasite enzyme has a regulatory site on the enzyme that binds fructose-2,6-bisphosphate (210). It is proposed that a tight binding inhibitor to the fructose-2,6-bisphosphate regulatory site would be an effective trypanocide. However, modeling studies on glycolysis predict that this enzyme is likely to be a mediocre drug target (158).

3.1.13 Additional Glycosomal Enzymes as Potential Drug Targets. Several additional enzymes are involved in carbohydrate metabolism of kinetoplastids. Phosphoenolpyruvate carboxykinase (PEPCK) is one such enzyme. In insect stages of T. brucei, T. cruzi, and Leishmania, glucose consumption is associated with CO_2 fixation and excretion of significant amounts of succinate. The glycosomes of these life-cycle stages have low amounts of phosphoglycerate kinase and highly elevated levels of PEPCK and malate dehydrogenase (MDH). The combined action of these two enzymes converts phosphoenolpyruvate to malate, with the concomitant oxidation of NADH to NAD^+ and conversion of ADP to ATP, thereby maintaining zero net internal balance of ATP and NAD^+ in the glycosome (211). Consequently, PEPCK is purported to be a prime candidate for inhibitor development (212, 213). The recently published crystal structure of the T. cruzi enzyme (214) will greatly aid efforts at structure-based drug design.

Pyruvate phosphate dikinase (PPDK) is another glycosomal enzyme that is expressed in T. cruzi, Leishmania, and the procyclic stage of T. brucei (but not bloodstream forms) and does not exist in higher eukaryotes. The enzyme has been cloned and its structure determined from several sources and the three-dimensional structure determined (215–217). The functional role and importance of this enzyme in glycosomal metabolism has not been determined. Compartmentalization of this enzyme in the glycosome is important since a futile cycle, resulting in net hydrolysis of ADP, would occur if PPDK were in the cytosol (Scheme 1).

In T. cruzi, PDDK could play a role in formation of phosphoenolpyruvate for the synthesis of phosphonopyruvate (catalyzed by phosphoenolpyruvate mutase) and its subsequent conversion to aminoethylphosphonate (AEP). Phosphonopyruvate mutase has been

$$\text{Pyruvate} + P_i + \text{ATP} \rightarrow \text{PEP} + \text{AMP} + PP_i \quad (1)$$

$$\text{PEP} + \text{ADP} \rightarrow \text{Pyruvate} + \text{ATP} \quad (2)$$

$$PP_i + H_2O \rightarrow 2P_i \quad (3)$$

$$\text{ADP} + H_2O \rightarrow \text{AMP} + 2P_i. \quad \text{Sum}$$

Scheme 1. Biochemical reactions expected if PPDK were in the cytosol. Where 1 = PPDK, 2 = PK, and 3 = pyrophosphatase.

cloned and expressed from T. cruzi (Sarkar and Fairlamb, unpublished), suggesting that the pathway is present. In contrast to T. brucei and Leishmania spp., AEP is an invariant component of the glycan core of the parasites' surface glycosylinositylphospholipids (GIPLs) and mucins, suggesting an important role in survival. However, further work is required to establish whether this pathway is a viable drug target.

An NADH-dependent fumarate reductase is present in the promastigote stage of Leishmania parasites and T. cruzi. Mammalian cells do not possess soluble fumarate reductase activity. Chalcones, components of Chinese licorice, have been found to inhibit fumarate reductase and to be lethal to L. major and L. donovani (218). Nielsen et al. (219) synthesized and performed three-dimensional quantitative structure-activity relationship (QSAR) analysis on a large series of chalcones as potential antileishmanial agents. Licochalcone A (26), a lead compound in these studies, has an antileishmanial IC_{50} value of 13 μM and is one of the more potent inhibitors of fumarate reductase found to date. In T. cruzi, soluble fumarate reductase activity is associated with dihydroorotate dehydrogenase, implicating fumarate reductase in de novo pyrimidine biosynthesis (220). It remains to be determined whether this is also the case in Leishmania.

3.2 Glycosomal Protein import as a Drug Target

Glycosomal enzymes are synthesized in the cytosol and thus have to be imported into the organelle for glycolysis to function correctly in a coordinated manner. As mentioned earlier, inappropriate expression of PGK in the cytosol disrupts normal glycolytic function in bloodstream T. brucei (205). Thus, inhibition of the glycosomal import process is predicted to be lethal. The initial hypothesis that "hot spots" of positive charges spread 40 Å apart on some glycosomal proteins were topogenic signals for import (221) was soon abandoned as details emerged concerning import of proteins into peroxisomes. Given that the requirements for glycosomal import are apparently more relaxed than those for peroxisomal import, selective inhibition of this process could represent a therapeutic strategy (222). Glycosomal import is accomplished by two mechanisms. The first relies on a specific C-terminal peptide sequence similar to the peroxisomal targeting signal (PTS1) that is used by peroxisomes to import proteins (223). Whereas peroxisomes have a rather strict requirement for a serine-lysine-leucine (SKL) carboxy-terminal sequence to undergo import by the PTS1 mechanism, kinetoplastids have a much more degenerate C-terminal target signal for the glycosomal import (224). With the SKL sequence as a starting point, S can be any other polar amino acid; K can be replaced by amino acids capable of forming H bonds; and L is replaceable by other hydrophobic amino acids. Blattner et al. found that a C-terminal SSL, which is not a targeting signal for peroxisomal import (225), is sufficient to direct the import of the cytosolic protein β-glucuronidase into glycosomes (226). Thus, it is possible to attain selective targeting of proteins to the glycosomes of trypanosomes vs. the peroxisomes of mammalian species. The second method for

importing proteins into peroxisomes and glycosomes uses the peroxisomal targeting signal (PTS2). The PTS2 targets an N-terminal or internal nonapeptide sequence of general structure H_2N-X-X-R/K-L/V/I-Q-X5-H/Q-L/A for glycosomal import.

Among the 11 T. ***b. brucei*** glycosomal proteins whose primary structures have been determined, seven have the C-terminal tripeptide sequences apparently capable of targeting them for glycosomal import through the PTS1: GAPDH [AKL; (227)], PGI [SHL; (169)], PGK [SSL; (226)], PEPCK [SRL; (228)], G3PDH [SKM; (186)], and PPDK [AKL; (216)]. The other four proteins, HK (156), TIM (175), ALDO (172), and 56-kDa PGK (229), possess C-terminal tripeptide sequences incapable of acting as targeting signals for glycosomal protein import. These proteins have N-terminal or internal peptide(s) PTS2 sequences as glycosomal targeting signals. The N-terminal 10 amino acid peptide in mammalian 3-ketoacyl-CoA thiolase that serves as a targeting signal for peroxisomal import (230) does not function in glycosomal protein import (228), demonstrating that selective targeting or inhibition of the transporter is possible. The recently solved structure of tetrameric aldolases from T. ***b. brucei*** and ***Leishmania*** reveal that two PTS2 nonapeptides interact to form a dimeric structure that could serve as a basis for the design of glycosome import inhibitors (174). A peptidomimetic based on the structure of the C-terminal tripeptide glycosomal targeting signal could function as an inhibitor of glycosomal peptide import and result in a new class of antitrypanosomal agents (231).

The PTS1 or PTS2 sequences of the glycosomal enzymes bind to membrane importer proteins (mPTS) that translocate them into or through the glycosomal membrane. Little is known about the receptor sites on the transporter proteins or the mechanism by which they internalize the glycosomal enzymes (223, 232).

3.3 Inhibitors of the Pentose-Phosphate Shunt

Inhibitors of the pentose-phosphate pathway (PPP) provide another potential target for the development of antitrypanosomal agents (233). The PPP provides NADPH that is essential for many biosynthetic reactions, including the regeneration of reduced trypanothione that is required for protection of the organisms from oxidative stress. A proportion of the first two enzymes of the PPP (glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase) are associated with the glycosome (234,2351, where they provide NADPH for alkoxyphospholipid biosynthesis (143, 236,237).

3.4 Polyamines and Trypanothione

The biosynthetic routes to polyamines and trypanothione are outlined in Figure 19.10. There are at least five validated or highly plausible targets for drug development in the polyamine and trypanothione synthesis pathway. In T. ***brucei*** and ***Leishmania*** ornithine decarboxylase (ODC) and *S*-adenosylmethionine decarboxylase (AdoMetDC) are co-rate-limiting enzymes in the synthesis of spermidine. T. ***cruzi*** lacks ODC activity (238) and obtains putrescine and spermidine from the host by means of inducible high affinity transporters (239). With the exception of T. ***cruzi,*** spermine is not synthesized in trypanosomes and spermidine is the major polyamine that is essential for fulfilling the diverse functions of polyamines in the trypanosome cell (240). In addition, spermidine has the unique function in the Kinetoplastida of serving as a substrate in the biosynthesis of trypanothione, an essential metabolite for the survival of these organisms. Thus, inhibitors of polyamine biosynthesis or polyamine utilization are potentially toxic to the Kinetoplastida.

3.4.1 Inhibitors of Ornithine Decarboxylase. As discussed previously in the section on the mechanism of action of eflornithine (4), the blood form of T. ***b. gambiense*** appears to be uniquely susceptible to inhibition of polyamine synthesis. The susceptibility of this one species may be a result of the slow turnover of ornithine decarboxylase (ODC) in T. ***b. gambiense,*** coupled with the low concentrations of polyamines in the blood, thus negating repletion of polyamines by active transport mechanisms (89). The therapeutic use of eflornithine is limited by its short serum half-life and its

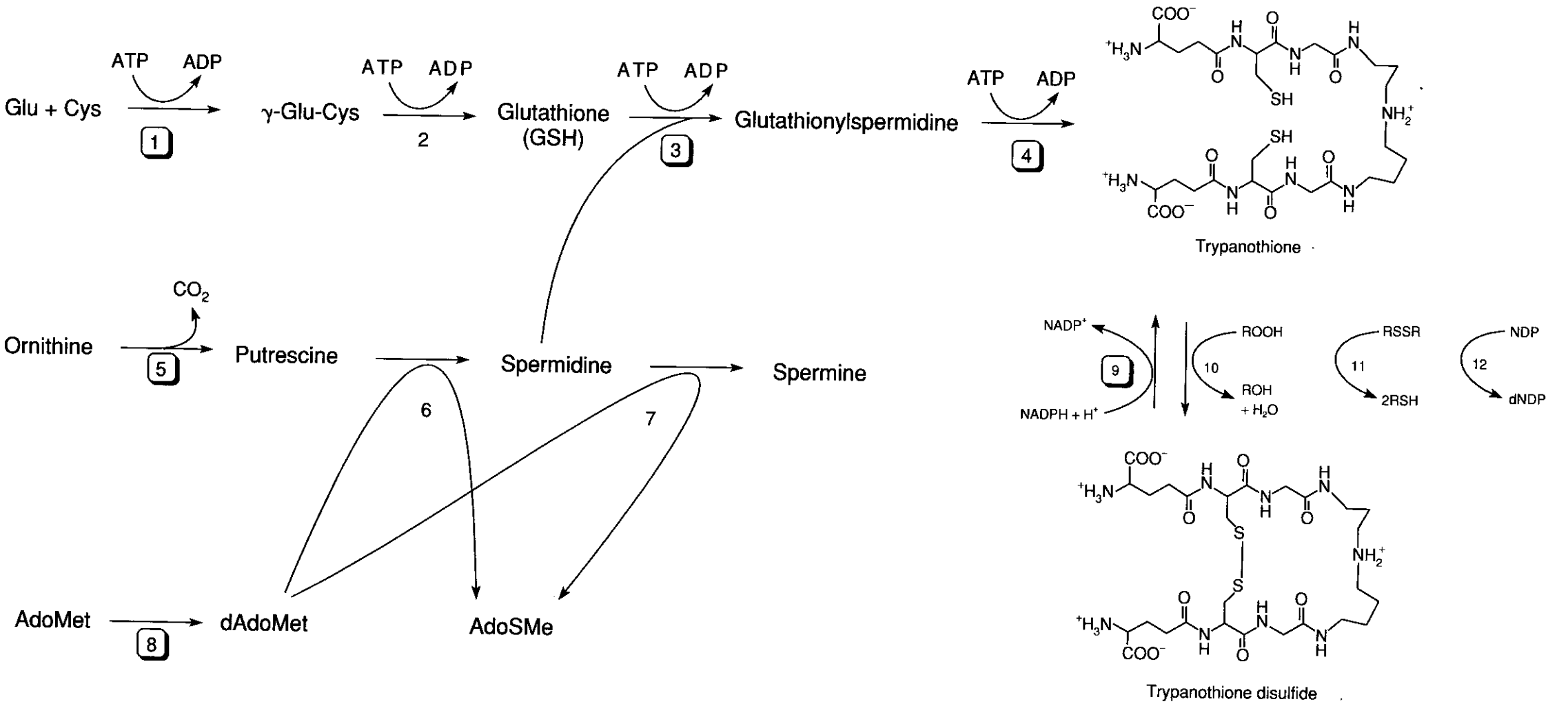


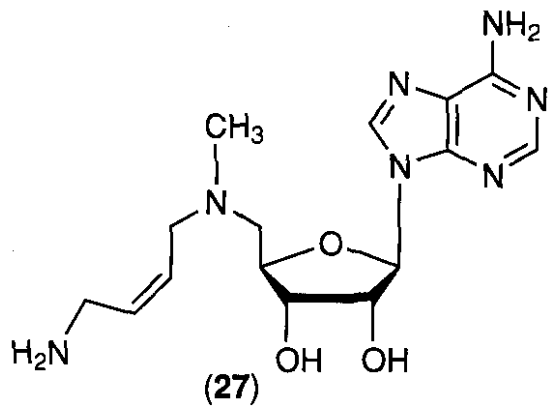
Figure 19.10. The biosynthetic pathways to polyamine and trypanothione in *T. brucei*. GSH, glutathione. Enzymes in boxes are validated targets for drug development: (1) γ-glutamylcysteine synthetase; (2) glutathione synthetase; (3) glutathionylspermidine synthetase; (4) trypanothione synthetase; (5) ornithine decarboxylase; (6) spermidine synthetase; (7) spermine synthetase; (8) AdoMet decarboxylase; (9) trypanothione reductase; (10) tryparedoxin peroxidase; (11) dithiol reductase; (12) ribonucleotide reductase.

poor oral bioavailability. A potent ODC inhibitor with a longer serum half-life and good oral bioavailability would certainly simplify treatment of late-stage T. b. gambiense infection and might be effective against Leishmania species in conjunction with inhibition of the polyamine transporters (241).

