CHAPTER ONE

Molecular Biology of Cancer

JESSE D. MARTINEZ
MICHELE TAYLOR PARKER
KIMBERLY E. FULTZ
NATALIA A. IGNATENKO
EUGENE W. GERNER
Departments of Radiation Oncology/Cancer Biology Section
Molecular and Cellular Biology
Biochemistry and Molecular Biophysics
Cancer Biology Graduate Program
The University of Arizona
Tuscon, Arizona

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1 INTRODUCTION

Cancer is a major human health problem worldwide and is the second leading cause of death in the United States (1). Over the past 30 years, significant progress has been achieved in understanding the molecular basis of cancer. The accumulation of this basic knowledge has established that cancer is a variety of distinct diseases and that defective genes cause these diseases. Further, gene defects are diverse in nature and can involve either loss or gain of gene functions. A number of inherited syndromes associated with increased risk of cancer have been identified.

This chapter will review our current understanding of the mechanisms of cancer development, or carcinogenesis, and the genetic basis of cancer. The roles of gene defects in both germline and somatic cells will be discussed as they relate to genetic and sporadic forms of cancer. Specific examples of oncogenes, or cancer-causing genes, and tumor suppressor genes will be presented, along with descriptions of the relevant pathways that signal normal and cancer phenotypes.

While cancer is clearly associated with an increase in cell number, alterations in mechanisms regulating new cell birth, or cell proliferation, are only one facet of the mechanisms of cancer. Decreased rates of cell death, or apoptosis, are now known to contribute to certain types of cancer. Cancer is distinctive from other tumor-forming processes because of its ability to invade surrounding tissues. This chapter will address mechanisms regulating the important cancer phenotypes of altered cell proliferation, apoptosis, and invasiveness.

Recently, it has become possible to exploit this basic information to develop mechanism-based strategies for cancer prevention and treatment. The success of both public and private efforts to sequence genomes, including human and other organisms, has contributed to this effort. Several examples of mechanism-based anti-cancer strategies will be discussed. Finally, potential strategies for gene therapy of cancer will also be addressed.

2 TUMORIGENESIS

2.1 Normal-Precancer-Cancer Sequence

Insight into tumor development first came from epidemiological studies that examined the relationship between age and cancer incidence that showed that cancer incidence increases with roughly the fifth power of elapsed age (2). Hence, it was predicted that at least five rate-limiting steps must be overcome before a clinically observable tumor could arise. It is now known that these rate-limiting steps

are genetic mutations that dysregulate the activities of genes that control cell growth, regulate sensitivity to programmed cell death, and maintain genetic stability. Hence, tumorigenesis is a multistep process.

Although the processes that occur during tumorigenesis are only incompletely understood, it is clear that the successive accumulation of mutations in key genes is the force that drives tumorigenesis. Each successive mutation is thought to provide the developing tumor cell with important growth advantages that allow cell clones to outgrow their more normal neighboring cells. Hence, tumor development can be thought of as Darwinian evolution on a microscopic scale with each successive generation of tumor cell more adapted to overcoming the social rules that regulate the growth of normal cells. This is called clonal evolution **(3).**

Given that tumorigenesis is the result of mutations in a select set of genes, much effort by cancer biologists has been focused on identifying these genes and understanding how they function to alter cell growth. Early efforts in this area were lead by virologists studying retrovirus-induced tumors in animal models. These studies led to cloning of the first oncogenes and the realization that oncogenes, indeed all cancer-related genes, are aberrant forms of genes that have important functions in regulating normal cell growth (4). In subsequent studies, these newly identified oncogenes were introduced into normal cells in an effort to reproduce tumorigenesis in vitro. Importantly, it was found that no single oncogene could confer all of the physiological traits of a transformed cell to a normal cell. Rather this required that at least two oncogenes acting cooperatively to give rise to cells with the fully transformed phenotype (5). This observation provides important insights into tumorigenesis. First, the multistep nature of tumorigenesis can be rationalized as mutations in different genes with each event providing a selective growth advantage. Second, oncogene cooperativity is likely to be cause by the requirement for dysregulation of cell growth at multiple levels.

Fearon and Vogelstein (6) have proposed a linear progression model (Fig. 1.1) to describe tumorigenesis using colon carcinogenesis in humans as the paradigm. They suggest that malignant colorectal tumors (carcinomas) evolve from preexisting benign tumors (adenomas) in a stepwise fashion with benign, less aggressive lesions giving rise to more lethal neoplasms. In their model, both genetic [e.g., adenomatous polyposis coli (APC) mutations] and epigenetic changes (e.g., DNA methylation affecting gene expression) accumulate over time, and it is the progressive accumulation of these changes that occur in a preferred, but not invariable, order that are associated with the evolution of colonic neoplasms. Other important features of this model are that at least four to five mutations are required for the formation of a malignant tumor, in agreement with the epidemiological data, with fewer changes giving rise to intermediate benign lesions, that tumors arise through the mutational activation of oncogenes and inactivation of tumor suppressor genes, and that it is the sum total of the effect of these mutations on tumor cell physiology that is important rather than the order in which they occur.

An important implication of the multistep model of tumorigenesis is that lethal neoplasms are preceded by less aggressive intermediate steps with predictable genetic alterations. This suggests that if the genetic defects which occur early in the process can be identified, a strategy that interferes with their function might prevent development of more advanced tumors. Moreover, preventive screening methods that can detect cells with the early genetic mutations may help to identify these lesions in their earliest and most curable stages. Consequently, identification of the genes that are mutated in cancers and elucidation of their mechanism of action is important not only to explain the characteristic phenotypes exhibited by tumor cells, but also to provide targets for development of therapeutic agents.

2.2 Carcinogenesis

Carcinogenesis is the process that leads to genetic mutations induced by physical or chemical agents. Conceptually, this process can be divided into three distinct stages: initiation, promotion, and progression (7). Initiation involves an irreversible genetic change, usually a mutation in a single gene. Promotion is gen-

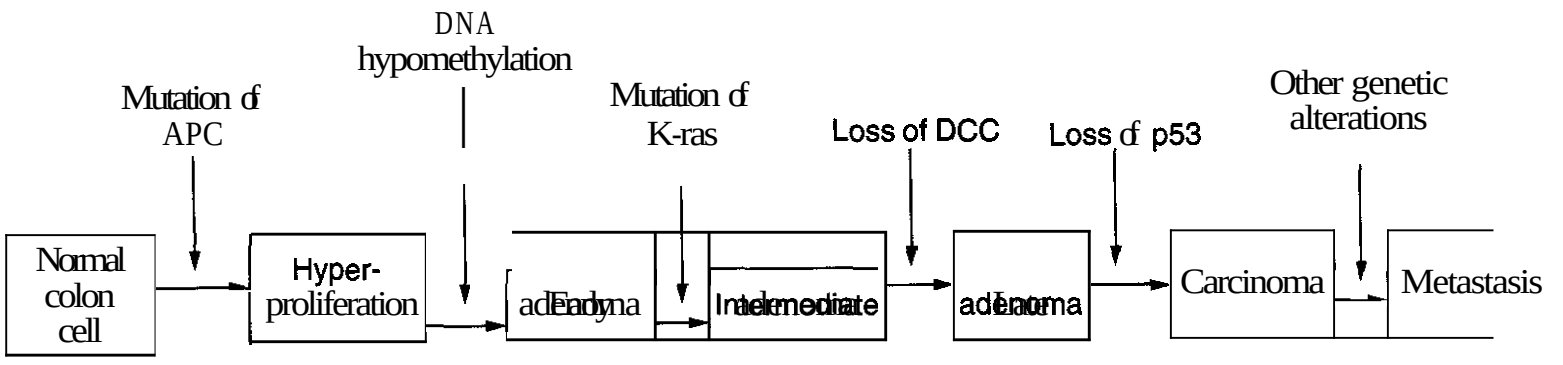


Figure 1.1. Adenoma-carcinoma sequence. Fearon and Vogelstein **(6)** proposed this classic model for the multistage progression of colorectal cancer. A mutation in the APC tumor suppressor gene is generally considered to be the initiation event. This is followed by the sequential accumulation of other epigenetic and genetic changes that eventually result in the progression from a normal cell to a metastatic tumor.

erally associated with increased proliferation of initiated cells, which increases the population of initiated cells. Progression is the accumulation of more genetic mutations that lead to the acquisition of the malignant or invasive phenotype.

In the best-characterized model of chemical carcinogenesis, the mouse skin model, initiation is an irreversible event that occurs when a genotoxic chemical, or its reactive metabolite, causes a DNA mutation in a critical growth controlling gene such as Ha-ras (8). Outwardly, initiated cells seem normal. However, they remain susceptible to promotion and further neoplastic development indefinitely. DNA mutations that occur in initiated cells can confer growth advantages, which allow them to evolve and/or grow faster bypassing normal cellular growth controls. The different types of mutations that can occur include point mutations, deletions, insertions, chromosomal translocations, and amplifications. Three important steps involved in initiation are carcinogen metabolism, DNA repair, and cell proliferation. Many chemical agents must be metabolically activated before they become carcinogenic. Most carcinogens, or their active metabolites, are strong electrophiles and bind to DNA to form adducts that must be removed by DNA repair mechanisms (9). Hence, DNA repair is essential to reverse adduct formation and to prevent DNA damage. Failure to repair chemical adducts, followed by cell proliferation, results in permanent alterations or mutation(~in the genome that can lead to oncogene activation or inactivation of tumor suppressor genes.

Promotion is a reversible process in which chemical agents stimulate proliferation of initiated cells. Typically, promoting agents are nongenotoxic, that is they are unable to form DNA adducts or cause DNA damage but are able to stimulate cell proliferation. Hence, exposure to tumor promoting agents results in rapid growth of the initiated cells and the eventual formation of non-invasive tumors. In the mouse skin tumorigenesis model, application of a single dose of an initiating agent does not usually result in tumor formation. However, when the initiation step is followed by repeated applications of a tumor promoting agent, such as 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), numerous skin tumors arise and eventually result in invasive carcinomas. Consequently, tumor promoters are thought to function by fostering clonal selection of cells with a more malignant phenotype. Importantly, tumor formation is dependent on repeated exposure to the tumor promoter. Halting application of the tumor promoter prevents or reduces the frequency with which tumors form. The sequence of exposure is important because tumors do not develop in the absence of an initiating agent even if the tumor promoting agent is applied repeatedly. Therefore, the genetic mutation caused by the initiating agent is essential for further neoplastic development under the influence of the promoting agent.

Progression refers to the process of acquiring additional mutations that lead to malignancy and metastasis. Many initiating agents can also lead to tumor progression, strong support for the notion that further mutations are

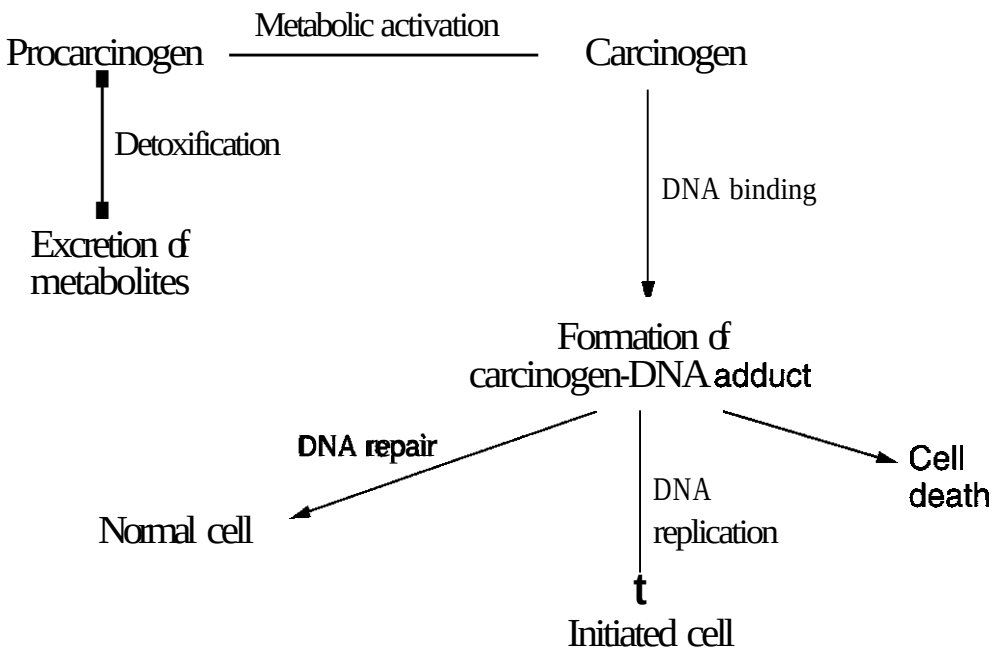


Figure 1.2. Possible outcomes of carcinogen metabolic activation. Once a carcinogen is metabolically activated it can bind to DNA and form carcinogen-DNA adducts. These adducts will ultimately lead to mutations if they are not repaired. If DNA repair does not occur, the cell will either undergo apoptosis or the DNA will be replicated, resulting in an initiated cell.

needed for cells to acquire the phenotypic characteristics of malignant tumor cells. Some of these agents include benzo(a)pyrene, β-napthylamine, 2-acetylaminofluorene, aflatoxin B_1, dimethylnitrosamine, 2-amino-3-methylimidazo(4,5-*f*)quinoline (IQ), benzidine, vinyl chloride, and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (10). These chemicals are converted into positively charged metabolites that bind to negatively charged groups on molecules like proteins and nucleic acids. This results in the formation of DNA adducts which, if not repaired, lead to mutations (9) (Fig. 1.2). The result of these mutations enables the tumors to grow, invade surrounding tissue, and metastasize.

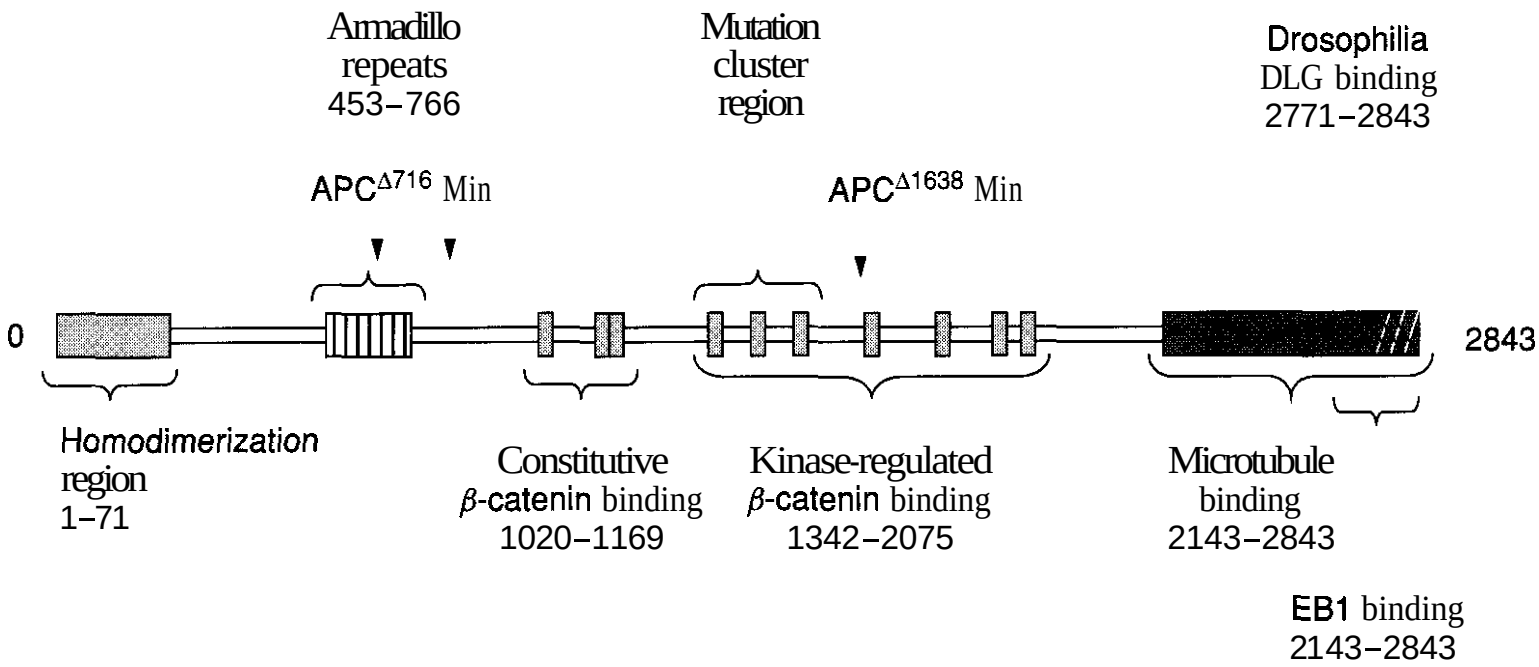
Damage to DNA and the genetic mutations that can result from them are a central theme in carcinogenesis. Hence, the environmental factors that cause DNA damage are of great interest. Environmental agents that can cause DNA damage include ionizing radiation, ultraviolet (UV) light, and chemical agents (11). Some of the DNA lesions that can result include single-strand breaks, double-strand breaks, base alterations, cross-links, insertion of incorrect bases, and addition/deletion of DNA sequences. Cells have evolved several different repair mechanisms that can reverse the lesions caused by these agents, which has been extensively reviewed elsewhere (12).

The metabolic processing of environmental carcinogens is also of key importance because this can determine the extent and duration to which an organism is exposed to a carcinogen. Phase I and phase II metabolizing enzymes play important roles in the metabolic activation and detoxification of carcinogenic agents. The phase I enzymes include monooxygenases, dehydrogenases, esterases, reductases, and oxidases. These enzymes introduce functional groups on the substrate. The most important superfamily of the phase I enzymes are the cytochrome P450 monooxygenases, which metabolize polyaromatic hydrocarbons, aromatic amines, heterocyclic amines, and nitrosamines. Phase II metabolizing enzymes are important for the detoxification and excretion of carcinogens. Some examples include epoxide hydrase, glutathione-S-transferase, and uridine 5'-diphosphate (UDP) glucuronide transferase. There are also some direct acting carcinogens that do not require metabolic activation. These include nitrogen mustard, dimethylcarbamyl chloride, and β-propiolactone.

2.3 Genetic Variability and Other Modifiers of Tumorigenesis

2.3.1 Genetic Variability Affecting Cancer.

Different types of cancers, as well as their severity, seem to correlate with the type of mutation acquired by a specific gene. Mutation "hot spots" are regions of genes that are frequently mutated compared with other regions within that gene. For example, observations that the majority of colon adenomas are associated with alterations in the adenomatous polyposis coli (APC) have been based on immunohistochemical analysis of p-catenin localization and formation of less than full



Murine models	Intestinal tumor number
$APC^{\Delta 716}$	200–600
Min (850 stopcodon)	60–80
$APC^{\Delta 1638}$	<10

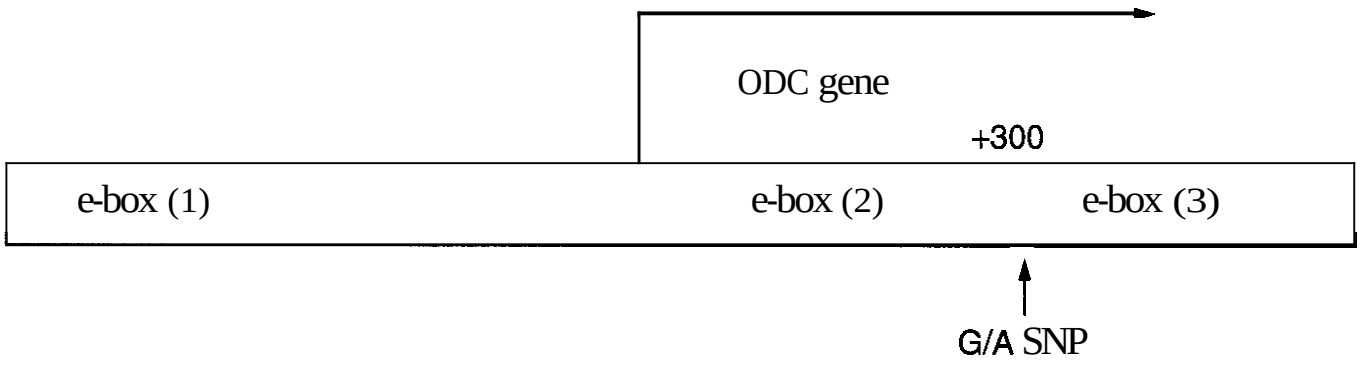
Figure 1.3. Diagram of APC protein regions, relating risk of intestinal carcinogenesis to length of APC peptide translated. APC contains 2833 amino acids. Mutation hot spot regions are found in areas between amino acids 1500–2000. Three genetically altered mouse models of APC-dependent intestinal carcinogenesis have been developed. Min mice have a stop codon mutation in codon 850 of the murine APC homolog. Two transgenic mice, $APC^{\Delta 716}$ and $APC^{\Delta 1635}$, also have been developed. Intestinal tumor number in these models is inversely related to size of the APC peptide translated.

length APC protein production after *in vitro* translation of colonic mucosal tissue RNA. These studies have not documented specific gene mutations in APC. This is important, because it is known from animal studies that the location of APC mutations can have a dramatic effect on the degree of intestinal carcinogenesis. Thus, it is possible that colon adenoma size, and subsequent risk of colon cancer could be dictated by location of specific mutations in APC (Fig. 1.3).

As suggested by the model depicted in Fig. 1.3, high risk might be associated with mutations causing stop codons in the amino terminal end of the protein. Low risk might be associated with mutations resulting in peptides of greater length. Current research is testing the hypothesis that specific genetic alterations in APC alone may be sufficient as a prognostic factor for risk of adenoma recurrence and subsequently, colon cancer development.

One type of genetic alteration that is gaining increasing attention is the single nucleotide polymorphism (SNP). This polymorphism results from a single base mutation that leads to the substitution of one base for another., SNPs occur quite frequently (about every 0.3–1 kb within the genome) and can be identified by several different techniques. A common method for the analysis of SNPs is based on the knowledge that single-base changes have the capability of destroying or creating a restriction enzyme site within a specific region of DNA. Digestion of a piece of DNA, containing the site in question, with the appropriate enzyme can distinguish between variants based on the resulting fragment sizes. This type of analysis is commonly referred to as restriction fragment length polymorphism (RFLP).

The importance of analyzing SNPs rests on the premise that individuals with a nucleotide at a specific position may display a normal phenotype, whereas individuals with a different nucleotide at this same position may exhibit increased predisposition for a certain disease or phenotype. Therefore, many studies are being conducted to determine the fre-



E-box (1)	SNP allele (Frequency)		Promoter activity
CACGTG	G	(90–95%)	1
CAGCTG	G	(90–95%)	0.5
CACGTG	A	(5–10%)	3–8

Figure 1.4. Influence of specific genetic changes on ODC promoter activity. These data were derived from transient transfection experiments in human colon tumor–derived HT29 cells. The arrow in this figure 1.4 shows the SNP. The SNP occurs between two E-boxes that are located **3'** of the transcription start site. The effects of this genetic change are taken from Guo et al. **(56).** It is important to point out that the constructs used to assess the promoter activity of the polymorphic region containing the SNP and E-boxes 2 and **3** contained some of the **5'** promoter region, but not E-box **1** (56). The constructs used to assess the role of E-box **1** in HT-29 contained the major, c-myc unresponsive allele between E-boxes 2 and **3.**

quency of specific SNPs in the general population and to use these findings to explain phenotypic variation.

For example, a recent study found an association between a polymorphism leading to an amino acid substitution (aspartate to valine) in codon 1822 of the APC gene and a reduced risk for cancer in people eating a low-fat diet (13). The variant valine had an allele frequency of 22.8% in a primarily Caucasian control population. This non-truncating mutation has not yet been shown to have functional significance. If functional, such a polymorphism could cooperate with single allele truncating mutations that occur with high frequency in sporadic colon adenomas (14), to increase colon cancer risk. This polymorphism is especially interesting, because dietary factors, specifically fat consumption, may contribute to risk in only specific genetic subsets.

2.3.2 Genetic Variability in c-myc–Dependent Expression of Ornithine Decarboxylase. The proliferation-associated polyamines are essential for cell growth but may contribute to carcinogenesis when in excess. Various studies have shown that inhibition of polyamine synthesis impedes carcinogenesis. Ornithine decarboxylase (ODC), the first enzyme in polyamine synthesis, may play a key role in tumor development. Therefore, elucidation of the mechanisms by which ODC is regulated is essential. The literature indicates that ODC is a downstream mediator of APC and suggests that ODC may be an APC modifier gene. Thus, polymorphisms in the ODC promoter affecting c-myc–dependent ODC transcription could be a mechanism of genetic variability of APC-dependent carcinogenesis.

O'Brien and colleagues (15) have measured the incidence in several human subgroups of a SNP in a region of the ODC promoter, 3' of the transcription start site, that is flanked by two E-boxes (CACGTG) (Fig. 1.4). The E-box is a DNA sequence where specific transcription factors bind. The two resulting alleles are identified by a polymorphic *Pst*I RFLP. The minor allele (A at position +317) is homozygous in 6–10% of individuals, whereas the major allele (G at position +317) is homozygous or heterozygous in 90–94% of these groups. They have also measured functionality of the polymorphisms. When ODC promoter-reporter constructs are expressed in rodent cells, the minor allele confers 3–8 times the promoter activity compared with the major allele. Further, expression of the minor allele is

enhanced by c-myc expression to a greater extent than the major allele.

2.4 Epigenetic Changes

Gene function can be disrupted either through genetic alterations, which directly mutate or delete genes, or epigenetic alterations, which alter the state of gene expression. Epigenetic mechanisms regulating gene expression include signal transduction pathways, DNA methylation, and chromatin remodeling. Methylation of DNA is a biochemical addition of a methyl group at position 5 of the pyrimidine ring of cytosine in the sequence CG. This modification occurs in two ways: *(1)* from a preexisting pattern on the coding strand or ***(2)*** by ***de*** novo addition of a methyl group to fully unmethylated DNA. Cleavage of DNA with the restriction endonuclease *Hpa*II, which cannot cut the central C in the sequence CCGG if it is methylated, allows detection of methylated sites in DNA. Small regions of DNA with methylated cytosine, called "CpG islands," have been found in the 5'-promoter region of about one-half of all human genes (including most housekeeping genes).

There are three DNA methyltransferases (Dnmt), Dnmtl, Dnmt3a, and Dnmt3b, that have been identified in mammalian cells (16). The most abundant and ubiquitous enzyme, Dnmtl, shows high affinity for hemimethylated DNA, suggesting a role of Dnmtl in the inheritance of preexisting patterns of DNA methylation after each round of DNA replication. The other two enzymes, Dnmt3a and Dnmt3b, are tissue specific and have been shown to be involved in ***de*** novo methylation. ***De*** novo CpG island methylation, however, is not a feature of proliferating cells, and can be considered a pathologic event in neoplasia.

Over the years, a number of different methyl-CpG binding proteins, such as methyl-CpG-binding domain-containing proteins (MBD1-4) were identified (17) that compete with transcription factors and prevent them from binding to promoter sequences. These methyl-CpG binding factors can also recruit histone deacetylases (HDACs), resulting in condensation of local chromatin structure (Fig. 1.5). This makes the methylated DNA less accessible to transcription factors and results in gene silencing.

Gene expression is inhibited by DNA methylation. DNA methylation patterns dramatically change at different stages of cell development and differentiation and correlate with changes in gene expression (18). Demethylation releases gene expression in the first days of embryogenesis. Later, ***de*** novo methylation establishes adult patterns of gene methylation. In differentiated cells, methylation status is retained by the activity of the Dnmtl enzyme. In normal tissues, DNA methylation is associated with gene silencing, chromosome X inactivation (19), and imprinting (20). Because the most normal methylation takes place within highly repeated transposable elements, it has been proposed that such methylation plays a role in genome defense by suppressing potentially harmful effects of expression at these sites.

Neoplastic cells are characterized by simultaneous global DNA hypomethylation, localized hypermethylation that involves CpG islands and increased HDAC activity (21). Hypomethylation has been linked to chromosomal instability in vitro and it seems to have the same effect in carcinogenesis (22). 5-Methylcytosine is a relatively unstable base because its spontaneous deamination leads to the formation of uracil. Such changes can also contribute to the appearance of germline mutations in inherited disease and somatic mutations in neoplasia. Aberrant CpG island hypermethylation in normally unmethylated regions around gene transcription start sites, which results in transcriptional silencing of genes, suggests that it plays an important role as an alternate mechanism by which tumor suppressor genes are inactivated in cancer (21). Hypermethylated genes identified in human cancers include the tumor suppressor genes that cause familial forms of human cancer when mutated in the germline, as well as genes that are not fully documented tumor suppressors (Table 1.1). Some of these genes, such as APC, the breast cancer gene BRCA-1, E-cadherin, mismatch repair gene hMLH1, and the Von Hippel-Lindau gene can exhibit this change in non-familial cancers.

Recent studies indicate that promoter hypermethylation is often an early event in tumor progression. It has been shown in the colon that genes that have increased hyper-

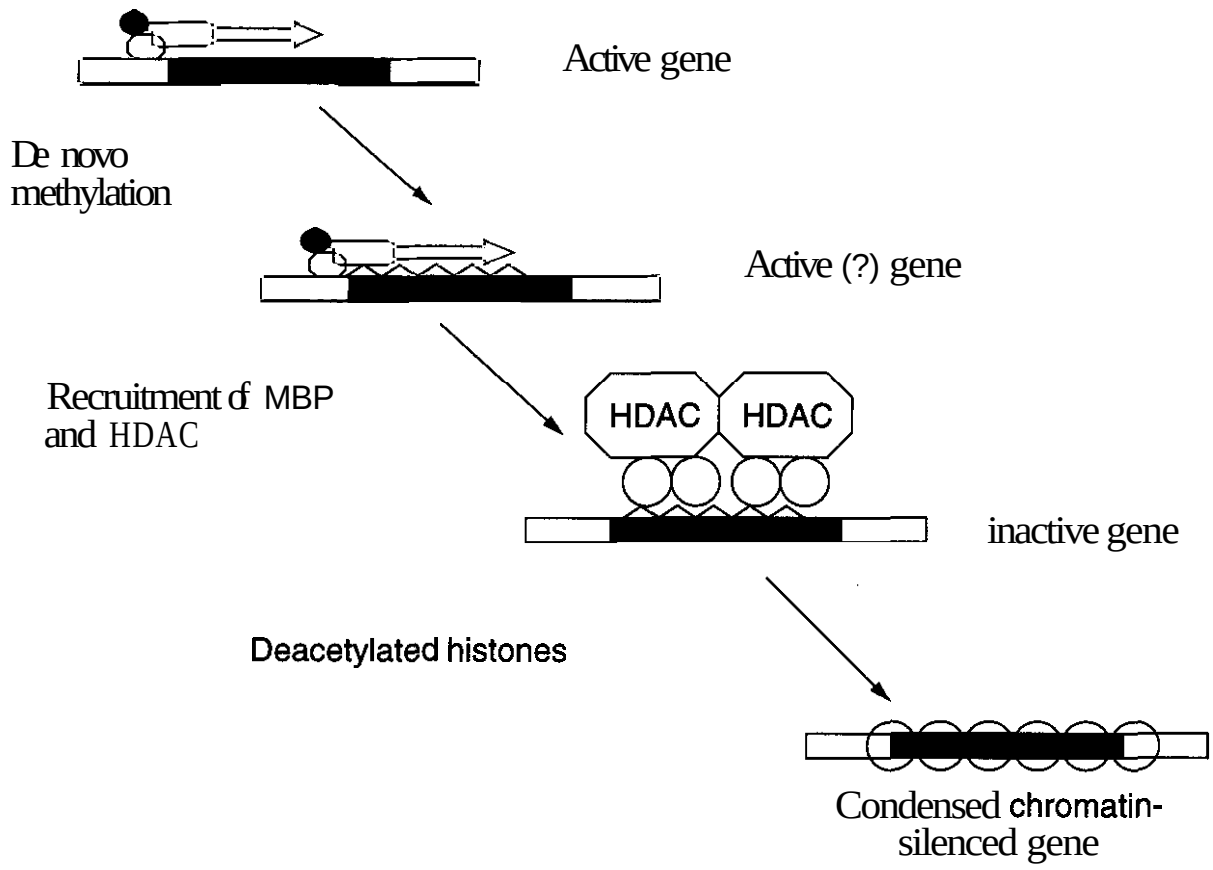


Figure 1.5. Effect of methylation and histone deacetylation on gene expression. When a gene is active, the promoter region is occupied by transcription factors that direct production of messenger RNA. ***De novo*** methylation has minimal effects on gene expression. However, methylated DNA attracts methyl-binding proteins (MBP). These methyl-binding proteins in turn attract a protein complex that contains histone deacetylase (HDAC). This results in inhibition of messenger RNA synthesis, and no functional protein can be made from the gene. Through the action of MBP and HDAC, the DNA structure changes to a compact, "condensed chromatin" configuration, which results in permanent inhibition of messenger RNA and protein synthesis (silencing).

methylation in the promoter region in normal tissue as a function of aging are the same as genes with the highest rate of promoter hypermethylation in tumors (9). Interestingly, this group of genes does not include classic tumor suppressor genes. Some genes, such as the estrogen receptor where age-related hypermethylation in the colon was first discovered, may be important for the modulation of cell growth and differentiation in the colonic mucosa.