3.4.2 Inhibitors of *S*-Adenosylmethionine Decarboxylase and Related Enzymes. AdoMetDC and spermidine synthase in trypanosomes are crucial for the synthesis of spermidine (19) and qualify as potential targets for antitrypanosomal chemotherapy. Trypanosomal AdoMetDC does not cross-react with a human AdoMetDC antiserum, indicating a considerable difference in the structures of the two enzymes (242). A number of adenosine analogs have been prepared that inhibit AdoMetDC, and have shown activity against the Kinetoplastida. MDL 73811, 5'-[(Z-4-amino-2-butenyl)methylamino]-5'-deoxyadenosine (**27**) (Fig. 19.11), is a time-dependent irreversible inhibitor to the AdoMetDC in T. b. brucei, with a K_i value of 1.5 μM and k_{inact} = 0.039 s^{-1} (243). Intracellular concentrations of putrescine and AdoMet were increased and the level of spermidine was decreased in trypanosomes treated with (**27**) (244). Compound (**27**) also proved to be active against trypanosome infections in animal models (245). The drug is efficiently imported into trypanosome to a high intracellular concentration of 1.9 mM by a parasite-specific adenosine transporter not present in mammalian cells (58, 246). The combination of selective import into trypanosomes and irreversible inhibition of AdoMetDC makes (27) worthy of further investigation.

Adenosine analog (28) inhibits AdoMetDC irreversibly, is a substrate for the purine transporter, and has an IC_{50} value of 0.9 μM against T. brucei in *vitro* (247). Nucleotide analogs (29) (hydroxyethylthioadenosine; HETA) and (30) have not been shown to inhibit AdoMetDC. These two agents strongly inhibit protein methylation enzymes (mostly carboxyl methylation) that use AdoMet as a cosubstrate. However, these two agents are transported into trypanosomes by the P1 system and both have submicromolar trypanocidal activity against T. b. brucei and strains of trypanosomes resistant to melarsoprol (248, 249).

Methylglyoxal bis(guanylhydrazone) (MGBG, 31) is a potent, but nonselective AdoMetDC inhibitor with additional antimitochondrial actions that has been used in cancer chemotherapy (250). In the early 1990s, scientists at Ciba-Geigy undertook the synthesis and biological evaluation of a large series of MGBG analogs in the hope of discovering new cancer chemotherapeutic AdoMetDC inhibitors (251–253). None of the compounds has been successfully developed into marketable anticancer agents. Tests for antitrypanosomal activity of the MGBG derivatives revealed a number of compounds that had trypanocidal activity below 0.5 μM in vitro (254), and that the activity was maintained in *vivo* (255). The best of the compounds was CGP 40215A (compound 32; Fig. 19.11), which cured acute infections of T. brucei in mice at a dose $\leq$ 25 mg/kg (255). The structural similarity of (**32**) and the older trypanocides pentamidine (2) and diminazene (7) is significant. All three of these compounds (**32**, **2**, and **7**), as well as AdoMet, use the P2 transporter in trypanosomes for active uptake (38, 256a,b). Once in trypanosomes, the agents all exert a trypanocidal action, although it is far from certain that this action is attributable to inhibition of AdoMetDC (50, 53). Compound (32) remains under development as a potential clinical antitrypanosomal agent. Two additional compounds with bis-guanidine-related structures are in development as antitrypanosomal agents. DB289 (**33b**), the bis(methyl carbamate) prodrug of furamidine (**33a**), is orally active against T. brucei and is one of two drugs being developed by the WHO on a grant from the Bill Gates Foundation (257). The drug is currently in Phase II clinical trials. Furamidine binds tightly to AT sequences in the minor groove of DNA (258a,b); however, its mechanism of antitrypanosomal activity remains undetermined. Trybizine (SIPI 1029, 34) was found to have IC_{50} values of 0.15 to 2.15 nM in four strains of cultured African trypanosomes (259–261). In the mouse in vivo screen, (**34**) cured 12 of 13 trypanosome isolates at a dose of $\leq$10 mg/kg i.p. and it showed



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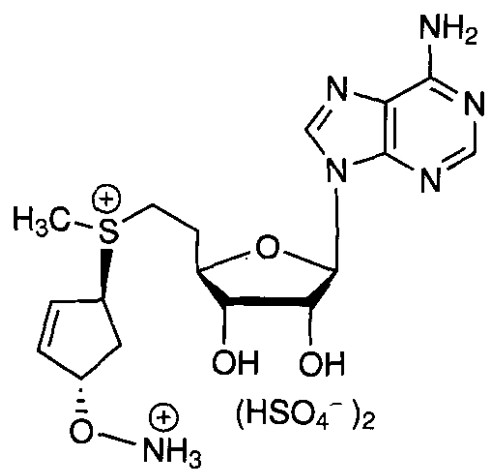

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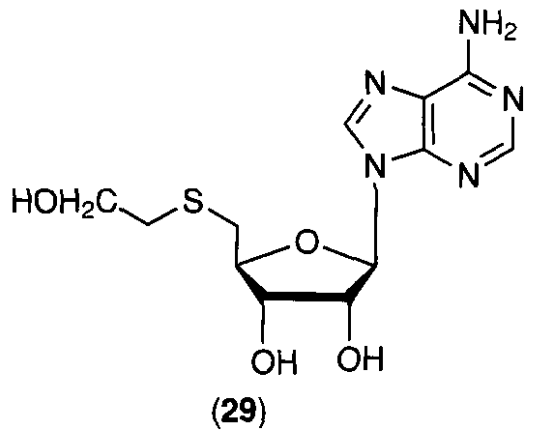

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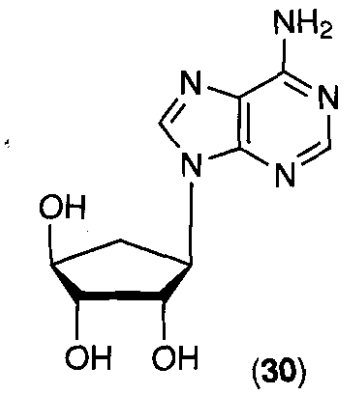

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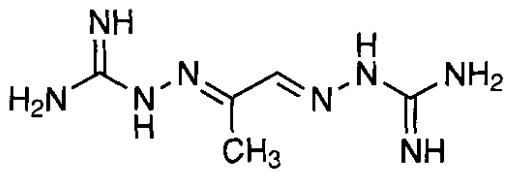
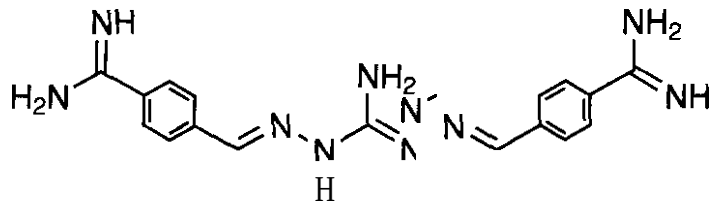
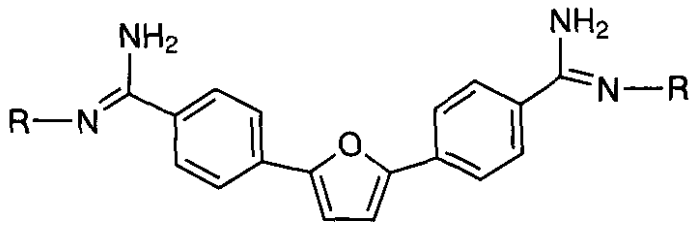


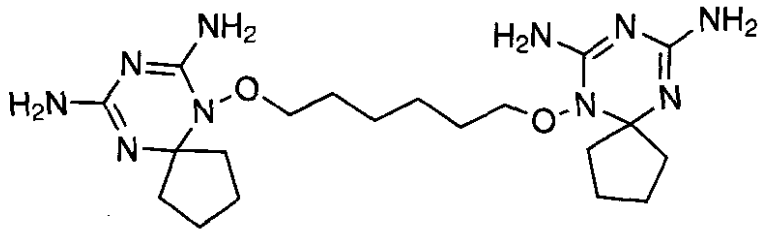
MGBG (31)



CGP 40215A (32)



R,R = H; Furamidine (**33a**)
R,R = -COOMe; DB289 (**33b**)



Trybizine (34)

Figure 19.11. Antitrypanosomal compounds that inhibit AdoMetDC. Several of the compounds have not been shown to be AdoMetDC inhibitors but are included because of their structural similarity to the known AdoMetDC inhibitors in the figure.

oral activity at higher doses. Compound (34) inhibits AdoMetDC, with an IC_{50} value of 38 μM (261).

3.4.3 Inhibition of the Biosynthesis of Trypanothione and of Trypanothione Reductase. Kinetoplastids biosynthesize spermidine and glutathione by the same process as that of mammalian species, yet they do not use glutathione as such. Instead, they conjugate glutathione and spermidine to make a unique substance named trypanothione (262, 263). The enzymes for trypanothione synthesis, glutathionylspermidine synthetase (GspS) and trypanothione synthetase (TryS), and trypanothione reductase (TryR) are unique to these

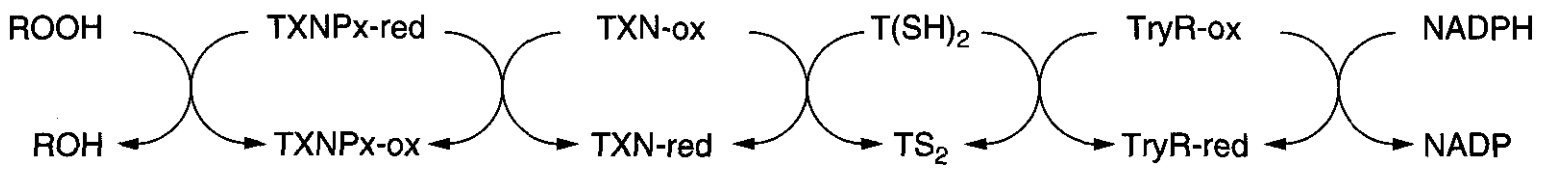


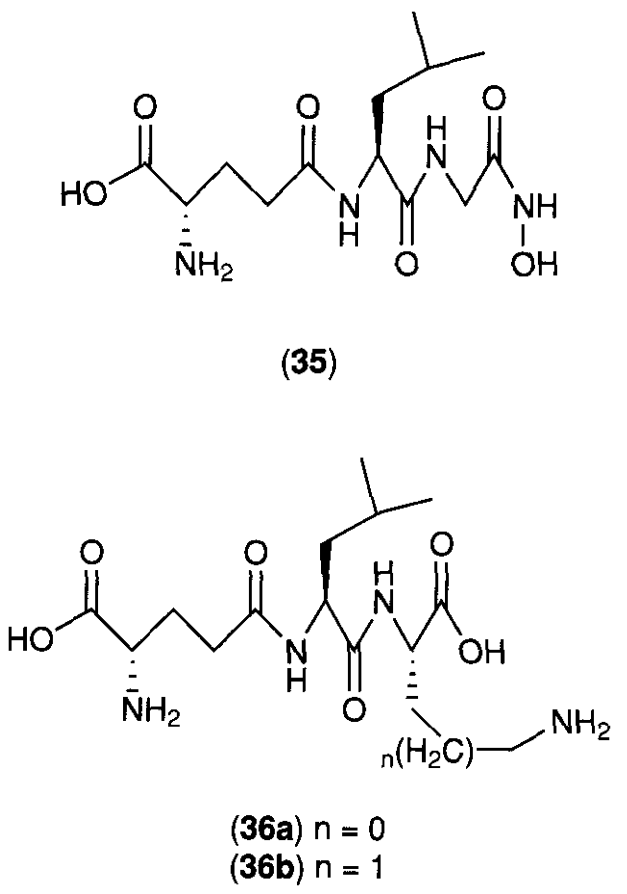
Figure 19.12. The trypanothione peroxidase system for detoxifying peroxides. Enzymes trypanothione reductase (TryR) and tryparedoxin peroxidase (TryP) are boxed. $T(SH)_2$ and $T(S)_2$ are trypanothione and trypanothione disulfide, respectively. $TryX[SH]_2$ and $TryX[S]_2$ are the thioredoxin-like proteins tryparedoxin and tryparedoxin disulfide, respectively. ROOH can be hydrogen peroxide or an alkyl or aryl hydroperoxide.

organisms (for reviews see refs. 264–266). Crystal structures of each protein component of the antioxidant pathway in Figure 19.12 have been determined (267–271). Kinetoplastids use trypanothione for most reactions where glutathione is used in mammalian cells. A major function of trypanothione is the protection of the organisms from oxidative stress (Fig. 19.12). Kinetoplastida lack catalase and are highly dependent on the redox cascade described in Figure 19.12 for neutralization of H_2O_2 and lipid peroxides (272). Tryparedoxin peroxidase and tryparedoxin are members of the thioredoxin family and they may be exploitable as drug targets (see Refs. 273–276 for reviews). The advantage of the biological substitution of trypanothione for glutathione in the kinetoplastids remains unclear. Trypanothione and glutathione have similar redox potentials and thiol-disulfide exchange reactions between them will occur nonenzymatically (265), although trypanothione is slightly more electronegative (−0.242 V for glutathione versus −0.230 V for trypanothione). An additional difference is that the thiols of trypanothione are more acidic than those of glutathione; thus, trypanothione exists more in the thiolate form (15% versus 1% for glutathione) and is more nucleophilic at pH 7.4. Trypanothione remains an effective nucleophile at pH 5.5, whereas glutathione does not (277).

Trypanothione forms stable 1:1 complexes with heavy metal ions such as trivalent arsenicals (61, 62). The stability of the complex is much weaker than that of 2,3-dimercaptopropanol or lipoic acid (61, 62), but probably stronger than with glutathione. The complex between trypanothione and the arsenic of melarsen oxide is believed not to be the major mechanism of action of this drug (63), but may be involved in resistance through export of the trypanothione-arsenical complex (278). Although trypanothione-antimony complexes cannot be separated by HPLC, NMR studies have demonstrated formation of a stable 1:1 complex at pH 7.4, in which trivalent antimony binds to two sulfur atoms and to the oxygen atom of a water molecule (279).

3.4.3.1 ***Inhibitors of Glutathionylspermidine Synthetase.*** The trypanothione biosynthetic enzymes GspS and TryS have been generally accepted as targets for antitrypanosomal chemotherapy. Both enzymes have been cloned and sequenced (280) from Crithidia fmciculata, a kinetoplastid that infects insects. Research on these enzymes has been sparse because of a lack of a ready source of enzymes for study. Oza et al. (281) recently reported the soluble expression of GspS from C. fasciculata in E. *coli*. GspS will function in the reverse direction, hydrolyzing N^1-glutathionylspermidine to glutathione and spermidine. The amidase activity of GspS lies in the N-terminus region of the enzyme and is dependent on a single sulfhydryl group of Cys79. The dual action of the enzyme supports the idea that GspS functions in part as a way to store and retrieve spermidine in the cell, as well as to supply glutathionylspermidine for the synthesis of trypanothione.