Promoter hypermethylation of genes, which are normally unmethylated at all ages, has also been found early in tumorigenesis. These epigenetic alterations can produce the early loss of cell cycle control, altered regulation of gene transcription factors, disruption of cell-cell interactions, and multiple types of genetic instability, which are all characteristic of neoplasia. For example, hypermethylation of the APC gene has recently been reported for a subset of colon cancers (23). Hypermethylation of hMLH1, which is associated with microsatellite instability in colon, endometrial, and gastric neoplasia, has been seen in early, stages of cancer progression (24). Finally, hypermethylation of the E-cadherin promoter frequently occurs in early stages of breast cancer and can trigger invasion (25).

Loss of gene function through epigenetic changes differs from genetic changes in terms of its consequences for tumor biology. First, gene function loss caused by aberrant promoter methylation may manifest in a more subtle, selective advantage than gene mutations during tumor progression. Second, although promoter hypermethylation causing gene silencing is usually stable in cancer cells, this change, unlike mutation, is potentially reversible. It has become evident that not only the mutagens, but various factors influencing cell metabolism, particularly methylation, lie at the origin of carcinogenesis.

Silencing of gene expression by methylation may be modulated by biochemical or biological manipulation. It has been shown that pharmacological inhibition of methyltrans-

Table 1.1 Hypermethylated Genes in Cancer

Gene	Function	Type of Tumor
Familial Cancers		
APC	Signal transduction	Colon cancer
BRCA1	DNA repair	Breast cancer
E-cadherin	Adhesion and metastasis	Multiple cancers
hMLH1	DNA mismatch repair	Colon, gastric, and endometrial cancer
p16/CDKN2A	Cell cycle regulation	Multiple cancers
RB1	Cell cycle regulation	Retinoblastoma
VHL	Cytoskeletal organization, angiogenesis inhibition	Renal-cell cancer
Other Cancers		
Androgen receptor	Growth and differentiation	Prostate cancer
c-ABL	Tyrosine kinase	Chronic myelogenous leukemia
Endothelin receptor B	Growth and differentiation	Prostate cancer
Estrogen receptor α	Transcription	Multiple cancers
FHIT	Detoxification	Esophageal cancer
GST-π	Drug transport	Prostate cancer
MDR1	Drug transport	Acute leukemias
06-MGMT	DNA repair	Multiple cancers
p14/ARF	Cell cycle regulation	Colon cancer
p15/CDKN2B	Cell cycle regulation	Malignant hematologic disease
Progesterone receptor	Growth and differentiation	Breast cancer
Retinoic acid receptor β	Growth and differentiation	Colon and breast cancer
THBS1	Angiogenesis inhibition	Colon cancer, glioblastoma multiforme
TIMP3	Metastasis	Multiple cancers

ferases resulted in reactivation of gene expression in vitro (26) and prevented tumor growth in animal models (27). These studies generated interest in the clinical uses of hypomethylating agents in humans.

3 MOLECULAR BASIS OF CANCER PHENOTYPES

Cancer is a multistep process that requires the accumulation of multiple genetic mutations in a single cell that bestow features characteristic of a neoplastic cell. Typically, tumor cells differ from normal cells in that they exhibit uncontrolled growth. Because features that distinguish tumor from normal cells may be key to understanding neoplastic cell behavior and may ultimately lead to therapies that can target tumor cells, considerable effort has been directed at identifying the phenotypic characteristics of in vitro–transformed cells and of tumor cells derived from natural sources. This work has resulted in a list of properties that are characteristic of tumor cells and that are now known to be the basis for the behaviors exhibited by neoplastic cells. Some of the features that will be discussed in detail include immortality, decreased dependence on growth factors to support proliferation, loss of anchorage-dependent growth, loss of cell cycle control, reduced sensitivity to apoptotic cell death, and increased genetic instability. Other morphological and biochemical characteristics used to identify the transformed phenotype are cytological changes, altered enzyme production, and the ability to produce tumors in experimental animals (28).

3.1 Immortality

Normal diploid fibroblasts have a limited capacity to grow and divide both in vivo and in vitro. Even if provided with optimal growth conditions, in vitro normal cells will cease dividing after 50–60 population doublings and then senesce and die. In contrast, malignant cells that have become established in culture

proliferate indefinitely and are said to be immortalized. The barrier that restricts the life span of normal cells is known as the Hayflick limit and was first described in experiments that attempted immortalization of rodent cells (29). Normal embryo-derived rodent cells, when cultured in vitro, initially divide rapidly. Eventually, however, these cultures undergo a crisis phase during which many of the cells senesce and die. After extended maintenance, however, proliferation in the cultures increases and cells that can divide indefinitely emerge. The molecular changes that take place during crisis have revealed at least two important restrictions that must be overcome for cells to become immortalized and both of these changes occur in natural tumor cells.

One barrier to cellular immortalization is the inability of the DNA replication machinery to efficiently replicate the linear ends of DNA at the 5' ends, which leadsto the shortening of the chromosome. In bacteria, the end-replication problem is solved with a circular chromosome. In human cells. the ends of chromosomes are capped with 5–15 kb of repetitive DNA sequences known as telomeres. Telomeres serve as a safety cap of noncoding DNA that is lost during normal cell division without consequence to normal function of the cell. However, because telomere length is shortened with each round of cell division, indefinite proliferation is impossible because eventually the inability to replicate chromosomal ends nibbles into DNA containing vital genes.

Telomeres seem to be lengthened during gametogenesis as a consequence of the activity of an enzyme called telomerase. Telomerase activity has been detected in normal ovarian epithelial tissue. More importantly, telomerase activity is elevated in the tumor tissue but not the normal tissue from the same patient. This implies that one mechanism by which tumor cells overcome the shortening telomere problem and acquire the capacity to proliferate indefinitely is through abnormal up-regulation of telomerase activity. The finding that telomerase activity is found almost exclusively in tumor cells is significant because it suggests that this enzyme may be a useful therapeutic target (30). Therapies aimed at suppressing telomerase would eliminate a feature essential for tumor cell survival and would be selective.

A second feature of immortalization is loss of growth control by elimination of tumor suppressor activity. Recent evidence suggests that inactivating mutations in both the Rb and p53 tumor suppressor genes occurs during crisis. Both of these genes are discussed in more detail later in this chapter and both function to inhibit cell proliferation by regulating cell cycle progression. Consequently, loss of tumor suppressor function also appears to be a critical event in immortalization.

3.2 Decreased Dependence on Growth Factors to Support Proliferation

Cells grown in culture require media supplemented with various growth factors to continue proliferating. In normal human tissues, growth factors are generally produced extracellularly at distant sites and then are either carried through the bloodstream or diffuse to their nearby target cells. The former mode of growth factor stimulation is termed endocrine stimulation, and the latter mode, paracrine stimulation. However, tumor cells often produce their own growth factors that bind to and stimulate the activity of receptors that are also present on the same tumor cells that are producing the growth factor. This results in a continuous self-generated proliferative signal known as autocrine stimulation that drives proliferation of the tumor cell continuously even in the absence of any exogenous proliferative signal. Autocrine stimulation is manifested as a reduced requirement for serum because serum is the source of many of the growth factors in the media used to propagate cells in vitro.

Because of the prominent role that growth factors and their cognate receptors play in tumor cell proliferation, they have also become favorite therapeutic targets. For example, the epidermal growth factor receptor (EGFR) is known to play a major role in the progression of most human epithelial tumors, and its overexpression is associated with poor prognosis. As a consequence, different approaches have been developed to block EGFR activation function in cancer cells, including anti-EGFR blocking monoclonal antibodies (MAb), epidermal growth factor (EGF) fused to toxins, and small molecules that inhibit the receptor's tyrosine kinase activity (RTK). Of these, an

orally active anilinoquinazoline, ZD1839 ("Iressa") shows the most promise as an antitumor agent by potentiating the antitumor activity of conventional chemotherapy (31).

3.3 Loss of Anchorage-Dependent Growth and Altered Cell Adhesion

Most normal mammalian cells do not grow, but instead undergo cell death if they become detached from a solid substrate. Tumor cells, however, frequently can grow in suspension or in a semisolid agar gel. The significance of the loss of this anchorage-dependent growth of cancer cells relates to the ability of the parent tumor cells to leave the primary tumor site and become established elsewhere in the body. The ability of cancer cells to invade and metastasize foreign tissues represents the final and most difficult-to-treat stage of tumor development, and it is this change that accompanies the conversion of a benign tumor to a life-threatening cancer.

Metastasis is a complex process that requires the acquisition of several new characteristics for tumor cells to successfully colonize distant sites in the body. Epithelial cells normally grow attached to a basement membrane that forms a boundary between the epithelial cell layers and the underlying supporting stroma separating the two tissues. This basement membrane consists of a complex array of extracellular matrix proteins including type IV collagen, proteoglycans, laminin, and fibronectin, which normally acts as a barrier to epithelial cells. A common feature of tumor cells with metastatic potential is the capacity to penetrate the basement membrane by proteolysis, to survive in the absence of attachment to this substrate, and to colonize and grow in a tissue that may be foreign relative to the original tissue of origin.

Consequently, metastasis is a multistep process that begins with detachment of tumor cells from the primary tumor and penetration through the basement membrane by degradation of the extracellular matrix (ECM) proteins. This capacity to proteolytically degrade basement membrane proteins is driven, in part, by the expression of matrix metalloproteinases. Matrix metalloproteinases, or MMPs, are a family of enzymes that are either secreted (MMPs 1–13, 18–20) or anchored in the cell membrane (MMPs 14–17) (Table 1.2). Regulation of MMPs occurs at several levels: transcription, proteolytic activation of the zymogen, and inhibition of the active enzyme (32). MMPs are typically absent in normal adult cells, but a variety of stimuli, such as cytokines, growth factors, and alterations in cell-cell and cell-ECM interactions, can induce their expression. The expression of MMPs in tumors is frequently localized to stromal cells surrounding malignant tumor cells. Most of the MMPs are secreted in their inactive (zymogen) form and require proteolytic cleavage to be activated. In some cases. MMPs have been shown to undergo mutual and/or autoactivation *in vitro* (33).

Several lines of evidence implicate MMPs in tumor progression and metastasis. First, MMPs are overexpressed in tumors from a variety of tissues and the expression of one, matrilysin, is clearly elevated in invasive prostate cancer epithelium (34–36). Second, reduction of tissue inhibitor of matrix metalloproteinases-1 (TIMP-1) expression in mouse fibroblasts (Swiss 3T3), using antisense RNA technology, increased the incidence of metastatic tumors in immunocompromised mice. Similarly, overexpression of the various MMPs has provided direct evidence for their role in metastasis. Importantly, synthetic MMP inhibitors have also been produced and they lead to a reduction in metastasis in several experimental models of melanoma, colorectal carcinoma, and mammary carcinoma, suggesting a mechanism by which the invasive potential of tumors may be reduced (37).

Once tumor cells escape through the basement membrane, they can metastasize through two major routes, the blood and lymphatic vessels. Tumors originating in different parts of the body have characteristic patterns of invasion. Some tumors, such as those of the head and neck, spread initially to regional lymph nodes. Others, such as breast tumors, have the ability to spread to distant sites relatively early. The site of the primary tumor generally dictates whether the invasion will occur through the lymphatic or blood vessel system. The cells that escape into the vasculature must evade host immune defense mechanisms to be successfully transported to regional or distal locations. Tumor cells then

Table 12 MMPs

MMP	Common Name	Substrates	Cell Surface
1	collagenase-1, interstitial collagenase	collagen I, II, III, VII, X, IGFBP	yes
2	gelatinase A	gelatin, collagen I, IV, V, X, laminin, IGFBP, latent TGF-β	yes
3	stromelysin-1	collagen III, IV, V, IX, X, gelatin, E-cadherin, IGFBP, fibronectin, elastin, laminin proteoglycans, perlecan, HB-EGF, proMMP-13	unknown
7	matrilysin	laminin, fibronedin, gelatin, collagen IV, proteoglycans FasL, proMMP-1, HB-EGF	yes
8	collagenase-2, neutrophil collagenase	collagen I, II, III, VII, X	unknown
9	gelatinase B	collagen I, IV, V, X, gelatin, IGFBP, latent TGF-b	yes
10	stromelysin-2	collagen III, IV, IX, X, gelatin, laminin, proteoglycans, proMMP-1, proMMP-13	unknown
11	stromelysin-3	IGFBP, a-1-antiprotease	unknown
12	metalloelastase	elastin, proMMP-13	unknown
13	collagenase-3	collagen I, II, III, IV, VII, X, XIV, fibronectin, proMMP-9, tenascin, aggrecan	unknown
14	MT1-MMP	gelatin, collagen I, fibrin, proteoglycans, laminin, fibronectin, proMMP-2	yes
15	MT2-MMP	larninin, fibronectin, proMMP-2, proMMP-13, tenascin	yes
16	MT3-MMP	gelatin, collagen III, fibronectin, proMMP-2	yes
17	MT4-MMP	unknown	yes
18/19	RASI-1	unknown	unknown
20	Enamelysin	amelogenin	unknown

exit blood vessels and escape into the host tissue by again compromising a basement membrane, this time the basement membrane of the blood vessel endothelium. Projections called invadopodia, which contain various proteases and adhesive molecules, adhere to the basement membrane, and this involves membrane components such as laminin, fibronectin, type IV collagen, and proteoglycans. The tumor cells then produce various proteolytic enzymes, including MMPs, which degrade the basement membrane and allow invasion of the host tissue. This process is referred to as extravasation.

The interaction between cells and extracellular matrix proteins occurs through cell-surface receptors, the best characterized of which is the fibronectin receptor that binds fibronectin. Other receptors bind collagen and laminin. Collectively these receptors are called integrins, and their interaction with matrix components conveys regulatory signals to the cell **(38).** They are heterodimeric molecules consisting of one of several alpha and beta subunits that may combine in any number of permutations to generate a receptor with distinct substrate preferences. Changes in the expression of integrin subunits is associated with invasive and metastatic cells facilitating invasion by shifting the cadre of integrins to integrins that preferentially bind the degraded subunits of extracellular matrix proteins produced by MMPs. Hence, integrin expression has served as a marker for the invasive phenotype and may be a logical target for novel therapies that interfere with the progress of advanced tumors.

In addition to their role in invasion, the evidence also indicates that MMPs may play a role in tumor initiation and in tumorigenicity. Expression of MMP-3 in normal mammary epithelial cells led to the formation of invasive tumors (39). A proposed mechanism for this initiation involves the ability of MMP-3 to

cleave E-cadherin. E-cadherin is a protein involved in cell-cell adhesion together with other proteins such as β-catenin and α-catinin. Loss of E-cadherin function is known to lead to tumorigenicity and invasiveness as a result of loss of cellular adhesion. Interestingly, inhibition of MMP-7 and MMP-11, using antisense approaches, did not affect invasiveness or metastatic potential in vitro. However, tumorigenicity was altered (40). Matrilysin, MMP-7 messenger RNA (mRNA), are present in benign tumors and malignant tumor cells of the colon. The relative level of matrilysin expression correlates with the stage of tumor progression.

3.4 Cell Cycle and Loss of Cell Cycle Control

Proliferation is a complex process consisting of multiple subroutines that collectively bring about cell division. At the heart of proliferation is the cell cycle, which consists of many processes that must be completed in a timely and sequence specific manner. Accordingly, regulation of cell cycle events is a multifaceted affair and consists of a series of checks and balances that monitor nutritional status, cell size, presence or absence of growth factors, and integrity of the genome. These cell cycle regulatory pathways and the signal transduction pathways that communicate with them are populated with oncogenes and tumor suppressor genes.

Cell division is divided into four phases: G1, S, G2, and M (Fig. 1.6). The entire process is punctuated by two spectacular events, the replication of DNA during S phase and chromosome segregation during mitosis or M phase. Of the four cell cycle phases, three can be assigned to replicatingcells and only the G1 phase, and a related quiesent phase, GO, are nonreplicative in nature. Normal cycling cells that cease to proliferate enter the resting phase, or G1, and their exit into the replicative phases is strongly dependent on the presence of growth factors and nutrients. However, once the cells enter the replicative phase of the cell cycle, they become irrevocably committed to completing cell division. Hence, the conditions that lead to exit from G1 and entry into S are tightly regulated and are frequently misregulated in neoplastic cells that exhibit uncontrolled proliferation. Studies first conducted by Arthur Pardee revealed the existence of a point in G1 that restricted the passage of cells into S phase, and this was postulated to be controlled by a labile protein factor (41). Passage across this restriction point, or R point, is now known to be sensitive to growth factor stimulation.

Movement through the cell cycle is controlled by two classes of cell cycle proteins, cyclins and cyclin dependent kinases (CDKs), which physically associate to form a protein kinase that drives the cell cycle forward (42). At least 8 cyclins and 12 CDKs have been identified in mammalian cells. The name "cyclin" derives from the characteristic rise and fall in abundance of cyclin B as cells progress through the cell cycle. The accumulation of cyclin proteins occurs through cell cycle-dependent induction of gene transcription, but elimination of cyclins occurs by carefully regulated degradation that is enabled through protein sequence tags known as destruction boxes and PEST sequences. Although not all of the cyclin types exhibit this oscillation in protein quantity, those cyclins that play key roles in progression through the cell cycle (cyclins E, A, and B) are most abundant during discrete phases of the cell cycle. Cyclin D1 is synthesized during G1 just before the restriction point and plays an important role in regulation of the R point. Cyclin E is most abundant during late G1 and early S and is essential for exit from G1 and progression into S phase. Elevated levels of these two G1 cyclins can result in uncontrolled proliferation. Indeed, both cyclin D1 and cyclin E are overexpressed in some tumor types, suggesting that the cyclins and other components of the cell cycle may be useful therapeutic targets (43).

The second component of the enzyme complex is CDK that, as the name implies, requires an associated cyclin to become active. At least 12 of the protein kinases have been isolated from humans, Xenopus, and *Drosophila*, and are numbered according to a standardized nomenclature beginning with CDK1, which for historical reasons, is most frequently referred to as cell division cycle 2 (cdc2). Unlike the cyclins, abundance of the CDK proteins remains relatively constant

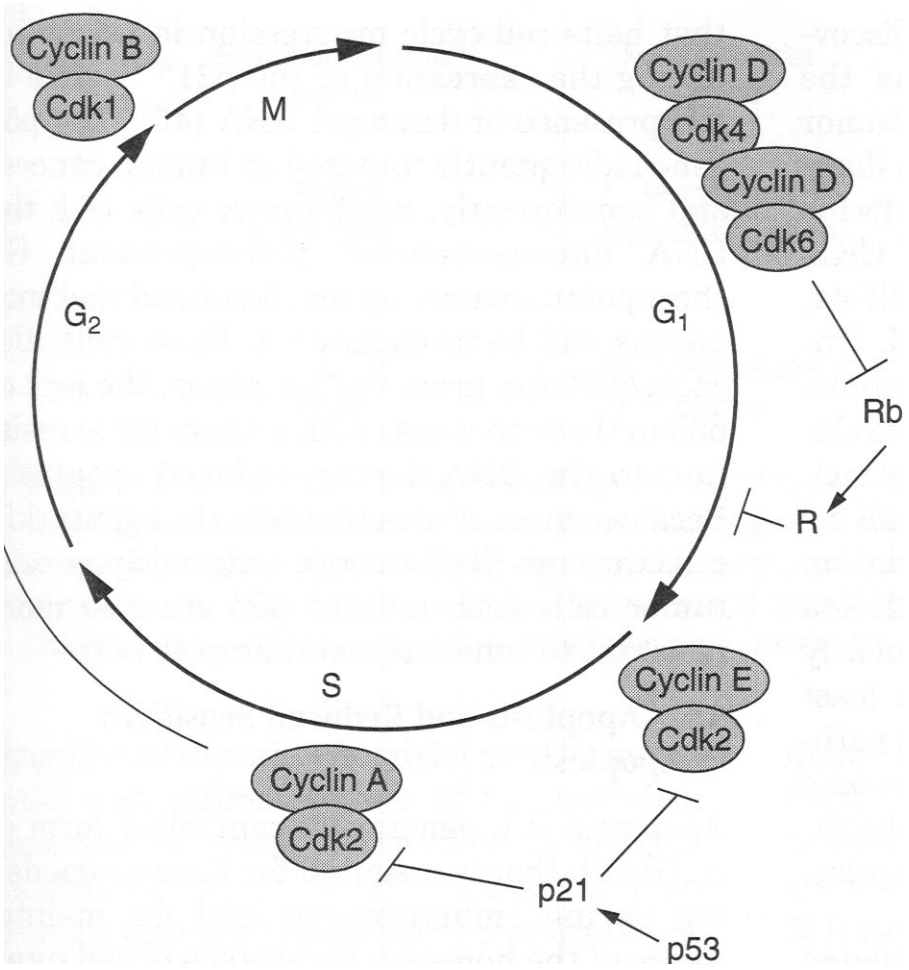


Figure 1.6. Model of the cell cycle and the cyclin/cdk complexes that are required at each cell cycle phase. CyclinD/cdk4-6 complexes suppress Rb function by phosphorylating the protein allowing transition across the restriction R-point. P53 suppresses cell cycle progression by stimulating the expression of the cyclin dependent kinase inhibitor p21, which binds with and inactivates a variety of cyclin/cdk complexes.

throughout the cell cycle. Instead, their activity changes during different phases of the cell cycle in accordance with whether or not an activating cyclin is present and whether or not the kinase itself is appropriately phosphorylated. Both cyclins and CDKs are highly conserved from yeast to man and function similarly, suggesting that the cell cycle is controlled by a universal cell cycle engine that operates through the action of evolutionarily conserved proteins. Hence, drug discovery studies aimed at identifying agents that regulate the cell cycle may be performed in model organisms, such as yeast, C. elegans, and *Drosophila* with some assurance that the targeted mechanisms will also be relevant to humans.

It is now clear that specific cyclinlcdk complexes are required during specific stages of the cell cycle. Cyclin D1/cdk4,6 activity is essential for crossing the restriction point and pushing cells into replication. A major substrate of the cyclin D1/cdk4,6 complex is the retinoblastoma (Rb) tumor suppressor protein, which when phosphorylated by this kinase complex, is inactivated. This frees the cell from the restrictions on cell proliferation imposed by the Rb protein. It is this event that is believed to be decisive in the stimulation of resting cells to undergo proliferation. Cyclin E/cdk2 plays a role later in the cell cycle for proliferating cells by pushing them from G1 into S phase. Cyclin E is overexpressed in some breast cancers where it may enhance the proliferative capacity of tumor cells. Cyclin A/cdk2 sustains DNA replication and is therefore required during S phase. Cyclin B/cdc2 is required by cells entering mitosis up through metaphase. At the end of metaphase, cyclin B is degraded, and cdc2 becomes inactivated, allowing mitotic cells to progress into anaphase and to complete mitosis. Sustaining the activity of cyclin B/cdc2 causes cells to arrest in metaphase. Hence, it is the collective result brought about by the activation and deactivation of cyclinlcdk complexes that pushes proliferating cells through the cell cycle.

Superimposed on the functions of the cell cycle engine is a complex network of both positive and negative regulatory pathways. Important negative regulators are the cyclin dependent kinase inhibitors or CKIs. There are two families of CKIs, the Cip/Kip family and the INK4 family (44). The Cip/Kip family consists of three members, p21/Cip1/waf1/Sdi1, p21/Kip1, and p57/Kip2. All of the proteins in this family have broad specificity and can bind to and inactivate most of the cyclin/cdk complexes that are essential for progression

through the cell cycle. $p21^{waf1}$, the first discovered and best characterized member of the Cip/Kip family, is stimulated by the p53 tumor suppressor protein in response to DNA damage and halts cell cycle progression to allow for DNA repair (45). The INK4 family of CKIs contains four member proteins, p16/INK4a, p15/INK4b, p18/INK4c, and p19/INK4d. Unlike the Cip/Kip family, the INK4 proteins have restricted binding and associate exclusively with cdk4/6. Consequently, their principal function is to regulate cyclin D1/cdk4/6 activity, and therefore, the phosphorylation status of the Rb tumor suppressor. p16/INK4a is itself a tumor suppressor that is frequently mutated in melanoma (46). Indeed, at least one component of the p16/cyclin D1/Rb pathways is either mutated or deregulated in some fashion in over 90% of lung cancers, emphasizing the importance of this pathway in regulating tumor cell proliferation.

Transit through the cell cycle is regulated by two types of controls. In the first type, the cumulative exposure to specific signals, such as growth factors, is assessed and if the sum of these signals satisfies the conditions required by the R point, proliferation ensues. In the second. feedback controls or checkpoints monitor whether the genome is intact and whether previous cell cycle steps have been completed. At least five cell cycle checkpoints have been identified, two that monitor integrity of the DNA and halt cell cycle progression in either G1 or *G2,* one that ensures DNA synthesis has been completed before mitosis begins, one that monitors completion of mitosis before allowing another round of DNA synthesis, and one that monitors chromosome alignment on the equatorial plate before initiation of anaphase. Of these, the two checkpoints that monitor integrity of DNA have been the most extensively studied, and as might be expected, these checkpoints and the genes that enforce them are critically important for the response that cells mount to genotoxic stresses. Abrogation of checkpoints leads to genomic instability and an increased mutation frequency (47).

Progress in elucidating the mechanisms of checkpoint function reveals that a number of checkpoint genes are frequently mutated in human cancers. For example, the p53 tumor suppressor functions as a cell cycle checkpoint that halts cell cycle progression in G1 by inducing the expression of the $p21^{waf1}$ gene in the presence of damaged DNA (45). The p53 gene is frequently mutated in human cancers and consequently, most tumor cells lack the DNA damage-induced p53-dependent G1 checkpoint, increasing the likelihood that mutations will be propagated in these cells. Because p53 also promotes apoptosis, the lack of p53 in these cells also makes them more resistant to the DNA damage-induced apoptosis. Because most chemotherapeutic agents kill cells through DNA damage-induced apoptosis, tumor cells with mutant p53 are also more resistant to conventional therapies (48).

3.5 Apoptosis and Reduced Sensitivity to Apoptosis

Apoptosis is a genetically controlled form of cell death that is essential for tissue remodeling during embryogenesis and for maintenance of the homeostatic balance of cell numbers later in adult life. The importance of apoptosis to human disease comes from the realization that disruption of the apoptotic process is thought to play a role in diverse human diseases ranging from malignancy to neurodegenerative disorders. Because apoptosis is a genetically controlled process, much effort has been spent on identifying these genetic components to better understand the apoptotic process as well as to identify potential therapeutic targets that might be manipulated in disease conditions where disruption of apoptosis occurs.

Although multiple forms of cell death have been described, apoptosis is characterized by morphological changes including cell shrinkage, membrane blebbing, chromatin condensation and nuclear fragmentation, loss of microvilli, and extensive degradation of chromosomal DNA. In general, the apoptotic program can be subdivided into three phases: the initiation phase, the decisiodeffector phase, and the degradationlexecution phase (Fig. 1.7). In the initiation phase, signal transduction pathways that are responsive to external stimuli, such as death receptor ligands, or to internal conditions, such as that produced by DNA damage, are activated. During the ensuing decisiodeffector phase, changes in the mitochondrial membrane occur that result in

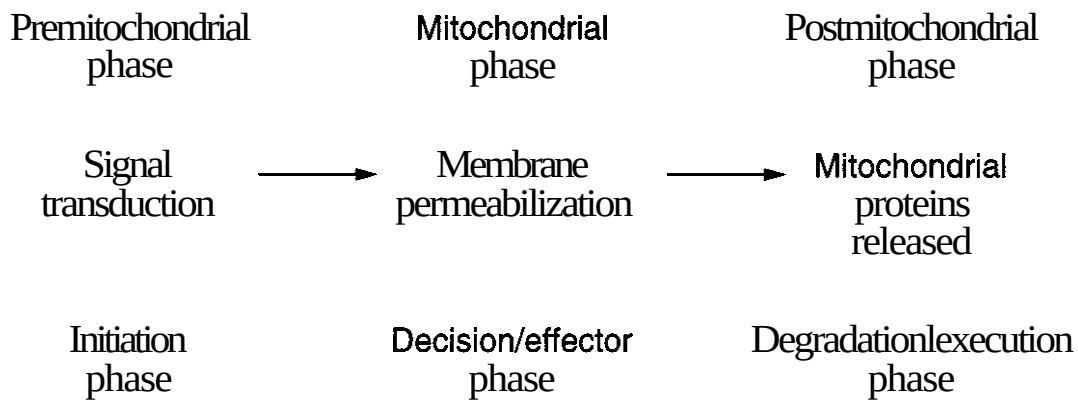


Figure 1.7. Mitochondria-mediated apoptosis. Mitochondria-mediated apoptosis is divided into three phases. Mitochondrial stress stimulates signal transduction and constitutes the initiation phase. During the second phase, changes in the structure of the mitochondrial membrane make it permeable to large proteins, allowing the release of cytochrome c and induction of the third and final phase, during which degradation of cellular proteins occurs.

disruption of the mitochondrial membrane potential and ultimately loss of mitochondrial membrane integrity. A key event in the decision/effector phase is the release of cytochrome c into the cytoplasm and activation of proteases and nucleases that signal the onset of the final degradation/execution phase. An important concept in understanding apoptosis is that the mitochrondrion is a key target of apoptotic stimuli and disruption of mitochondrial function is central to subsequent events that lead to degradation of vital cellular components.

Of the signal transduction pathways that initiate apoptosis, the best understood at the molecular level involves the death receptors including Fas/cluster of differentiation 95 (CD95), tumor necrosis factor receptor 1 (TNFR1), and death receptors 3, 4, and 5 (DR 3,4,5) (Fig. 1.8). All death receptors share an amino acid sequence known as the death domain (DD) that functions as a binding site for a specific set of death signaling proteins. Stimulation of these transmembrane receptors can be induced by interaction with its cognate ligand or by binding to an agonistic antibody, which results in receptor trimerization and recruitment of intracellular death molecules and stimulation of downstream signaling events. Here death receptors are classified as either CD95-like (Fas/CD95, DR4, and DR5) or TNFR1-like (TNF-R1, DR3, and DR6) based on the downstream signaling events that are induced as a consequence of receptor activation.

Activation of Fas/CD95 leads to clustering and recruitment of Fas-associated death domain (FADD; sometimes called Mort1) to the Fas/CD95 intracellular DD (49). FADD contains a C-terminal DD that enables it to interact with trimerized Fas receptor as well as an *N*-terminal death effector domain (DED), which can associate with the prodomain of the serine protease, caspase-8. This complex is referred to as the death-inducing signaling complex (DISC). As more procaspase-8 is recruited to this complex, caspase-8 undergoes transcatalytic cleavage to generate active protease. Activation of TNFR1-like death receptors results in similar events except that the first protein to be recruited to the activated receptor is the TNFR-associated death domain (TRADD) adaptor protein that subsequently recruits FADD and procaspase-8. Signaling through the TNFR1-like receptors is more complex and includes recruitment of other factors that do not interact with Fas/CD95. For example, TRADD also couples with the receptor interacting protein (RIP), which links stimulation of TNFR1 to signal transduction mechanisms, leading to activation of nuclear factor-kappa B (NF-κB). Because RIP does not interact with Fas/CD95, this class of receptors does not activate NF-kappa B.

The critical downstream effectors of death receptor activation are the caspases, and these are considered the engine of apoptotic cell death (50). Caspases are a family of cysteine proteases with at least 14 members. They are synthesized in the cells as inactive enzymes that must be processed by proteolytic cleavage at aspartic acid residues. These cleavage sites are between the N-terminal prodomain, the

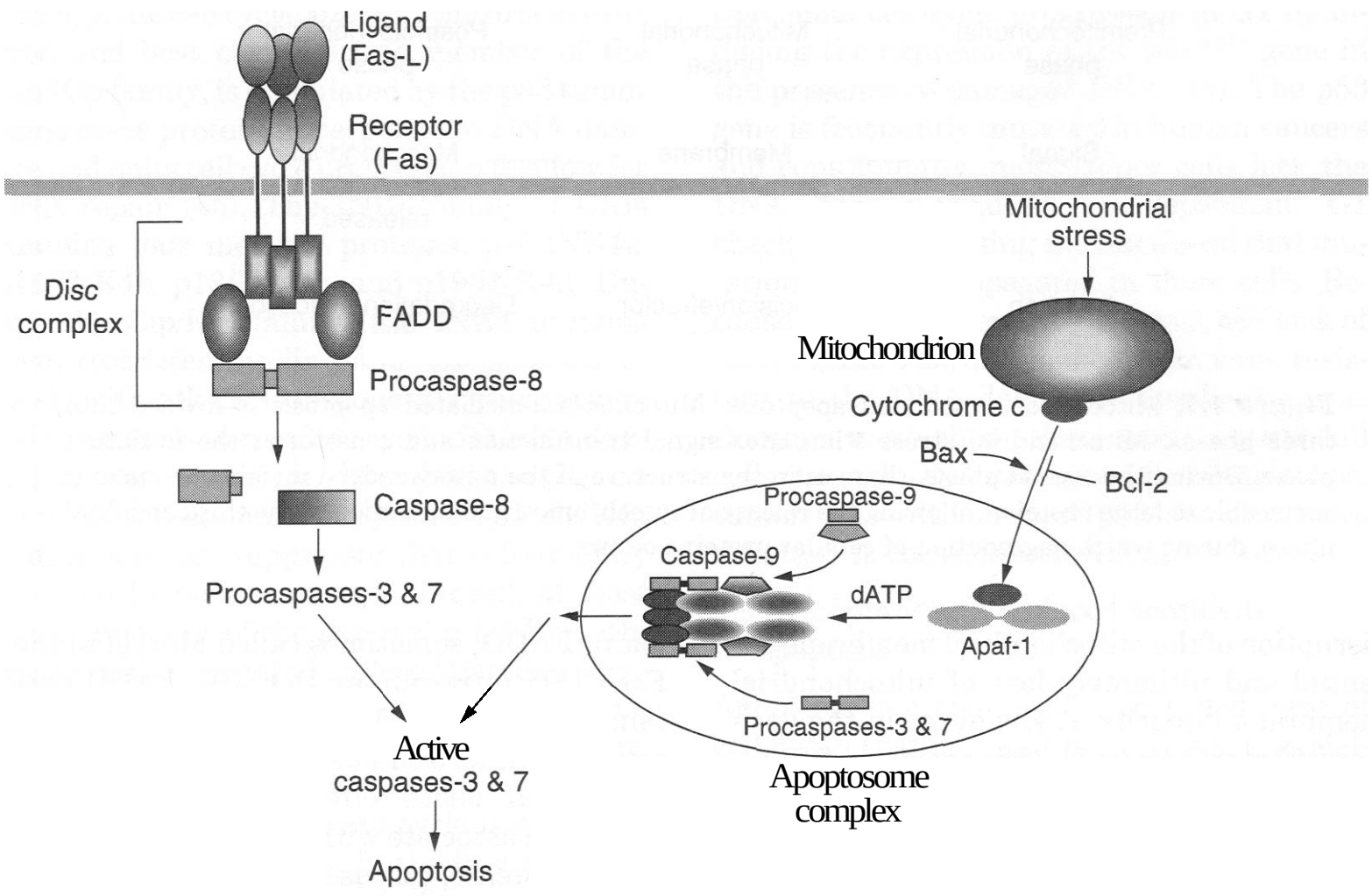


Figure 1.8. Apoptosis—receptor-mediated and mitochondrial apoptosis cascades. Trimerization of the Fas receptor initiates recruitment of the death domain-containing adaptor protein FADD, which binds to procaspase-8 promoting trans-catalytic cleavage of prodomain. Caspase-8 initiates the caspase cascade by acting on downstream effector caspases 3 and 7. In mitochondria-mediated apoptosis cytochrome c, release is a key event in apoptosis and is stimulated by Bax and suppressed by Bcl-2. The released cytochrome c binds with Apaf-1 and in conjunction with dATP induces a conformational change in Apaf-1 that permits oligomerization into a ~700-kDa complex, which is called the apoptosome complex and is capable of recruiting caspases-9, -3, and -7.

large P20, and small P10 domains. The activated proteases cleave other proteins by recognizing an aspartic acid residue at the cleavage site and are consistent with an auto- or trans-cleavage processing mechanism for activation when recruited to activated death receptors.

Importantly, biochemical studies support the notion of a caspase hierarchy that consists of initators and effectors that are activated in a cascade fashion. Initiator caspases such as caspase-8 and -9 are activated directly by apoptotic stimuli and function, in part, by activating effector caspases such as caspase-3, -6, and -7 by proteolytic cleavage. It is the effector caspases that result in highly specific cleavage of various cellular proteins and the biochemical and morphological degradation associated with apoptosis.

In contrast to death receptor-mediated apoptosis that functions through a well-defined pathway, mediators of stress-induced apoptosis such as growth factors, cytokines, and DNA damage activate diverse signaling pathways that converge on the mitochondrial membrane (51). Many proapoptotic agents have been shown to disrupt the mitochondrial membrane potential ($\Delta\Psi_m$), leading to an increase in membrane permeability and release of cytochrome c into the cytosol. Cytochrome c release is a common occurrence in apoptosis and is thought to be mediated by opening of the permeability transmembrane pore complex (PTPC), a large multiprotein complex that consists of at least 50 different proteins. The cytosolic cytochrome c interacts with apoptosis activating factor-1 (Apaf-1), dATP/ATP, and procaspase-9 to form a complex known as the apoptosome. Cytochrome c and dATP/ATP stimulate Apaf-1 self-oligomerization and trans-catalytic activation of pro-

caspase-9 to the active enzyme. Active caspase-9 activates effector caspases-3 and -7 and leads to the cellular protein degradation characteristic of apoptosis.

As release of cytochrome c can have dire consequences for viability of the cell, its release is tightly regulated. Indeed, a whole family of proteins, of which B-cell lymphoma-2 (Bcl-2)is the founding member, that share homology in regions called the Bcl-2 homology domains are dedicated to regulation of cytochrome c release from the mitochondria (52). Both positive regulators (Bax, Bak, Bik, and Bid) that promote apoptosis and negative regulators (Bcl-2 and Bcl-x,), which suppress apoptosis, act by regulating permeability of the mitochondria1 membrane to cytochrome c. Bcl-2 family members have been found in both the cytosol and associated with membranes. Bax is normally found in the cytosol, but subcellular localization changes during apoptosis. Bax has been shown to insert into the mitochondrial membrane where, because of its structure that is similar to other pore-forming proteins, it is thought to promote release of cytochrome c. Bcl-2 functions by inhibiting insertion of Bax into the mitochondrial membrane. Hence, a key factor that determines whether a cell will undergo apoptosis is the ratio of proapoptotic to antiapoptotic Bcl-2 family proteins.

Because apoptosis serves to eliminate cells with a high neoplastic potential, cancer cells have evolved to evade apoptosis primarily through two mechanisms. In the first of these, Bcl-2, which suppresses apoptosis, is overexpressed. The Bcl-2 oncogene was first identified as a break point in chromosomal translocations that frequently occurred in B-cell–derived human tumors. Characterization of the rearrangements revealed that the Bcl-2 gene is overexpressed by virtue of being placed adjacent to the powerful IgH promoter. Cloning of the Bcl-2 gene and overexpression in cells of B-cell lineage reduced the sensitivity of these cells to apoptosis and allowed them to survive under conditions that ordinarily caused normal cells to die.

The second mechanism that provides cancer cells with resistance to apoptosis is the suppression of the Fas receptor. As with other receptors, mutations can occur in either the ligand binding domain or in the intracellular domain interfering with activation of the death signaling pathway. More recently a novel mechanism for suppressing Fas-receptor activation has been identified in which cancer cells synthesize decoy receptors to which ligands can bind but are unable to induce apoptosis (53).

3.6 Increased Genetic Instability

A hallmark of tumor cells is genetic instability that is manifested at the chromosomal level as either aneuploidy (the gain or loss of one or more specific chromosomes) or polyloidy (the accumulation of an entire extra set of chromosomes). Acquisition of extra chromosomes is one mechanism by which extra copies of a growth promoting gene can be acquired by cancer cells, providing them with a selective growth advantage. Structural abnormalities are also common in advanced tumors that lead to various types of chromosomal rearrangements. Translocations and random insertion of genetic material into one chromosome from another can place genes that are not normally located adjacent to one another in close proximity usually leading to abnormal gene expression. Some of these rearrangements are routinely observed in some cancers such as in Burkitt's lymphoma where rearrangements involving chromosome 8 and 14 lead to abnormal expression of the c-myc protooncogene as a consequence of being placed adjacent to the immunoglobulin heavy chain promoter.

In chronic myelogenous leukemia (CML), an abnormal chromosome known as the Philadelphia chromosome results from a translocation involving chromosomes 9 and 22. The genes for two unrelated proteins, c-Abl and Bcr, a tyrosine kinase, and a GTPase activating protein (GAP), are spliced together, forming a chimeric protein that results in a powerful and constitutively active kinase that drives proliferation of the cells in which it is expressed.

Other forms of genetic instability include gene amplification. Under normal conditions, all DNA within the cell is replicated uniformly and only once per cell cycle. However, in cancer cells some regions of a chromosome can undergo multiple rounds of replication such that multiple copies of a growth-promoting

gene(s) is obtained. These can result in chromosomes with regions of DNA that stain uniformly during karyotype analysis of a tumor cell or in the production of extrachromosomal DNA-containing bodies known as double minute chromosomes. A typical example of this type of amplification targets the N-myc gene, which is amplified in ~30% of advanced neuroblastomas (54).

More subtle changes at the sequence level affecting growth-controlling genes is also common in human tumors. Mutations can occur as a consequence of either defects in DNA repair or decreased fidelity during DNA replication. The components of these pathways are critical for maintenance of genome integrity and inherited mutations in the genes of DNA repair proteins and proteins that repair misreplicated DNA explains some inherited cancer-prone syndromes (55).

3.7 Angiogenesis

Without the production of new blood vessels, tumor growth is limited to a volume of a few cubic millimeters by the distance that oxygen and other nutrients can diffuse through tissues. As tumor size increases, intratumoral O_2 levels fall and the center of the mass becomes hypoxic, leading to up-regulation of the hypoxia inducible factor (HIF1). HIFl is a heterodimeric transcription factor composed of a constitutively expressed HIF-1 beta subunit and an O_2 regulatable HIF-1 alpha subunit (56). Under normoxic conditions, levels of HIFl are kept low through the actions of the VHL tumor suppressor protein, which functions as a ubiquitin ligase that promotes degradation through a proteosome mediated pathway (57). An important transcriptional target of HIFl is the VEGF growth factor, which in conjunction with other cytokines, induces neovascularization of tumors and allows them to grow beyond the size limitation imposed by oxygen diffusion. This increased production of proangiogenic factors and reduction of anti-angiogenic factors is known as the "angiogenic switch" and is a significant milestone in tumorigenesis that leads to the development of more lethal tumors.

Angiogenesis is the sprouting of capillaries from preexisting vessels during embryonic development and is almost absent in adult tissues with the exception of transient angiogenesis during the female reproductive cycle and wound healing, and the soluble factor that plays a critical role in promoting angiogenesis is vascular endothelial growth factor (VEGF) (58). VEGF was first implicated in angiogenesis when it was identified as a factor secreted by tumor cells, which caused normal blood vessels to become hyperpermeable (59). The following evidence supports a role for VEGF in tumor angiogenesis.

1. VEGF is present in almost every type of human tumor. It is especially high in concentration around tumor blood vessels and in hypoxic regions of the tumor.
2. VEGF receptors are found in blood vessels within or near tumors.
3. Monoclonal neutralizing antibodies for VEGF can suppress the growth of VEGF-expressing solid tumors in mice. These lack any effect in cell culture where angiogenesis is not needed.

Ferrara and Henzel (60) identified VEGF as a growth factor capable of inducing proliferation of endothelial cells but not fibroblasts or epithelial cells. Inhibition of one of the identified VEGF receptors, FLK1, inhibits the growth of a variety of solid tumors (61). Similarly, the injection of an antibody to VEGF strongly suppresses the growth of solid tumors of the subcutaneously implanted human fibrosarcoma cell line HT-1080 (62).

There are several forms of VEGF that seem to have different functions in angiogenesis. These isoforms are VEGF, VEGF-B, VEFG-C, and VEGF-D. VEGF-B is found in a variety of normal organs, particularly the heart and skeletal muscle. It can form heterodimers with VEGF and can affect the availability of VEGF for receptor binding (63). VEGF-D seems to be regulated by c-fos and is strongly expressed in the fetal lung (64). However, in the adult it is mainly expressed in skeletal muscle, heart, lung, and intestine. VEGF-D is also able to stimulate endothelial cell proliferation (65).

VEGF-C is about 30% homologous to VEGF. Unlike both VEGF and VEGF-B, VEGF-C does not bind to heparin. It is able to

increase vascular permeability and stimulate the migration and proliferation of endothelial cells, although at a significantly higher concentration than VEGF. VEGF-C is expressed during embryonal development where lymphatics sprout from venous vessels (66). It is also present in adult tissues and may play a role in lymphatic endothelial differentiation. Flt-4, the receptor for VEGF-C, is expressed in angioblasts, veins, and lymphatics during embryogenesis, but it is mostly restricted to the lymphatic endothelium in adult tissues. Because of these expression patterns, VEGF-C and Flt-4 may be involved in lymphangiogenesis. This is the process of lymphatic generation. Lymphatic vasculature is very important because of its involvement in lymphatic drainage, immune function, inflammation, and tumor metastasis.

Other cytokines and growth factors also play an important role in promoting angiogenesis. Some of these act directly on endothelial cells, whereas others stimulate adjacent inflammatory cells. Some can cause migration but not division of endothelial cells such as angiotropin, macrophage-derived factor, and TNFα, or stimulate proliferation such as EGF, acidic and basic fibroblast growth factors (aFGF, bFGF), transforming growth factor β (TGFβ), and VEGF (67). Tumors secrete these factors, which stimulate endothelial migration, proliferation, proteolytic activity, and capillary morphogenesis (68).

Several angiogenic factors have been identified that can be secreted from tumors. Many of these are growth factors that are described as heparin-binding growth factors. Specifically, these include VEGF, FGFs, TGF-β, and the hepatocyte growth factor (HGF). The binding of these factors to heparin sulphate proteoglycans (HSPG) may be a mechanism for bringing the growth factors to the cell surface and presenting them to their appropriate receptors in the proper conformation. This facilitates the interaction between the growth factors and receptors. Studies have shown that tumor growth is adversely affected by agents that block angiogenesis (69) but is stimulated by factors that enhance angiogenesis (70).

Angiogenesis may be useful as a prognostic indicator. Tumor sections can be stained immunohistochemically for angiogenic determinants, such as VEGF, to determine the density of vasculature within the tumor, and there is a strong correlation between high vessel density and poor prognosis (71). This correlation implies a relationship between angiogenesis and metastasis.

4 CANCER-RELATED GENES

4.1 Oncogenes

Oncogenes are derived from normal host genes, also called protooncogenes, that become dysregulated as a consequence of mutation. Oncogenes contribute to the transformation process by driving cell proliferation or reducing sensitivity to cell death. Historically, oncogenes were identified in four major ways: chromosomal translocation, gene amplification, RNA tumor viruses, and gene transfer experiments. Gene transfer experiments consist of transfecting DNA isolated from tumor cells into normal rodent cells (usually NIH-3T3 cells) and observing any morphological changes. These morphological changes became the hallmarks for cell transformation, the process of becoming tumorigenic. As previously discussed, the characteristics of transformed cells are as follows: (*1*) the ability to form foci instead of a monolayer in tissue culture; (2)the ability to grow without adherence to a matrix, or "anchorage-independent growth"; and **(3)** the ability to form tumors when injected into immunologically compromised animals.

There are seven classes of oncogenes, classified by their location in the cell and their biochemical activity (Table **1.3).** All of these oncogenes have different properties that can lead to cancer. The classes of oncogenes are growth factors, growth factor receptors, membrane-associated guanine nucleotide-binding proteins, serine-threonine protein kinases, cytoplasmic tyrosine kinases, nuclear proteins, and cytoplasmic proteins that affect cell survival.

4.1.1 Growth Factors and Growth Factor Receptors. Cell growth and proliferation are subject to regulation by external signals that are typically transmitted to the cell in the

Table 1.3 Oncogenes

Oncogenes	Protein Function	Neoplasm(s)
Growth Factors		
sis	Platelet-derived growth factor	fibrosarcoma
int-2	Fibroblast growth factor	breast
trk	Nerve growth factor	neuroblastoma
Growth Factor Receptors		
erb-B1	Epidermal growth factor receptor	squamous cell carcinoma
erb-B2/HER2/neu	Heregulin	breast carcinoma
fms	Hematopoietic colony stimulating factor	sarcoma
ros	Insulin receptor	astrocytoma
Tyrosine kinases		
bcr-abl	Tyrosine kinase	chronic myelogenous leukemia
src	Tyrosine kinase	colon
lck	Tyrosine kinase	colon
Serine-Threonine protein kinases		
raf	Serine-threonine kinase	sarcoma
mos	Serine-threonine kinase	sarcoma
Guanine nucleotide binding proteins		
H-ras	GTPase	melanoma; lung, pancreas
K-ras	GTPase	leukemias; colon, lung, pancreas
N-ras	GTPase	carcinoma of the genitourinary tract and thyroid; melanoma
Cytoplasmic proteins		
bcl-2	Anti-apoptotic protein	non-Hodgkin's B-cell lymphoma
Nuclear proteins		
myc	Transcription factor	Burkitt's lymphoma
jun	Transcription factor (AP-1)	osteosarcoma
fos	Transcription factor (AP-1)	sarcoma

form of growth factors that bind to and activate specific growth factor receptors. Predictably, one class of oncogenes consists of growth factors that can stimulate tumor cell growth. In normal cells and tissues, growth factors are produced by one cell type that then act on another cell type. This is termed paracrine stimulation. However, many cancer cells secrete their own growth factors as well as express the cognate receptors that are stimulated by those factors. Because of this autocrine stimulation, cancer cells are less dependent on external sources of growth factors for proliferation and their growth is unregulated. Examples of oncogenic growth factors include v-sis, which is the viral homolog of the platelet-derived growth factor (PDGF) gene. PDGF stimulates the proliferation of cells derived from connective tissue such as fibroblasts, smooth muscle cells, and glial cells. Thus, tumors caused by excess stimulation by v-sis include fibrosarcomas and gliomas.

The receptors that interact with growth factors are also another large family of oncogenes. Growth factor receptors are composed of three domains: an extracellular domain that contains the ligand binding domain that interacts with the appropriate growth factor, a hydrophobic transmembrane domain, and a cytoplasmic domain that typically contains a kinase domain that can phosphorylate tyrosine residues in other proteins. Hence, these receptors are frequently referred to as receptor tyrosine kinases (RTK). It is this kinase

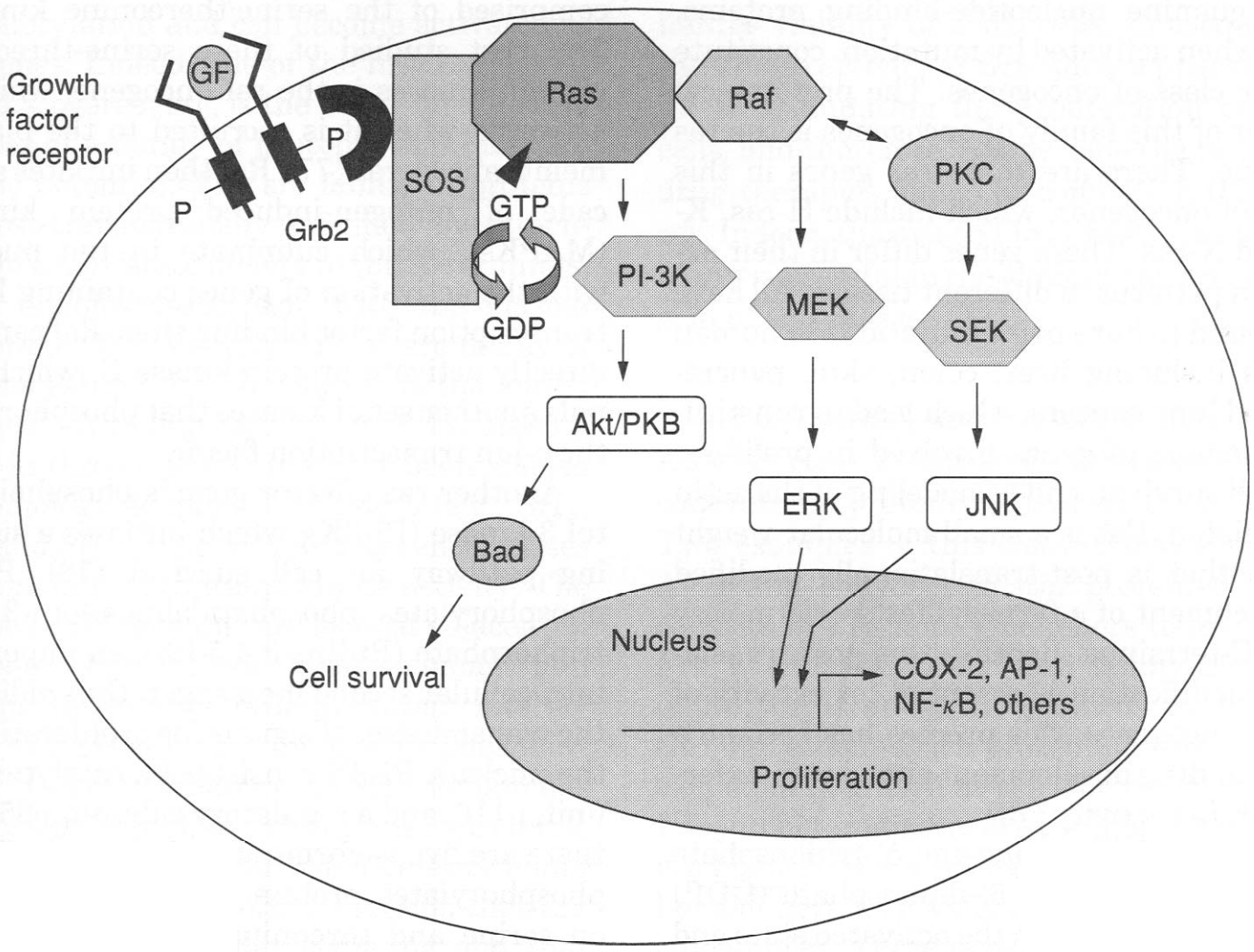


Figure **1.9.** Ras signaling pathway. Growth factor (GF) binds to its receptor and initiates dimerization and autophosphorylation. Grb2 interacts with SOS, which activates ras by promoting the GTP-bound form. Ras recruits Raf to the plasma membrane and initiates the Raf/MAPK signaling cascade. Protein kinase C also stimulates this pathway as well as another cascade of stress-activated kinases (SEK/JNK). Both of these signaling pathways promote cell proliferation by stimulating the transcription of genes like cyclooxygenase-2, activator protein-1, and nuclear factor-κB. Ras also signals phosphoinositol-3-kinase and Akt/protein kinase B for cell survival.

activity that is essential to the intracellular signaling that is stimulated by an activated receptor and in all oncogenic receptors mutations that lead to constitutive intracellular signaling promote unregulated cellular proliferation. RTKs can become oncogenically activated by mutations in each of the protein domains. Genetic mutations that result in the production of an epidermal growth factor receptor (EGFR) lacking the extracellular ligand binding domain leads to constitutive signaling. This oncogenic EGFR is known as erb-B1 (Fig. 1.9).

Normally, EGF binds to the extracellular portion of the EGFR and causes dimerization of the intracellular part of the receptor and association with adaptor proteins, Son of Sevenless (SOS), and growth factor receptor binding protein 2 (Grb 2). These proteins interact through src-homology (SH) domains SH2 and SH3, respectively. Through an unknown mechanism, the SOS-Grb 2 complex activates the oncogene ras. Ras induces an intracellular cascade of kinases to promote proliferation. These signaling cascades become constitutive when the extracellular portion of the EGFR becomes truncated, as in the case of erb-B1. Oncogenic activation of a related RTK, erb-B2, occurs as a consequence of a single point mutation that falls within the transmembrane region of this receptor (72). This mutated receptor is frequently found in breast cancers. Finally, mutations in the cytoplasmic kinase domain can also cause constitutive activity leading to constitutive signaling.

4.1.2 G Proteins. In many cases, signaling that is initiated by growth factors activating their receptors passes next to membrane asso-

ciated guanine nucleotide-binding proteins, which when activated by mutation, constitute another class of oncogenes. The prototypical member of this family of oncogenes is the ras oncogene. There are three ras genes in this family of oncogenes, which include H-ras, K-ras, and N-ras. These genes differ in their expression patterns in different tissues. All have been found to have point mutations in human cancers including liver, colon, skin, pancreatic, and lung cancers, which lead to constitutive signaling of genes involved in proliferation, cell survival, and remodeling of the actin cytoskeleton. Ras is a small molecular weight protein that is post-translationally modified by attachment of a farnasyl fatty acid moiety to the C-terminus. Because this post-translational modification is essential for activity of the ras oncogenes, this process has become a target for drug development aimed at interfering with ras activity (73).

Ras binds both guanosine 5′-triphosphate (GTP) and guanosine 5'-diphosphate (GDP) reversibly but is only in the activated state and capable of signaling when bound to GTP. The activated, GTP-bound form of ras signals a variety of mitogen-induced and stress-induced pathways, leading to transcription of genes necessary for cell growth and proliferation (74). Mitogens such as growth factors can activate ras through the epidermal growth factor receptor, and stress factors affecting ras include ultraviolet light, heat, and genotoxins. Guanine nucleotide exchange factors (GEFs) foster ras activation by promoting the exchange of GDP for GTP. In contrast, GTPase activating proteins (GAPs) suppress ras activity by promoting GTP hydrolysis by ras, resulting in the GDP-bound inactive form of ras (75). Importantly, because GAPs function to suppress cell proliferation, they can be thought of as tumor suppressors. Indeed, the neurofibromatosis gene, NF-1, is a GAP that acts as a tumor suppressor gene and can be inherited in a mutated and nonfunctional form giving rise to the Von Recklinghausen neurofibromatosis or neurofibromatosis type 1 cancer syndrome (76).

4.1.3 Serine/Threonine Kinases. Once activated, ras then transmits the growth signal to a third class of signaling molecules that is comprised of the serine/thereonine kinases. The best studied of these serine-threonine protein kinases is the raf oncogene, which is activated when it is recruited to the plasma membrane by ras (77). Raf then initiates a cascade of mitogen-induced protein kinases (MAPKs), which culminate in the nucleus with the activation of genes containing Elk-1 transcription factor binding sites. Raf can also directly activate protein kinase C, which signals another set of kinases that phosphorylate the c-jun transcription factor.

Another ras effector gene is phosphoinositol 3-kinase (PI-3K), which initiates a signaling pathway for cell survival (78). PI-3K phosphorylates phosphatidalinositol (3,4,5)-triphosphate (PtdIns-3,4,5-P3), an important intracellular second messenger, thus aiding in the transmission of signals for proliferation to the nucleus. PI-3K consists of a catalytic subunit, p110, and a regulatory subunit, p85, and there are five isoforms of each subunit. PI-3K phosphorylates protein kinase B (Akt/PKB) on serine and threonine residues, which in turn modulate cellular processes like glycolysis and translation initiation and elongation. Akt/PKB also phosphorylates Bad, a pro-apoptotic protein. When Bad is phosphorylated, it is sequestered by the 14-3-3 protein, rendering it incapable of binding to the anti-apoptotic protein, bcl-2, and thus, results in apoptosis. Akt's phosphorylation of Bad serves to inhibit apoptosis and promote cell survival. This has deleterious effects for the organism because tumor cells are not permitted to undergo apoptosis and will survive and divide.

PI-3K has been linked to the development of colon cancer by a study showing that genetic inactivation of the p110gamma catalytic subunit of PI-3K leads to the development of invasive colorectal adenocarcinomas in mice (79). This pathway is not completely separate from the Raf/MAPK pathway, because Akt has been found to inhibit Raf activity. In fact, none of the aforementioned ras-mediated pathways operate completely independently; there are multiple examples of crosstalk between these signaling pathways.

4.1.4 Nonreceptor Tyrosine Kinases. In addition to growth factor receptors, other nonreceptor kinases target protein tyrosines for

phosphorylation and can become activated as oncogenes. Indeed, one of the first oncogenes to be discovered, src, is the best characterized member of a family of proteins that have oncogenic potential. The src family of proteins are post-translationally modified by attachment of a myristate moiety to the N-terminus, which enables association with the plasma membrane. The members of the src family of proteins exhibit 75% homology at the amino acid level with the greatest degree of similarity found in three regions that have been labeled src homology domains 1, 2, and 3 (e.g., SH1, SH2, and SH3). The SH1 domain encompases the domain that contains kinase activity. The SH2 and SH3 domains are located adjacent to and N-terminal to the kinase domain and function to promote protein/protein interactions. The SH2 domain binds with phosphorylated tyorsines, whereas the SH3 domain has affinity for the proline rich regions of proteins. Importantly, SH2 and SH3 domains are found in a large number of other proteins that are involved in intracellular signaling and that have oncogenic potential, and the structure of these domains are strongly conserved. Because SH2 and SH3 domains serve to potentiate signal transduction, they have also become targets for drug discovery programs aimed at disrupting the constitutive signaling generated by oncogenic activity (80).

A second oncogenic protein tyrosine kinase of considerable clinical importance is the Bcr-Abl oncogene. The Bcr-Abl protein is a chimeric fusion protein formed by a reciprocal translocation involving chromosomes 9 and 22. This chromosomal rearrangement is diagnostic for the hematopoietic malignancy, chronic myelogenous leukemia (CML), and the rearranged chromosome is known as the Philadelphia chromosome (81). The c-Abl gene maps to chromosome 9 and is a tyrosine kinase, whereas the BCR gene is now known to be GTPase-activating protein (GAP), which when fused to Abl results in an unregulated tyrosine kinase that functions to promote cellular proliferation (82). The bcr-abl protein interacts with SH2 domains on Grb 2 and relocates to the cytoskeleton and initiates ras signaling, a primary mode of tumorigenic potential. Bcr-abl reduces growth factor dependence, alters adhesion properties, and enhances viability of CML cells. Consequently, the kinase activity of Bcr-Abl is a primary factor in stimulating the proliferation of CML cells, and therefore, has become the target for drug therapies aimed at combating this cancer. Indeed, the drug STI571 has been spectacularly successful in the clinic at causing remission of this disease (83).

4.1.5 Transcription Factors as Oncogenes. Another class of oncogenes are those that encode nuclear proteins, or transcription factors. Two examples of this class of oncogenes are AP-1 and c-myc. Activator protein-1 (AP-1) consists of Fos family members (c-fos, fos B, Fra 1, and Fra 2) and Jun family members (c-jun, jun B, and jun D), which can dimerize through a lucine rich proteidprotein interaction domain known as the leucine zipper (84). Fos-jun heterodimers are the most active, jun-jun homodimers are weakly active, and fos-fos homodimers form only in extremely rare circumstances. These dimers bind to AP-1 DNA binding sites, which are also called the tumor promoter TPA-responsive element (TRE) or glucocorticoid response element (GRE). AP-1 can be activated by ionizing and ultraviolet irradiation, DNA damage, cytokines, and oxidative and cellular stresses (85).

AP-1 has several functions in the cell, including the promotion of cell proliferation and metastasis. AP-1 is a nuclear target for growth factor-induced signaling such as the aforementioned EGFR-mediated kinase cascade. AP-1–regulated genes include genes necessary for metastasis, and invasion like the MMPs matrilysin and stromelysin, as well as collagenase two proteins that aid in cell migration through connective tissue.

Deregulation of c-myc often occurs either by gene rearrangement or amplification in human cancers. Here again the hematologic cancers are instructive. In Burkitt's lymphoma, a frequent reciprocal translocation between chromosomes 8 and 14 leads to juxtapositioning of the myc gene adjacent to the Ig heavy chain promoter/enhancer complex, causing uncontrolled expression and production of the myc protein (86). Translocations between chromosomes 2 and 8 and between 8 and 22 also occur and involve other immunoglobulin

producing gene complexes. In all cases the overproduction of myc results in uncontrolled cell proliferation.

Myc overexpression also occurs in solid tumors, but is usually the result of gene amplification (87). The oncogenic potential of c-myc has been studied most widely as it pertains to the development of colon cancer. Both c-myc RNA and protein are overexpressed at the early and late stages of colorectal tumorigenesis. The cause for this overexpression is still unknown, but a strong possibility may be that it is regulated by the APC pathway. The APC tumor suppressor gene is mutated in approximately 90% of colorectal tumors, both sporadic and inherited forms. AFC will be discussed in detail in the "tumor suppressor" section of this chapter.

He et al. (88) found that when APC expression was induced in stably transfected $APC^{-/-}$ colon cancer cells (using an inducible metallothionine promoter linked to the APC gene), they observed a time-dependent decrease in the RNA and protein levels of c-myc. This suggested that c-myc may be regulated by AFC through the β-catenin/T-cell factor-4 (Tcf-4) transcription complex. They also showed that constitutive expression of mutant β-catenin (mutated so that it is insensitive to APC) in embryonic kidney cells resulted in a significant increase of c-myc expression. Analysis of the c-myc gene revealed two possible Tcf-4 transcription factor binding sites. Mobility shift assays demonstrated that Tcf-4 binds to both of the potential binding sites, leading to c-myc gene expression. Expression of dominant-negative Tcf-4 in HCT116 (mutant β-catenin) or SW480 (mutant AFC) reduced endogenous levels of c-myc (88).

The c-myc protein binds to DNA through its basic, helix-loop-helix/leucine zipper domain. Many target genes of c-myc have been identified that are involved in cell growth and proliferation. Some of these genes include ODC, cell cycle genes cyclins A, E, and D1, as well as cdc2, cdc25, eukaryotic initiation factor 4E (eIF4E), heat shock protein 70 (hsp70), and dihydrofolate reductase. Overexpression of c-myc may therefore affect the transcription of these genes, thus promoting hyperproliferation and tumorigenesis.

C-myc is also found to be amplified in promyelocytic leukemia and small cell lung cancer. The c-myc protein requires dimerization with Max to initiate transcription, and Max homodimers serve as an antagonist of transcription. The formation of Mad-Max dimers also suppresses transcription. It is also interesting to note that the full oncogenic potential of c-myc relies on cooperation with other oncogenes like ras.

4.1.6 Cytoplasmic Proteins. Bcl-2 is an example of a cytoplasmic oncogene that has antiapoptotic potential. Increased production of bcl-2 protein is seen in a variety of tumor types and is associated with poor prognosis in carcinomas of the colon and prostate. The function of bcl-2 is explained in detail in the "apoptosis" section of this chapter.