The biosynthesis of trypanothione in T. *cruzi* differs from *C.* fasciculata in that a single enzyme with the greatest similarity to TryS from C. fmciculata catalyzes formation of trypanothione from glutathione and spermidine (Oza, Tetaud, and Fairlamb, unpublished results). The enzyme shows less specificity toward the polyamine substrate and can also synthesize the homotrypanothione analog (238) from aminopropylcadaverine and glutathione. Like the Crithidia, GspS, the T.



(35)

(36a) n = 0
(36b) n = 1

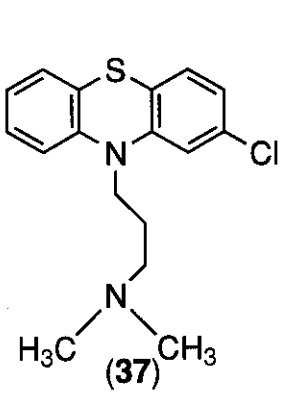
Figure 19.13. Inhibitors of glutathionylspermidine synthetase.

cruzi TryS, displays weak amidase activity with glutathionylspermidine, trypanothione, and homotrypanothione.

Arnssoms et al. (282,283) reported a series of glutathione-like tripeptides (γ-Glu-Leu-Gly-X) as potential inhibitors of GspS. The hydroxamic acid derivatization of the glycine carboxylate (35, Fig. 19.13) has a K_i value of 2.5 μM and substitution of the glycine by diaminopropropanoic acid (**36a**) or lysine (**36b**) gave inhibitors with K_i values of 7.2 and 6.4 μM, respectively.

3.4.3.2 ***Inhibitors of Trypanothione Reductase.*** Trypanothione is an essential metabolite for mediating the redox balance in trypanosomes and trypanothione reductase (TryR) is required to return trypanothione disulfide to its reduced state. TryR uses FAD as a cofactor and NADPH as an electron donor (120). The enzyme was originally purified from C. fasciculata (284) and subsequently from other species (120, 285, 286). Gene sequences are available for C. fasciculata and medically and veterinary-relevant species (287–291). Detailed X-ray characterizations of the three-dimensional structure were completed on the recombinant TryR from C. *fasciculata* (292). The crystal structure, refined to 2.6-Å resolution (293), revealed significant differences from that of glutathione reductase, which accounts for the pronounced discriminatory properties of the enzymes for their disulfide substrates (120,284,285). Subsequently, crystal structures of TryR (268, 294) and TryR complexed with mepacrine (295) from T. cruzi have been reported. The binding site for trypanothione is more open because of the rotations of two helical domains that form part of the active site. In addition, the highly positive charged and hydrophilic region of glutathione reductase, where the glycine carboxylate of glutathione interacts with the enzyme, is replaced by a hydrophobic and negatively charged pocket in trypanothione reductase (292). TryR has a glutamic acid at position 17 that could either form a hydrogen bond with one of the amide linkages between the spermidine and glycine carboxylate (267) or interact with the positively charged secondary amine of spermidine. The marked differences between glutathione and trypanothione reductases in their disulfide binding sites provide an excellent basis for the discovery of specific TryR inhibitors by structure-based drug design methods (296). Validation of TryR as a drug target has been provided by a variety of genetic experiments (297–299), including conditional gene knockouts in T. brucei (300). Agents that are proposed to act as inhibitors of TryR are shown in Fig. 19.14.

A number of tricyclic analogs were selected from the computer model of TryR and docking experiments with phenothiazine-based structures (301). Compounds from this lead were obtained or synthesized and evaluated for inhibitory activity on TryR. The first strong lead for an inhibitor was chlorpromazine (**37**), which has an IC_{50} value of 35 μM against recombinant trypanothione reductase from T. cruzi (302). Open-ring compound (38) was made in an attempt to separate the neuroleptic properties of (37) from its TryR inhibition activity and resulted in a slight increase in TryR inhibitory activity (46% inhibition of TryR from T. cruzi versus 16% for 37) (303). A series of tricyclic agents related to the antidepressant drug clomipramine (39) was prepared and evaluated for TryR inhibitory activity. Clomipramine (IC_{50} = 6.5 μM) was the most active compound against TryR from T.



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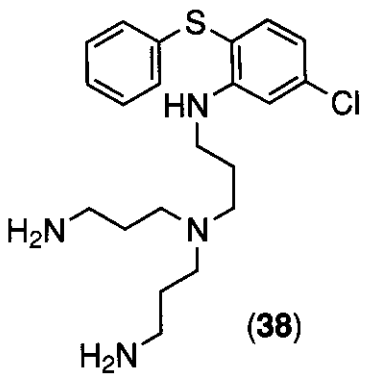

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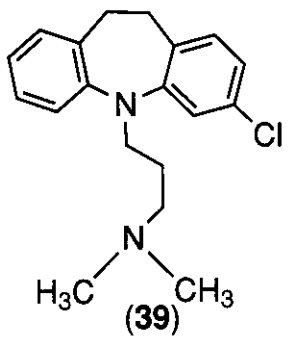

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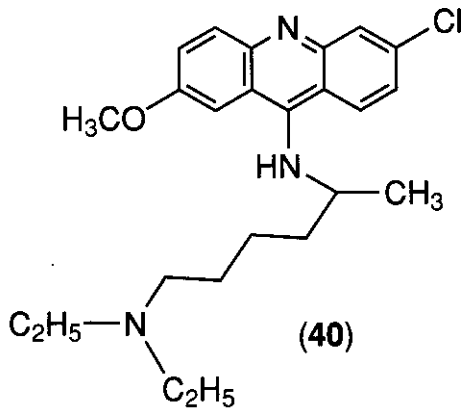

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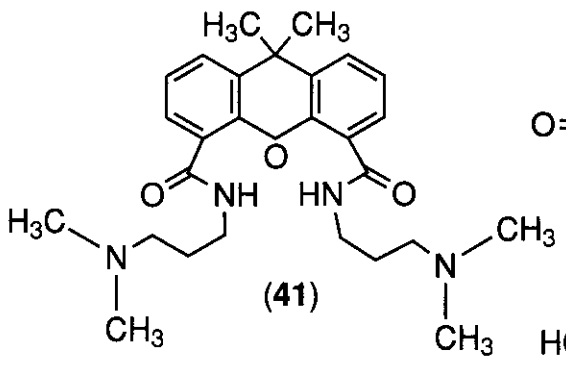

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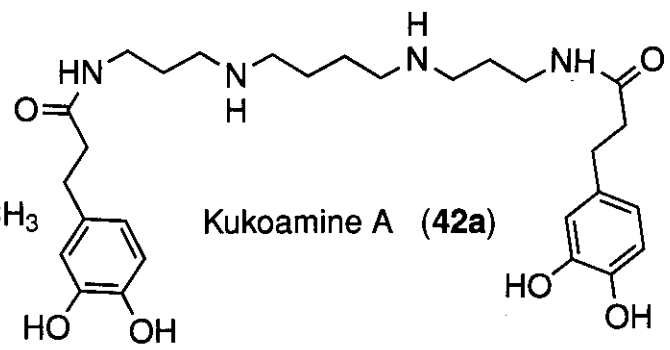
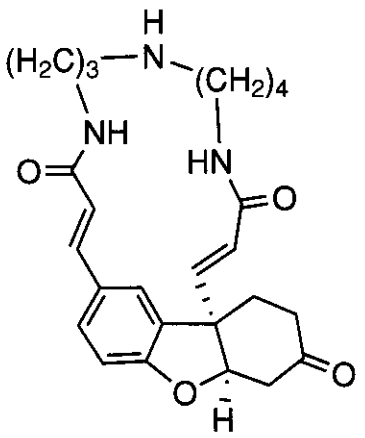
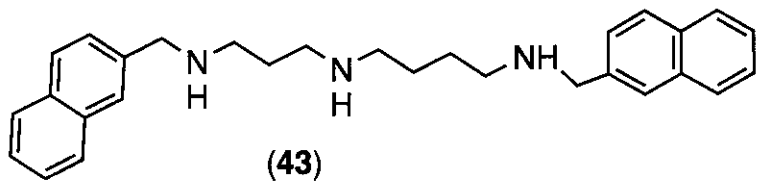


Kukoamine A (**42a**)



Lunarine (**42b**)



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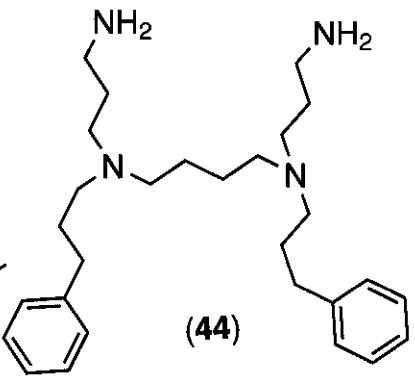

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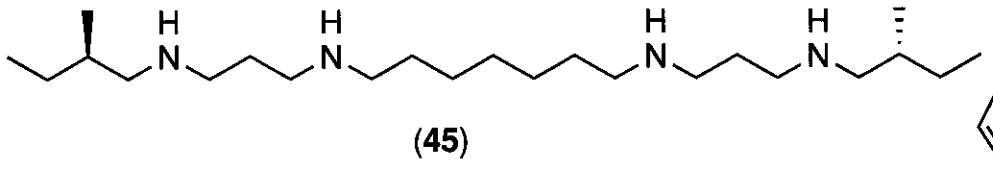

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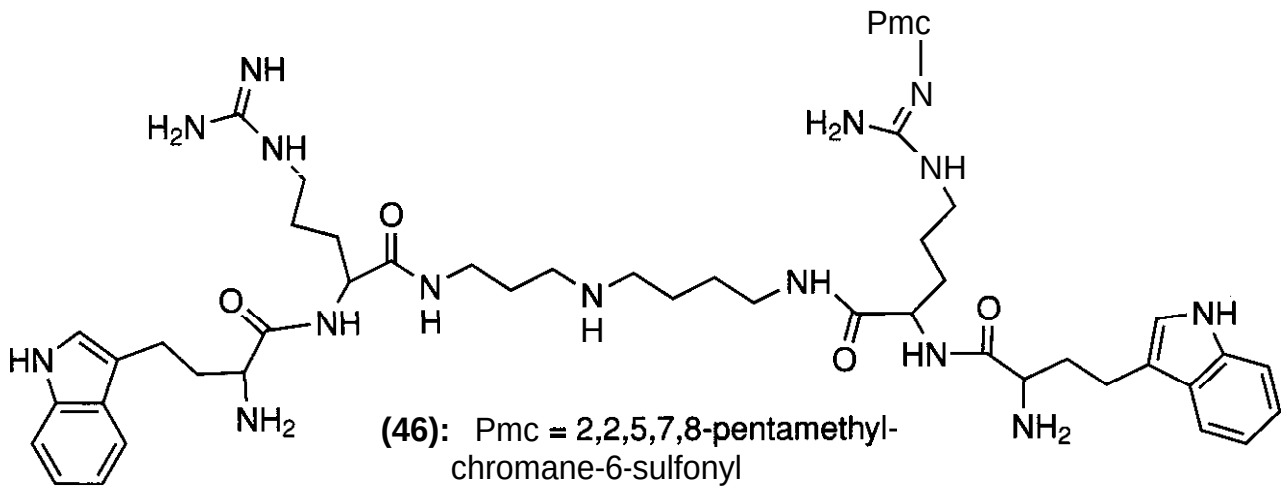
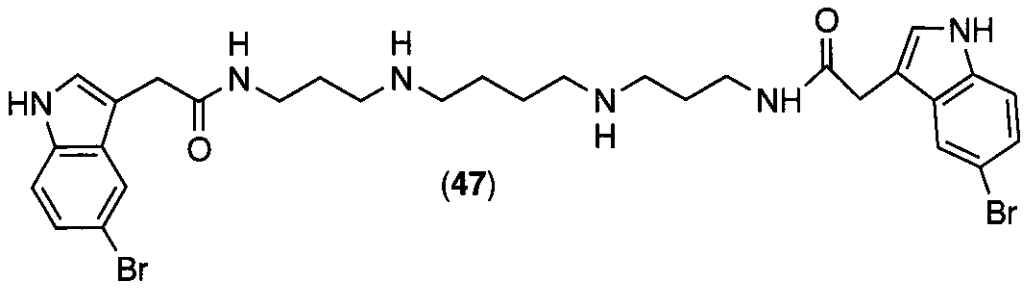


(46): Pmc = 2,2,5,7,8-pentamethyl-chromane-6-sulfonyl



(47)

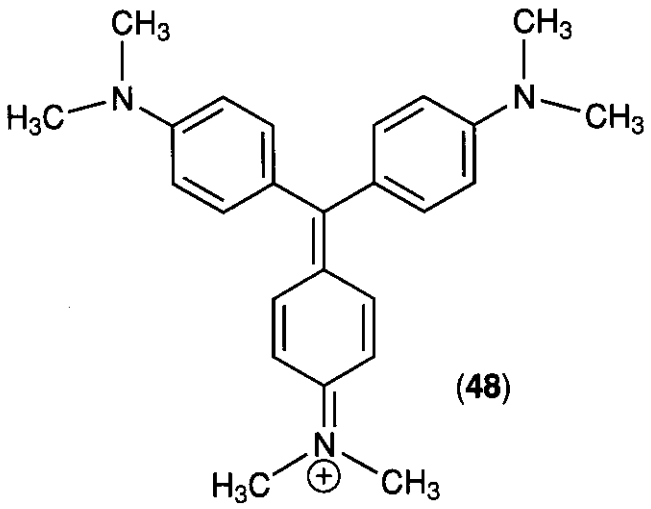
Figure 19.14. Inhibitors of trypanothione reductase.

cruzi (304). Tricyclic acridine derivatives related to known antiparasitic drugs were investigated for TryR inhibitory activity. The most active compound found was the mepacrine homolog (**40**), which has a K_i value of 5.5 μM, although simple acridines lacking the entire aminoalkyl substituent from the 9 amino group were nearly as potent (305). The 9-aminoacridines related to **(38)** were competitive inhibitors of TryR, with more than one molecule of inhibitor able to bind to the enzyme. Two 9-thioacridine compounds were prepared and gave K_i values similar to those of the 9-aminoacridines but they exhibited mixed-type kinetics. These findings demonstrate the difficulty in drug design through the use of computer-aided structure fitting or in the development of QSAR for TR inhibitors (305). Chibale et al. (306) prepared 9,9-dimethylxanthines as potential TryR inhibitors. The most active compound in the series was (**41**) (K_i = 13.8 μM).