4.2 Tumor Suppressor Genes

In contrast to oncogenes, tumor suppressor genes can directly or indirectly inhibit cell growth. Those that directly inhibit cell growth or promote cell death are known as "gatekeepers" and their activity is rate limiting for tumor cell proliferation. Hence, both copies of gatekeeper tumor suppressors must be functionally eliminated for tumors to develop. This characteristic requirement is a hallmark of tumor suppressor genes. Mutations that inactivate one allele of a gatekeeper gene can be inherited through the germline, which in conjunction with somatic mutation of the remaining allele, leads to cancer predisposition syndromes. For example, mutations of the APC gene lead to colon tumors. Somatic mutations that inactivate both gatekeeper alleles occur in sporadic tumors.

Those tumor suppressor genes that do not directly suppress proliferation, but function to promote genetic stability are known as "caretakers." Caretakers function in DNA repair pathways and elimination of caretakers results in increased mutation rates. Because numerous mutations are required for the full development of a tumor, elimination of caretaker tumor suppressors can greatly accelerate tumor progression. As with gatekeepers, mutations can be inherited through the germline and can give rise to cancer predisposition syndromes. An example of a caretaker gene is

Table 1.4 Tumor Suppressor Genes

TS Gene	Protein Function	Neoplasm(s)
APC	cell adhesion	colon
BRCA 1	transcription factor	breast and ovary
BRCA 2	DNA repair	breast and ovary
CDK4	cyclin D kinase	melanoma
hMLH1	DNA mismatch repair	HNPCC[a]
hMSH2	DNA mismatch repair	HNPCC
hPMS1	DNA mismatch repair	HNPCC
hPMS2	DNA mismatch repair	HNPCC
MEN1[b]	Ret receptor	thyroid
NF1	GTPase	neuroblastoma
p53	transcription factor	colon, lung, breast
Rb	cell cycle checkpoint	retinoblastoma
WT-1	transcription factor	childhood kidney

[a]Hereditary non-polyposis colon cancer.
[b]Multiple endocrine neoplasia.

MSH2, which functions in the mismatch DNA repair system, and inherited mutations in this gene gives rise to the hereditary nonpolyposis colorectal cancer (HNPCC) syndrome (Table 1.4).

4.2.1 Retinoblastoma. Retinoblastoma (Rb)is a childhood disease. There are both hereditary and nonhereditary forms of the disease. Approximately 60% of patients develop the nonhereditary form and present with unilateral tumor development (one eye is affected). About 40%of Rb patients have a germline mutation that predisposes them to the disease. Of these patients, 80% of the cases are bilateral, 15%are unilateral, and about 5%are asymptomaticcarriers of the mutation. It is an autosomal dominant trait and is caused by mutations in the Rb gene on chromosome 13. Abnormalities of the Rb gene have also been seen in breast, lung, and bladder cancers.

Retinoblastoma arises when both of the Rb alleles are inactivated. In the inherited form, one parental chromosome carries a defect (most often a deletion) at the Rb locus. A second somatic mutation must occur in retinal cells to cause the loss of the other (normal) Rb allele. In sporadic cases, both of the parental chromosomes are normal and both Rb alleles are lost as a result of individual somatic mutations. Approximately one-half of all retinoblastoma cases show a deletion at the Rb locus. The locus is very large, >150 kb, and therefore may be more susceptible to mutations because it is such a large target.

Rb was the first human tumor suppressor gene identified, and the loss of RB protein function leads to malignancy. The RB protein is localized in the nucleus where it is either phosphorylated or unphosphorylated (Fig. 1.10). When unphosphorylated, RB binds to the E2F transcription factor and prevents transcriptional activation of E2F target genes. This normally occurs during the M and early G1 phases of the cell cycle. During late G1, S, and G2 phases, RB is phosphorylated. When phosphorylated, RB can no longer bind to E2F. This release from inhibition allows E2F to activate transcription of S-phase genes and the cell cycle progresses. When loss of RB function occurs because of various mutations in the Rb gene, the cell cycle becomes deregulated, and uncontrolled cell division results. This is because RB can no longer bind to and inhibit E2F. Therefore, the transcription factor can constitutively activate its target genes. This ultimately leads to tumor development (89).

4.2.2 p53. The p53 tumor suppressor is activated in response to a wide variety of cellular stresses including DNA damage, ribonucleotide depletion, redox modulation, hypoxia, changes in cell adhesion, and the stresses created by activated oncogenes. The p53 protein functions as a transcription factor that, when

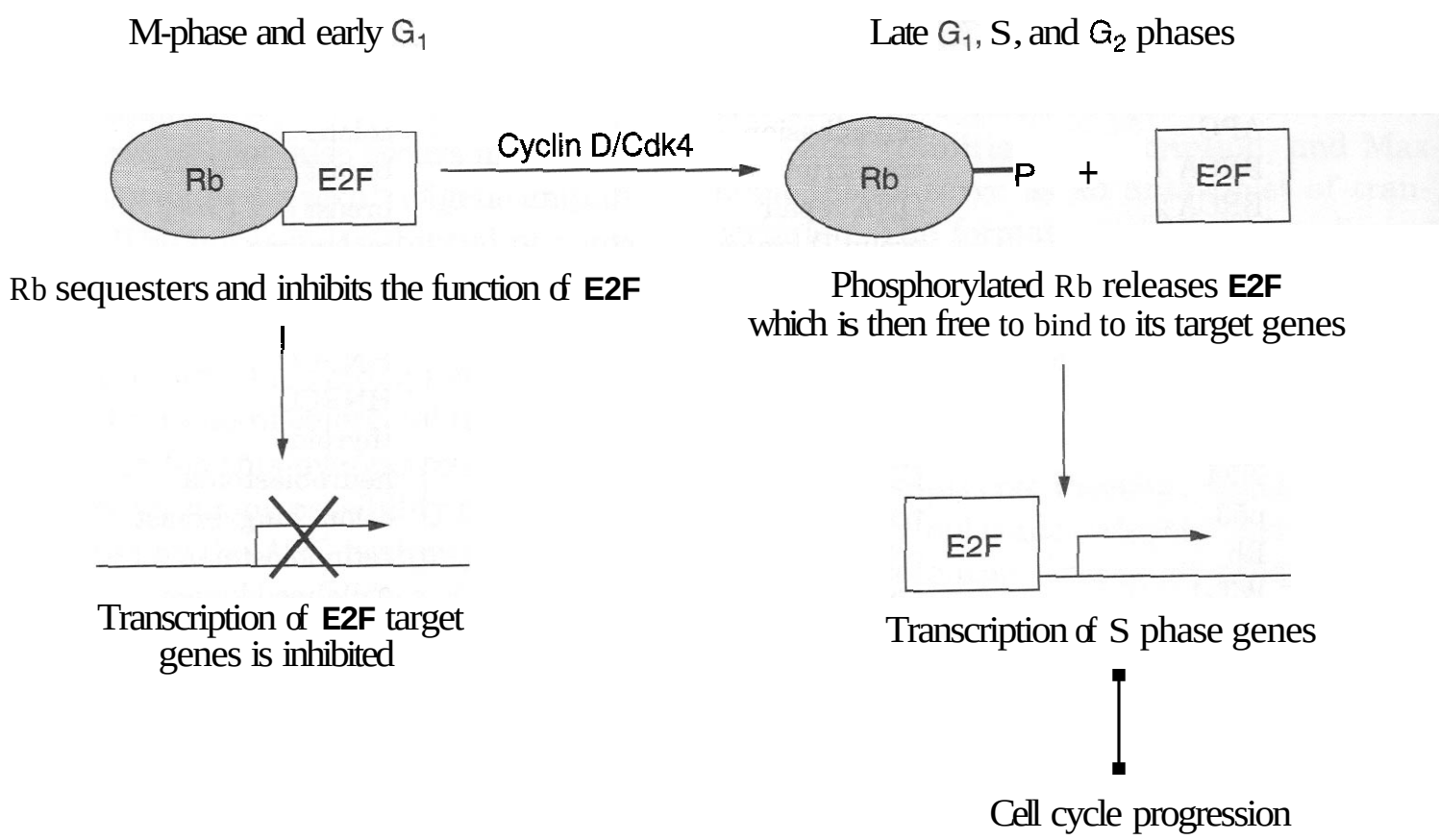


Figure 1.10. Cell cycle control by the retinoblastoma (Rb) tumor suppressor protein. Unphosphorylated Rb negatively regulates progression into the S phase of the cell cycle by binding to the **E2F** transcription factor. In this complex, **E2F** is prevented from activating transcription of its target genes. During late G1, Rb is phosphorylated by the cyclin D/Cdk4 complex and can no longer sequester the **E2F** transcription factor. **E2F** then binds to its target S-phase genes, promoting their transcription and allowing the cell cycle to progress.

activated, stimulates the expression of a variety of effectors that bring about growth arrest, promote DNA repair, and stimulate cell death by apoptosis. Collectively these activities act to maintain genomic stability. Elimination of p53 function leads to increased rates of mutation and resistance to apoptosis. Thus, p53 sits at the crux of several biochemical pathways that are disrupted during tumorigenesis. Consequently, mutations in p53 are the most frequent genetic change encountered in human cancers.

p53 activity can be eliminated by at least three mechanisms. The most common event that leads to a nonfunctioning protein is mutation of the p53 gene, which occurs in about 50% of all sporadic human tumors. As with other tumor suppressors, mutations can occur in somatic tissues or can be inherited through the germline. Inherited p53 mutations give rise to the Li-Fraumeni syndrome in which affected individuals develop bone or soft-tissue sarcomas at an early age. In addition, nonmutational inactivation of p53 can occur in the presence of viral transforming antigens. For example, the simian virus 40 (SV40) large T antigen binds with p53 and forms an inactive complex, whereas the papilloma virus E6 protein eliminates p53 by causing premature degradation of the protein through the 26S proteosome. Clearly, the interaction between these transforming antigens and p53 is critical because viral antigens that are incapable of doing so lose their transforming ability. The third mechanism by which p53 activity can be eliminated is by cytoplasmic sequestration. p53 that is unable to enter the nucleus cannot induce the expression of downstream effector genes that are necessary for mounting the cellular response to genotoxic stress.

Activation of p53 by ionizing radiation (IR) and other DNA damaging agents involves a complex set of interdependent post-translational modifications that control protein/protein associations, protein turnover, and subcellular localization. Under normal conditions, levels of p53 are kept minimal by ubiquitination and proteosome-mediated degradation that contributes to the short half-life (3–20 min) of the protein. A key player in

maintenance of low p53 levels is mdm2. Mdm2 performs this function by interacting with p53 at its N-terminus and targets p53 for proteosome-mediated degradation. Exposure to IR results in a series of, as yet incompletely understood, phosphorylation events in p53's N-terminus, which inhibits Mdm2 binding and results in increased intracellular p53 levels. Mdm2 and p53 function in a feedback loop where activated p53 stimulates the expression of Mdm2, which in turn reduces the duration of up-regulated p53 activity. Overexpression of Mdm2 suppresses p53 by preventing its accumulation in response to DNA damage. Consequently, Mdm2 can function as an oncogene that acts in much the same way as the papilloma virus E6 protein. In fact, Mdm2 is overexpressed in some tumors such as osteosarcomas.

The p53 protein can be divided into three structural domains that are essential for tumor suppressor function. The N-terminus consists of a transactivation domain that interacts with various basal transcription factors and cellular and viral proteins that modify its function. The central domain contains the sequence specific DNA binding activity. Most mutations in the p53 gene fall within this domain that disrupts the structure of this region and eliminates DNA binding activity. The importance of DNA binding is emphasized by the fact that mutations accumulate preferentially in several amino acids that are involved in directly contacting DNA. The C-terminus has been assigned several activities including non-specific DNA binding activity, acting as a binding site for other p53 molecules, and formation of p53 tetramers, and functioning as a pseudosubstrate domain that occludes the central DNA binding domain.

Because of the frequency with which p53 is mutated in human tumors, much attention has been directed at developing methods that compensate for the loss of wild-type function or can reactivate wild-type p53 activity in mutant proteins. For example, strategies aimed at manipulating the conformation of mutant proteins have led to the discovery that peptides that bind the C-terminus can reactivate wild-type function in some mutant proteins. Strategies that take advantage of the vast knowledge of virus biology and p53 function have lead to the construction of viral vectors that can introduce a wild-type p53 into tumor cells. One clever approach takes advantage of the fact that adenoviruses with a defective E1B 55K protein cannot replicate in normal human cells. For adenoviruses to replicate in cells, they must suppress p53 activity, which functions to limit the uncontrolled DNA replication that is required for production of virus genomes. However, adenoviruses with a defective E1B 55K gene can replicate in tumor cells because they lack a functional p53. Thus, these viruses kill tumor cells specifically and leave normal cells untouched (90).

4.2.3 Adenomatous Polyposis Coli. The tumor suppressor gene, APC, is mutated in almost 90% of human colon cancers and 30% of melanoma skin cancers. The inherited loss of APC tumor suppressor function results in familial adenomatous polyposis (FAP). FAP patients develop hundreds to thousands of colon polyps by their second or third decade of life. By age 40, one or two of these polyps usually develops into a malignant carcinoma, and thus, many of these patients choose to have a colectomy to prevent carcinoma formation. Mutations in APC occur in the majority of sporadic colon cancers too.

APC mutation is an early event in colon carcinogenesis, and is therefore, considered to be the initiating event. Loss of this tumor suppressor gene results in constitutive activity of the oncogene, c-myc, through an intricate collection of protein-protein interactions. Briefly, APC interacts with other cellular proteins, including the oncogene p-catenin (Fig. 1.11). Axin, an inhibitor of Wnt signaling, forms a complex with glycogen synthase kinase 3β (GSK3β), p-catenin, and APC and stimulates the phosphorylation of β-catenin by GSK3β, thus causing down-regulation of gene expression mediated by β-catenin/Tcf complexes (91). Dissociation of the axin, GSK3β, β-catenin, and APC complex by Wnt family members leads to stabilization of p-catenin and activation of Tcf-mediated transcription. Deletion of APC alleles, or mutations causing truncations in APC that influence its interaction with p-catenin, also leads to stabilization of β-catenin and activation of Tcf/lymphoid enhancing factor (Lef)–dependent gene expression. At

least one member of the Tcf/Lef family of transcriptional activators has been identified in human colon mucosal tissues. This member is termed hTcf-4. Several target genes for Tcf/Lef have been identified, including the c-myc oncogene. Overexpression of wild-type APC cDNA in human colon tumor-derived HT29 cells, which lack a normal APC allele, causes down-regulation of c-myc transcription. Up-regulation of β-catenin in cells expressing normal APC alleles causes increased c-myc expression. Thus, wild-type APC serves to suppress c-myc expression. Either normal regulation by Wnt signaling, or mutation/deletion of APC, activates c-myc expression. In many colon cancers, the APC gene is not necessarily mutated, but the mutation in the pathway is found in β-catenin, which yields the same constitutive signaling from the pathway.

APC regulates the rates of proliferation and apoptosis by several different mechanisms. Wild-type APC is important for cytoskeletal integrity, cellular adhesion, and Wnt signaling. APC plays a role in the G1/S transition of the cell cycle by modulating expression levels of c-myc and cyclin D1. Wild-type, full length APC is also important in maintaining intestinal cell migration up the crypt and inducing apoptosis.

4.2.4 Phosphatase and Tensin Homologue. The phosphatase and tensin homologue (PTEN) or mutated in multiple advanced cancers (MMAC) tumor suppressor gene was first identified in the most aggressive form of brain cancer, glioblastoma multiform. PTEN also is mutated in a significant fraction of endometrial carcinomas, prostate carcinomas, and melanomas. PTEN's primary functions as a tumor suppressor gene are the induction of cell cycle arrest and apoptosis (92). PTEN is a dual-specificity phosphatase, meaning that it can dephosphorylate proteins on serine, threonine, and tyrosine residues. It specifically dephosphorylates PtdIns-3,4,5-P3, antagonizing the function of PI-3K. PTEN, therefore, acts as a negative regulator of Akt activation. Because Akt can suppress apoptosis by the phosphorylation of the pro-apoptotic protein Bad, PTEN can induce apoptosis of mutated or stressed cells to prevent tumor formation.

In addition to modulating apoptosis, PTEN plays a role in angiogenesis. PTEN suppresses the PI-3K-mediated induction of blood vessel growth factors like VEGF. EGF and ras act to induce genes regulated by the hypoxia-induced factor (HIF-1), which is blocked by PTEN activity. PTEN also inhibits cell migration and formation of focal adhesions when overexpressed in glioblastoma cell lines, suggesting that it helps to inhibit metastasis as well (93).

PTEN also inhibits signaling from the insulin growth factor receptor (IGF-R). Insulin receptor substrates-112 (IRS-1/2) are docking proteins that are recruited by the insulin receptor and in turn, recruit PI-3K for signal transduction. The tumor suppressor function of PTEN helps to prevent aberrant signaling when insulin binds to its cell surface receptor.

4.2.5 Transforming Growth Factor-β. Transforming growth factor-β (TGF-β) is growth stimulatory in endothelial cells but growth inhibitory for epithelial cells, rendering it a tumor suppressor gene in epithelial-derived cancers. The TGF-β family of growth factors binds to two unique receptors, TGF-β type I and type II. Tumor cells lose their response to the growth factor and mutations in the receptors also contribute to carcinogenesis. Ligand binding to the TGF-β receptors causes intracellular signaling of other tumor suppressor genes, the Smad proteins. Smads help to initiate TGF-β-mediated gene transcription.

TGF-β1 normally inhibits growth of human colonic cells, but in the process of becoming tumorigenic, these cells obtain a decreased response to the growth inhibitory actions of TGF-β. TGF-β1 also serves as an inhibitor of immune surveillance (94). TGF-β1 indirectly suppresses the function of the immune system by inhibiting the production of TNF-α and by inhibiting the expression of class II major histocompatibility complex (MHC) molecules. TGF-β1 also promotes tumor progression by modulating processes necessary for metastasis such as degradation of the extracellular matrix, tumor cell invasion and VEGF-mediated angiogenesis.

The TGF-β receptor type II (TβRII) is mutated in association with microsatellite instability in most colorectal carcinomas (95). As

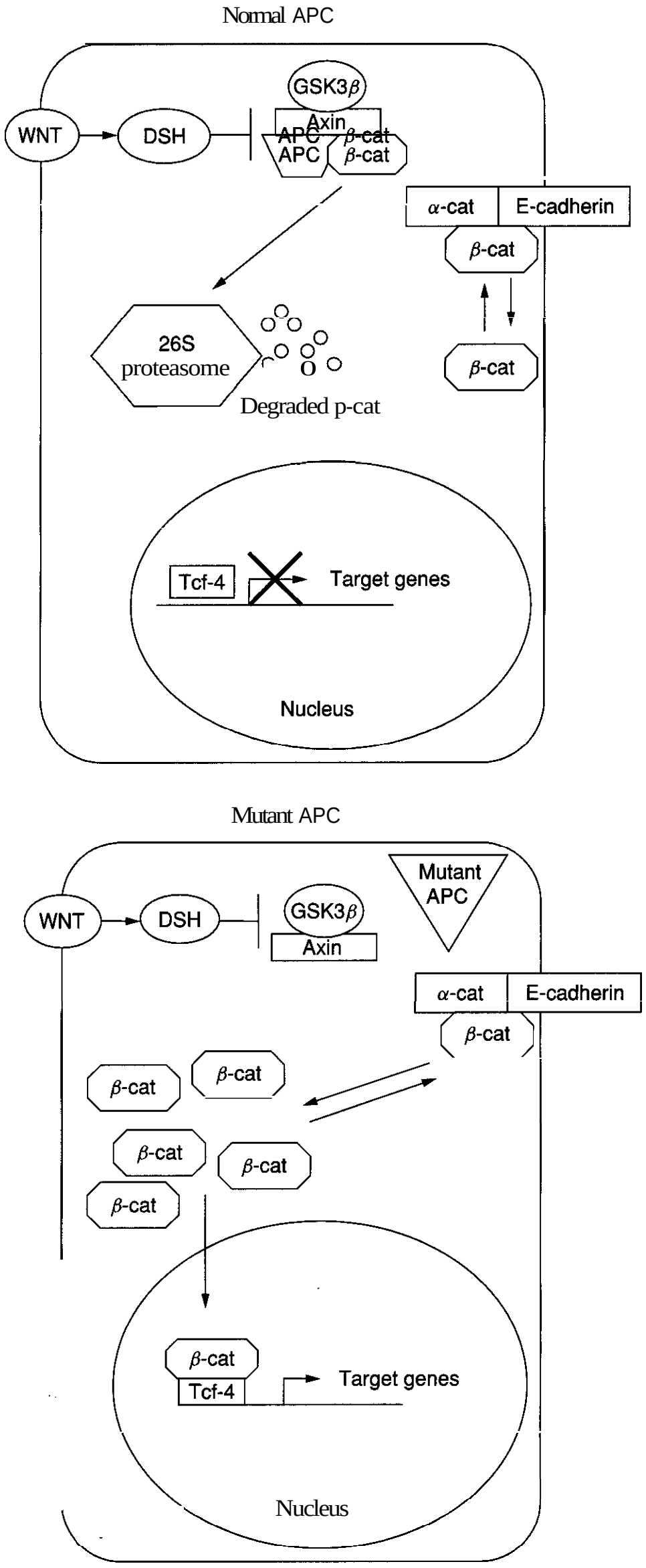


Figure 1.11. The APC signaling pathway. In a normal cell, APC forms a complex with **axin**, **GSK-3β**, and p-catenin. This promotes proteosomal degradation of **β-catenin** and prevents transcription of **β-catenin/Tcf4** target genes. When APC is mutated, the multi-protein complex cannot form and p-catenin is not degraded. Instead, **β-catenin** is translocated to the nucleus where it binds with Tcf4 to activate transcription of various target genes. Some of the known target genes, like c-myc and cyclin D1, play important roles in cell proliferation.

many as 25% of colon cancers have missense mutations in the kinase domain of this receptor. A missense mutation in the kinase domain of the TβRI has also been identified in metastatic breast cancer. It was also found that the expression of the TGF-β2 receptor is suppressed in metastatic oral squamous cell carcinomas compared with the primary tumor.

4.2.6 Heritable Cancer Syndromes. There are several known inheritable DNA repair-deficiency diseases. Four of these are autosomal recessive diseases and include Xeroderma pigmentosum (XP), ataxia telangiectasia (AT), Fanconi's anemia (FA), and Bloom's syndrome (BS). XP patients are very sensitive to UV light and have increased predisposition to skin cancer (approximately 1000-fold) (96). AT patients exhibit a high incidence of lymphomas, and the incidence of lymphoma development is also increased for both FA and BS patients.

HNPCC arises due to a defect in mismatch repair (MMR). The incidence of HNPCC is often quoted as 1–10% of all colorectal cancers (97). It is an autosomal dominant disease and results in early onset of colorectal adenocarcinoma. Many of these tumors demonstrate microsatellite instability and are termed replication error positive (RER+). Endometrial and ovarian cancers are the second and third most common cancers in families with the HNPCC gene defect.

The most common mutations in HNPCC are in the mismatch repair genes, MSH2 and MLH1 (>80%) (98). The mismatch repair system normally corrects errors of 1–5 base pairs made during replication. Therefore, defects in this system result in many errors and create microsatellite instability. A suggested model for HNPCC development starts with a mutation in the MMR genes followed by another mutation in a gene such as APC. These two events lead to cellular hyperproliferation. Next, a mutation occurs leading to the inactivation of the wild-type allele of the MMR gene. Because of this MMR defect, mutations in other genes involved in tumor progression, such as deleted in colon cancer (DCC), p53, and K-ras, occur.

A variety of genes are responsible for the different inherited forms of GI cancers. For example, individuals with FAP, bearing germline mutations/deletions in the APC tumor suppressor gene, account for only a small fraction of colon cancers in the United States (<1%). However, the majority of sporadic colon adenomas have also been found to contain single allele alterations in APC and exhibit altered signaling of p-catenin, a protein negatively regulated by APC. Altered β-catenin signaling is inferred from immunohistochemical studies demonstrating that β-catenin is translocated to the nucleus in the majority of epithelial cells in adenomas, whereas β-catenin is generally seen associated with the cell membrane in normal colonic epithelia. These data suggest that the process of adenoma development selects for alterations in APC.

5 INTERVENTIONS

5.1 Prevention Strategies

Numerous investigators are taking advantage of our current knowledge of the mechanisms of carcinogenesis in human epithelial tissues to develop strategies for disrupting this process and thereby preventing cancer. As discussed earlier in this chapter, carcinogenesis proceeds by a multistep process, in which normal epithelial tissues acquire aberrant growth properties. These neoplastic cells progress to become invasive cancer. Historically, cancer therapy has addressed only the last phase of this process. Prevention strategies are now focusing on pre-invasive, yet neoplastic lesions.

Prevention strategies generally influence one or more of five processes in carcinogenesis (99). One strategy has been to inhibit carcinogen-induced initiation events, which lead to DNA damage. An important caveat to this strategy is that the intervention must be present at the time of carcinogen exposure to be effective. Once irreversible DNA damage has occurred, this type of strategy is ineffective in preventing cancer development.

Another strategy has been to inhibit initiated cell proliferation associated with the promotion stage of carcinogenesis. An advantage to this type of strategy is that interventions affecting promotion are effective after initiating events have occurred. Because humans are exposed to carcinogenic agents (e.g., chem-

icals in tobacco smoke, automobile exhaust) throughout their lifetimes, cancer preventive agents that work after initiating events have occurred are desirable. Two strategies of decreasing cell proliferation are induction of apoptosis, or cell death, and differentiation, which may or may not be associated with apoptosis. Induction of either differentiation or apoptosis will stabilize or decrease, respectively, overall cell number in a tissue.

A final strategy for preventing cancer is to inhibit development of the invasive phenotype in benign, or non-invasive, precancers that occur during the process of epithelial carcinogenesis.

Investigators are beginning to address the possibility that the efficacy of cancer prevention strategies may depend on both genetic and environmental risk factors affecting specific individuals. Mutationsldeletion of the APC tumor suppressor gene, discussed earlier, causes intestinal tumor formation in both rodents and humans. Increasing levels of dietary fat increases intestinal tumor number in rodent models (100). However, mice with a defective APC gene develop tumors even on low-fat diets. Thus, dietary modifications may reduce carcinogenesis in individuals without, but may be ineffective in individuals with, certain genetic risk factors for specific cancers. Recently, several large randomized studies conducted in the United States have failed to detect any protective effect of dietary fiber increase or dietary fat decrease on colon polyp recurrence (101).

5.2 Targets

Targets for cancer prevention strategies can be either biochemical species produced by the action of a physical or chemical carcinogen or an enzyme/protein aberrantly expressed as a consequence of a genetic or environmental risk factor (the latter would include exposure to environmental carcinogens). In developing mechanism-based prevention or treatment strategies based on specific "targets," it is crucial to establish that the "target" is present in the target tissue (or cells influencing target tissue behaviors), causatively involved in the disease process in question and modulated by the intervention.

5.2.1 Biochemical Targets. One example of a biochemical target produced by carcinogens is reactive oxygen species (ROS). Ionizing radiation is a complete carcinogen and produces much of its DNA damage through ROS (102). Several strategies for preventing ROS-induced cell damage have been developed. The aminothiol, amifostine, inhibits radiation-induced DNA damage to a large degree by scavenging free radicals produced by ionizing radiation. Amifostine and its derivatives suppress ionizing radiation-induced transformation and carcinogenesis. Antioxidants, including protein and non-protein sulfhydrals and certain vitamins, are effective modulators of ROS produced by physical and chemical carcinogens (103). Antioxidants are effective in inhibiting carcinogenesis in some experimental models, but their roles in human cancer prevention remains unclear. At least some agents with antioxidant activity may increase carcinogenesis in some tissues. Heavy smokers receiving combinations of beta-carotene and vitamin A had excess lung cancer incidence and mortality, compared with control groups not receiving this intervention (104).

Other examples of biochemical targets are the dihydroxy bile acids, which are tumor promoters of colon cancer (105). Both genetic and dietary factors are known to influence intestinal luminal levels of these steroid-like molecules. whose levels are associated with colon cancer risk. Calcium reduces intestinal luminal bile acid levels by several possible mechanisms, and dietary calcium supplementation is associated with a small (~25%), but statistically significant, reduction in colon polyp recurrence (106). This result requires cautious evaluation, however, as similar levels of calcium supplementation have been associated with increased risk of prostate cancer (107). This example and the result of the beta-carotene study mentioned above underscore the tissue-specific differences in carcinogenesis and the difficulties of applying common dietary components (e.g., calcium, antioxidants) in cancer prevention strategies in humans.

5.2.2 Cyclooxygenase-2 and Cancer. Cyclooxygenase (COX) enzymes catalyze prostaglandins from arachidonic acid. Prostaglandins play a role in biological processes

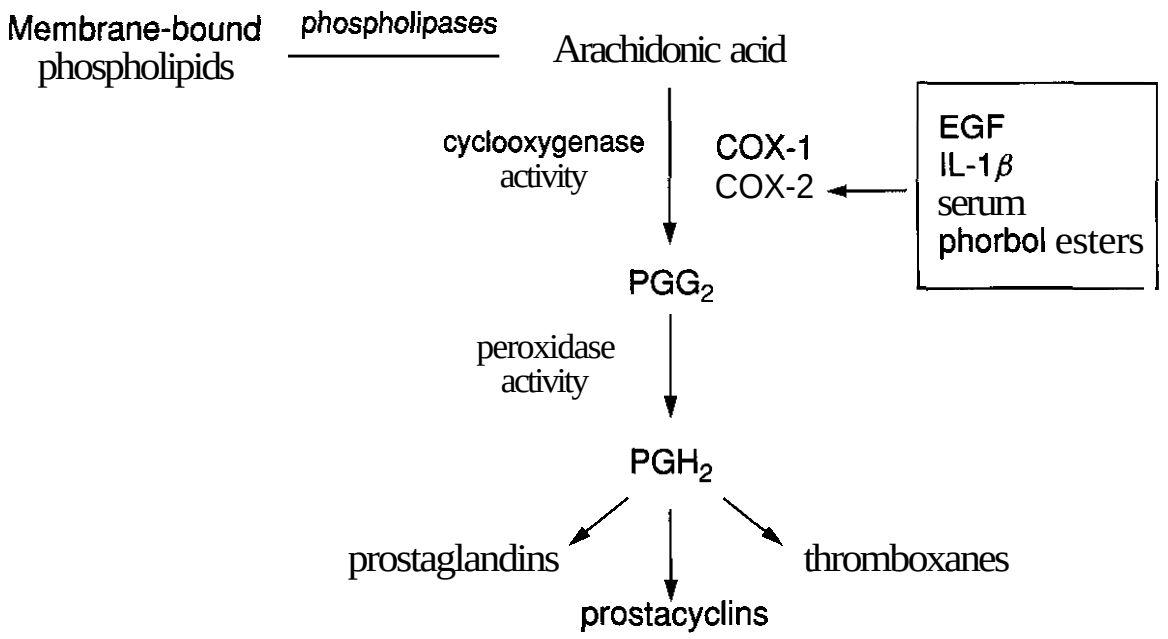


Figure 1.12. Cyclooxygenases catalyze prostaglandins from arachidonic acid. COX-2 is inducible by a variety of stimuli including growth factors, cytokines, and tumor promoters. PGH2 forms three classes of eicosanoids: prostaglandins, prostacyclins, and thromboxanes. Adapted from C. S. Williams and R. N. DuBois, *Am. J. Physiol.*, 270, G393 (1996).

including blood clotting, ovulation, bone metabolism, nerve growth and development, and immune responses (108). There are two COX isoforms, COX-1 and COX-2. COX-1 is constitutively expressed in most cell types and is necessary for homeostasis of colonic epithelium and platelet aggregation. COX-2, on the other hand, is inducible by a variety of stimuli including growth factors, stress conditions, and cytokines (Fig. 1.12).

Several studies have implicated COX-2 in carcinogenesis. COX-2 protein levels, and therefore, prostaglandin production, are up-regulated in many tumor types, including pancreatic, gastric, breast, skin, and colon cancers. Several lines of evidence suggest that overexpression of COX-2 plays an important role in colonic polyp formation and cancer progression. COX-2 modulates metastatic potential by inducing MMPs, which can be directly inhibited by COX-2 inhibitors. In addition, cells overexpressing COX-2 secrete increased levels of angiogenic factors like VEGF and bFGF. COX-2 not only aids in invasion but also inhibits apoptosis by up-regulating Bcl-2.

'COX-2 has come under intensive study as a target for colon cancer prevention. Multiple studies have illustrated that COX-2 selective inhibitors suppress tumorigenesis in multiple intestinal neoplasia (Min) mice. COX-2 inhibitors also inhibit tumor cell growth in immunocompromised mice (109). The same phenomena has been illustrated in human chemoprevention trials. Recent studies have linked prolonged use of nonsteroidal anti-inflammatory drugs (NSAIDs) to decreased colon cancer risk and mortality. NSAIDs inhibit the cyclooxygenase enzymes, and new COX-2 selective agents are gaining popularity in the treatment of inflammation. NSAIDs that inhibit both COX-1 and COX-2 have been associated with reduced cancer risk in several large epidemiology studies. Whether inhibition of COX-1 and/or COX-2 is the optimal strategy for reducing risks of certain cancers is unknown.