A popular approach to the preparation of TryR inhibitors has been the substitution of various groups onto a polyarnine scaffold. Natural product lead compounds kukoamine A (**42a**) and lunarine (**42b**) are analogs of spermine and have K_i values of 1.8 μM (307) and 144 μM (k_{inact} = 0.116 s^{-1}) against TryR from T. *cruzi*, respectively (267). O'Sullivan et al. (308, 309) reported several series of aryl alkyl spermidine derivatives. Of the compounds prepared, (**43**) (K_i = 9.5 **a**) and (**44**) (K_i = 3.5 μM) were the most active. A series of bis-alkyl and aryl-alkyl terminal N-substituted spermine-related compounds, having a 3–7-3 pattern of hydrocarbon chain lengths between the nitrogen atoms, has been prepared. Compound (**45**) is one of the most potent of the compounds, having IC$_{50}$ values of 40 and 165 nM against the K243 and resistant K243-As-10–3 strains of trypanosomes, respectively (310). The N^1,N^{17}-bis(benzyl) analog of (45) was nearly as potent. Smith and Bradley (311) prepared three 576-compound libraries of spermidine-amino acid conjugates and screened the compounds for their ability to inhibit TryR. Compound (46) was the most potent, having a K_i value of 100 nM. This work was followed by additional solid-phase synthesis attempting to optimize the activity of the lead compounds. The N^1,N^{12}-bis-(5-bromo-3-indole acetic acid) amide derivative of spermine (47) had a K_i value of 76 nM when tested against TryR from T. *cruzi* (312).

For compounds in the studies described in the preceding paragraph, it should be noted that there is not a good correlation between their ability to inhibit TryR and their toxicity against cultured trypanosomes. Thus, the mechanism by which these compounds exert a trypanocidal action remains in doubt and multiple mechanisms of activity may be involved. In addition, all of the compounds that have been tested *in vivo* for their ability to cure mice acutely infected with trypanosomes have yielded disappointing results. It is not known whether the lack of *in vivo* potency is attributed to poor uptake by trypanosomes, to rapid metabolism of the compounds by the host, or to other unidentified factors. It should be borne in mind that strictly competitive inhibitors of low nanomolar potency may be required to maintain high levels of inhibition in the face of possibly millimolar levels of trypanothione disulfide accumulating intracellularly as a result of effective inhibition of TryR. Irreversible tight-binding inhibitions may be required. Certainly, the inability of medicinal chemists to find a drug to attack what appears to be a prime target for selective toxicity is frustrating.

3.4.3.3 ***Subversive Substrates.*** Henderson et al. attempted the design of subversive substrates for TryR (104). The toxic action of bleomycin on cancer cells might be considered an analogy to the subversive substrate approach to drug design (313). The objective of this strategy is to find a compound that will bind to the active site of the target enzyme (TryR in this case) and subvert electrons from their normal flow from NADPH by way of FAD to trypanothione disulfide (Fig. 19.12). The compound should accept electrons from the FAD cofactor of TryR and divert them into a redox cycling-based mechanism of cell toxicity, as described in Fig. 19.6. The resulting oxygen-derived free radicals would damage the enzyme, or important structures in the surrounding cell, and result in a trypanocidal action. In addition, the normal function of the redox cascade from NADPH to peroxides is disrupted, which in itself might be sufficient for a trypanocidal action. This method of at-



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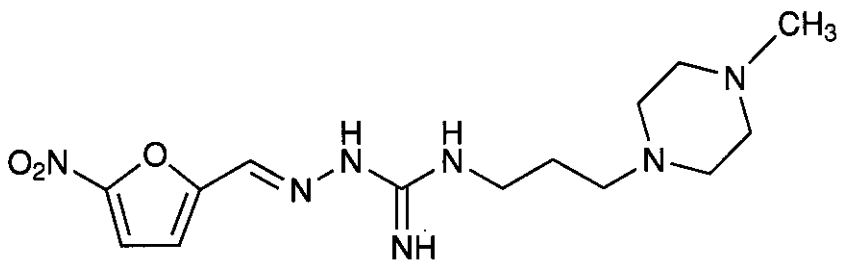

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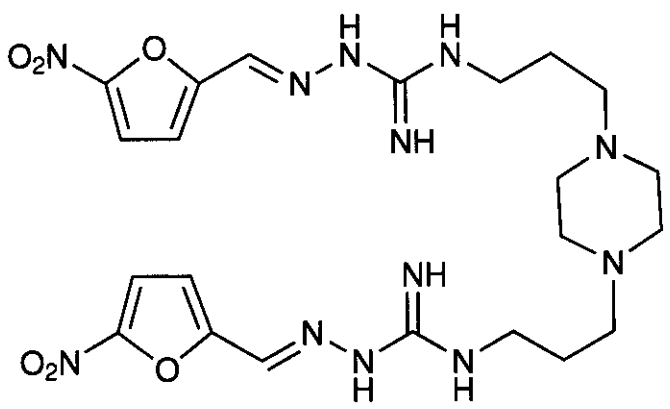

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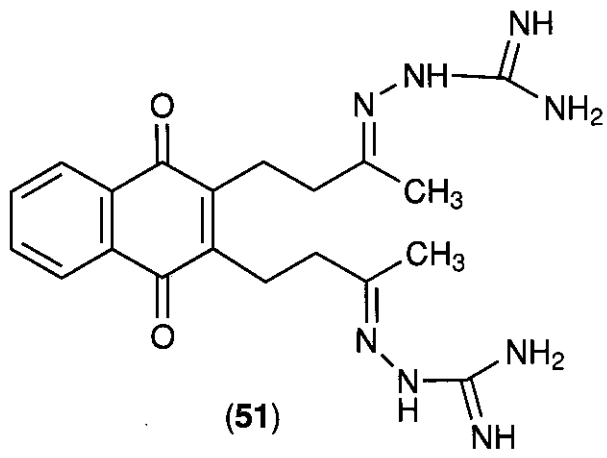

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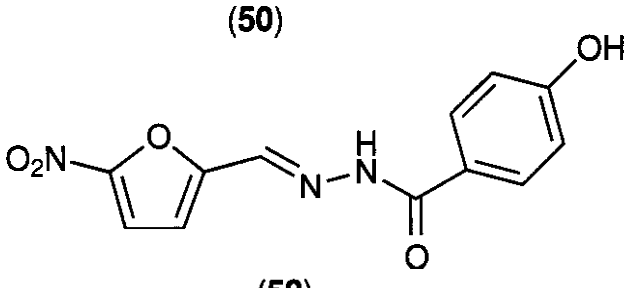

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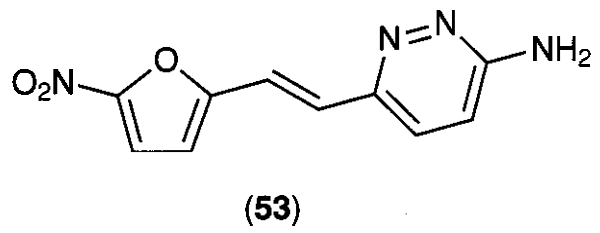

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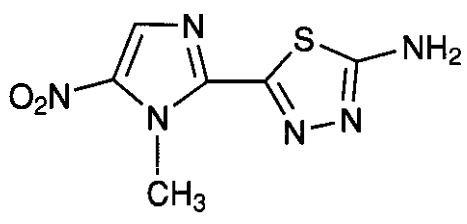
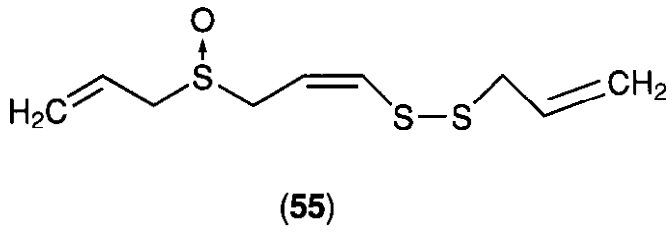


Megazol (54)



(55)

Figure 19.15. Subversive substrates.

tack seemed reasonable on the basis of the known antitrypanosomal activity of compounds like nifurtimox **(12)** and benznidazole **(13).** Structures of other agents that may have a mechanism of action by serving as subversive substrates are given in Fig. 19.15. Interestingly, crystal violet **(48)** is a potent inducer of oxidative stress and has long been used in blood banking to kill T. *cruzi* trypomastigotes and thus prevent the spread of Chagas' disease through blood transfusions (314).

Attempts to find an effective trypanocidal subversive substrate for TryR have been only partially successful. In the initial work, two nitrofuran derivatives **(49** and **50)** and the naphthoquinone **(51)** were identified as

trypanocidal subversive substrates. All three of these compounds were effective at a concentration range of 1–5 μM in preventing T. cruzi trypomastigotes from infecting human saphenous vein smooth muscle cells in culture (104). None of the compounds is very active in in vivo screens for trypanocidal activity. Other nitrofuran derivatives such as nifuroxazide (52) and nifurprazine (53) are effective subversive substrates for TryR and possess trypanocidal activity in vitro (315). Cenas et al. (316) published on the mechanism of reduction of quinones by TryR from T. congolense. A library of 1360 1,4-naphthquinone derivatives was synthesized and tested for activity against TryR from T. cruzi. The most potent compounds from this series had IC_{50} values of 0.3–1.1 μM and were highly selective for TryR vs. glutathione reductase (317).

Megazol (**54**), structurally related to the nitrofurans and other subversive substrates, is an important compound that is in development as an antitrypanosomal agent. The mechanism of the drug is not known, but it probably is associated with its ability to undergo cyclical one-electron reduction, thereby generating reactive oxygen species in *vivo*. Megazol has not been shown to inhibit any specific trypanosomal enzyme. Megazol is effective in curing T. *b.* brucei infections in mice (318) and is effective in combination with melarsoprol (**319**) and suramin (320). Megazol owes part of its effectiveness to its active uptake into trypanosomes by the P2 transporter (321). Studies on the pharmacokinetics, metabolism, and excretion of megazol have been published (322, 323).

Ajoene (**55**), a component of garlic, has been found to be an effective irreversible inhibitor of both TryR and glutathione reductase. Ajoene forms a mixed disulfide bond with Cys58 of TryR (324). In addition, ajoene is a substrate for TryR and leads to the accumulation of reactive oxygen species.

3.5 Inhibitors of Lipid and Clycolipid Metabolism

With the exception of bloodstream T. brucei, which obtains cholesterol from host LDL, the sterol biosynthesis in other kinetoplastids resembles that of fungi much more closely than that of mammalian species. Ergosterol is the major sterol used by this order in membrane stabilization. Thus, agents that have proved to be effective in treating systemic fungal infections are worthy of investigation for activity against T. cruzi and Leishmania spp. Amphotericin B (**18**) has proved to be highly effective for the treatment of visceral leishmaniasis. Structures of agents acting as inhibitors of sterol and lipid biosynthesis are given in Figure 19.16.

3.5.1 Inhibitors of 14α-Demethylase and Other Enzymes Related to Sterol Biosynthesis. The azole class of inhibitors of 14α-demethylase, blocking the conversion of lanosterol to 4,4-dimethylcholesta-8,14,24-triol, in the biosynthetic pathway to ergosterol, has yielded varied effectiveness in the treatment of leishmaniasis and Chagas' disease. The gene for 14α-demethylase from T. brucei has been cloned and characterized (325). In most cases, treatment with an azole agent slows progression of the disease but rarely results in a long-term cure (see Refs. 326–328 and references cited therein). Recent results with newer agents and combination therapy are more encouraging. Alrajhi et al. (328) reported the results of a clinical trial of a 6-week oral fluconazole (56) treatment of cutaneous infections caused by L. major. The median time to healing in the fluconazole treated group was 8.5 weeks compared to 11.2 weeks in the placebo group ($P < 0.001$). It was concluded that the fluconazole therapy was safe and useful. Ketoconazole (57) has been shown to have a synergistic effect in combination with lysophospholipid analogs related to miltefosine (17) against the epimastigote form of T. cruzi grown in culture (142). A combination of benznidazole (**13**) and (**57**) was found to be effective in curing resistant T. cruzi infections in mouse model experiments (329). Third-generation triazole antifungal agents have a more potent trypanocidal action on T. cruzi than the older triazoles. Urbina et al. (330) found that DO870 (58) is able to cure acute and chronic T. cruzi infections in mice and were the first compounds ever shown to have this activity. Resistant strains of T. cruzi were cured in mice by (58) incorporated into polyethyleneglycol-polylactide nanospheres (331). Unfortunately, the clinical development of (58) has been dis-

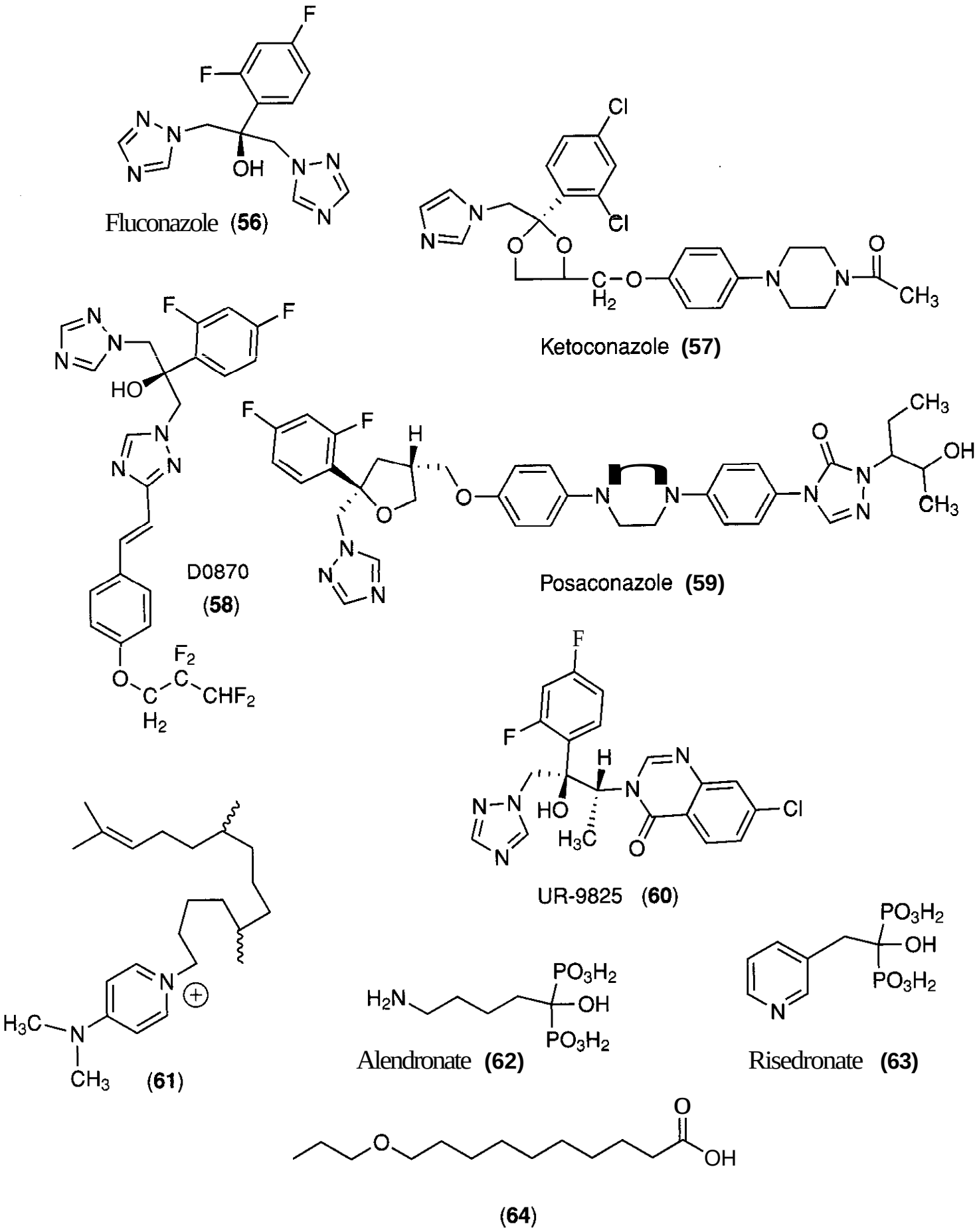


Figure 19.16. Investigational agents affecting lipid metabolism.

continued by Zeneca because of adverse reactions. Posaconazole (**59**), another third-generation triazole, has shown activity similar to that of **(58)** and clinical trials with this agent are planned **(332, 333).** The investigational triazole agent **UR-9825** (60) also has been found to be extremely potent, with MIC values of 30 and **10** n*M* against **T.** *cruzi* epimastigotes and amastigotes in cell culture, respectively (334).

Other enzymes in the sterol pathway may offer targets for antitrypanosomal chemother-

apeutic agents. Urbina et al. (335) and Rodrigues et al. (336) reported that a 22,26-azasterol analog has potent inhibitory activity against delta$^{24(25)}$ sterol methyl transferase from T. ***cruzi.*** Sterol biosynthesis is disrupted and parasites die when exposed to 100 n*M* concentration of inhibitor in the culture medium. Compound (**61**), an inhibitor of oxidosqualene cyclase, the enzyme that converts 2,3-oxidosqualene to lanosterol, had ED$_{50}$ values of 2–6 n*M* against four strains of T. ***cruzi*** in cell culture, whereas the ED$_{50}$ value for inhibition of murine 373 fibroblasts was 880 n*M* (337).

3.5.2 Inhibitiors of Prenylation and Protein Anchoring. Bisphosphonates, used medicinally as inhibitors of bone resorption, have been found to be active both ***in vitro*** and ***in vivo*** against kinetoplastids at concentrations causing little toxicity to host cells (338). The mode of action of the bisphosphonates is thought to involve the inhibition of prenyldiphosphate synthetase and the prenylation of proteins (339–342). The selectivity of the bisphosphonates may be attributed in part to their accumulation in acidocalcisomes, organelles storing high concentrations of pyrophosphate that are specific to trypanosomatids and apicomplexan parasites (338, 343). Alendronate (62) and risedronate (63) were among the most active bisphosphonates tested for inhibition of T. ***cruzi*** farnesyl pyrophosphate synthetase, having IC$_{50}$ values of 0.77 and 0.037 μM, respectively (342).

The variant surface glycoprotein (VSG) coat of bloodstream African trypanosomes is anchored to the cell surface by a glycosyl phosphatidylinositol (GPI) that contains myristate as its only fatty acid component (344,345). *T. **brucei*** has several hundred VSG genes, only a fraction of which have been cloned (346). In a process termed fatty acid remodeling, two fatty acids with longer carbon chains than those of myristate on the GPI precursor are sequentially replaced by two myristate molecules (347). Alternatively, myristoylation can occur by an acyl exchange reaction. Antigenic variations enable the trypanosomes to evade the host immune response and are essential for parasite survival. The process of GPI myristate exchange or fatty acid remodeling does not occur in humans. Thus, a drug that would block the process of fatty acid remodeling, acyl exchange, or replace the myristate with a dysfunctional fatty acid might be an effective trypanocidal agent. Doering et al. (348) studied a set of 244 myristate analogs, on the basis of 11-oxatetradecanoic acid (64) as a lead compound, for their ability to incorporate into the GPI both in a cell-free system and in intact trypanosomes. Twenty compounds that were toxic to trypanosomes at 10 μM were discovered from the series. Compound (64) had an LD$_{50}$ value for the cultured T. ***brucei*** of 1.0 μM and some of the compounds in the series were even more toxic to trypanosome growth. None was subsequently found to be active in mice. The trypanocidal activity of the compounds correlated roughly with their degree of incorporation into the VSG; however, the mechanism of action for the compounds could not be determined with certainty.

In a study related to the VSG, Roper et al. (349) cloned the gene UDP-glucose-4'-epimerase, which is responsible for the conversion of UDP-glucose to UDP-galactose in T. ***brucei.*** *A* UDP-glucose-4'-epimerase knockout in T. ***brucei*** was also constructed. The hexose transporter of T. ***brucei*** will not carry galactose and it was believed that this enzyme might be the only source of galactose in the trypanosome. Galactose is an essential component of the VSG coat. Deletion of UDP-glucose-4'-epimerase was lethal to the trypanosomes, thus validating the enzyme as a target for drug design.

3.6 Cysteine Protease Inhibitors

The major proteases of the order Kinetoplastida are of the cathepsin B-like and cathepsin L-like subfamilies of the papain family of cysteine proteases. Sajid and McKerrow (350) published an excellent review of this topic. T. ***cruzi*** expresses a large amount of cysteine protease most closely related to cathepsin L. The enzyme has been named cruzain and is synonymous with cruzipain. Cruzain is expressed in varying amounts in all life forms to T. ***cruzi*** (351, 352). Chagasin, a 12-kDa protein isolated from T. ***cruzi*** epimastigote lysates, is a tight-binding inhibitor of cruzain and apparently functions as a natural regulator of cruzain's activity (353). Cruzain has been cloned (354) and the three-dimensional structure determined (355,356). Cruzain has been

validated as a target for drug development in studies by Engel et al. (357). The catalytic mechanism of the cysteine proteases is well understood from extensive studies on papain and related enzymes. Resistance to cysteine protease inhibitors can be induced in T. ***cruzi*** through the mechanism of enhancement of a protein secretory pathway (358).

T. brucei expresses a cysteine protease, of the cathepsin L subfamily, similar to cruzain. The enzyme, called brucipain (or trypanopain-Tb), has been cloned and expressed in E. ***coli*** (359). Brucipain and cruzain have a 59% sequence homology and a approximately 45% homology with mammalian cathepsin L enzymes. Brucipain has been validated as a target for drug development (360); however, selectivity for parasite vs. host enzymes remains a problem.

Leishmania spp. express three genes for cysteine proteases: the cathepsin L-like and cathepsin B-like proteases plus calpain-like protease. Inhibitors effective on cruzain and brucipain are effective on the enzyme(s) from ***Leishmania*** spp. (361), but usually less active on this species because of the multiple forms of the proteases present.

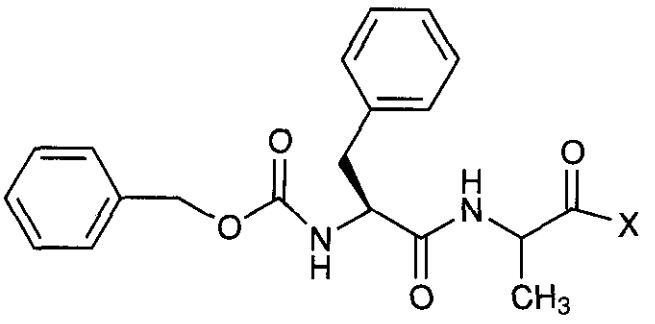
A notable difference between mammalian and Kinetoplastid cathepsin enzymes lies in the carboxy-terminus, where the kinetoplastid enzyrnes have an extension of about a 100 amino acids, made up of polythreonine residues in cruzain and polyproline residues in brucipain. The function of the additional amino acids is not known, but they are not necessary for the catalytic activity of the enzymes. An additional difference of the parasitic enzymes is their pH-activity profile. Human cathepsin L resides predominantly in lysosomes and has a pH maximum at 4.5, whereas cruzain functions well at higher pH and has a pH maximum near 8.0 (350).

Structures of investigational agents that work through a mechanism of cysteine protease inhibition are given in Figure 19.17. The first cysteine protease inhibitors shown to have activity against trypanosomes were the dipeptide fluoromethylketone derivative (**65a**) and the related diazomethylketone (**65b**) (360, 362). Compound (**65b**) was the more active compound ($ED_{,,}$ = 0.18 μM) against T. ***brucei*** cultures. These compounds were able to cure parasite infections in mice but produced unacceptable toxicities at high doses (350). A series of dipeptidyl-epoxide derivatives were investigated for their trypanocidal activity. Epoxide (66) had an IC_{50} value of 10 nM against cruzain, but no activity of the compound toward trypanosomes has been reported (363). The most promising trypanocidal cysteine protease inhibitor reported to date is the vinyl sulfone (67) (364a,b). This drug is highly effective in curing acute and chronic T. ***cruzi*** in mice. Compound (67) showed little or no toxicity to the animals at the treatment doses employed. It is projected that (67) will enter clinical trials for the treatment of Chagas' disease in the near future (350). Huang et al. (365) reported a series of mercaptomethyl ketones as potent and selective inhibitors of cruzain. Compound (68) had K_i values of 1.1, 1700, and 144 nM for recombinant cruzain, cathepsin B, and cathepsin L, respectively. Compound (68) showed time-dependent inhibition and tight binding to cruzain.

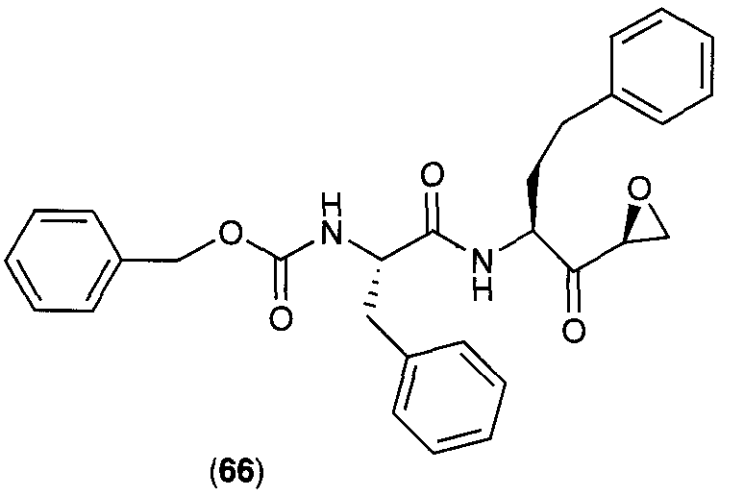
Compounds (**65–68**) are all irreversible inhibitors of cysteine proteases. In an attempt to discover competitive inhibitors for the enzyme, an ***in silico*** docking search of a chemical databank for fit to the cruzain active site was performed. Lead compounds have been prepared and tested, and potency optimization is being pursued. The most potent compounds reported from these studies to date are the hydrazides (69) and (**70**), which have $ED_{,,}$ values against bloodstream T. ***brucei in vitro*** of 0.3 and 0.6 μM, respectively (366).

3.7 Inhibitors of Dihydrofolate Reductase/Pteridine Reductase

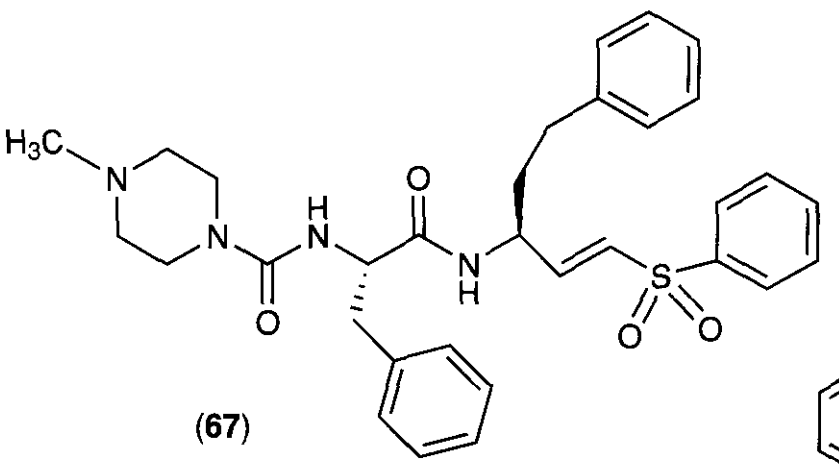
It has been determined that ***Leishmania*** spp. are resistant to dihydrofolate reductase (DHFR) inhibitors because they amplify the production of pteridine reductase (PTR), which can reduce folates and bypass the inhibition of DHFR (367). An attempt to find compounds capable of inhibiting these enzymes simultaneously led to the discovery of structures that inhibit both DHFR and PTR (368). Compound (71) (Fig. 19.18) was one of several compounds that showed submicromolar IC_{50} values against PTR, DHFR, and cultured ***L. major.*** Gilbert's group used the Cambridge



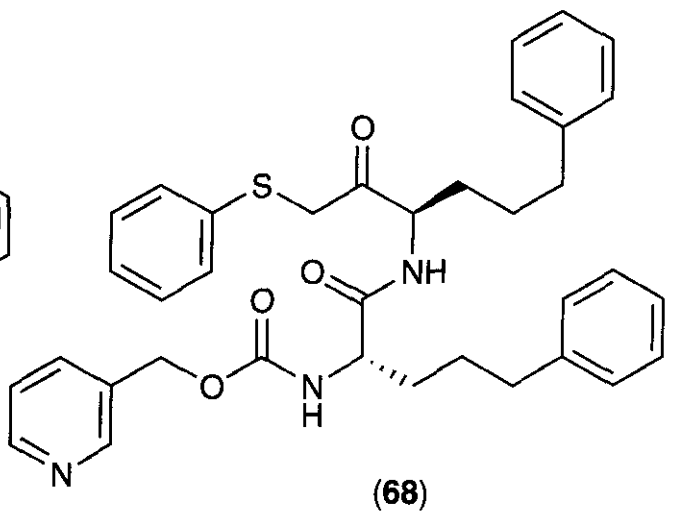
(**65a**), X = CH_2F
(**65b**), X = CHN_2