Because COX-2 is induced in certain neoplastic tissues, the molecular regulation of its expression is being studied in a variety of experimental models. Human and rodent cell lines expressing various levels of COX-2 are being studied for genetic modifications that lead to the dysregulation of COX-2. COX-2 regulation occurs both transcriptionally and translationally, and this regulation differs depending on the species studied and the mutational status of the cell lines.

Signaling pathways leading to modulation of COX-2 expression are also being investigated. Both oncogenes and tumor suppressor genes have been shown to modulate COX-2 in cell model systems. The activation of the H-ras and K-ras oncogenes leads to induction of COX-2 expression in colon cancer cells. This

induction is mediated by the stabilization of COX-2 mRNA. Wild-type, full-length APC suppresses COX-2 expression, suggesting that normal activity of this tumor suppressor gene may prevent cancer by inhibiting expression of cancer-promoting genes like COX-2. APC down-regulates COX-2 protein without affecting COX-2 mRNA levels. Thus, both ras and APC regulate COX-2 expression by post-transcriptional mechanisms. TGF-β1 is another tumor suppressor gene that influences expression of COX-2. TGF-β1-mediated transformation of rodent intestinal epithelial cells causes a significant induction of COX-2 protein expression. TGF-β1 synergistically enhances ras-induced COX-2 expression by stabilizing COX-2 mRNA. COX-2 expression is also influenced by the PI-3K pathway. Pharmacological inhibition of PI-3K or downstream PKB/Akt, as well as dominant-negative forms of Akt dramatically reduce COX-2 protein levels.

5.2.3 Other Targets. Technologies such as DNA microarrays are identifying genes that are aberrantly up-regulated in human intraepithelial neoplasia (IEN). As discussed earlier, ODC, the first enzyme in polyamine synthesis, is up-regulated in a variety of IEN as a consequence of specific genetic alterations. Difluoromethylornithine (DFMO), an enzyme activated irreversible inhibitor of ODC, is a potent suppressor of several experimental models of epithelial carcinogenesis and is being evaluated in human cancer prevention trials (110). Pathways signaling cell behaviors are also activated in specific cancers. A number of agents, including NSAIDs and components of green and black teas, have been shown to inhibit certain signaling pathways in cell-type and tissue-specific manners.

5.3 Therapy

5.3.1 Importance of Studying Gene Expression. Cancer, among other diseases, is caused by the deregulation of gene expression. Some genes are overexpressed, producing abundant supplies of their gene products, whereas other crucial genes are suppressed or even deleted. The expression levels of genes associated with cancer influence processes such as cell proliferation, apoptosis, and invasion. Genes involved in growth, for example, are often overexpressed in tumor tissues compared with normal adjacent tissue from the same organ. It is imperative to elucidate which genes are overexpressed or down-regulated in tumors because these genes represent critical therapeutic targets.

Researchers today generally concentrate on a few particular genes and study their regulation, expression, and downstream signaling using conventional molecular biology tools. With the onslaught of new genome data, and the development of the GeneChip, scientists are now able to study the expression levels of numerous genes simultaneously. The ability to analyze global profiles of gene expression in normal tissue compared with tumor tissue can help reveal how gene expression affects the overall process of carcinogenesis.

5.3.2 cDNA Microarray Technology. cDNA microarray technology is based on the simple concept of DNA base pairing. cDNA from tumor samples hybridize with the complementary DNA sequences on the chip. The DNA sequences are the target genes that will be studied for expression levels in particular tissues. These sequences, or probes, can be in the form of known oligos, DNA encoding \$he full-length gene, open reading frames (ORFs), or sometimes even the entire genome of an organism like Saccharomyces cerevisiae. Genes can be chosen by their proximity to each other on a chromosome or their similar functions. cDNA probes are then spotted onto a glass slide or computer chip (GeneChip), using a variety of different robotic techniques. A typical microarray slide will contain approximately 5000 genes.

cDNA microarray is particularly useful to the field of cancer biology because it allows scientists to study changes in gene expression caused when a normal tissue becomes neoplastic. In addition, normal tissue can be compared with preneoplastic lesions as well as metastatic cancer, to fully examine the entire tumorigenic process. The mRNA is extracted from cell lines or tissue and is reverse transcribed into the more stable form of cDNA. The cDNA is then labeled with reporters containing two colored dyes, rhodamine red, Cy3,

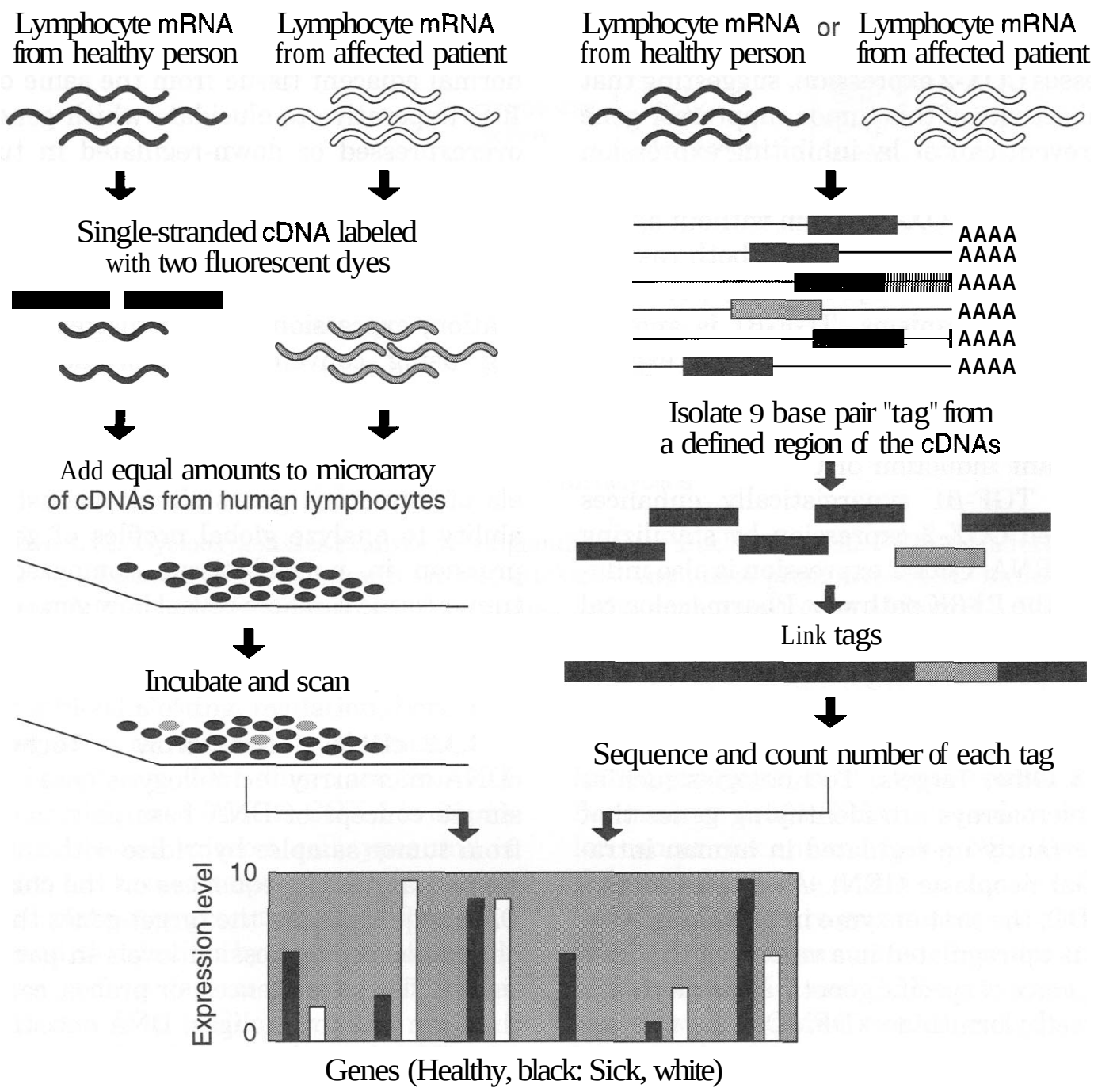


Figure 1.13. Comparison of cDNA microarray and SAGE technologies. At left is a diagram of the microarray assay for gene expression; the SAGE technique is illustrated at right. Here, the procedures assess how gene expression differs in lymphocytes from a healthy person and those from a person fighting off an infection. Reprinted with permission from K. Sutliff, ***Science, 270, 368*** (1995). American Association for the Advancement of Science.

and fluorescien green, Cy5. The cDNA is then hybridized to the DNA on the microarray slide. The slides are exposed to a laser beam, causing the dyes to give off their respective emissions and the relative expression levels of that gene are read and processed.

A similar technique to cDNA microarray that allows for multigene expression analysis is serial analysis of gene expression (SAGE) (Fig. 1.13). SAGE is based on the principle that a 9–10 nucleotide sequence contains sufficient information to identify a gene. These short nucleotide sequences are amplified by polymerase chain reaction (PCR) and then 30–50 of these SAGE "tags" are linked together as a single DNA molecule. These long DNA molecules are sequenced and the number of times that a single "tag" appears correlates to that gene's expression level. Proof of concept for this technique was illustrated in a study of gene expression in pancreatic cells. The most abundant "tags" found were those that encoded highly expressed pancreatic enzymes like trypsinogen 2. cDNA microarray methodology has also been validated by studies showing that expression data for tumor cell lines grown in tissue culture conditions can be classified according to their tissue of origin.

5.3.3 Discoveries from cDNA Microarray Data. The contribution of microarray technology is influential in both the basic understanding of cancer pathology as well as in drug discovery and development. These studies reveal genes that may prove to be important diagnostic or prognostic markers of disease. They also can be used to predict adverse reactions to chemotherapies if mRNA from drug-treated cells is hybridized to panels of genes related to liver toxicity or the immune response.

Microarray technology also corroborates many in *vitro* cell studies that are criticized for ignoring the important role of other cell types in the tumor microenvironment. This technology can aid in distinguishing between cell type–specific or tumor-specific gene expression. For example, SAGE analysis of colon tumors and colon cancer cell lines showed 72% of the transcripts expressed at reduced levels in colon tumors were also expressed at reduced levels in the cell lines. One interesting finding from this study was that two commonly mutated oncogenes, c-fos and c-erbB3, were found to be expressed at higher levels in normal colonic epithelium than in colonic tumors; this contradicts reports that these oncogenes are up-regulated in transformed cells compared with normal cells. Again, microarray analysis is helping to merge cell biology studies with whole tumor biology.

Activation of the c-myc oncogene is a common genetic alteration occurring in many cancers. A cDNA microarray study found that c-myc activation leads to down-regulation of genes encoding extracellular matrix proteins, and thus, may play a role in regulating cell adhesion and structure. C-myc has also been associated with cell proliferation, which was illustrated by up-regulation of the genes eIF-5A and ODC. Another study of colon tumors revealed that only 1.8% of the 6000 transcripts studied were differentially expressed in normal tissues and tumors (111). Studies such as these suggest the critical importance of these differentially regulated genes in the cancer phenotype.

In addition to oncogene activation, the effects of tumor suppressor genes have been investigated through microarray technology. Over 30 novel transcripts were identified as regulated by p53 induction (112). Such a great number of genes simultaneously linked to p53 expression would not have been possible without SAGE technology. However, only 8% of these new genes were induced in normal cells compared with p53 knockout cells, suggesting that most of these p53-dependent genes are also dependent on other transcription factors. This is just one example of how microarray technology may be able to look at crosstalk in signaling pathways.

5.3.4 Limitations of Microarray Technologies. Although cDNA microarray and SAGE technologies are quickly identifying new genes involved in tumorigenesis, there are significant limitations to these strategies. First, the expression pattern of a gene only provides indirect information about its function; a new gene may be classified as necessary for a certain biological process, but its exact role in that process cannot be determined. Second, mRNA levels do not always correlate with protein levels, and even protein expression may not translate into a physiological effect. Third, the up-regulation or suppression of a gene may be either the cause or the effect of a disease state and microarray technology does not distinguish between the two possibilities.

Both cDNA microarray and SAGE analyses require verification of changes in gene expression by Northern blots. Modest changes in gene expression are often overlooked when data is reported in terms of fourfold or greater changes. Because the ability to detect differences in gene expression is dependent on the magnitude of variance, a small induction or suppression of a gene may be discarded as inconsequential when it may actually be critical for downstream signaling of other genes.

5.4 Modifying Cell Adhesion

5.4.1 MMP Inhibitors. Several MMP inhibitors are currently being developed for cancer treatment. If MMPs do play an integral role in malignant progression, then pharmacological inhibition of MMPs could inhibit tumor invasiveness. The inhibition of MMP function is currently the focus of most antimetastatic efforts. MMP inhibitors fall into three categories: (1) collagen peptidomimetics and non-

peptidomimetics, (2) tetracycline derivatives, and (3)bisphosphonates. The peptidomimetic MMP inhibitors have a structure that mimics that of collagen at the site where the MMP binds to it. Batimastat, a peptidomimetic inhibitor, was the first MMP inhibitor to be evaluated in cancer patients and is not orally available. Matimastat is orally available and is currently in phase II and III clinical trials (113). When bound to the MMP, these inhibitors chelate the zinc atom in the enzyme's active site. There are several nonpeptidomimetic inhibitors that are also in various phases of clinical trials. These are more specific than their peptidic counterparts and have exhibited antitumor activity in preclinical studies (113).

Tetracycline derivatives inhibit both the activity of the MMPs and their production. They can inhibit MMP-1, -3, and -13 (the collagenases) and MMP-2 and -9 (the gelatinases) by several different mechanisms. These mechanisms include (1) blocking MMP activity by chelation of zinc at the enzyme active site, (2) inhibiting the proteolytic activation of the pro-MMP, (3) decreasing the expression of the MMPs, and (4) preventing proteolytic and oxidative degradation of the MMPs.

The mechanism of action of the bisphosphonates has not been elucidated, but they have been used extensively for disorders in calcium homeostasis and recently in breast cancer and multiple myeloma patients to prevent bone metastases (114). Clodronate, a bisphosphonate, inhibited expression of MT1-MMP RNA and protein in a fibrosarcoma cell line and effectively reduced the invasion of melanoma and fibrosarcoma cell lines through artificial basement membranes (115).

5.4.2 Anticoagulants. One theory surrounding the invasion process is that blood-clotting components may play a role in metastasis by either trapping the tumor cells in capillaries or by facilitating their adherence to capillary walls. Large numbers of tumor cells are released into the bloodstream during the metastatic process, and they must be able to survive the wide range of host defense mechanisms. Tumor cells have been shown to interact with platelets, lymphocytes, and leukocytes, and this may serve to promote metastasis. Studies have been done that inhibit tumor cell-platelet interactions, and these have resulted in a decreased probability of metastasis formation. It has also been shown that fibrin is always located in and around cancerous lesions, which may indicate that the cells use the fibrin structure as a support on which to attach themselves and grow. It may also serve as protection against host inflammatory cells so that the tumor is not destroyed.

Treating hepatic metastases of a human pancreatic cancer in a nude (lacking a thymus) mouse with prostacyclin, a potent inhibitor of platelet aggregation, led to a significant reduction in the mean surface area of the liver covered with tumor compared with the untreated control group (116). Many other groups have reported a reduction in metastatic potential with treatment of prostacyclin and prostacyclin-analogues, such as iloprost and cicaprost. There are currently over 50 different clinical trials in varying phases underway to determine the efficacy of these anticoagulant therapies. Most of these trials are in combination with other conventional anti-cancer regimens. So far, the experimental evidence indicates that anticoagulants or inhibitors of platelet aggregation are useful in the prevention of metastases.

5.4.3 Inhibitors of Angiogenesis. The growth and expansion of tumors and their metastases are dependent on angiogenesis, or new blood vessel formation. Angiogenesis is regulated by a complex of stimulators and inhibitors (Fig. 1.14). The balance between the positive and negative regulators of angiogenesis inside a tumor environment is important for the homeostasis of microvessels. Tumor cells can secrete proangiogenic paracrine factors, which stimulate endothelial cells to form new blood vessels. The use of angiogenesis inhibitors may be a potential mode of therapy and is still in early clinical trials. This type of therapy would be a way of controlling the disease rather than eliminating it. Whereas toxicity may not be a major problem, adverse effects may be expected in fertility and wound healing.

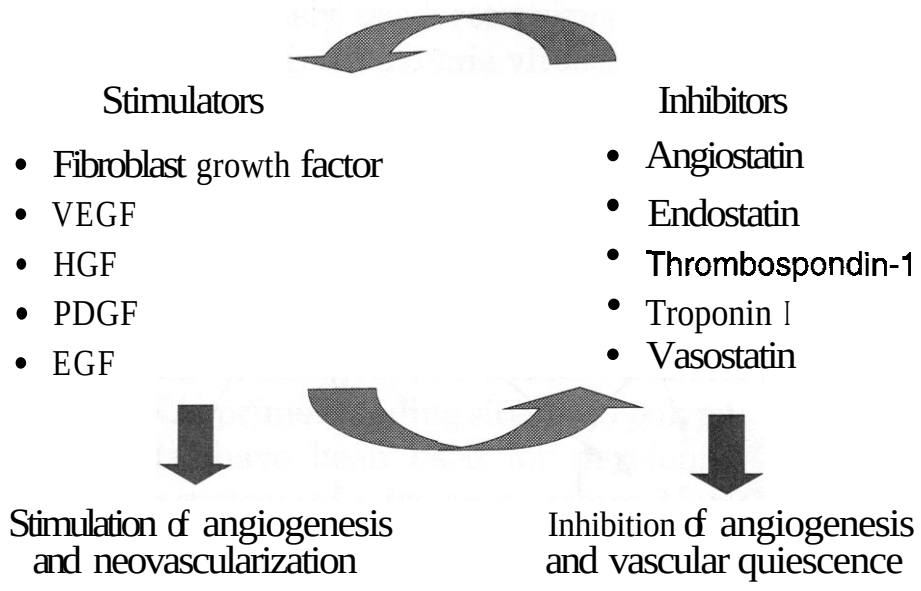


Figure 1.14. Stimulators and inhibitors of angiogenesis. Under physiological conditions, the balance of factors that affect angiogenesis is precisely regulated. However, under pathophysiological conditions, normal angiogenesis is disturbed because of the continued production of stimulators.

5.5 Prospects for Gene Therapy of Cancer

Gene therapy is the transfer of genetic material into cells for therapeutic purpose. Gene transfer technology has become available after extensive study of molecular mechanisms of many diseases and improvement of techniques for manipulating genetic materials in the laboratory. Concepts for genetic therapy of cancer were developed based on knowledge that neoplasia is a molecular disorder resulting from loss of expression of recessive tumor suppressor genes and activation of dominant oncogenes.

Cancer gene therapy is aimed at correcting genetic mutations found in malignant cells or delivering biologically active material against cancer cells. One approach used in gene therapy of cancer is gene replacement/correction to restore the function of a defective homologous gene or to down-regulate oncogenic expression in somatic cells. Another approach is immune modulation by introduction of therapeutic genes, such as cytokines, into the target cells to treat cancer by stimulating an immune response against the tumor. Molecular therapy by activating prodrugs (e.g., ganciclovir, 5-fluorocytosine) within tumor cells and suicide gene therapy approaches have already been successful in early clinical trials. The high performance of these approaches fully depends on the efficacy and specificity of therapeutic gene expressing and delivery systems.

5.5.1 Cene Delivery Systems. The exogenous genetic material (the transgene) is usually introduced into tumor cells by a vector. A vector, or plasmid, is a circular DNA sequence that is designed to replicate inserted foreign DNA for the purpose of producing more protein product. Plasmids designed for gene therapy applications usually contain the gene of interest and regulatory elements that enhance the gene's expression. The ideal vector for gene therapy is one that would be safe, have high transfection efficiency, and be easy to manipulate and produce in large quantities. It would be efficient at delivering genetic material and selectively transducing cells within a tumor mass. The vector would be immunogenic for the recipient and would express the gene in a regulated fashion and at high levels as long as required.

There are two main approaches for the insertion of gene expressing systems into cells. In the ex *vivo* technique, cells affected by the disease are transfected with a therapeutic gene in vitro for the expression of exogenous genetic material. After viral propagation, replication is rendered incompetent and these cells can be transplanted into the recipient. In the in vivo technique, vectors are inserted directly into target tissue by systemic injections of the gene expressing system.

The simplest delivery system is a plasmid by itself, or so-called naked DNA. Direct injections of DNA have been successfully used to transfect tissues with low levels of nuclease activity in muscle tissue (117), liver (118), and experimental melanoma (119). Systemic injection of naked DNA is, in general, much less efficient because serum nucleases degrade plasmid DNA in the blood within minutes (120).

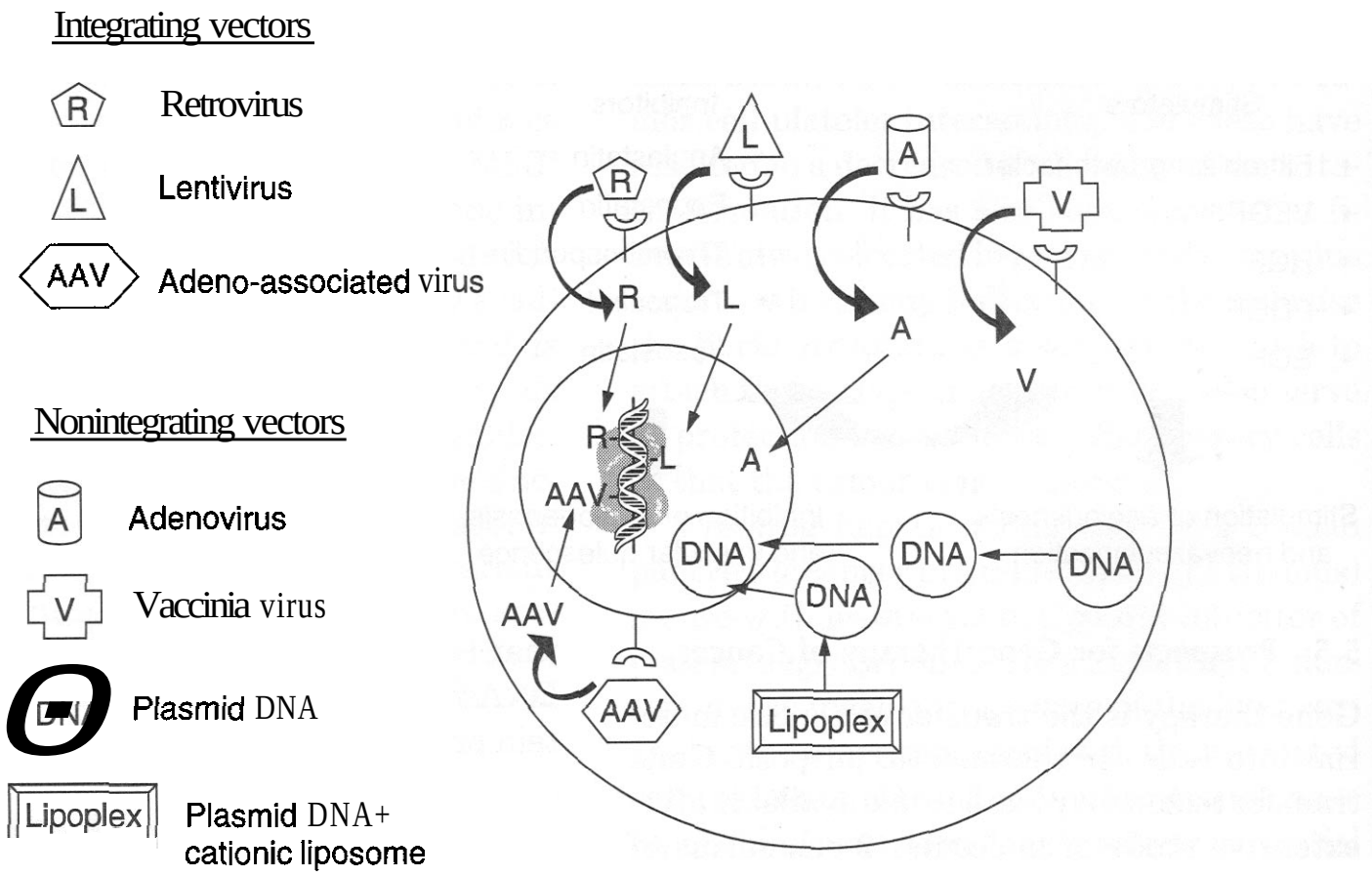


Figure 1.15. Virus particles bind to specific receptors on the surface of target cells. These vectors are internalized and their genome enters the cells. In the case of retroviruses, the single-stranded RNA genome is converted into double-stranded DNA by the reverse transcriptase enzyme encoded by the virus. The double-stranded DNA is taken up by the nucleus and integrated within the host genome as a provirus. The integration is random for retroviruses. Lentiviruses have a similar life cycle. Adenovirus binds to specific receptors on the surface of susceptible cells and are then absorbed and internalized by receptor-mediated endocytosis. The viral genome enters the cytoplasm of the cell and the double-stranded DNA genome is taken up by the nucleus. Vaccinia virus replicates in the cytoplasm of cells. DNA delivered by lipoplex and other nonviral systems enters cells through electrostatic interactions (endocytosis, phagocytosis, pinocytosis, and direct fusion with cell membrane). DNA is. released before entry into the nucleus, where it stays as an episome.

To protect DNA on systemic application, it is usually complexed with viruses or with cationic lipids, polymers, or peptides. The resulting complex protects the DNA from the attack of nucleases and potentially improves transfection efficiency and specificity on multiple levels through interaction of DNA complexes with the various biological barriers.

The choice of viral or non-viral (synthetic) delivery strategy depends on localization and type of affected tissue, as well as on therapeutic approach. Viral vectors use the ability of viruses to overcome the cellular barriers and introduce genetic material either through the integration of the vector into the host genome (retroviruses, lentiviruses, adeno-associated viruses) or by episomal delivery (adenoviruses) followed by stable gene expression (Fig. 1.15).

5.5.1.1 Viral Vecfors. Retroviral vectors have been used for *ex vivo* gene delivery and are the most useful vectors for stably integrating foreign DNA into target cells. Retroviruses are enveloped viruses that contain 7- to 12-kb RNA genomes. After the virus enters the cells through specific cell surface receptors, its genome is reverse transcribed into double-stranded DNA and subsequently integrated into the host chromosome in the form of a provirus. The provirus replicates along with the host chromosome and is transmitted to all of the host cell progeny. Because the retrovirus genome is relatively small and well characterized, it was possible to engineer a vector encoding only the transgene without replication competent viruses (RCV) or virus structural genes.

The most widely used retrovirus vectors are based on murine leukemia viruses (MLV). The lack of specificity of these vectors is a major obstacle for appropriate and controlled expression of foreign genes. Retroviruses are not efficient for direct in vivo injection because of inactivation by the host immune system (121). To circumvent this, cis-acting viral sequences, such as long terminal repeats (LTRs), transfer RNA (tRNA) primer binding sites, and polypurine tracts, have been used for developing packaging systems of retrovirus vectors. Many recombinant retrovirus vectors are designed to express two genes, one of which is often a selectable marker. New strategies for expression, such as splicing, transcription from heterologous promoters, and translation directed by an internal ribosome entry signal (IRES), have been used for expression of the second gene. Attempts have also been made to achieve efficient gene delivery by targeting retroviral integration through modifying protein sequences in the viral envelope (122). These modifications include various targeting ligands, particularly ligands for the human EGFR, erythropoetin receptor, and single chain antibody fragments against the low-density lipoprotein (LDL) receptor.

Examples of lentiviruses are the human immunodeficiency virus (HIV-1), the equine infectious anemia virus, and the feline immunodeficiency virus (123). Although lentiviruses also have an RNA genome, their advantage compared with other retroviruses is the ability to infect and stably integrate into nondividing cells. To create a safe gene transfer vector based on the HIV-1 genome, the genome was altered and mutated to produce replication-defective particles. Several studies, both in vitro and in vivo, have shown successful gene transfer, including transduction of non-dividing hematopoetic cells at high efficiencies (up to 90%) and stable gene expression in several target tissues of interest such as liver (8 weeks) and muscle and brain (6 months) with no detectable immune response (124). At the same time, the safety concern still remains for in vivo applications of this vector.

Vaccinia virus is a member of the Poxviridae family, which possesses a complex DNA genome encoding more than 200 proteins. The advantages of using vaccinia viruses for gene transfer include their ability to accommodate large or multiple gene inserts, to infect cells during different stages of the cell cycle, and their unique feature to replicate in the cytoplasm. Recombinant vaccinia vectors can be constructed using homologous recombination after transfection of vaccinia virus-infected cells with plasmid DNA constructs. This vector has been used in clinical trials to deliver genes encoding tumor antigens such as melanoma antigen (MAGE-1), carcinoembryonic antigen (CEA), prostate-specific antigen (PSA), interleukins (e.g., IL-1β, IL-12), and costimulatory molecule B7 (125).

In recent years, there has been a great interest in the use of adenoviral vectors for cancer gene therapy. The main reasons for this are the ease in construction of adenoviruses in the laboratory and their ability to grow to high titers, infect a variety of cell types, and produce the heterologous protein of interest in dividing and non-dividing cells. Adenoviruses are also characterized by efficient receptor-mediated endocytosis, mediated by its fiber protein, and on infection of cancer cells, they exhibit high levels of transgene expression (126). They often are used to transfer genes of large sizes because of their high packaging capacity (up to 36 kb). Adenoviral vectors do pot integrate into the host chromosomes, and therefore, they are degraded by the host. This results in a short-term expression of the transduced gene, which, nevertheless, could be sufficient to achieve the cancer gene therapy efficacy. Adenoviruses are widely used for direct in vivo injections. Adenoviruses are DNA-containing, non-enveloped viruses.

The two most commonly used adenoviruses for recombinant vectors are Ad2 and Ad5, mainly because their genomes have been best characterized and because these viruses have never been shown to induce tumors. Adenoviruses, like other viral vectors, lack cell and tissue specificity. To improve targeted gene delivery, attempts have been made to couple ligands or antibodies to the adenovirus capsid proteins (127). Specificity of the transgene expression also can be introduced by using tissue specific antigens, such as CEA for the treatment of pancreatic and colon cancers, mucin (MUC-1) promoters for breast cancer cells, al-

Table 1.5 Cell-Type Specific Promoters for Targeted Gene Expression

Promoter	Target Cell/Tissue	Therapeutic Gene
PEPCK promoter	Hepatocytes	Neomycin phosphotransferase, Growth hormone
AFP promoter	Hepatocellular carcinoma	HSV-tk, VZV-tk
MMTV-LTR	Mammary carcinoma	TNFα
WAP promoter	Mammary carcinoma	Recombinant protein C
β-casein	Mammary carcinoma	In development
CEA promoter	Colon and lung carcinoma	HSV-tk, CD
SLPI promoter	Carcinomas	HSV-tk, CD
Tyrosinase promoter	Melanomas	HSV-tk, IL-2
c-erbB2 promoter	Breast, pancreatic, gastric carcinomas	CD, HSV-tk
Myc-max-responsive element	Lung	HSV-tk
	Therapy-Inducible Tissues	
Egr-1 promoter	Irradiated tumors	TNFα
Grp78 promoter	Anoxic, acidic tumor tissue	Neomycin phosphotransferase
MDR1 promoter	Chemotherapy-treated tumors	TNFα
HSP70	Hyperthermy-treated tumors	IL-2

pha-fetoprotein promoters for hepatocellular carcinoma, and the tyrosinase promoter for melanoma (126). *In* vivo administration of adenoviral vector has been extensively used in preclinical and clinical cancer therapy (128).

There are many regulatory elements controlling cell type–specific gene expression and inducible sequences within promoters that have been used in construction of viral vectors for cancer therapy. Vector systems that include cell type–specific promoters or elements responding to regulatory signals represent a way for a safe, selective, and controlled expression of therapeutic genes that could increase efficacy and stability of gene expression (Table 1.5).

Vectors based on adenoassociated viruses (AAV) also have been successfully used to transfer genes. AAV is a small, single-stranded DNA virus that requires a helper virus for infection, usually an adenovirus or herpesvirus. AAV vectors can be used for the delivery of antisense genes, "suicide" gene therapy, and recently, for the delivery of anti-angiogenic factors. Recent studies in the area of vector design have been focused on conditional expression that can be induced by antibiotics (129), heat shock (130), or other small molecules (131).

5.5.1.2 Non-Viral Gene Delivery Systems. Non-viral gene delivery systems are based on non-covalent bonds between cationic carrier molecules (e.g., lipids or polymers) and the negatively charged plasmid DNA. Complexes of DNA with three main groups of materials, i.e., cationic lipids (lipoplex) such as CTAB and DMRI, polymers (polyplex) such as poly-L-lysine and polyathylenimine, or peptides have been evaluated as synthetic gene delivery systems (132). The formation of these complexes, which is generally based on electrostatic interactions with the plasmid DNA, is difficult to control as they depend on both the stoichiometry of DNA and complexing agent and on kinetic parameters (e.g., speed of mixing and volumes). It has been shown that DNA is efficiently condensed and protected from nucleases at higher lipid:DNA ratios, proving that the positive charges of the complexes are important for the interaction with cells in vitro and *in* vivo. Although the resulting particles are stable, they have a high tendency to interact non-specifically with biological surfaces and molecules.

Lipoplexes are actively used in clinical trials for *in* vivo and *ex* vivo delivery of genes encoding cytokines, immunostimulatory molecules, and adenoviral genes (133). In vivo,

these interactions may compromise the tissue-specific delivery of the complexes, creating uneven biodistribution and transgene expression in the body, particularly, in lungs. To overcome this problem, the complexes can be injected either into the vasculature or directly into the affected organ (134).