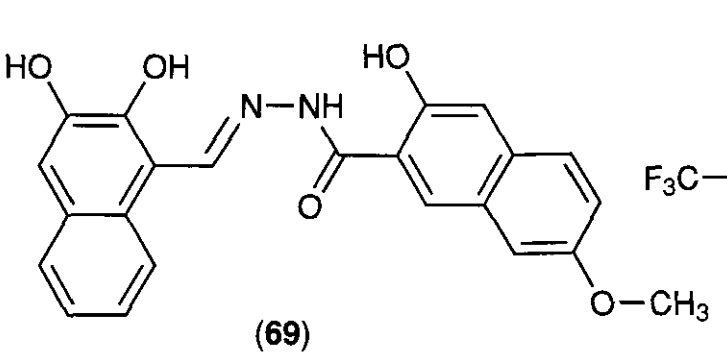
(**66**)



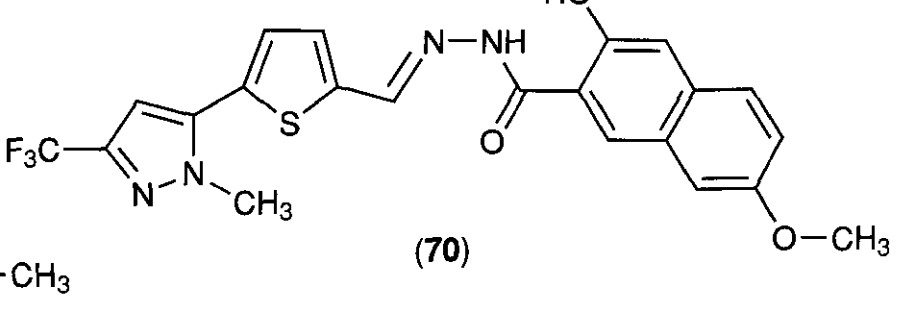
(**67**)



(**68**)



(**69**)



(**70**)

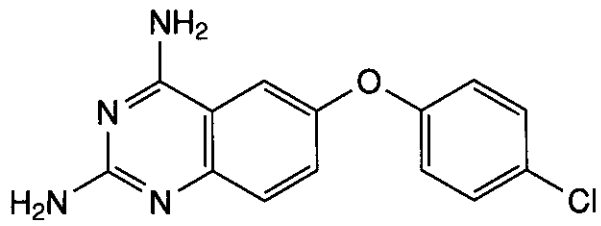
Figure 19.17. Cysteine protease inhibitors effective as trypanocides.

Structural Database and the computer program Dock **3.5** to perform a virtual screen on a homology model of T. cruzi DHFR. Leads from the virtual screen were tested for activity against T. ***cruzi*** DHFR and the most potent compounds were assayed against cultured T. ***b. rhodesiense.*** Structures **(72)** and **(73)** have been identified as two of the more active compounds, each having activity in the low micromolar range **(369, 370).**

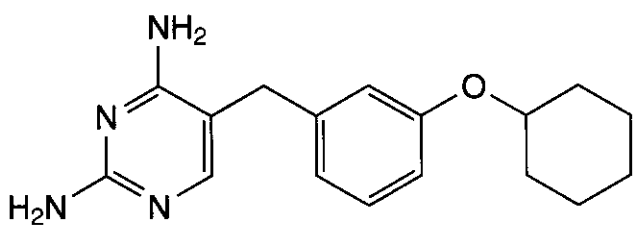
3.8 Inhibition of Purine Interconversions

African trypanosomes lack the capability of de *novo* synthesis of purine nucleotides **(371).** Carter and Fairlamb **(58)** characterized P1 and P2 adenosine transporters in T. ***b. brucei*** that function to scavenger purines from the serum of the host. Melarsen oxide, pentamidine, megazol, and other useful antitrypanosomal agents use the P1 or **P2** transporters to concentrate their toxic effects in the trypanosomal cells. In addition to the use of the transporters for drug targeting, it has been proposed that potent inhibitors to the transporters might yield trypanocidal drugs. However, the multiplicity of nucleoside and nucleobase transporters make this an extremely challenging proposition.

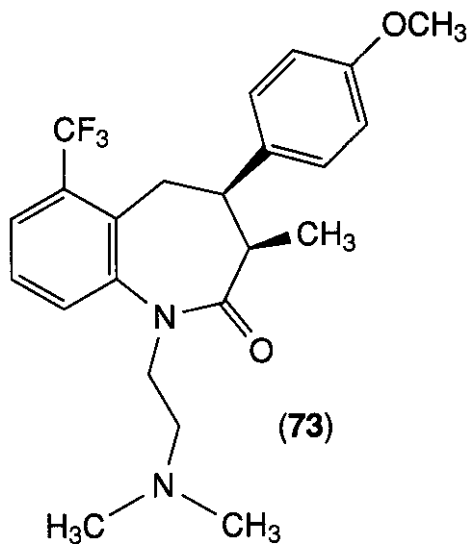
Once inside the cell, the purines must be converted to useful nucleotides by the purine salvage enzymes in trypanosomes. Thus, the purine salvage enzymes are credible targets



(71)



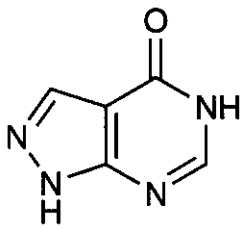
(72)



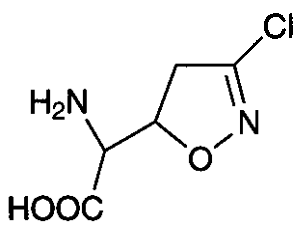
(73)

Figure 19.18. Inhibitors of trypanosomal dihydrofolate reductase.

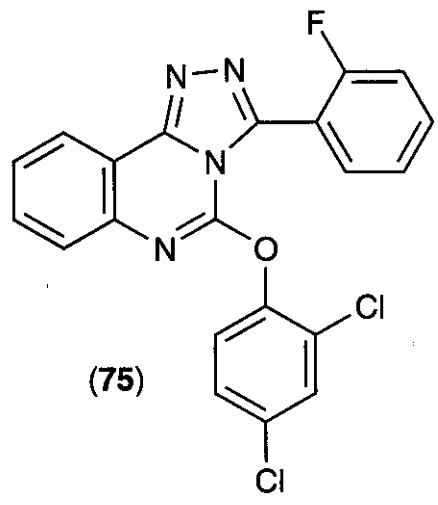
for antitrypanosomal chemotherapy. Again, the considerable redundancy in the salvage and interconversion pathways presents a major challenge for drug design. To date, the most successful purine analogs are prodrugs that are metabolically activated through several enzyme-catalyzed steps. The structures of several drugs that affect purine or pyrimidine interconversions are shown in Fig. 19.19. The most successful example of this approach was the identification of antileishmanial activity in allopurinol (74) (372). Allopurinol is a sub-



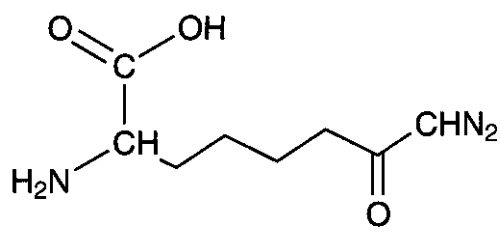
Allopurinol
(74)



Acivicin
(76)



(75)



DON (77)

Figure 19.19. Inhibitors of purine and pyrimidine interconversion.

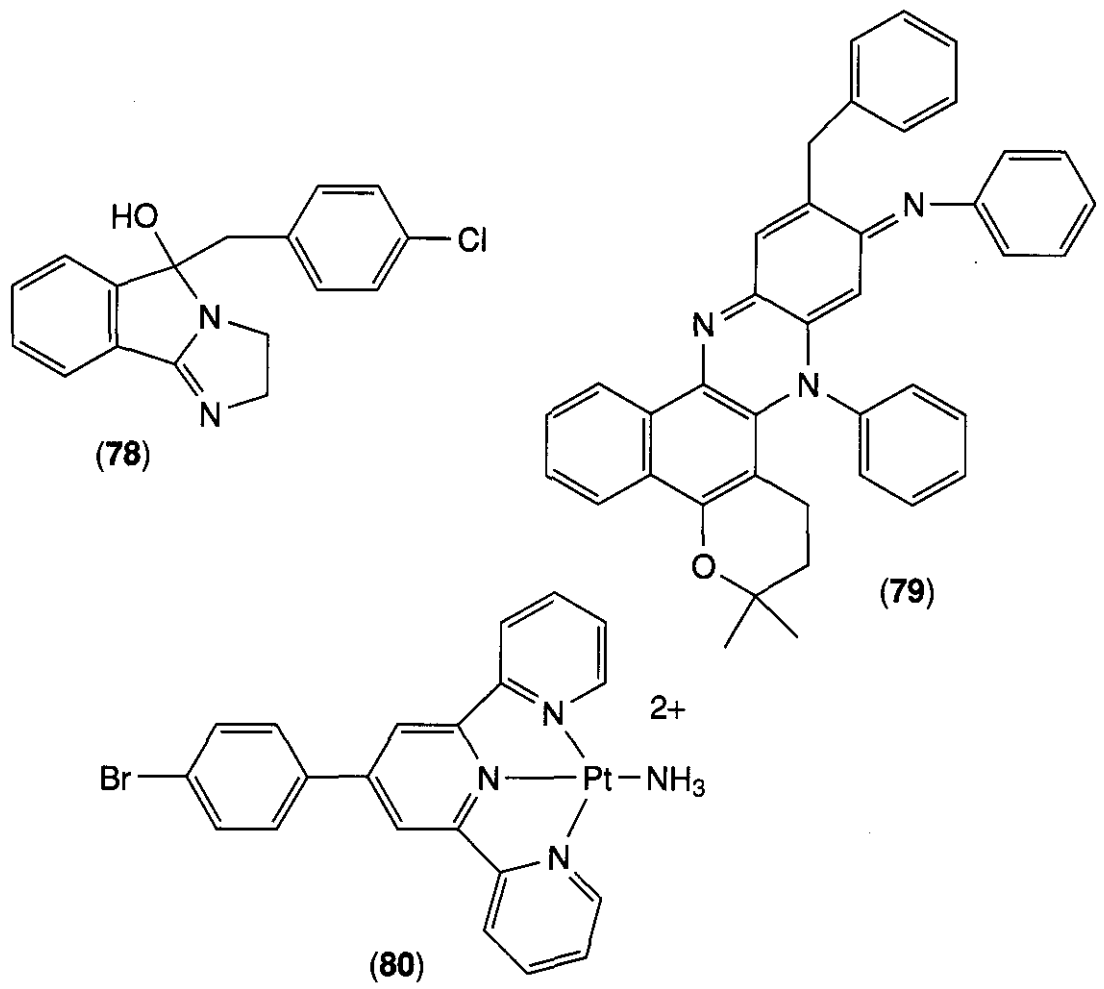


Figure 19.20. Miscellaneous new leads for antitrypanosomal drugs.

strate for hypoxanthine phosphoribosyltransferase (HPRT) from L. *donovani*, forming allopurinol-ribose-5'-phosphate (allo-5'-RP). Allo-5′-RP is a substrate for the rest of the parasite enzymes involved in converting IMP to ATP and incorporation of ATP into RNA (204). Allo-5'-RP is an inhibitor of GMP reductase and IMP dehydrogenase, and the allo-5'-RP compounds cause an increase in the breakdown of RNA in L. *donovani* (373). The antileishmanial activity of allopurinol is probably a sum of all of these toxic effects on purine interconversion. The clinical efficacy of allopurinol by itself is weak (374); however, the activity of allopurinol improves when it is combined with fluconazole (375).

The crystal structure of HPRT has been determined from multiple sources (see refs. 376–378 and references cited therein). A computer model of the active site of HPRT from T. cruzi was used in a docking search against a database of 34 million compounds (379). Twenty compounds were selected for best fit and screened for inhibitory activity against HPRT from T. cruzi. Sixteen of the compounds had IC_{50} values in the range 0.5 to 17 μM. Four compounds from the 16 were selected for screening against cultured T. cruzi in irradiated mouse macrophages. At 10 μM, two of the compounds showed potent trypanocidal activity. Structure (75) is one of the best leads from this study (379). Hofer et al. (380) investigated CTP synthetase in T. brucei as a potential target for drug development. They found that CTP pools are very low in T. brucei and that this parasite compensates for inhibition of CTP synthetase by the salvage of cytidine. Two inhibitors of CTP synthetase, acivicin (76) and DON (**77**), were tested against T. brucei in cultured cells and in T. *brucei*-infected mice. Both compounds were effective as trypanocidal agents in these tests (380).

3.9 Miscellaneous Active Compounds

Structures of several miscellaneous drugs affecting trypanosomal growth are given in Fig. 19.20. Compound (**78**), synthesized on the basis of a stilbenoid lead, had IC_{50} values of 10 and 7.9 μM against cultured T. cruzi epimastigotes and trypomastigotes, respectively (381, 382). The mechanism of action of these compounds is undetermined. Neves-Pinto et al. (383) reported derivatives of β-lapachone, of which the most active compound was (79)

(ED_{50} = 61 μM against T. *cruzi* trypomastigotes). The activity of (79) may be related to its structural resemblance to 5-methylphenazinium, a known inducer of free radicals. The (terpyridine) platinum (II) complex (**80**) showed potent trypanocidal action against L. *donouani* (100% at 1 μM), T. *cruzi* (65% at 1 μM), and T. *brucei* (100% at 30 nM). The mechanism of action of (80) is thought to be platination of parasitic DNA (384,385).

4 CONCLUSIONS

Despite their devastating impact on world health, few new drugs to treat the Kinetoplastida diseases have been introduced in the last 25 years. Several drugs are currently in clinical trials. Miltefosine offers hope as the first effective oral treatment to cure visceral leishmaniasis and other members of the lysophospholipid class of agents show promising trypanocidal activity. Megazol has shown good oral activity against African trypanosomiasis and is currently in clinical trials. The third-generation triazole antifungal agents posaconazole and UR-9825 have shown potent *in vivo* activity against *T. cruzi* infections in mice. Members of the existing bisphosphonates class of bone resorption inhibitors have potent trypanocidal activity *in vivo* and may provide new chemotherapeutic drugs for the Kinetoplastida diseases. A potent irreversible vinyl sulfonyl peptidomimetic inhibitor of cysteine proteases is scheduled to begin clinical trials for treatment of Chagas' disease (*T. cruzi*). DB289 and CGP 40215A are two additional agents that have shown potent action against trypanosomatids and are in various stages of drug development.