The combinatorial gene delivery approach uses the whole virus, either replication deficient or inactivated, or only essential viral components, together with the non-viral system. Systems, based on adenovirus ("adenofection"), or viral proteins that are required to trigger efficient endosomal escape, and polyplex and lipoplex non-viral systems have shown improvement in transfection efficiency and resistance to endosomal degradation (135).

5.6 Gene Therapy Approaches

5.6.1 immunomodulation. This approach employs the patient's physiological immune response cascade to amplify therapeutic effects (136). Most patients with cancer lack an effective immune response to their tumors. This could be caused by defects in antigen presentation, stimulation, or differentiation of activated T cells into functional effector cells. Antitumor immunity response requires participation of different immune cells, including helper effector T-cells (Th), cytotoxic T-lymphocytes (CTLs), and natural killer (NK) cells. Activation of $CD4^+$ and $CD8^+$ T-cells requires at least two major signals. The first signal is triggered by binding of complexes of T-cell receptor (TCR) and specific antigenic peptide with MHC-class II or I molecules, respectively. The second signal for $CD4^+$ T-cells is provided by engagement of CD28 on the T-cell surface by members of the B7 family of costimulatory molecules on the surface of professional antigen-presenting cells. The nature of second signal for $CD8^+$ T-cells has not been completely understood but requires the presence of helper $CD4^+$ T-cells. Following activation and clonal expansion, activated $CD4^+$ T-cells differentiate into helper effector cells of either the Th1 or Th2 phenotype. Th1 cells produce cytokines, such as IL-2, interferon-y, and TNF, that stimulate monocytes and NK cells and promote the differentiation of activated $CD8^+$ T-cells into CTLs.

The growing understanding of the biological basis of antigen-specific cellular recognition and experimental studies of an antitumor effect mediated through the cellular immune system helped to develop various immunomodulation strategies. Modulation of immune response can be achieved through stimulation and modification of immune effector cells, enabling them to recognize and reject cells that carry a tumor antigen. Additionally, tumor cells can be genetically modified to increase immunogenicity and trigger an immune response.

Cytokine levels are relatively low in cancer patients. To correct for this deficiency, cytokines can be introduced as recombinant molecules, and this is advantageous in controlling their blood concentration and biological activity. Because cytokines are relatively unstable *in vivo,* cancer patients have to receive a large amount of the recombinant protein to maintain the required blood concentration for biological activity. Administration of the protein is often toxic to the patients. Another therapeutic approach is the introduction of genes encoding various cytokines, costimulatory molecules, allogenic antigens, and tumor-associated antigens into tumors (137). Previous preclinical studies have shown that cytokines that facilitate Th1 cell-mediated immune reactions but not Th2 cell-mediated reactions, when produced in tumors, are effective for antitumor responses. In addition, cytokines or costimulatory molecules delivered to tumor cells may enhance the transfer of tumor antigens to antigen-presenting cells. The most potent known antigen-presenting cells for actively stimulating specific cellular immune responses are dendritic cells. *Ex vivo* gene delivery to cultured dendritic cells or direct *in vivo* gene delivery to antigen-presenting cells can be more efficient in stimulating cellular antitumor immunity (138).

Several technical problems of expressing sufficient amounts of immunostimulatory proteins in appropriate target cells remain unsolved, but the potential of immune modulation gene therapy is high. Immunotherapy trials also contribute to the present knowledge of

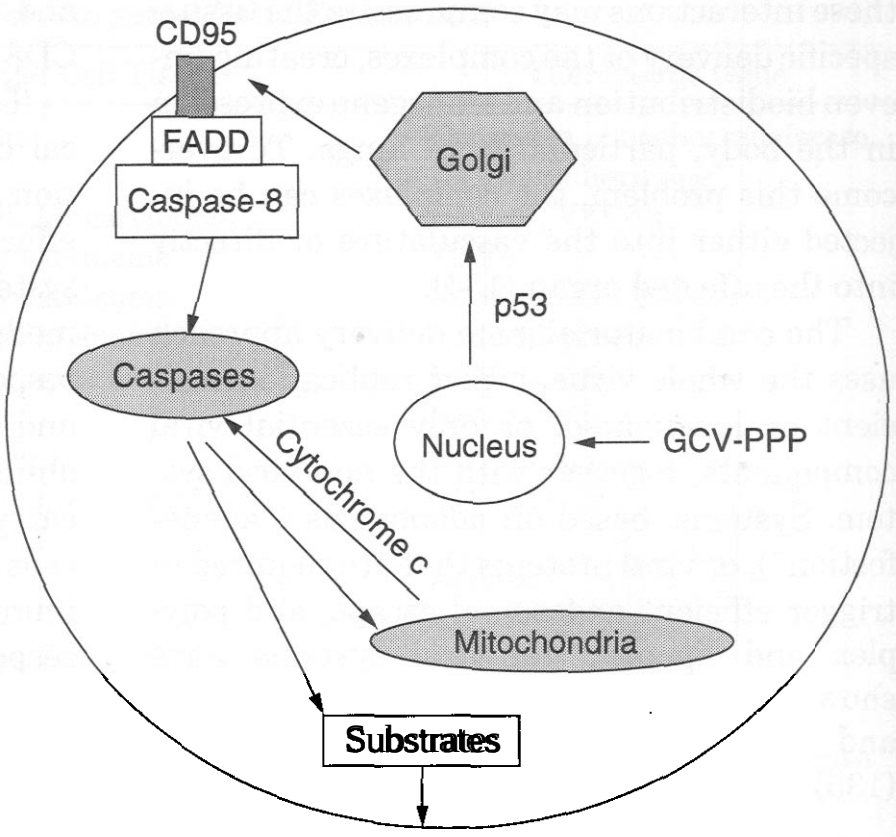


Figure 1.16. Mechanisms of thymidine kinase (TK) ganciclovir (GCV)-induced apoptosis. TK phosphorylates the nontoxic prodrug GCV to GCV-triphosphate (GCV-PPP), which causes chain termination and single-strand breaks on incorporation into DNA. TWGCV induces p53 accumulation, which can cause translocation of preformed death receptor CD95 from the Golgi apparatus to the cell surface without inducing de *novo* synthesis of CD95. The signaling complex then is formed by CD95, the adapter molecule Fas-associated death domain (FADD) protein, and the initiator caspase-8, which leads to cleavage of caspases causing apoptosis. TWGCV also leads to mitochondria damage, including loss of mitochondrial membrane potential and the release of cytochrome c inducing caspase activation and nuclear fragmentation.

how antitumor responses can be effectively produced in cancer patients.

5.6.2 Suicidal Gene Approach. Elimination of cancer cells can be accomplished by the introduction of vectors that specifically express death promoting genes in tumor cells. One method, called suicide gene therapy, involves the expression of a gene encoding an enzyme, normally not present in human cells, that converts a systemically delivered nontoxic prodrug into a toxic agent. The toxin should kill the cancer cells expressing the gene as well as the surrounding cells not expressing the gene (bystander effect).

The herpes simplex virus thymidine kinase type 1 (HSV-tk) gene was initially used for long-term replacement gene therapy because it is about 1000-fold more efficient than mammalian thymidine kinase at phosphorylating the nontoxic prodrug ganciclovir (GCV) into its toxic metabolite ganciclovir triphosphate. The efficacy of HSV-tk transduction of tumors followed by ganciclovir therapy has been confirmed by systemic administration of ganciclovir after intratumoral injection of fibroblasts transduced with an HSV-tk retroviral vector in several preclinical models (139). The molecular mechanism of HSV-tk therapy is based on induction of apoptosis in target cells through accumulation of p53 protein (Fig. 1.16). Clinical trials of HSV-tk suicide gene therapy, where ganciclovir was given after the retroviral or adenoviral introduction of HSV-tk gene, have been conducted in patients with brain tumors, melanoma, or mesothelioma (140–142) (Fig. 1.16).

Another suicide gene under active investigation for cancer therapy is the cytosine deaminase (CD) gene. CD converts the nontoxic fluoropyrimidine 5-fluorocytosine to 5-fluorouracil. Transduction of the CD renders tumor cells sensitive to 5-fluorocytosine *in* vitro and *in vivo*. The CD/5-fluorocytosine system has been used in a clinical trial, where adenovirus expressing the CD gene was injected intratumorally into hepatic metastases from colorectal cancer (143). As with HSV-tk gene transfer, evidence exists that cytosine-deaminase gene transfer into tumor cells promotes antitumor immune responses. The malignancies targeted with suicide gene therapy in the field of pediatric oncology are brain tumors, neuroblastoma, and acute lymphoblastic leukemia (144).

5.6.3 Targeting Loss of Tumor Suppressor Function and Oncogene Overexpression. Several tumor suppressor genes, including p53, Rb, and APC, have been identified by their association with hereditary cancers. Many sporadic tumors harbor inactivating or recessive mutations in one or more tumor suppressor genes. Gene transfer techniques can be ap-

plied to introduce wild-type copies of tumor suppressor genes into malignant cells, thus potentially reversing the neoplastic phenotype. The p53 tumor suppressor gene has been of interest because p53 mutation occurs commonly in a variety of human cancers, including breast, lung, colon, prostate, bladder, and cervix. The use of adenoviral vectors to deliver the p53 transgene to human tumors is now under evaluation in several clinical trials (145).The overexpression of Fas ligand caused by adenovirus-mediated wild-type p53 gene transfer induces neutrophil infiltration into human colorectal tumors, which may play a critical role in the bystander effect of p53 gene therapy (146).

Besides the p53 gene, other tumor suppressor genes that regulate the cell cycle have been used in cancer gene therapy. Among them are Rb, BRCA1, PTEN, p16, E2F, and fragile histidine triad (FHIT) genes. Clinical trials with BRCAl and Rb have been initiated (147).

Protooncogenes, in contrast to tumor suppressor genes, gain dominant mutation resulting in excessive expression of their protein products, which lead to development of the malignant phenotype. Three members of the Ras family of oncogenes (H-ras, K-ras, and N-ras) are among the most commonly activated oncogenes in human cancers. Several strategies have been designed to combat K-ras mutations, including antisense nucleotide, ribozymes (148–150), and intracellular single-chain antibodies (151). cDNA encoding antisense RNA can be delivered using the viral vector system approach. In vivo gene therapy with K-ras, c-fos, and c-myc antisense nucleotides is currently being applied in clinical trials.

5.6.4 Angiogenesis Control. Gene therapy offers a new strategy for the delivery of angiogenesis inhibitors. By engineering and delivering vectors that carry the coding sequence for an antiangiogenic protein, it is possible to produce high levels of antiangiogenic factors in the tumor location or to systemically prevent the growth of distant metastasis. Several angiogenic inhibitors, such as angiostatin (152), endostatin (153), plasminogen activator inhibitor type 1 (154), and truncated VEGF receptor (155), have been tested using this approach. These studies have demonstrated that retroviral and adenoviral vectors could be used to inhibit endothelial cell growth in vitro and angiogenesis in vivo. The inhibition of tumor-associated angiogenesis results in increased apoptotic tumor cell death, leading to inhibition of tumor growth.

5.6.5 Matrix Metalloproteinase. As mentioned earlier in the chapter, MMPs are capable of proteolytic degradation of stromal ECM, which is essential in cancer cell migration and invasion, as well as in tumor-induced angiogenesis. The activity of MMPs in vivo is inhibited by TIMPs, small secreted proteins with molecular weight of between 20 and 30 kDa. TIMPs inhibit MMPs by binding to both the latent and active forms of MMPs. The following properties of TIMPs such as secretion, diffusion (TIMP-1, -2 and -4), induction of apoptosis (TIMP-3), and inhibition of multiple MMPs make them very attractive tools for gene therapy application.

Inhibition of cancer cell invasion after overexpression of TIMPs using different gene delivery vectors has been shown in vitro in gastric cancer cells and mammary carcinoma cells (156,157). Overexpression in vitro of TIMP-2, which was delivered by a recombinant adenovirus (AdTIMP-2), inhibited the invasion of both tumor and endothelial cells in three murine models without affecting cell proliferation (158). Its in vivo efficiency has been evaluated in the LLC murine lung cancer model, the colon cancer C51 model, as well as in MDA-MB231 human breast cancer in athymic mice. Preinfection of tumor cells by AdTIMP-2 resulted in an inhibition of tumor establishment in more than 50% of mice in LLC and C51 models and in 100% of mice in the MDA-MB231 model. A single local injection of AdTIMP-2 into preestablished tumors of these three tumor types reduced tumor growth rates by 60–80%, and the tumor-associated angiogenesis index by 25–75%. Lung metastasis of LLC tumors was inhibited by >90%. In addition, AdTIMP-2-treated mice showed a significantly prolonged survival in all the cancer models tested. These data demonstrate the potential of adenovirus-mediated TIMP-2 therapy in cancer treatment.

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CHAPTER TWO

Synthetic DNA-Targeted chemotherapeutic Agents and Related Tumor-Activated Prodrugs

WILLIAM A. DENNY
Auckland Cancer Society Research Centre
Faculty of Medical and Health Sciences
The University of Auckland
Auckland, New Zealand

Contents

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1 GENERAL INTRODUCTION

Synthetic drugs have always played an important role in cancer therapy. In fact, systemic chemotherapy for cancer began in the 1940s and 1950s with the nitrogen mustards developed from war gases (1) and with antimetabolites developed from early knowledge about DNA metabolism (2). Large-scale random screening programs over the next 25 years (mainly by the U.S. National Cancer Institute) (3) seeking cytotoxic agents resulted in the identification of a number of cytotoxic natural products that target DNA. Many of these (e.g., anthracyclines, epipodophylloxins, and vinca alkaloids) became very useful drugs that are still widely used today. Most of the natural products were so complex that neither they nor close analogs could be economically produced by synthesis, limiting the role of synthetic chemistry in optimizing their potencies or pharmacokinetic properties. However, the discovery of their activity and mechanism of action sparked much work on simpler synthetic analogs. One result was the development of the large class of synthetic topoisomerase inhibitors that are now an important group of drugs. More recently, the increasing power of organic synthesis has greatly improved chances that quite complex natural product leads can be synthesized economically, and therefore that close analogs can be made to try and optimize physicochemical properties; recent examples are cyclopropylindolines and epothilones. However, the primary focus in this chapter are synthetic com-

pounds that have not been derived from a natural product lead. Finally, our increasing understandingof tumor physiology and genetics has allowed the development of a new class of synthetic agents, tumor-activated prodrugs. These attempt to exploit tumor-specific phenomena, such as unique antigen expression, low pH, and hypoxia, to activate prodrugs of the more classical cytotoxins only in tumors, thus increasing their therapeutic range.

2 ALKYLATINC AGENTS

2.1 Introduction

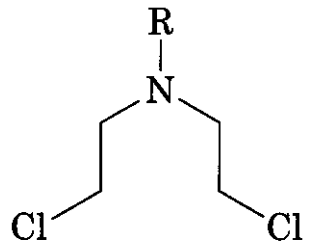
Compounds that alkylate DNA have long been of interest as anticancer drugs. Alkylating agents can be strictly defined as electrophiles that can replace a hydrogen atom by an alkyl group under physiological conditions, but the term is usually more broadly interpreted to include any compound that can replace hydrogen under these conditions, including metal complexes forming coordinate bonds. Many different types of chemical are able to alkylate DNA, and several are used as anticancer drugs, but the most important classes of such agents in clinical use are the nitrogen mustards and the platinum complexes, the nitrosoureas, and the triazene-based DNA-methylating agents. The DNA minor groove-alkylating cyclopropylindoles are also a fascinating group of compounds that may not yet have found their correct niche in cancer therapy. Other important classes of DNA alkylating agents, the pyrrolobenzodiazepines and the mitosenes, are covered in a different chapter, although the bioreductive properties of the mitosenes are mentioned in Section 5.3.

2.2 Clinical Examples of Alkylating Agents

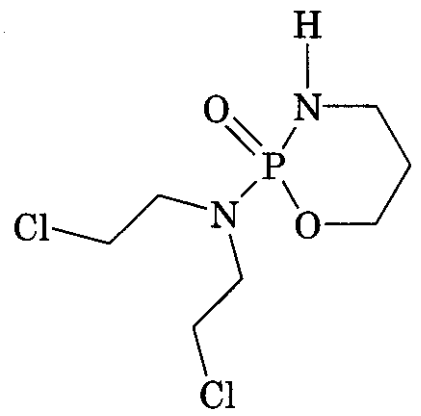
The most commonly used mustards and platinum complexes are listed in Table 2.1, along with other recent DNA-alkylating agents that have received clinical trial. These compounds are invariably used in combination with other agents in multidrug therapy regimens.

2.3 Mustards

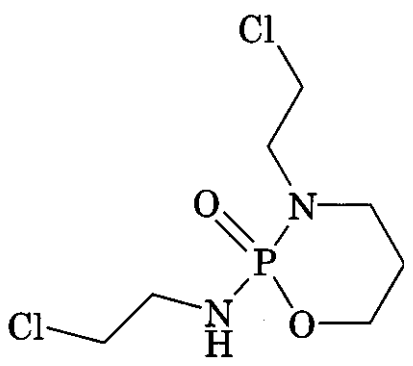
2.3.1 History. As noted above, the mustards were among the very earliest class of anticancer agents developed, and they have been extensively reviewed. Mechlorethamine (1) (4) was the first systemic agent approved for use in cancer therapy in 1949. Chlorambucil (2) (5) was approved in 1957, melphalan (3) (6) in 1964, cyclophosphamide (4) (7) in 1959, and ifosfamide (5)(8)in 1988. The phosphoramide mustard cyclophosphamide (4) is currently the most widely used mustard, while chlorambucil (2) and melphalan (3)are still in use as components of many combination chemotherapy regimens.



(1) R = CH_3
(2) R = $(CH_2)_3CO_2H$
(3) R = $CH(NH_2)CO_2H$



(4)



(5)

2.3.2 Mechanism and SAR. The biologically important initial lesion formed by mustards in cells is interstrand cross-links between different DNA bases (9), although there is also evidence that they cause termination of

Table 2.1 Alkylating Agents Used in Cancer Chemotherapy

Generic Name (Structure)	Trade Name	Originator	Chemical Class
Mustards			
mechlorethamine (**1**)	Mustargen	Merck	aliphatic mustard
chlorambucil (**2**)	Leukeran		aromatic mustard
melphalan (**3**)	Alkeran		aromatic mustard
cyclophosphamide (**4**)	Cytoxan	Bristol-Myers	phosphoramide mustard
ifosfamide (**5**)	Ifex	Bristol-Myers	phosphoramide mustard
Platinum complexes			
cisplatin (14)	Cisplatin	Bristol-Myers	platinum complex
carboplatin (15)	Paraplatin	Bristol-Myers	platinum complex
tetraplatin (16)	Ormaplatin		platinum complex
oxaliplatin (17)	JM-83	Sanofi	platinum complex
ZD-0473 (**18**)		AstraZeneca	platinum complex
satraplatin (19)	JM-216	Johnson-Matthey	platinum complex
BBR 3464 (20)		Boehringer	triplatinum complex
Cyclopropylindoles			
Adozelesin (22)		Upjohn	cyclopropylindole
Carzelesin (23)		Upjohn	cyclopropylindole
KW 2189 (24)		Kyowa Hakko	cyclopropylindole
Nitrosoureas			
CCNU (**29**)	Lomustine	Bristol-Myers	nitrosourea
BCNU (**30**)	Carmustine	Bristol-Myers	nitrosourea
Streptozotocin (**31**)	Zanosar	Upjohn	nitrosourea
Methylating agents			
dacarbazine (**29**)	DTIC		triazene
mitozolomide (30)	azolastone		triazene
temozolomide (**31**)	Temodar	Shering-Plough	triazene

transcription (10). The overall process of alkylation is a two-step sequence involved formation of a cyclic cationic intermediate, followed by nucleophilic attack on that intermediate by DNA (Fig. 2.1a). Mustards can be divided into two broad classes, depending on the mechanism of the rate-determining step in this process. The less basic compounds aromatic mustards have formation of the solvated cyclic carbocation (which is in equilibrium with the aziridinium cation) as the rate-determining step, following first-order kinetics (11) (Fig. 2.1b). Nucleophilic attack on this is then rapid, so that the cyclic form does not accumulate, and the overall reaction is first-order (S_N1), with the rate depending only on the concentration of the mustard (Equation 2.1).

$$R\text{—}X \rightarrow R^+ \rightarrow R\text{—}DNA \qquad (2.1)$$

For the more basic aliphatic mustards, the first step (formation of the aziridinium cation), is rapid, and the rate-determining step is a second-order nucleophilic substitution on this by DNA (11). In these cases, the aziridinium cation can be detected as an intermediate, and the overall reaction is second-order (S_N2), with the rate depending on the concentrations of both the mustard and the DNA (Equation 2.2).

$$DNA + R\text{—}X \rightarrow DNA \ldots ; R \ldots H \rightarrow R\text{—}DNA + X \qquad (2.2)$$

This kinetic classification is only broad, but it is useful as a rough predictor of the spectrum of adducts formed. Generally, S_N1-type compounds are expected to be less discriminating in their pattern of alkylation (reaction at N, P, and O sites on DNA), whereas most S_N2 type compounds tend to alkylate only at N sites on the DNA bases (12).

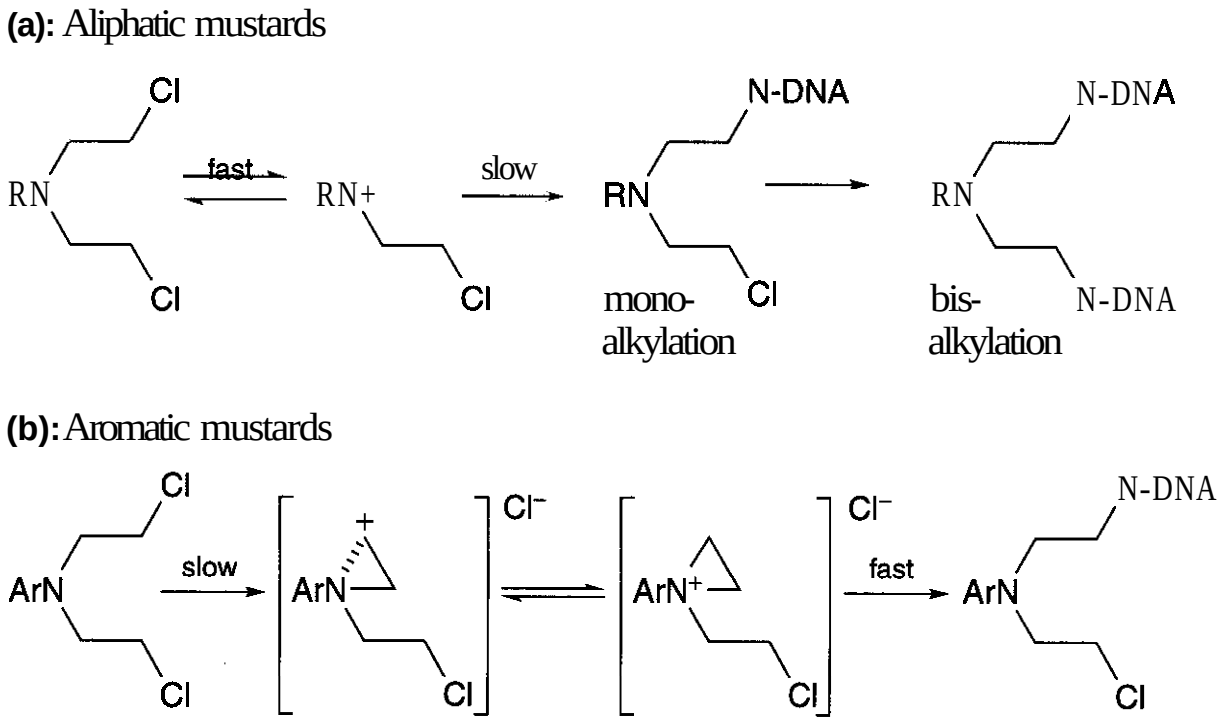


Figure 2.1. Mechanism of alkylation by mustards.

The primary site of DNA alkylation by mustards is at the N7 position of guanine, particularly at guanines in contiguous runs of guanines (13), which have the lowest molecular electrostatic potentials (14). However, the level of selectivity of the initial attack by mustards (to form monoadducts) is quite low, with evidence (15) that most guanines are attacked. Studies with alkyl mustards have also shown significant levels of alkylation at the N3 position of adenine (16, 17). However, the sequence selectivity of cross-link formation by mustards is necessarily higher, because of the requirement to have two suitable sites juxtaposed. Early work (18) on the interaction of mechlorethamine (1) with DNA resulted in isolation of the 7-linked bis-guanine adduct, and it has been widely assumed that the cross-links were between adjacent guanines (i.e., at 5'-GC or 5'-CG sites). However, later work (19) showed that the preferred cross-links are between non-adjacent guanines (i.e., at 5'-GNC sites).

Cyclophosphamide (4) is a non-specific prodrug of the active metabolite phosphoramide mustard, requiring enzymic activation by cellular mixed function oxidases (primarily in the liver) to 4-hydroxycyclophosphamide, which is in equilibrium with the open-chain aldophosphamide. Spontaneous elimination of this then gives acrolein and phosphoramide mustard (Fig. 2.2). The isomeric ifosfamide (5) is activated more slowly, but in a broadly similar fashion to give the analogous isophosphoramide mustard (20). A significant difference in the metabolism between the two isomers is a higher level of dechloroethylation with ifosfamide, which may account for its greater neurotoxicity (21).

The rates of the various reactions of aromatic nitrogen mustards (hydrolysis, alkylation of DNA) can be correlated closely with the basicity of the nitrogen, that in turn, can be systematically altered by ring substituents. The rates of hydrolysis (K_H) of a series of substituted aromatic nitrogen mustards in aqueous acetone can be described (22) by Equation 2.3, where a is the Hammett electronic parameter.

$$\log K_H = -1.84\sigma - 4.02 \qquad (2.3)$$

The negative slope is evidence for an S_N1 mechanism, indicating that electron-releasing substituents (negative a values) increase the rate of hydrolysis by accelerating formation of the carbocation. The same broad correlations hold for how well the compounds alkylate DNA, with a similar equation (Equation 2.4) describing the rates of alkylation (K) of 4-(4-nitrobenzyl)pyridine (a nucleophile similar to DNA nucleophilic sites) by substituted aromatic nitrogen mustards (22), where σ^- is an electronic parameter closely related to *a.*

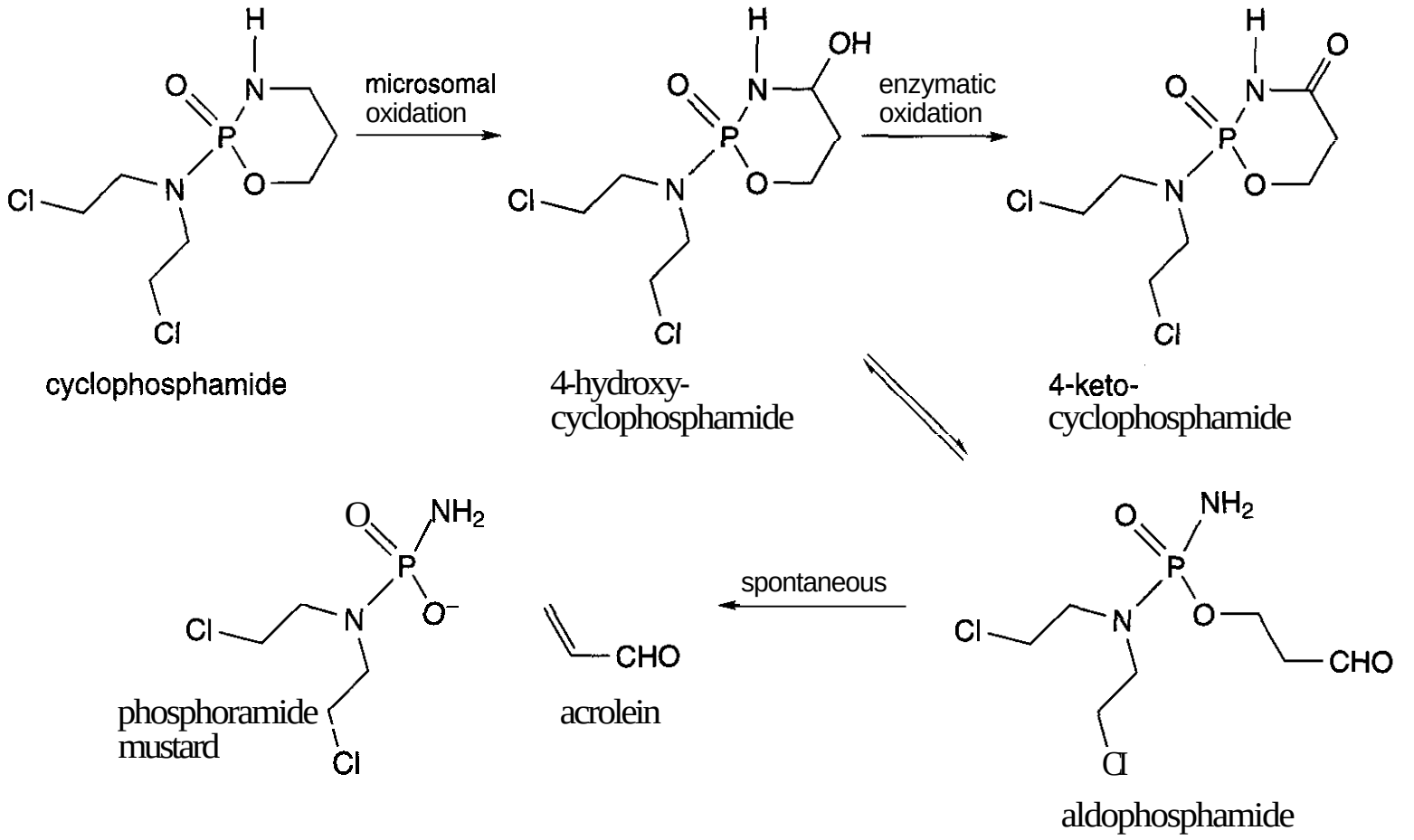


Figure 2.2. Metabolism of cyclophosphamide.

$$\log K = -1.92\sigma^{-} - 1.17 \qquad (2.4)$$

The cytotoxicities of the above compounds ($1/IC_{50}$ values) also correlate well with substituent σ values, with the more reactive compounds (bearing electron-donating substituents) being the more cytotoxic, as in Equation 2.5 (23).

$$\log(1/IC_{50}) = -2.46\sigma + 0.53 \qquad (2.5)$$

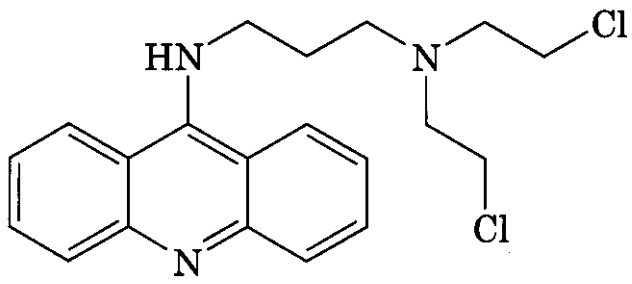
The cytotoxicity of aromatic mustards can thus be predictably varied over a very wide range by controlling the basicity of the mustard nitrogen through ring substitution or other means.

2.3.3 Biological Activity and Side Effects. The (necessarily) high chemical reactivity of mustards leads to rapid loss of drug by interaction with other cellular nucleophiles, particularly proteins and low molecular weight thiols. This results in the development of cellular reistance by increases in the levels of low molecular weight thiols (particularly glutathione) (24,251. Of equal importance for efficacy, much of the drug can reach the DNA with only one alkylating moiety intact, leading to monoalkylation events which are considered to be genotoxic rather than cytotoxic (26). The fact that cross-linking is a two-step process adds to the proportion of (genotoxic) monoalkylation events, because the second step is very dependent on spatial availability of a second nucleophilic DNA site. Mustards have no intrinsic biochemical or pharmacological selectivity for cancer cells, and they act as classical antiproliferative drugs, whose therapeutic effects are primarily cytokinetic. They target rapidly dividing cells rather than cancer cells, and this, together with their generally systemic distribution, causes killing of rapidly dividing normal cell populations in the bone marrow and gut, usually resulting in myelosupression, the dose-limiting side effect. Because of their genotoxicity, there is a risk of the development of second cancers from their mutagenic effects (27). The most frequent alkylator-induced malignancy is acute leukemia, usually occurring a long period (3–7 years) after treatment. These usually demonstrate deletions of chromosome 13 and loss of parts or all of chromosomes 5 or 7 (loss of the coding regions for tumor-suppressor genes). The induced tumors

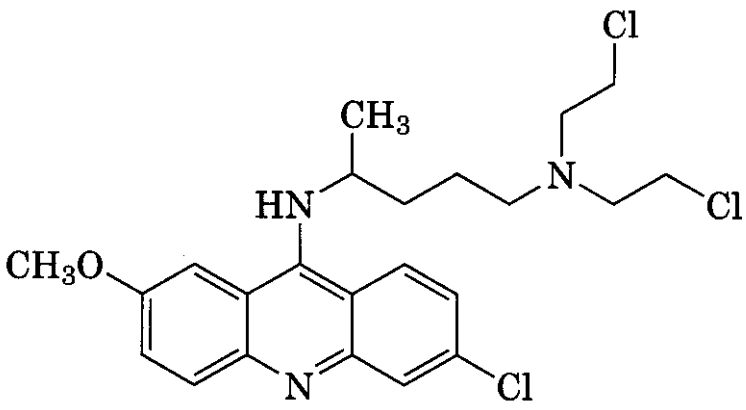
are typically myelodysplasias (28). In one study (29), 6% of all myeloid leukemias were therapy-related, with mustards, nitrosoureas, and procarbazine producing the greatest levels of induction.