Active programs to map the genetic code of all the major parasitic pathogens are in progress (see references to web sites). A plethora of hopeful and often validated targets, based on differences in the biochemistry of the parasites vs. the human hosts, have been identified. However, although essentiality of a target can often be demonstrated by genetic methods, insufficient consideration is frequently given to the issue of selectivity between host and parasite enzyme. Modern methods of drug discovery and development (i.e., high throughput screening methods, *in silico* screening, structure-based drug design, parallel synthesis techniques, the preparation of combinatorial libraries, etc.) are being applied to the Kinetoplastida diseases. There is good reason to be hopeful that major advancements in the treatment of these parasitic diseases will occur in the next 10 years, providing there is the political will and economic resources to move some excellent scientific discoveries on to the clinic.

5 WEBSITES OF INTEREST

- Information on parasite genome projects, http://parsunl.path.cam.ac.uW
- World Health Organization, www.who.int
- Leishmania Genome Network, www.ebi.ac.uk
- Centers for Disease Control and Prevention, Emerging Infectious Diseases, www.cdc.gov/ncidod/eid/index.htm
- Parasitological Research Groups worldwide, www.uniduesseldorf.de/WWW/MathNat/Parasitology/paen_ags.htm
- Sanger Center maintains data on nucleotide sequences for many microorganisms, www.sanger.ac.uk./Projects/
- Institute for Genomic Research, http://www.tigr.org
- Website of C. Laveissiere and L. Penchenier on African sleeping sickness, http://www.sleeping-sickness.com

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CHAPTER TWENTY

Anthelmintics

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Contents

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1 INTRODUCTION

Helminths are parasitic worms, which infect an estimated 2 billion people worldwide, nearly all in poor developing tropical or semitropical countries. Schistosomiasis and other helmintic infections account for more than 40% of all tropical disease excluding malaria (1). Although fatal helminth infections are relatively rare, many persons may be simultaneously infected with more than one parasite and these diseases inflict an enormous morbidity burden on some of the poorest of the world's countries, a burden that falls particularly heavily on children. Helminth infections contribute to malnutrition, anemia, stunted growth, cognitive impairment, and increased susceptibility to other diseases. In addition to the human burden, domestic animals are also very susceptible to helminth infections, which adds to the economic burden of developing countries and is also a problem for agriculture in many developed countries. A number of anthelmintics are used in both veterinary practice and human chemotherapy.

Helminths are metazoans broadly classified into the classes of nematodes [roundworms and two types of flatworms (trematodes and flukes)] and cestodes (tapeworms). These various organisms have enormously variable life cycles, routes of infection, and susceptibility to chemotherapy. Most require an intermediate host to complete their life cycles and invade human hosts through the skin or ingestion. *Strongyloides* and *Echinococcus* are exceptions; they can live their entire life cycle within a human host. Filaria, from the Latin word for thread, filum, are a particular group of thread-like nematodes of the family Onchocercidae, which live as adults in human tissue or fluids but require an intermediate host of blood-sucking arthropods. These organisms cause a variety of diseases known collectively as filariasis. References 2 and 3 offer excellent overviews of helmintic parasites and the diseases they cause.

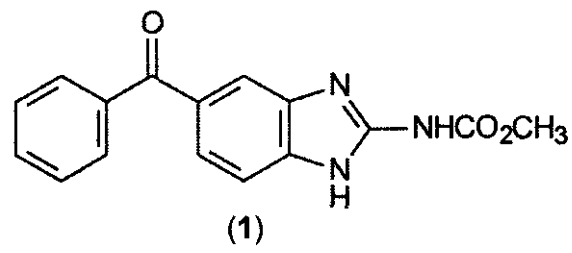
There are only about a dozen or so safe and effective drugs for treating helminth infections, but fortunately many of these are very effective and inexpensive. However, there has been no significant new anthelmintic introduced into clinical use since ivermectin entered clinical trials over 20 years ago.

2 CLINICAL USE OF AGENTS

The anthelmintics currently available in the United States are listed in Table 20.1. Of the drugs listed in Table 20.1, only pyrantel is available without a prescription in the United States, and three of them, bithionol, diethylcarbamazine, and suramin, are only available to physicians through the Centers for Disease Control under a compassionate use permit. A summary of the drugs effective against some of the more common helminth infections are summarized in Table 20.2.

2.1 Benzimidazoles

There are three benzimidazoles available in the United States, mebendazole (1), albendazole (2), and thiabendazole (3). The discovery in 1961 by Merck that thiabendazole (3) had broad spectrum activity against a variety of nematodes infecting the gastrointestinal tract was a major advance in anthelmintic therapy and was followed by the discovery and introduction of the benzimidazole carbamates, me-



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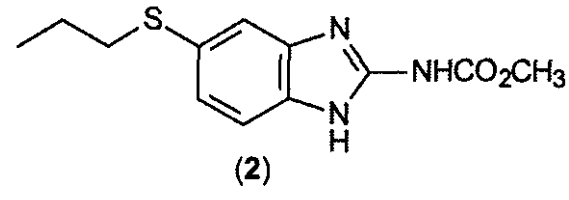

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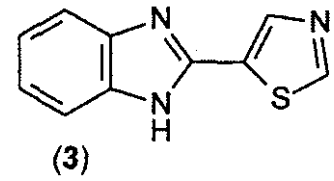


(3)

Table 20.1 **Anthelmintic Drugs**

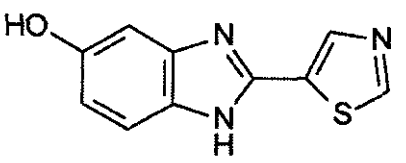
Generic Name	Trade Name	Originator	Chemical Class	Dose[a]
Mebendazole (1)	Vermox	Janssen	Benzimidazole	100 mg, 2× daily
Albendazole (2)	Albenza	SmithKlein	Benzimidazole	400 mg, 2× daily
Thiabendazole (3)	Mintezol	Merck	Benzimidazole	22 mg/kg, 2× daily
Praziquante (14)	Biltricide	Bayer	Pyrazinoisoquinoline	20–25 mgkg, 3×, one day
Ivermectin (19)	Stromectol	Merck	Macrocyclic lactone	150–200 μg/kg, once
Pyrantel (20)	Antiminth	Pfizer	Tetrahydropyrimidine	11 mg/kg, 1× daily
Oxamniquine (21)	Vansil, Mansil	Pfizer	Tetrahydroquinoline	12–15 mg/kg, 1× daily
Piperazine (24)	Multifuge		Piperazine	3.5 g/day for 2 days
Bithionol (25) (from CDC)	Lorothidol	Tanabe	Diphenylsulfide	40–66 mgkg, for 1 or 2 days
Diethylcarbamazine (26) (from CDC)	Hetrazan	Lederle	Piperidine	13 mg/kg, 1× daily for filariasis 2 mg/kg, 3× daily for ascariasis
Suramin (11) (from CDC, not recommended)	Bayer 205; Antrypol	Bayer	Bisnaphthalene sulfonic acid	1 g/week for 5–10 weeks

[a] Adult dose.

bendazole, albendazole, and others. Thiabendazole use has declined substantially since it was first introduced because more recently developed agents are equally or more effective and less toxic. It remains useful however for topical applications in the treatment of cutaneous larva migrans (creeping eruption). Mebendazole and the more recently developed albendazole are widely effective against both intestinal nematodes and most tissue nematodes.

The benzimidazole mechanism of action is inhibition of formation of microtubules through binding to β-tubulin monomers and preventing polymerization (4). Selectivity for parasite toxicity over host toxicity is caused by tighter binding to the parasite tubulin. There is evidence that susceptibility of a given organism to benzimidazoles is proportional to benzimidazole binding to the organism's tubulin. Further evidence has accumulated recently that benzimidazole resistance is caused by mutations in tubulin (5–9).

When taken orally, thiabendazole is rapidly absorbed and extensively metabolized by the liver (10). Thiabendazole was 12–15% excreted unchanged, and the predominant metabolite is 5-hydroxythiabendazole (4) found as both its free form (22–24%) and glucuronide and sulfate conjugates, 28–29% and 30–31%, respectively. Trace amounts of *N*-methylthiabendazole (5) were also found. All of the metabolites are inactive.

(3) → 

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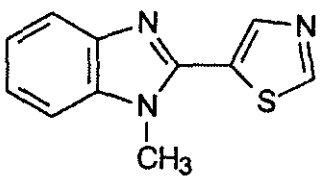
5-OH-conjugates

(4)

\+ 

(5)

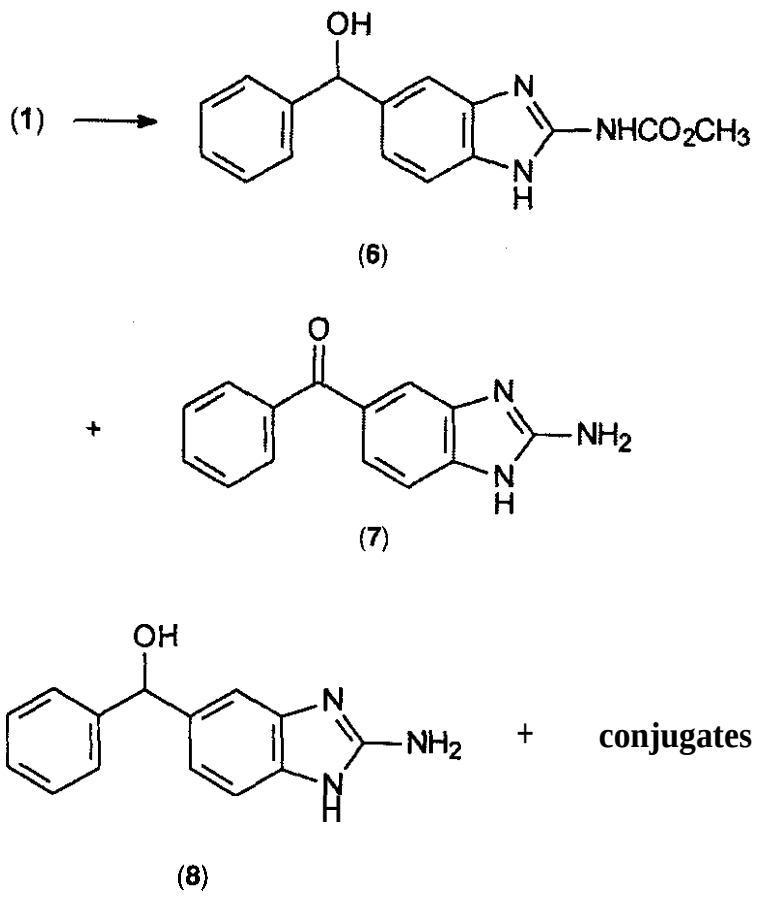
Mebendazole, on the other hand, has poor bioavailability (22%) after oral administration because of irregular absorption and extensive first-pass metabolism (11). The metabolites are inactive (12). The major metabolites are the product of ketone reduction (6) and carbamate hydrolysis (7), isolated both free and as conjugates. A minor metabolite is (8), the product of both reduction and hydrolysis.

Albendazole (2), the most recently developed benzimidazole anthelmintic, is also erratically absorbed and extensively metabo-

Table 20.2 Major Parasitic Infections and Drugs of Choice[a]

Infection (Common Name)	Organism	Drug(s) of Choice
Intestinal nematodes		
Ascariasis (roundworm)	*Ascaris* lumbricoides	Mebendazole, thiabendazole, pyrantel pamoate, or diethylcarbamazine
Uncinariasis (hookworm)	Ancylostoma duodenale, Necator americanus	Mebendazole or pyrantel[b]
Strongyloidiasis (threadworm)	*Strongyloides* stercoralis	Thiabendazole, ivermectin
Trichuriasis (whipworm)	Trichuris trichiura	Mebendazole
Enterobiasis (pinworm)	Enterobius *vermicularis*	Mebendazole, albendazole, thiabendazole, or pyrantel
Capillariasis	Capillaria philippinensis	Mebendazole, thiabendazole, or albendazole
Tissue nematodes		
Trichinosis	*Trichinela spiralis*	Albendazole, or mebendazole,[b] plus steroids for severe symptoms
Cutaneous larva migrans (Creeping eruption)	Ancylostoma *braziliense* and others	Thiabendazole, albendazole, or ivermectin
Onchoceriasis (River blindness)	*Onchocerca volvulus*	Ivermectin, diethylcarbamazine, or suramin[c]
Dracontiasis (Guinea worm)	*Dracunculus* medinensis	Thiabendazole or mebendazole
Angiostrongyliasis (rat lungworm)	*Angiostrongylus* cantonensis	Thiabendazole or mebendazole
Loiasis	*Loa* loa	Diethylcarbamazine
Cestodes		
Taeniasis (beef tapeworm)	Taenia saginata	Praziquantel[b]
Taeniasis (pork tapeworm)	Taenia solium	Praziquantel[b] or albendazole
Diphyllobothriasis (fish tapeworm)	*Diphyllobothrium* latum	Praziquantel[b]
Dog tapeworm	Dipylidium caninum	Praziquantel[b]
Hymenolepiasis (dwarf tapeworm)	Hymenolepsis *nana*	Praziquantel[b]
Hydatid cysts	Echinococcus *granulosus*	Albendazole or praziquantel[b]
Trematodes		
Schistosomiasis	Schistosoma mansoni	Praziquantel or oxamniquine
	Schistosoma japonicum	Praziquantel
	Schistosoma haematobium	Praziquantel
	Schistosoma mekongi	Praziquantel
Hermaphroditic flukes		
Fasciolopsiasis (intestinal fluke)	Fasciolopsis buski	Praziquantel
	Heterophyes heterophyes	Praziquantel
	Metagonimus yokogawai	Praziquantel
Clonorchiasis (Chinese liver fluke)	*Clonorchis sinensis*	Praziquantel
Fascioliisis (sheep liver fluke)	*Fasciola* hepatica	Praziquantel or bithionol[d]
Opisthorchiiis (liver fluke)	*Opisthorchis viverrini*	Praziquantel
Paragonimiasis (lung fluke)	*Paragonimus westermani*	Praziquantel or bithionol[d] (alternate)