2.3.4 Recent Developments: Minor Groove Targeting. Many of the limitations noted above could in principle be ameliorated by targeting the mustard moiety more specifically to the DNA-affinic carrier molecule. This has resulted in much work where mustards have been attached to DNA-affinic compounds (30–32). This could mean less chance of losing active drug by reaction with other cell components, rendering less effective the development of cellular resistance by elevation of thiol levels. A higher proportion of bifunctional alkylating agent delivered intact to the DNA would also contribute to a higher proportion of cross-links over to monoalkylation events. The use of a carrier with sequence-specific reversible binding ability should also result in greater specificity of alkylation, both sequence-specifically (at the favored reversible binding site of the carrier) and regio-specifically (at particular atoms on the DNA bases).

Attachment to DNA-intercalating carriers goes back to the work of Creech et al. (33), who originally suggested that the attachment to acridine carriers might serve to target the reactive center to DNA. They showed that such "targeted mustards" such as (6) were more potent than the corresponding untargeted moiety against ascitic tumors in vivo, but these proved to be exceptionally potent frameshift mutagens in bacteria, and this property has tended to dominate the perception of these compounds. Later work showed that such targeting by an intercalator could also drastically modify the pattern of DNA alkylation by the mustard. Thus, whereas untargeted mustards react largely at the N7 of guanines in runs of guanines, quinacrine mustard (7) also alkylates at guanines in 5'-GT sites (13). Isolation and identification of DNA adducts showed that whereas the acridine-linked mustard (8) formed primarily guanine N7 adducts, the similar analog (9) formed exclusively adenine N1 adducts (34), showing the extent of which DNA targeting by attachment to carrier molecules can alter the usual pattern of DNA alkylation by mustards.



(6)



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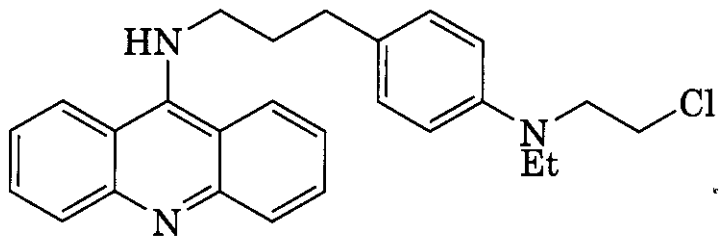
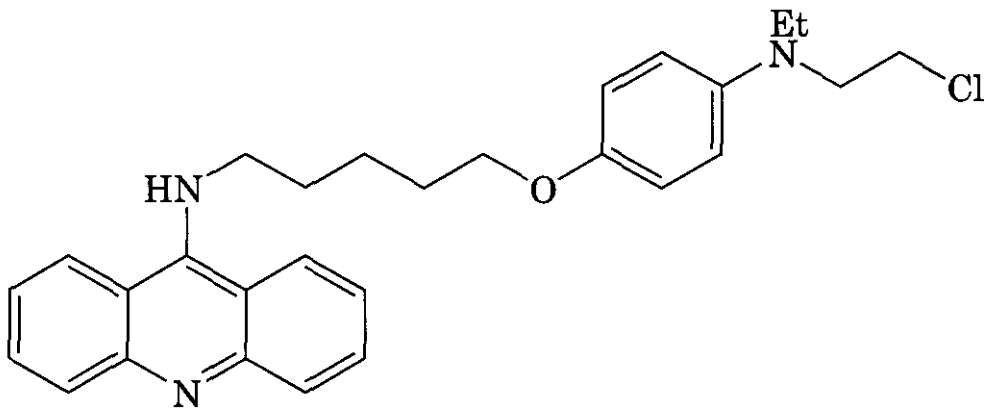


(8)

However, most DNA-targeting of mustards has been done using minor groove-binding carriers. These ligands offer much larger binding site sizes (up to 5–6 base pairs) than intercalators, together with a highly defined binding orientation. Whereas several other minor groove-binding carriers have been used (35–37), most work has employed polypyrrole and related ligands. These compounds have been well documented as reversible AT-specific minor groove binders (38), and early work using a variety of alkylating units (e.g., bromoacetyl) showed highly specific alkylation at adenines in runs of adenines (39). The benzoic acid mustard derivative tallimustine (10; FCE 24517) was selected for further development on the basis of its broad-spectrum solid tumor activity (40). Despite possessing a difunctional

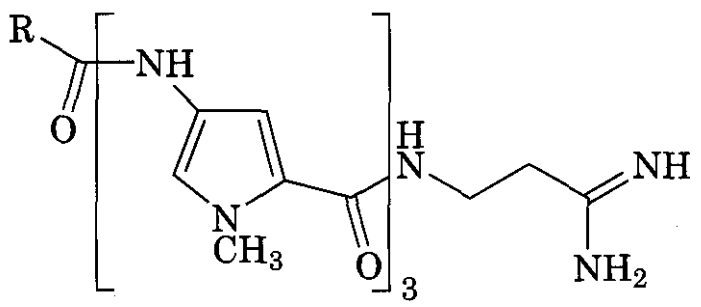


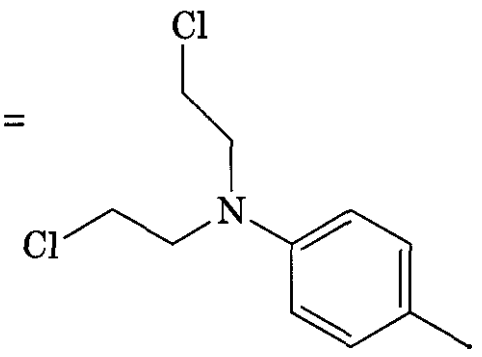
(9)

alkylator, this compound monoalkylates DNA at the N3 of adenine in the minor groove, almost exclusively at the sequence 5'-TTTTGA (41), with a single base modification in the hexarner completely abolishing alkylation (42). The number of pyrroleamide units also affected the pattern of DNA alkylation, with a monopyrrole analog showing mainly guanine-N7 alkylation similar to that of the untargeted mustard, but with additional adenine-N3 lesions (43). Di- and tripyrrole conjugates alkylated only in AT tracts, with increasing specificity for alkylation at the 3′-terminal units in two 5′-TTTTGG and 5′-TTTTGA sequences (guanine N3 and adenine N3 lesions, respectively).

Tallimustine was developed for clinical trial (44) and shows biological effects somewhat different to those of mustards like melphalan. It induces blockage of the cell cycle in G2 but without the delay through S-phase normally seen with untargeted mustards, suggesting a different mechanism of cytotoxicity through monoadduct formation (45). As a highly sequence-specific alkylator, it selectively blocks the binding of transcription binding protein and complexes to their AT-rich cognate sequences (46). Clinical trials of tallimustine (47, 48) reported severe myelosuppression as the dose-limiting toxicity. Recent work with halogenoacrylic derivatives (e.g., 11) show these may work differently, possibly through Michael-type reactions (49), with much better cytotoxicity/myelotoxicity indices (50).

Perhaps the ultimate in targeting mustards to specific DNA sites has been achieved by Dervan and co-workers, who have developed the "hairpin polyamide" concept where





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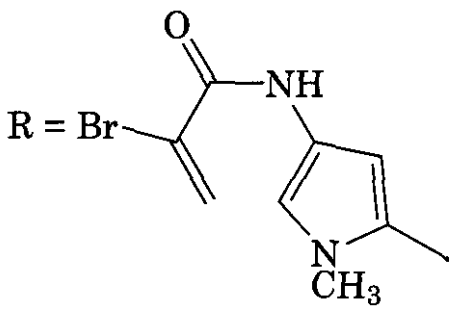
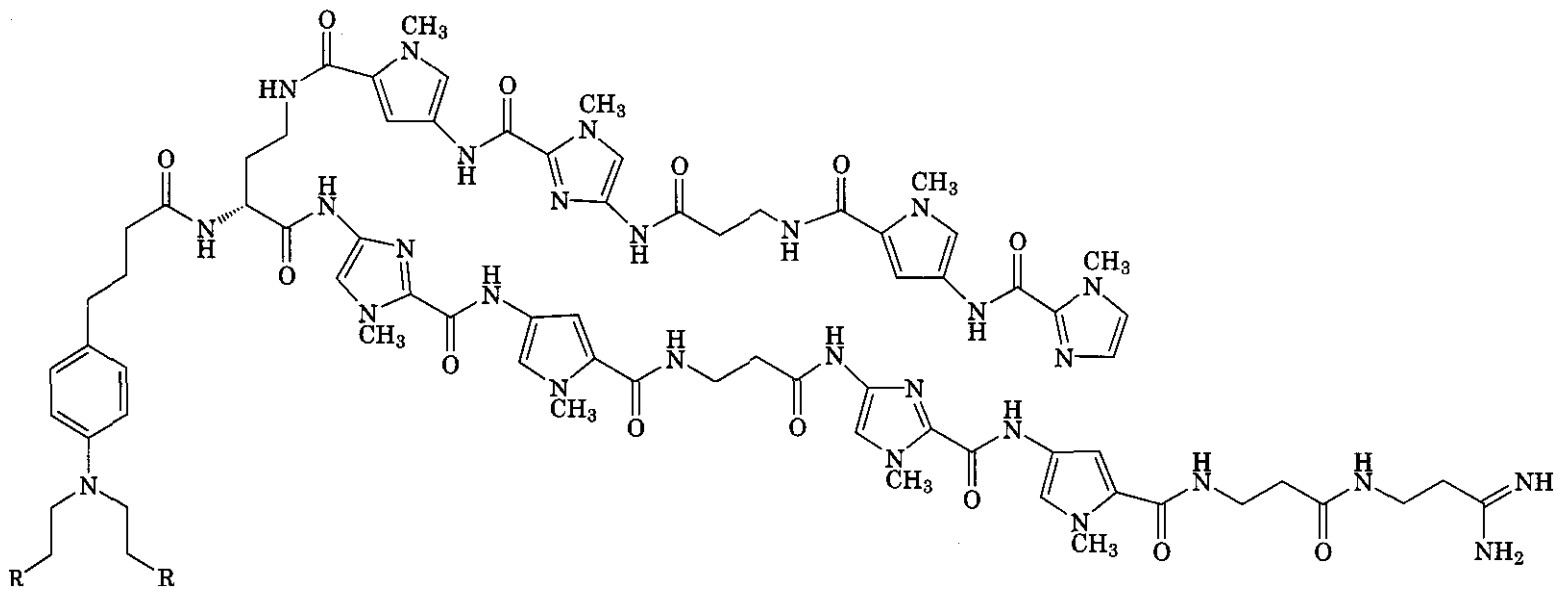


(11)

poly(pyrrole/imidazole) compounds bind in a side-by-side manner in the minor groove (51). These compounds can bind tightly and selectively to individual designated sequences of up to 12 bp long (52). As an example, polyamide 12, with an incipient mustard side chain attached, binds to its designated sites 5′-AGCT-GCT and 5'-TGCAGCA with equilibrium association constants K of 1.6 and 1.3 $\times$ 10^{10} M^{-1}, respectively, and >100-fold less strongly

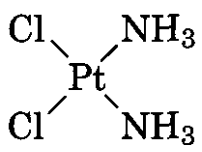
to double mismatch sites (53). The corresponding mustard (13) alkylated at adenine N3 sited in target 5′-(A/T)GC(A/T)GC(A/T) sequences on a 241-bp HIV-1 promotor sequence in high yield and about 20-fold selectively over double mismatch sites (53).



(12) R = OH
(13) R-Cl

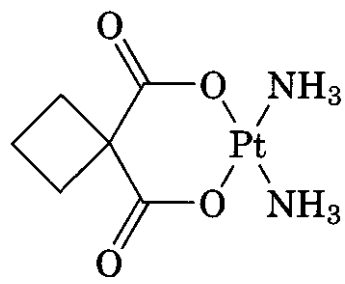
2.4 Platinum Complexes

2.4.1 History. The complex *cis*-diamminodichloroplatinum (II) (cisplatin; 14) was first described in 1845, but it was not until 1969 that it was reported to have antitumor activity. These studies were sparked by experiments by Rosenberg on the effects of electric fields on bacteria, when the peculiar effects seen with E. coli cells were shown to be caused by the electrochemical synthesis of cisplatin from the ammonium chloride electrolyte and the platinum electrodes (54). Clinical trials began in 1972, and after slow progress because of high toxicity, cisplatin became one of the most widely used anticancer drugs; it is the main reason for the spectacular successes in drug treatment of testicular and ovarian cancer. Thousands of analogs of cisplatin have been made and evaluated, with two major driving forces.



(14)

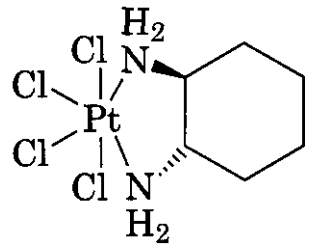
The first has been to seek compounds with lower neurotoxicity than cisplatin. Whereas better clinical management has improved things, one of the main drivers of analog development has been agents with less neurotoxicity. Carboplatin (15) has carboxylate instead of chloride-leaving groups. These hydrolyze much less rapidly, resulting in lower nephro- and neurotoxicity (the dose-limiting toxicity of carboplatin is myelosuppression), while retaining the broad spectrum of activity of cisplatin (55).



(15)

The second impetus to analog development has been to seek agents active in cell lines that become resistant to cisplatin. One mechanism of resistance to cisplatin is an increased ability to repair the DNA adducts formed (56), and analogs such tetraplatin (16; ormaplatin) and oxaliplatin (17), with trans-1,2-diaminocyclohexane (DACH) ligands, were shown to be more effective against such resistant cell lines (57). These compounds proved to be neurotoxic and tetraplatin was difficult to formulate, but oxaliplatin has shown promise (58),

especially in colorectal cancer, where it is synergistic with 5-fluorouracil (59). A second significant mechanism of resistance is elevation of thiol levels (primarily glutathione) in cells (60). The drug ZD-0473 (18;JM-473) is more resistant than cisplatin to thiols, possibly because of steric hindrance by the pyridine ligand (61) and is in phase II clinical trials as an N formulation; an oral formulation is also in development (62).



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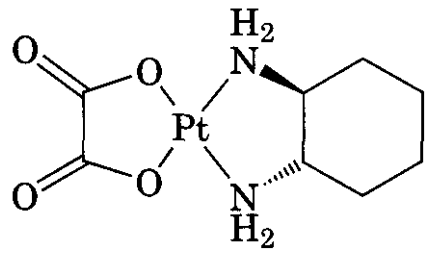

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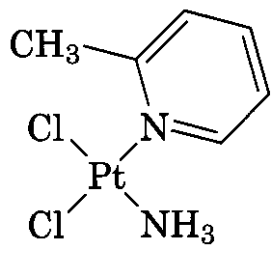
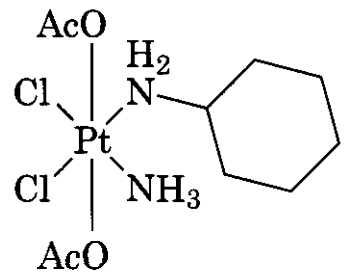


(18)

Satraplatin (19; JM216) is also being developed as an orally available platinum agent (63). It has potent in vitro cytotoxicity against a variety of tumor cell lines and also had oral antitumor activity against a variety of murine and human subcutaneous tumor models in vivo, broadly comparable with the level of activity obtainable with parenterally administered cisplatin (64). Satraplatin has shown activity in phase I trials in lung cancer, with no neurotoxicity or nephrotoxicity (63), and responses have also been seen in small cell lung cancer and hormone refractory prostate cancer.



(19)

2.4.2 Mechanism and SAR. As with the nitrogen mustards, the mechanism of action of the platinum complexes involves formation of DNA cross-links. In the platinum complexes, the chloride or carboxylato ligands are the leaving groups, with the amine ligands being substitutionally inert and serving to modulate other properties. The bonds formed and broken in this case are coordinate metal-ligand bonds that are not permanent but have characteristic half-lives (although these may be very long), making the chemistry quite different to that of the mustards. Thus the Pt-Cl bonds in cisplatin (14) are more stable in the relatively high chloride conditions in plasma than they are in the lower chloride conditions inside cells, where the reaction with water to form aquo species is more facile (65). The cationically charged aquo species have higher affinity for DNA, and react primarily at guanine N7 sites in the major groove to form long-lived ammine complexes (Fig. 2.3).

Cisplatin reacts with DNA to form a number of different adducts. However, by far the most common are intrastrand guanine N7-guanine N7 adducts between adjacent guanines on the same strand (ca. 65%), followed by similar intrastrand guanine N7-adenine N7 adducts (ca. 25%), with DNA-protein cross-links and monofunctional adducts making up less than 10% and DNA interstrand adducts less than 1% of the total adducts. A major difference between mustards and platinum complexes is that whereas hydrolysis in the former case is a deactivating event, leading to loss of bifunctionality (and thus cross-linking ability), with platinum complexes, formation of the aquo species is a necessary activating process. Thus there is a much higher proportion of cross-links to monoadducts formed with platinum complexes than with mustards. The use of [^{1}H, ^{15}N] heteronuclear single quantum coherence (HSQC) 2D

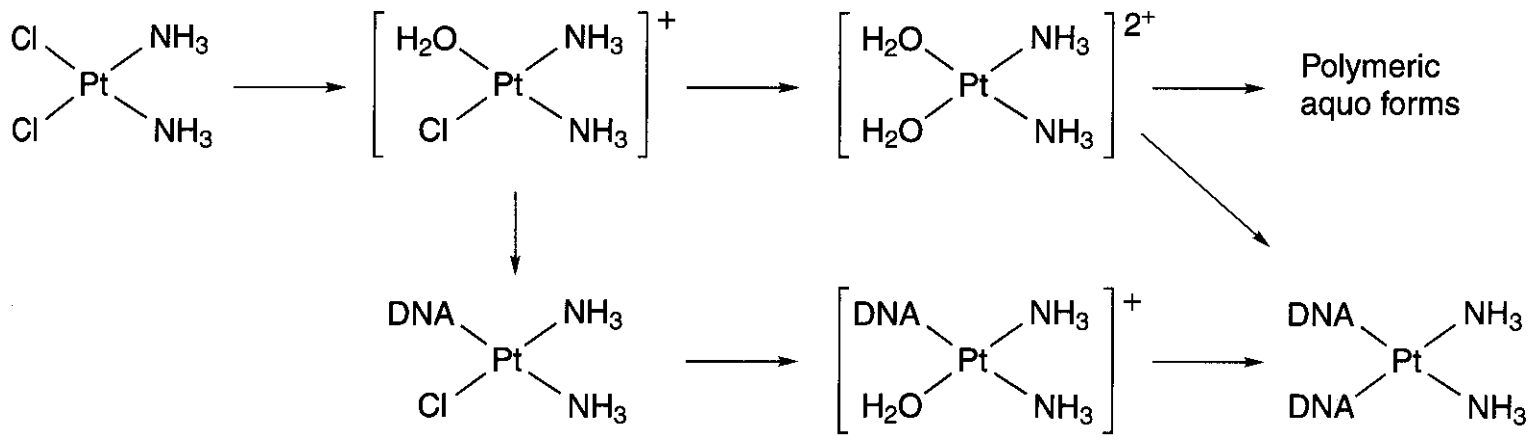


Figure 2.3. Reaction of platinum species with DNA.

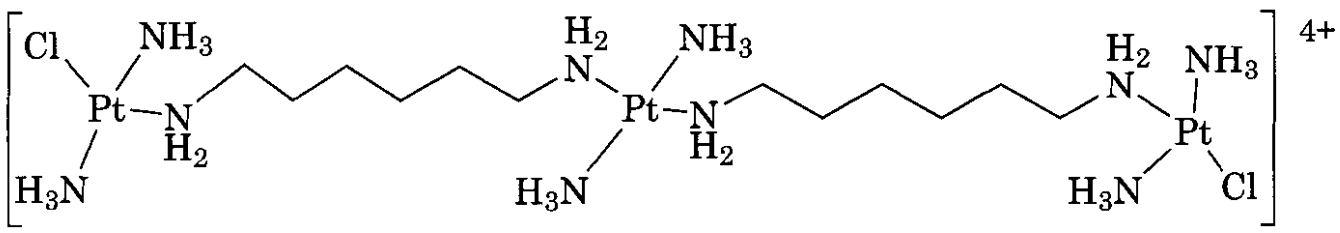
NMR has recently allowed a better understanding of the kinetics of the multiple processes involved in the reaction of platinum drugs with DNA (66).

2.4.3 Biological Activity and Side Effects. While cisplatin is an extremely useful drug, it has many side effects. In addition to the myelosuppressive activity typical of a DNA alkylating agent, it also showed severe renal and neural toxicity. The analog development work described above has been aimed primarily to overcoming some of the these side effects. Thus carboplatin is less nephro- and neurotoxic; tetraplatin, oxaliplatin, and ZD-0473 are more effective against various types of resistance mechanisms; and satraplatin is orally effective. However, none of these compounds are overall more effective than cisplatin, and they do not show major differences in their interaction with DNA.

2.4.4 Recent Developments: Increased Interstrand Crosslinking. The tetracationic triplatinum complex BBR 3464 (20) seems to represent a new structural class of DNA-modifying anticancer agents (67). It reacts with DNA faster than does cisplatin, suggesting rapid cellular uptake and nuclear access (68), to give a different profile of adducts than cisplatin, with about 20% being interstrand *GG* cross-links. DNA modified by BBR3464 cross-reacted with antibodies raised to transplatin-adducted DNA but not to antibodies raised to cisplatin-adducted DNA (67). BBR 3464 was 30-fold more cytotoxic than cisplatin in L1210 cells and showed no cross-resistance in sublines resistant to cisplatin because of impaired accumulation and lower DNA binding (69). Consistent with this, it was also highly active in a panel of cisplatin-resistant xenografts, giving longer growth delays (70). Unlike cisplatin, BBR 3464 was able to induce the p53/p21 pathway to a similar extent in both cisplatin-sensitive and -resistant cells (71), and had a quite different sensitivity profile to cisplatin in the U.S. National Cancer Institute's 60-cell-line screening panel (70). In a phase I trial using a single-dose schedule, no significant neural or renal toxicity was observed; the side-limiting toxicity at 0.17 mg/m^2 was short-lasting neutropenia (72).

2.5 Cyclopropylindoles

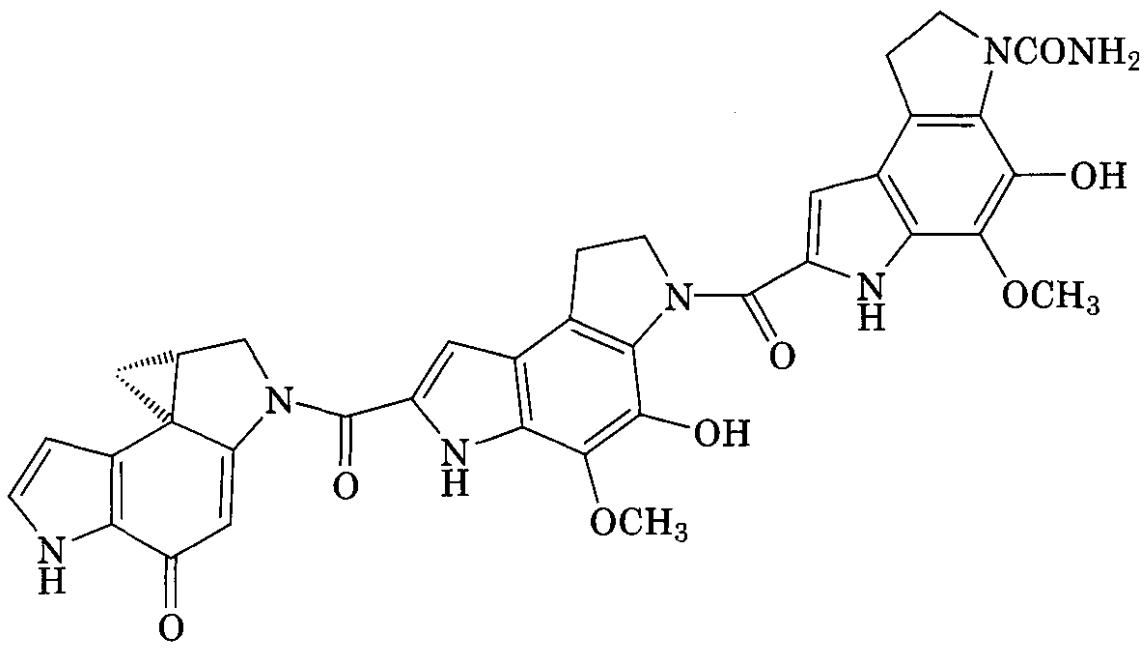
2.5.1 History. Interest in DNA minor-groove alkylating agents was stimulated by the discovery (73) of the natural product CC-1065 (21) from ***Streptomyces zelensis*** (74), which showed extraordinary potency in a number of animal tumor models (75) but with concomitant fatal delayed hepatotoxicity at therapeutic doses (76). An extensive synthesis program at Upjohn prepared a large number of analogs in an attempt to understand structure-activity relationships for the class (77) and succeeded in developing the structurally simpler agents adozelesin (22) and the open-chain form carzelesin (23), which did not show the delayed hepatotoxicity of CC-1065 (78, 79). The related semi-synthetic duocarmycin analog KW 2189 (24) is a carbamate prodrug, releasing the active moiety DU-86 (25) by esterase hydrolysis (80). KW 2189 has been prepared on a large scale by a three-step synthesis with an overall 55% yield from natural duocarmycin B2 (81). Although it is less potent than duocarmycin in cell culture assays,



(20)

it has high activity in a wide range of human solid tumor xenografts in mice and lacks the delayed lethal toxicity seen with some other cyclopropylindoles (82).

2.5.2 Mechanism and SAR. These compounds bind initially reversibly in the minor groove of **DNA** with minimal structural distortion, and subsequently, alkylate specifically at the **N3** position of adenine **(83).** This provides further evidence that targeting alkylating functionality to the **DNA** minor groove can provide compounds of very high cytotoxic potency. Whereas the lead compound is a natural



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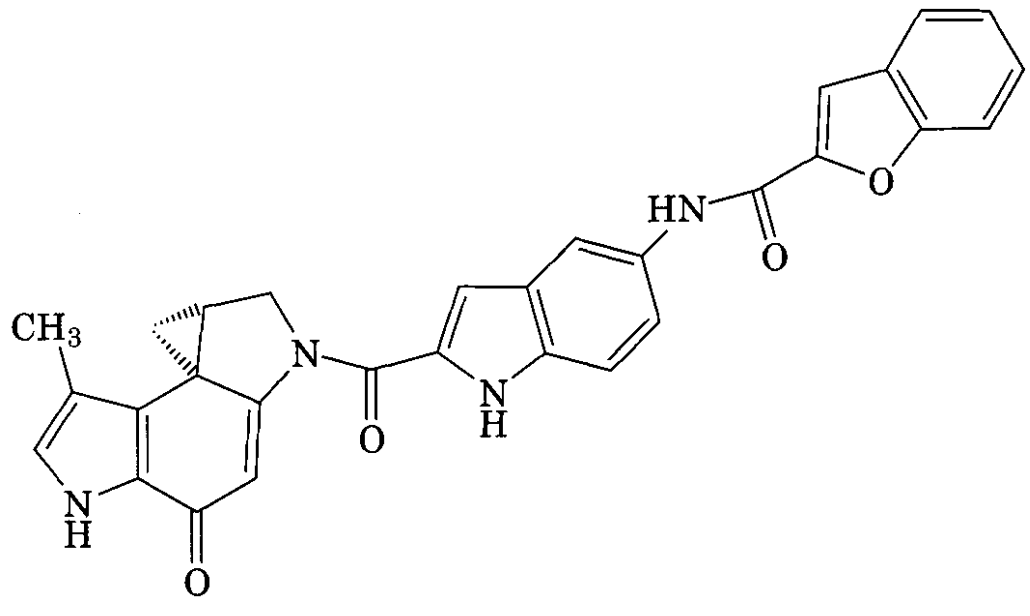

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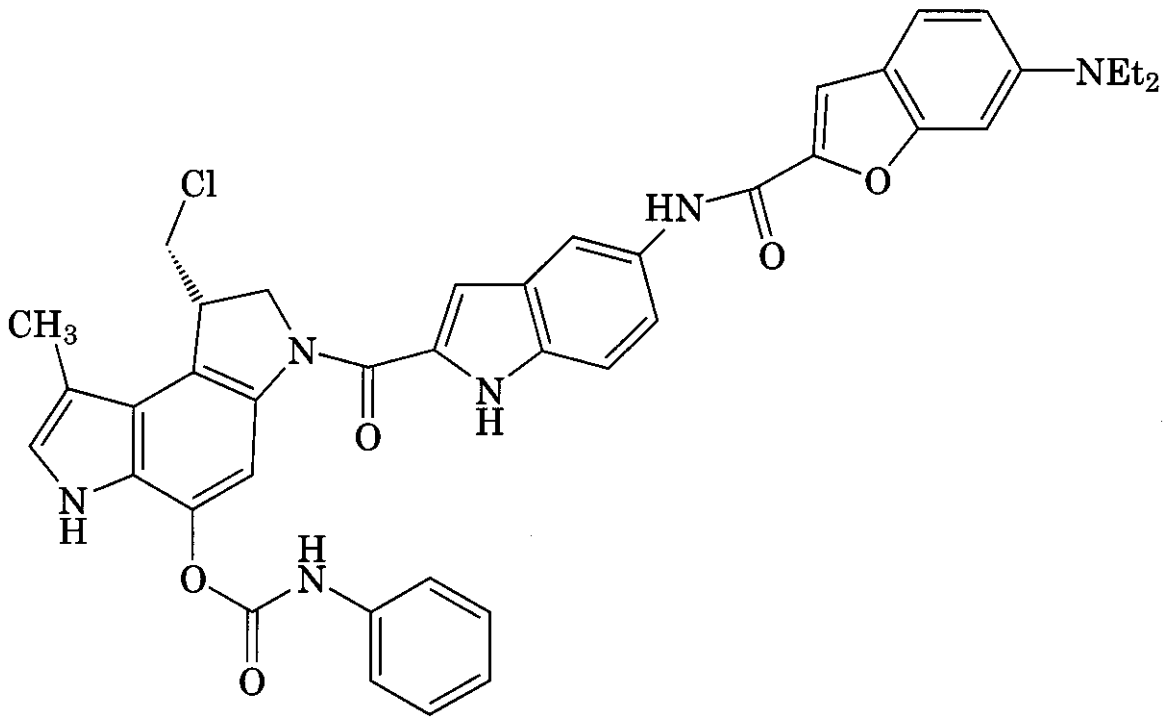

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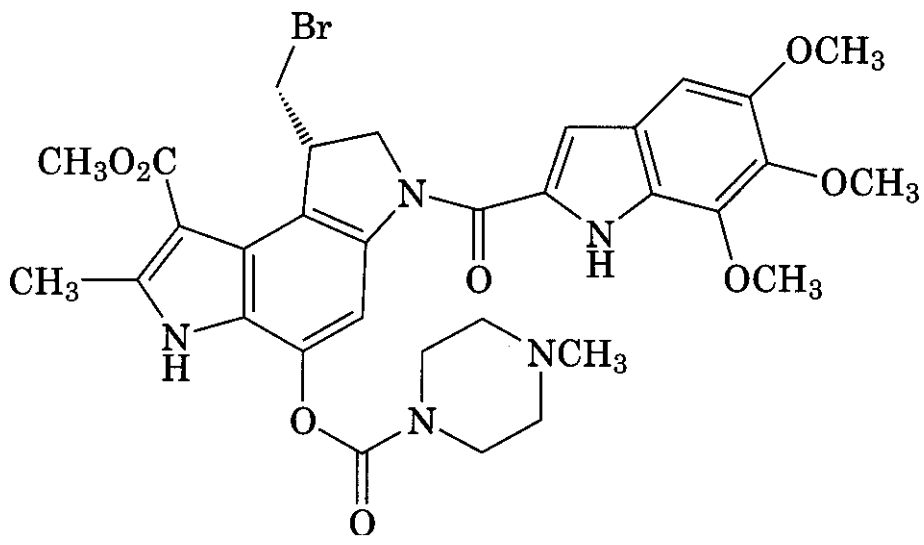

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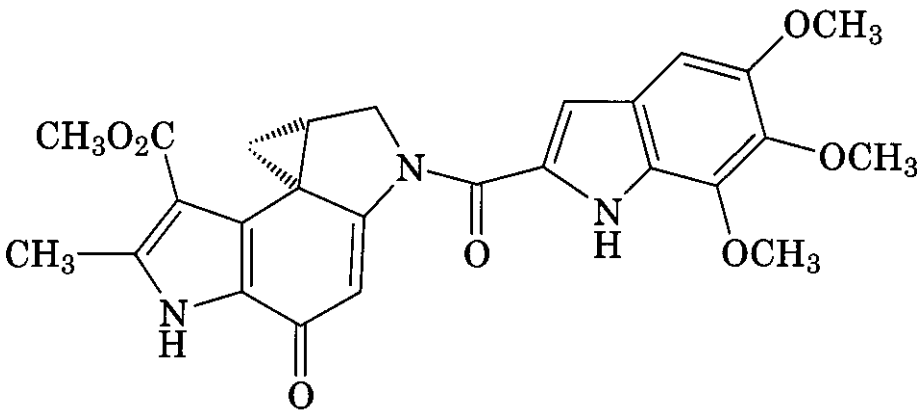


(25)

product, it has sparked a vast amount of synthetic chemistry, and the analogs developed for clinical studies are synthetic. It has been proposed (84) that binding of these compounds in the minor groove of DNA requires a propellor twist of the cyclopropyldienone and indole subunits around the amide bond, and that this interrupts the vinylogous amide sta-

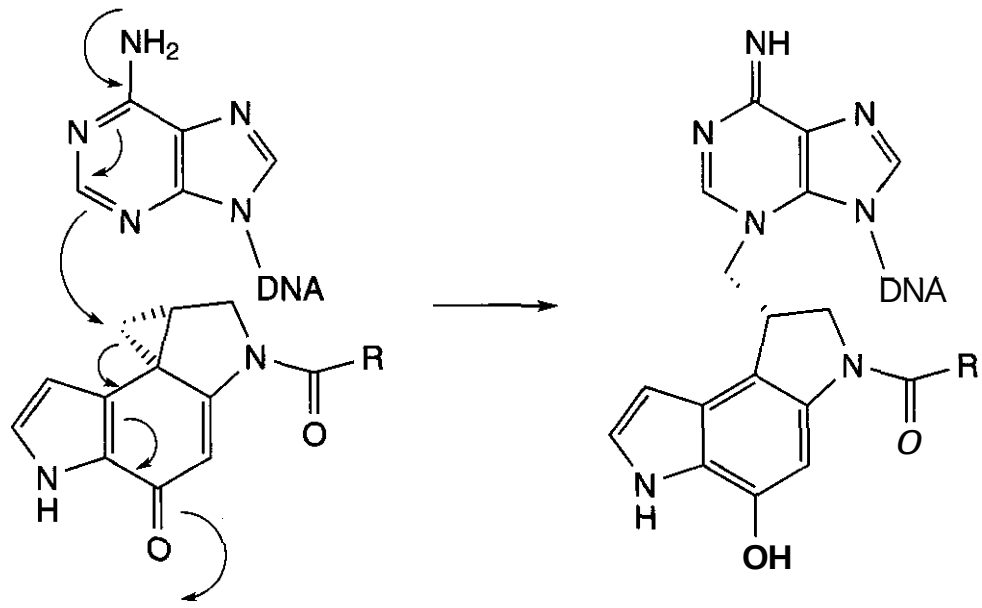
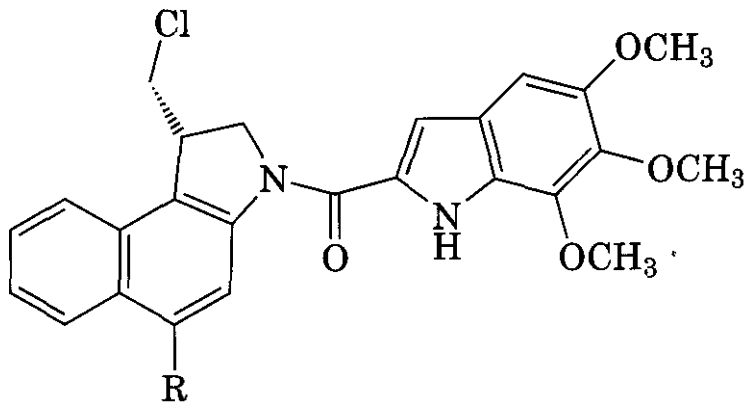


Figure 2.4. Alkylation of DNA by cyclopropylindoles.

bilization of the cyclopropyldienone, activating the conjugated cyclopropane electrophile (Fig. 2.4). Changes in the DNA binding side chain have only minor effects on the sequence selectivity of alkylation; both adozelesin (22) and carzelesin (23) alkylate DNA at the consensus sequences 5′-(A/T)(A/T)A and 5'-(A/T)(G/C)(A/T)A are broadly similar (85) to the consensus sequence 5′-(A/T)(A/T)A for CC-1065. A series of analogs of KW 2189 with water-solubilizing cinnamate side-chains were reported to have potent *in vivo* antitumor activity and low peripheral blood toxicity compared with the trimethoxyindole congeners (86), and more potent ring A dialkylaminoalkyl derivatives have also been reported (87).