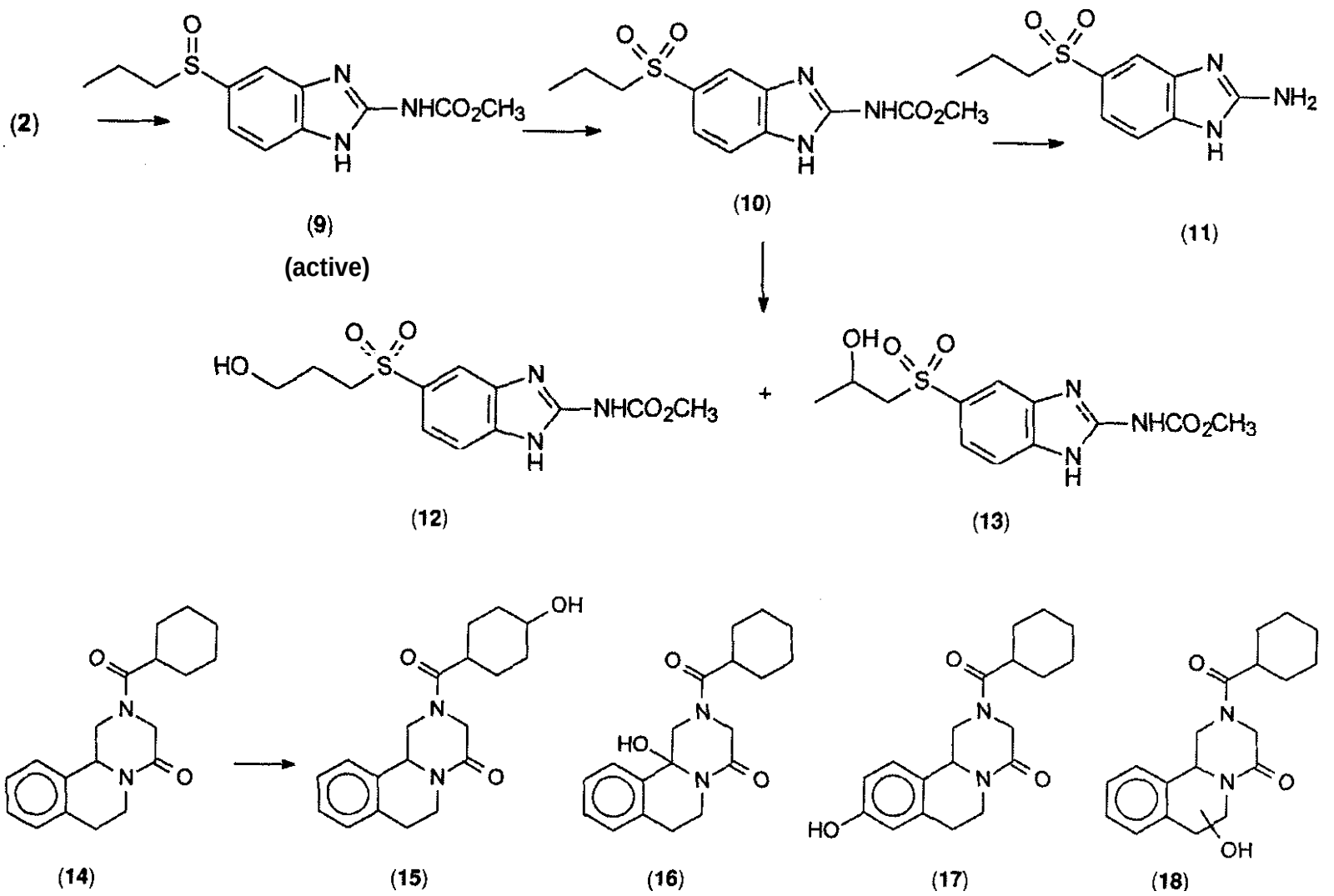
[a]Table modified from Drug Facts and Comparisons 2000 [2002, vol. 1101 with permission.
[b]Unlabeled use.
[c]Available from the Center for Disease Control, although generally not recommended.
[d]Available from the Center for Disease Control.

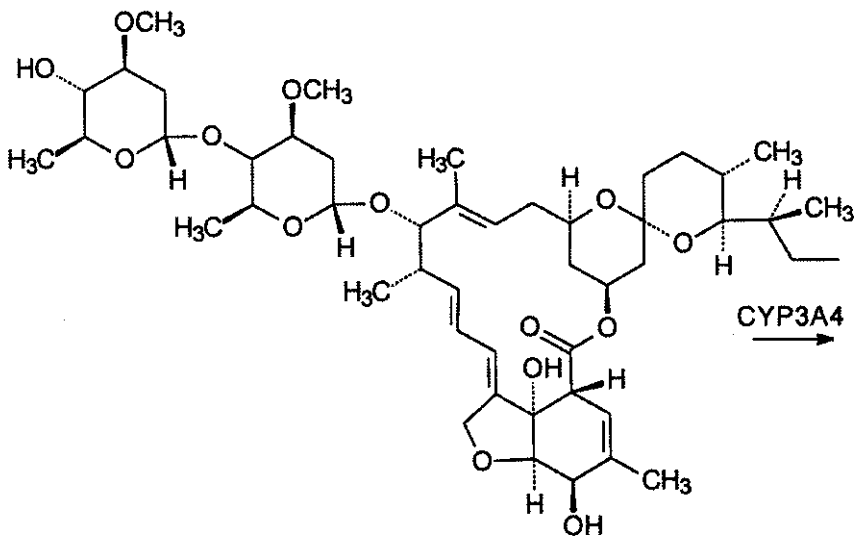


lized as shown (12). However the primary metabolite, albendazole sulfoxide (9) is active, and because parent albendazole is barely detectable in plasma, albendazole sulfoxide probably accounts for all the anthelmintic activity of the drug (13, 14). Formation of albendazole sulfoxide is carried out by both liver CYP450 and by microsomal flavin-linked monooxygenase. Sulfoxides are chiral, and the flavin enzyme favors formation of the (+)isomer and P450 favors formation of the (−)isomer (15). Both sulfoxide enantiomers are further oxidized to the inactive sulfone (10) and on to other minor inactive metabolites (**11–13**).

2.2 Praziquantel

Praziquantel (**14**) is a widely employed anthelmintic initially found to be active against schistosomiasis (16) and subsequently found to widely effective against a variety of trematodes and cestodes (17). Both safe and effective, praziquantel is the drug of choice for treating all forms of schistosomiasis. The mechanism of action of praziquantel is complex and as yet not clearly understood (18). Praziquantel rapidly penetrates the tegument of trematodes and cestodes but does not seem to be able to cross the thicker tegument of nematodes, which may account for its selectivity. Once inside, praziquantel alters calcium





CYP3A4 → 10 identified hydroxylated and demethylated metabolites

(19)

ion flux in the parasite, leading to muscle contractions, and spastic paralysis, leading to detachment of the parasite from the host. Praziquantel also causes damage to the tegument permitting host defenses to attack the weakened parasite. Parasites dislodged from their anchors to host tissue are carried to the liver and attacked by granulocytes (18).

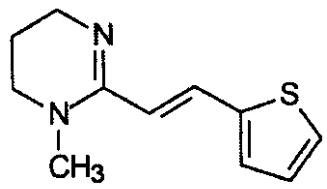
Praziquantel is extensively metabolized by the liver to hydroxylated products (19). The principal product (35%)is the cyclohexane-4-hydroxyl derivative (15)of which 80% is the cis-isomer and 20% the trans-isomer (20). Inhibitor studies indicate the metabolism is likely carried out predominantly by isozymes CYP2B1 and CYP3A (21). Other identified but minor hydroxylated metabolites (and their conjugates) are (16–18). All the metabolites are inactive.

2.3 Ivermectin

Ivermectin, (**19**), produced from avermectin by reduction of one double bond, is another breakthrough drug in anthelmintic chemotherapy. Active against most nematodes and introduced in the mid-1980s, ivermectin was the first safe and effective agent against *Onchocerca volvulus*, the causative agent of River Blindness (22). It is currently the drug of choice for treating onchocerciasis and strongyloides (23). Recent studies have shown ivermectin efficacious in treating lymphatic filarial infections such as those associated with both Wuchereria bancrofti and Brugia malayi infections of humans that cause elephantiasis (24). Ivermectin is replacing the far more toxic diethylcarbamazine in the treatment of many nematode infections. The antiparasitic mechanism of ivermectin involves disruption of the function of glutamate-gated chloride channels to induce a tonic paralysis of the nematode musculature (25–28). Cestodes and trematodes lack high affinity ivermectin-binding proteins, which may explain why these helminth groups are not sensitive to ivermectin (29). Ivermectin is well absorbed orally and metabolized by the liver CP450 system to at least 10 hydroxylated and demethylated products (30). The principal enzyme involved is CYP3A4.

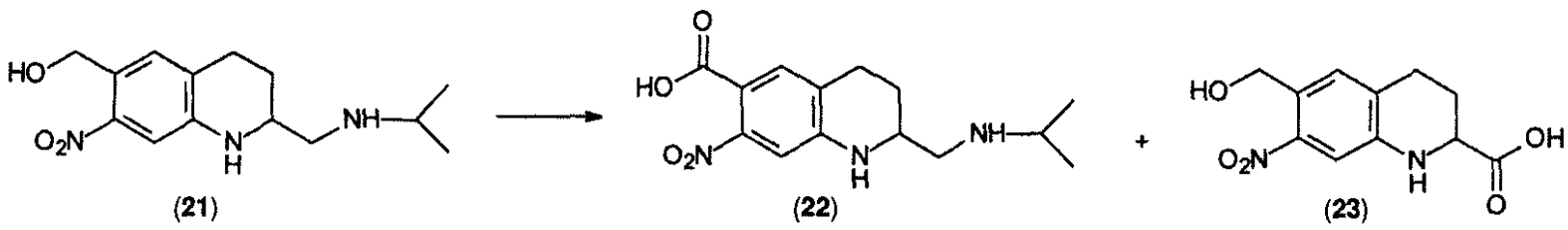
2.4 Pyrantel

Pyrantel (20) is the only anthelmintic available without a prescription in the United States. Introduced in the mid-1960s, pyrantel



(20)

quickly found use in treating intestinal nematodes (31) and is currently used for treating pinworm, (enterobiasis), roundworm (ascariasis), and hookworm (uncinariasis). The drug acts on nicotinic acetylcholine receptors to cause spastic paralysis of the worms that detach from the host and are swept out in feces (32). The drug is very poorly absorbed, which probably accounts for it's safety for human



(21) (22) (23)

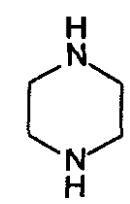
use without a prescription. Less than 15% is excreted in urine as parent drug or metabolites. Most is excreted unchanged in feces.

2.5 Oxamniquine

Oxamniquine (21) is an old drug with an intriguingly narrow spectrum of activity. Oxamniquine is an antischistosomal active only against S. *mansoni* (33) and has no effect on the other schistosomes at therapeutic doses. For reasons unknown, male *S. mansoni* concentrate the drug and die, leaving surviving females unable to lay eggs. The mechanism of action is unknown, although the drug exhibits some anticholinergic activity. Oxamniquine is readily absorbed and metabolized to the 6-carboxylic **acid** (34) (22) and 2-carboxylic acid (**23**).

2.6 Piperazine

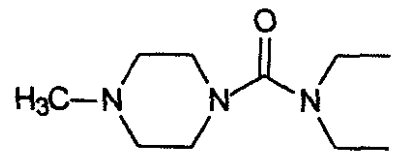
Piperazine (24) is useful as an alternative to mebendazole or pyrantel for treating combined ascariasis and Enterobius infections (35). Piperazine acts as a gamma-amino-butyric acid (GABA)-agonist, increasing membrane permeability to chloride ion and inducing a flaccid paralysis in the parasites (36). The drug is well absorbed, and about 20% is excreted unchanged in the urine (37).



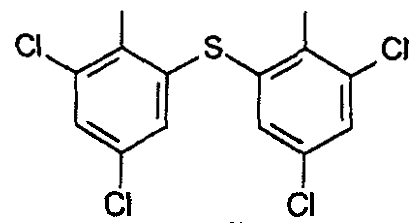
(24)

2.7 Bithionol, Diethylcarbamazine, and Suramin

Diethylcarbamazine (**25**), bithionol (**26**), and suramin (27) are available in the United States only through the government's Centers

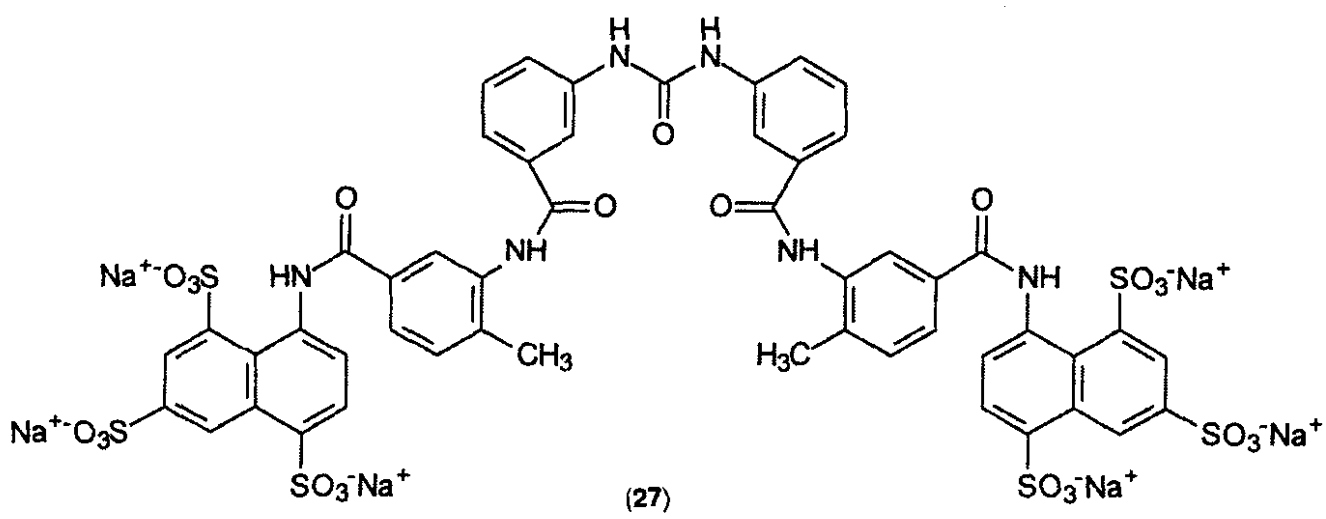


(25)



(26)

for Disease Control. These drugs are all very old, have limited efficacy, and have high toxicity. Diethylcarbamazine was at one time the drug of choice for filariasis infections, but has been replaced in most applications by ivermec-



(27)

tin. Suramin was at one time the drug of choice for onchocerciasis but has also been replaced by ivermectin or combinations of ivermectin with albendazole. These agents are available as drugs of last resort when other therapeutic regimens fail.

3 HISTORY

An exhaustive history of the development of anthelmintic drugs through the late 1970s is available in a previous edition of this work (38). Ivermectin is the only drug of significance to enter therapeutics in the last 20 years.

4 FUTURE DEVELOPMENTS

There is a paucity of research into new anthelmintics for several reasons, most notably that there is very little profit to be made from drugs in this class, and there are few viable molecular targets for application of modern techniques of drug design and high-throughput screening.

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