2.5.3 Biological Activity and Side Effects. Many of the synthetic compounds developed from the original natural product lead were also extremely potent and showed broad-spectrum activity in human tumor colony–forming assays (88), and both adozelesin (22) and carzelesin (23) proceeded to clinical trial. However, adozelsin had only marginal efficacy in a phase II trial of untreated metastatic breast carcinoma (89). Similarly, carzelesin showed no activity in a phase II trial in patients with a variety of advanced solid tumors (90). A phase I trial of KW 2189 (24) established the maximum tolerated dose at 0.04 mg/m^2/d when given daily for 5 days, with leukopenia, neutropenia, and thrombocytopenia as dose-limiting toxicities (91). A phase II pilot study in metastatic renal cell carcinoma showed a good safety profile but no activity (92).

2.5.4 Recent Developments. Amino analogs (e.g., 26) of the corresponding phenolic open-chain forms (e.g., 27) were reported to have comparable cytotoxicity (93) and similar patterns of DNA interaction, alkylating preferentially at 5′-A(A/T)AN sequences (94). These have been proposed as effectors for tumor-activated prodrugs (see Section 5).

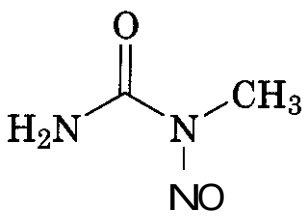


(26) R = NH_2
(27) R = OH

2.6 Nitrosoureas

2.6.1 History. This class of compounds has a long history, and extensive reviews exist on all aspects of their chemistry and biology (95). The initial impetus for their development came from screening at the U.S. National Cancer Institute, where 1-methyl-3-nitro-1-nitrosourea (28) showed some activity in the *in vivo* leukemia screen (96). Development of this lead resulted in the urea-based clinical agents BCNU (29; carmustine) and CCNU (30; lomustine). These reactive compounds have very short half-lives (a few minutes) (97), but their very lipophilic nature suggested they

might cross the blood-brain barrier and be useful in brain tumors (98). The more hydrophilic streptozotocin (31) is a natural product isolated from *Streptomyces* species. It was evaluated initially as an antibacterial agent but proved to be too toxic (99).



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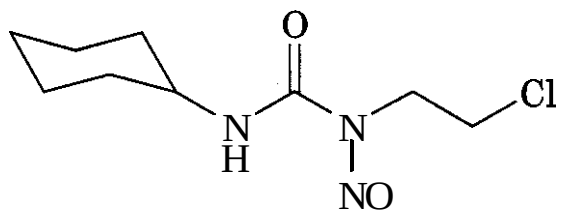

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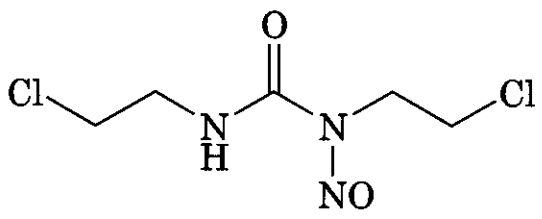

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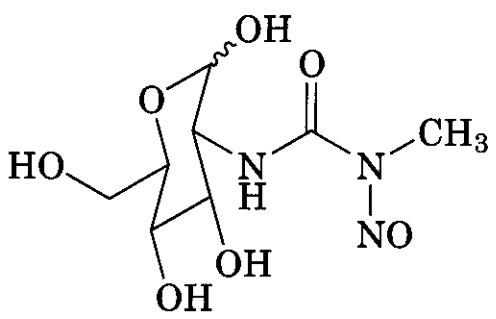


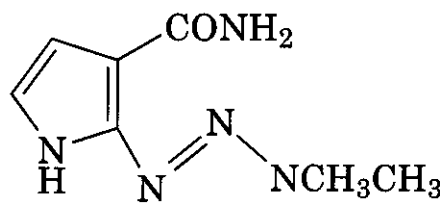
(31)

2.6.2 Mechanism and SAR. The mechanism of the nitrosoureas is complex. They possess both alkylating and carbamoylating activities (100). Decomposition occurs spontaneously in aqueous media by cleavage of the N—CO bond to give a diazoacetate (alkylating agent) and an isocyanic acid (carbamoylating agent) (101) (Fig. 2.5).

2.6.3 Biological Activity and Side Effects. Streptozotocin has been used as a component of multidrug protocols for Hodgkin's disease (102), and for pancreatic (103) and colorectal (104) carcinomas, where some responses were seen, but the drug is not now widely used. A recent report (105) notes activity of a streptozocin/o,p′-DDD combination in adrenocortical cancer.

2.7 Triazenes

2.7.1 History. Dacarbazine (32; DTIC) came from studies by Shealy and co-workers, and has been well reviewed (106, 107). Mitozolomide (33) was developed by Stevens and co-workers as a potential prodrug of linear triazenes such as [5-(3-3-dimethyl-1-triazenyl)imidazole-4-carboxamide] (DTIC) (108). The same workers later followed up with the development of the related temozolomide (34), which lacks the 2-chloroethyl group (109).



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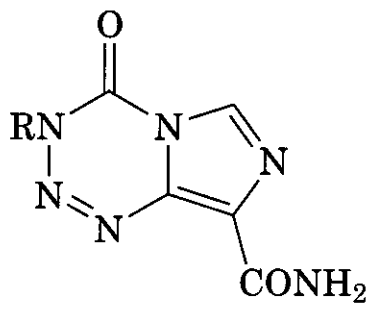


(33) R = CH_2CH_2Cl
(34) R = CH_3

2.7.2 Mechanism and SAR. The cyclic triazenes undergo base-catalyzed ring opening, followed by spontaneous decarboxylation. Thus, temozolomide (34) forms the open-chain triazene [5(3-methyl-l-triazenyl)imidazole-4-carboxamide] (MTIC), which then fragments to a methyldiazonium species, the DNA-methylating agent (Fig. 2.6) (110). Temozolomide alkylates DNA primarily at the

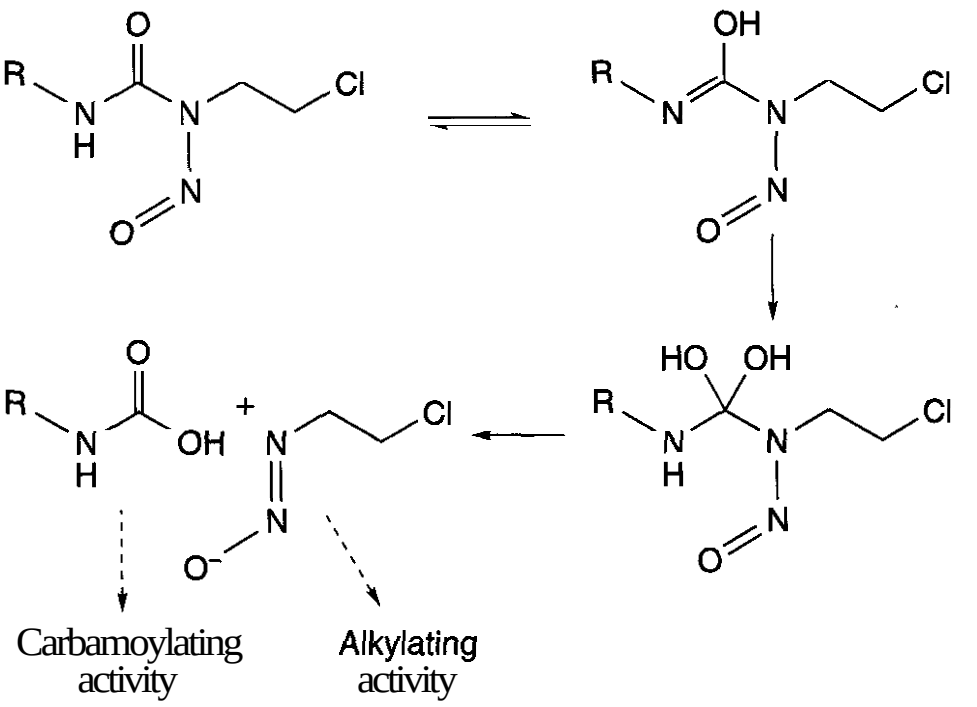


Figure 2.5. Mechanism of action of nitrosoureas.

N7 of guanine, but 0 6 guanine alkylation also occurs. The rate of conversion to the alkylating species is not influenced by the presence of DNA, suggesting no or very weak binding of the prodrug (110). As with mitozolomide, cytotoxicity correlates with the alkylation of the O6-position of guanine (111). L1210 cells treated with mitozolomide form DNA interstrand cross-links, presumably through the 2-chloroethyldiazo metabolite, suggesting this is a major mechanism of cytotoxicity (112). Mitozolomide preferentially alkylates DNA at guanines in runs of guanines, forming 7-hydroxyethyl and 7-chloroethyl adducts (113).

2.7.3 Biological Activity and Side Effects. Dacarbazine has been widely used for many years, and in particular, has been the cornerstone of drug therapy for malignant melanoma (114, 115). It is metabolized by *N*-hydroxylation, followed by N-demethylation, to give a monomethyltriazene that then methylates DNA (116). No surprisingly, dacarbazine is strongly carcinogenic in animal models (117), suggesting it may also be a human carcinogen.

Mitozolomide proved curative against a broad range of murine tumor models in *vivo* (118)and showed very pronounced antitumor effects in a range of human tumor xenografts (119). Cell lines with constitutive levels of 06-methylguanine-DNA methyl-transferase (Mer+ phenotype) were less sensitive to the cytotoxic effects of mitozolomide, consistent with alkylation of the O6-position of guanine being the cytotoxic event (111). In 1998, mito-

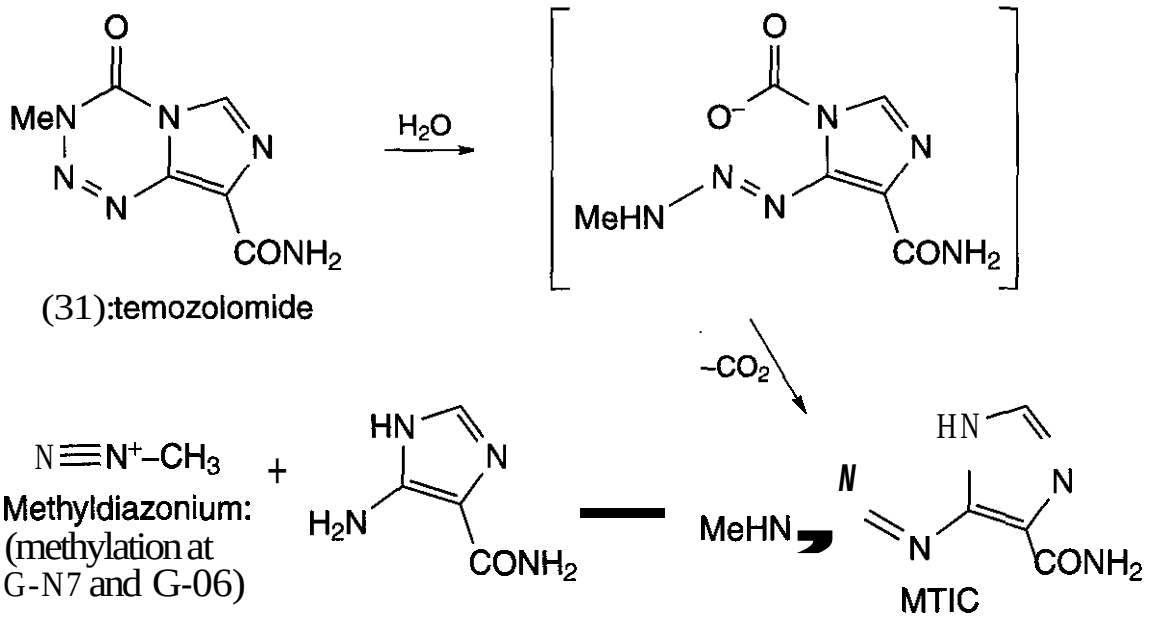


Figure 2.6. Mechanism of activation of temozolomide.

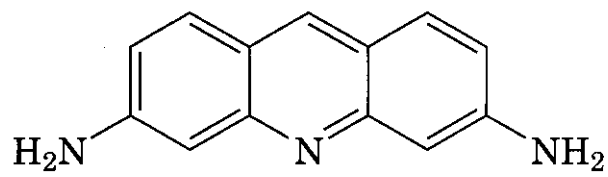
zolomide entered phase I clinical trials (120), but despite demonstrable activity in small-cell lung cancer (SCLC) and melanoma (121), unpredictable myelosuppression precluded further development (121,122). However, recent work in the successful transduction of human hematopoietic progenitor cells with variants of this enzyme has led to suggestions that this could be used clinically to protect against myelosuppression, to allow safer use of agents like mitozolomide and temozolomide in conjunction with 06-benzylguanine (123, 124).

Temozolomide also demonstrated good in *vivo* activity against a variety of mouse tumor models, including the TLX5 lymphoma (125), and excellent antitumor activity, including cures, on oral administration to athymic mice bearing both subcutaneous and intracerebral human brain tumor xenografts (126). Many later studies confirmed the good activity in brain tumor models, and this, together with the lesser myelosupression seen in toxicology screens, led to phase I trials (127). Trials in radiotherapy-resistant astrocytomas confirmed animal data suggesting that temozolomide efficiently passes the blood-brain barrier (128). Recent reviews show that oral temozolomide has almost 100% bioavailability, acceptable non-cumulative myelosuppression, and is clinically useful in the treatment of gliomas (129, 130) and brain metastases in advanced melanoma (131).

3 SYNTHETIC DNA-INTERCALATING TOPOISOMERASE INHIBITORS

3.1 Introduction

Intercalation as a mode of the reversible binding of ligands to DNA was first described by Lerman (132) for the acridine proflavine (35). Intercalation involves insertion of the chromophore between the base pairs, and is now understood to be the major DNA binding mode of virtually any flat polyaromatic ligand of sufficiently large surface area and suitable steric properties. Intercalative binding is driven primarily by stacking (charge-transfer and dipole-induced dipole) and electrostatic interactions, with entropy (dislodgement of ordered water around the DNA) of lesser and variable importance (133). A great deal of work has been done delineating the ligand structural properties that favor intercalation, the geometry, kinetics, and DNA sequence-selectivity of the binding process, and the effect of such binding on the structure of the DNA substrate (134). A very large number of compounds have been shown to be DNA intercalating agents, and many of these show cytotoxic activity. In the early 1980s, it was shown that these cytotoxic effects were primarily caused by the compounds forming ternary complexes with DNA and the enzyme topoisomerase II, altering the position of equilibrium and trapping a reaction intermediate termed the "cleavable complex" (135, 136). The DNA intercalators are now recognized collectively as a major class of topoisomerase poisons.



(35)

The topoisomerases are enzymes that regulate DNA topology by successive cleavage-religation reactions and are a major target for anticancer drugs. Topoisomerase (topo) II is a homodimeric protein, associated with the mitotic chromosome scaffold. It initially binds to DNA reversibly and then executes a series of concerted strand-breaking and religation processes to relieve torsional stresses generated during DNA replication (137). Whereas many types of agents interfere to some extent with the normal function of topo II, DNA-intercalating agents in particular have the ability to cause lethal DNA double-strand breaks. The observation of these breaks, characterized by protein covalently attached to the 5'-ends, first led to suggestions (135) that a topoisomerase enzyme was involved. It is now clear that the primary mode of cytotoxicity of most DNA intercalating agents involves inhibition of the religation step of the action of the enzyme mammalian DNA topo II (138,139). The topo II enzyme has major isozymes coded by two separate genes (140). The IIα isozyme (170 kDa) maps (141,142) to chromosome 17, is the regulated during the cell cycle, and is the target of virtually all of the DNA intercalators.

Table 2.2 Synthetic Topoisomerase (*topo*) Inhibitors Used in Cancer Chemotherapy

Generic Name (Structure)	Trade Name	Originator	Chemical Class
Topo II inhibitors			
amsacrine (**39**)	Amsidyl	Warner-Lambert	9-anilinoacridine
asulacrine (40)		Sparta	9-anilinoacridine
mitoxantrone (**41**)	Novantrone	Wyeth	anthracenedione
BBR 2778 (43)		Boehringer	
losoxantrone (45)		Warner-Lambert	
piroxantrone (46)		Warner-Lambert	
Dual topo I/II inhibitors			
DACA (51)	XR-5000	Xenova	Acridine
intoplicine (52)		Ilex	Pyridoindole
TAS 103 (**53**)		Taisho	Indenoquinolone
DMP 840 (**58**)		Knoll	Bis(naphthalimide)

The IIβ isozyme (180 kDa) maps (143) to chromosome 3 and becomes the predominant isozyme in both non-cycling cells and in cells resistant to "classical" topo II agents (138, 143).

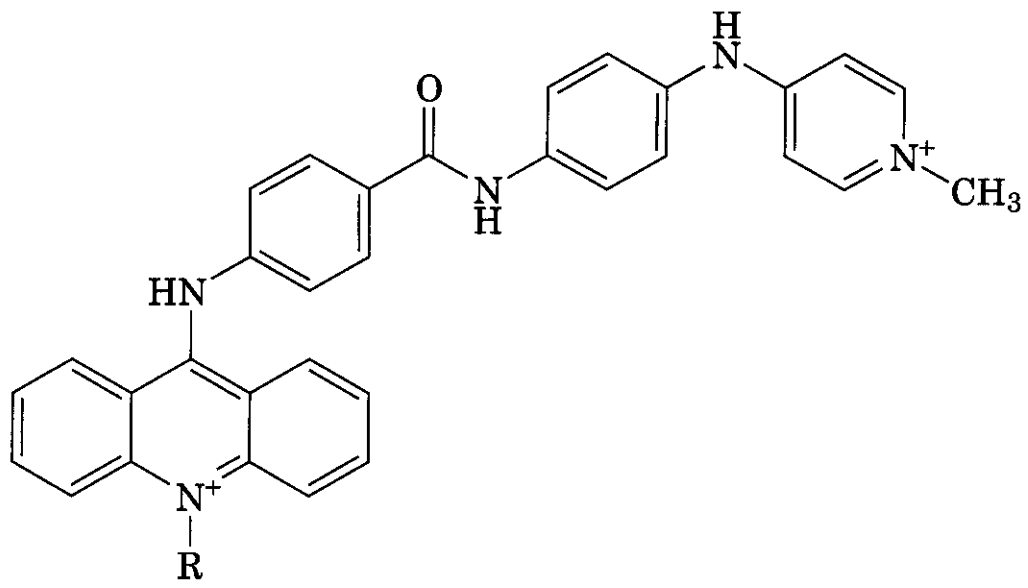
3.2 Clinical Use of Agents

The most commonly used synthetic topoisomerase inhibitors are listed in Table 2.2, together with some interesting new agents in early clinical development. A large number of synthetic DNA-intercalating agents have been developed, representing a broad range of chemistries. Many of these have been evaluated in early clinical trials, but relatively few have shown useful activity. Apart from synthetic analogs of natural products such as the anthracyclines (which will not be covered here), the major subclasses of clinically useful synthetic DNA intercalators are the acridines and the anthracenediones. In contrast, the most important topo I inhibitors are derived from the natural product camptothecin and will also not be covered here.

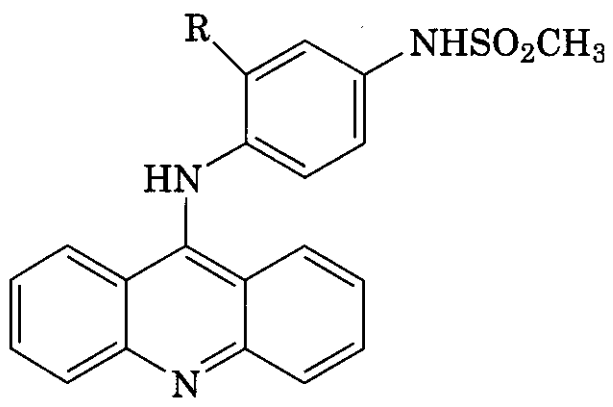
3.3 Topo II Inhibitors

3.3.1 History. The two main classes of synthetic topo II inhibitors are the 9-anilinoacridines and the anthracenediones. The 9-anilinoacridine amsacrine evolved from work carried out by Cain and associates on anti-leukemic quinolinium-type agents (144), which they suggested intercalated following initial minor groove binding of the remainder of the molecule (145). A series of acridinium analogs (e.g., 36) were more active (146), and later work showed that these compounds, with the larger chromophore, did intercalate DNA (147). The acridinium series proved unique in that unquarternized derivatives (e.g., 37) were also active, and a series of progressively simpler analogs led to the methanesulfonamide (38) (148), which had superior water-solubility, stability, and biological activity. Further work, on the basis of a theory that high electron density at the 6'-position was favored (149), showed that a 3'-methoxy group greatly increased potency, resulting in amsacrine (**39**; m-AMSA). Following detailed animal testing, the U.S. National Cancer Institute initiated clinical trials in 1974 (150). Encouraging results in both leukemias and lymphomas (150), with an apparent lack of cross-resistance to doxorubicin (151), resulted in amsacrine becoming the first synthetic DNA intercalator to show clinical efficacy (152).

The success of amsacrine led to a search for analogs with a broader spectrum of action. Because the high p*K*a (8.02) of amsacrine was thought to play a part in limiting its distribution, analogs with a lower p*K*a that still retained high DNA binding and had improved aqueous solubility were sought. QSAR studies (153) suggested that the anilino side chain was close to optimal, and focused attention on the 4- and 5-positions as being the most suitable for modification. Carboxamide substituents were seen as favourable for lowering p*K*a, and several 4- and 3-carboxamides, including the 4-methyl-5-methylcarboxamide (40; asula-



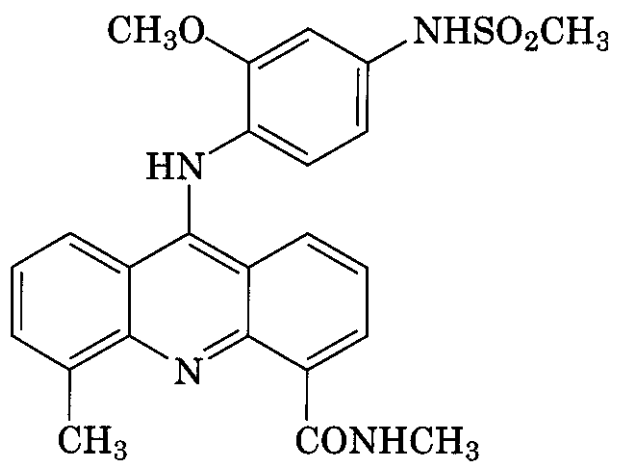
(36) R = CH_3
(37) R = H



(38) R = H
(39) R = OCH_3

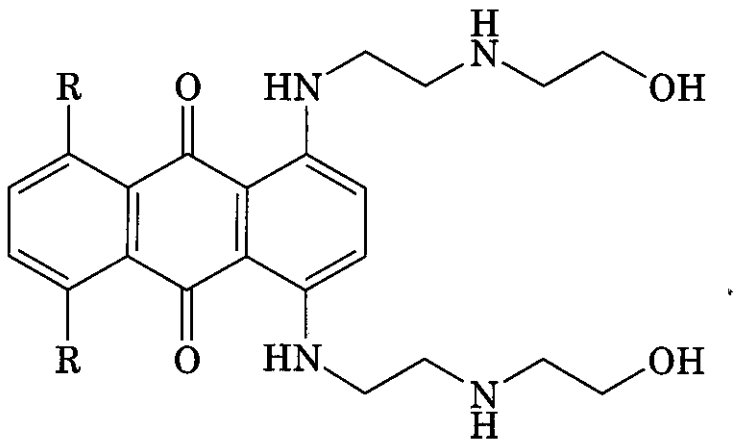
crine) were studied in detail (154). This proved the most active of a series evaluated for oral activity (155) was the best against both a human solid tumor cell-line panel (156) and a wide range of murine solid tumors in *vivo* (157), and was selected for clinical trial.

Mitoxantrone (41) was discovered through screening of industrial dye compounds (158).



(40)

Both it and the des-hydroxy analog amet-antrone (42) showed broad-spectrum activity in animal tumor models (159), and mitox-antrone has become probably the most widely used synthetic DNA intercalating agent.



(41) R = OH
(42) R = H

3.3.2 Mechanism of Action and SAR. Drugs aimed at topoisomerases can work in one or both of two ways; by inhibiting the ability of the enzyme to relax DNA by preventing its initial cleavage function or by preventing religation of the transient "cleavable complex" (stabilization of the cleavable complex), resultingin enhanced strand breaks. The second is the more cytotoxic process and is the mechanism by which the majority of topo inhibitors (or, more accurately, topo poisons) work. Much of the early structure-activity relationship (SAR) work on the DNA intercalator class of drugs focused around their interaction with DNA, delineating the requirements for suc-

cessful intercalation and tight binding (134). Early SAR studies for several classes of intercalators showed positive relationships between cytotoxic potency and strength of DNA binding (160, 161) and long residence times for the intercalators at individual DNA sites (162). However, the discovery that the primary mode of cytotoxicity of these compounds was inhibition of topo II through formation of a ternary drug/DNA/protein complex (138, 139) made it clear that drug design through modeling of DNA binding properties alone could be misleading.

Amsacrine binds to DNA by reversible, enthalpy-driven (163) intercalation of the acridine chromophore, with an association constant of $1.8 \times 10^5 M^{-1}$ for calf thymus DNA in 0.01 M salt (164). By analogy with the crystal structure determined for 9-aminoacridine binding to a dinucleotide (165), amsacrine was postulated to bind with the anilino ring lodged in the minor groove, with the 1′-substituent pointing tangentially away from the helix, and the possibility of it thus interacting with another (protein) macromolecule to form a ternary complex was noted (164). This conformation was also supported by energy calculations (133,166). In the ternary cleavable complexes, DNA intercalation of the acridine occurs, and the anilino side-chain seems to specifically interact with the enzyme as well (167,168).

Amsacrine causes comparable levels of cell killing in yeast transfected with either human topo IIα or IIβ, whereas etoposide, doxorubicin, and mitoxantrone produced higher degrees of cell killing with topo IIα (169). However, the sensitivity of a panel of human breast cancer cell lines to amsacrine was shown to correlate better with the level of expression of the topo IIα protein (although not with the level of topo IIα mRNA) (170), suggesting that the former is the most important mechanism of resistance to these topo II inhibitors. A human SCLC line (GLC4) with acquired resistance to amsacrine did not overexpress P-glycoprotein, and it had an 82% decrease in topoIIβ protein but no change in topoIIα protein level (171). A classification of antitumor drugs by their topo 11-induced DNA cleavage activity and sequence preference placed amsacrine in the class that enhanced the stabilization of cleavable complexes at a single major site, actingupstream of the DNA cleavage step through enhancement of cleavage (172). Amsacrine seems unique among topo II poisons in that its ability to trap both topo IIα– and topo IIβ–induced lesions is only modestly reduced in ATP-depleted cells; it is suggested that amsacrine produces mainly prestrand passage DNA lesions, whereas other topo II poisons only stabilize poststrand passage DNA lesions in intact cells (173). Studies with amsacrine and other topo II poisons in HeLa (174) and AHH-1 human lymphoblastoid (175) cells suggest that these compounds can induce cell death by apoptosis. However, whereas amsacrine induced apoptosis in wild-type SCLC cells, it did not do so in an amsacrine/camptothecin-resistant subline, and no significant difference in the expression of several genes (*c-myc, bc12,* c–jun, *p53*) involved in the apoptotic process was seen in either the parental and resistant cells after drug treatment. These data suggest that modulation in the apoptotic pathway could be an additional mechanism of resistance to amsacrine and other topo II agents (176).

Mitoxantrone also binds reversibly to DNA by intercalation (177), with an unwinding angle of 23°, probably with the chromophore inserted perpendicular to the base-pair axis with the side-chains lying in the major groove (178), although this has not been rigorously proven. Footprinting studies show the preferred intercalation site for mitoxantrone to be 5′-(A/T)CG or 5′-(A/T)CA sites (179). Mitoxantrone and the related ametantrone (42) bind tightly and about equally well to DNA with association constants of about $5 \times 10^6 M^{-1}$ at physiological salt concentrations (180), but mitoxantrone has about fourfold slower dissociation kinetics (177,181). The higher cytotoxicity of mitoxantrone compared with ametantrone correlated with its higher capacity to induce topo 11-mediated cleavable complexes, suggested because of greater stability of the ternary complex (182). However, whereas mitoxantrone showed a similar capacity to amsacrine at inducing cleavable complexes, it is considerably more potent and able to induce much more long-lived blocks at the G2 stage of the cell cycle (183). It induces DNA fragmentation and activates caspases, demonstrating that the ultimate cytotoxic effect is

induction of apoptosis (184). Mitoxantrone is readily oxidized (for example by human myeloperoxidase) to metabolites that covalently bind to DNA (185). It also, like the anthracyclines, forms formaldehyde-induced adducts that function as virtual interstrand cross-links (186). Both of these properties may be relevant to its biological activity. However, as with many other intercalators, mitoxantrone's cytotoxicity is caused largely by inhibition of topo II.

3.3.3 Biological Activity and Side Effects. The clinical use of amsacrine is mainly in acute myeloid leukemia. Amsacrine/etoposide therapy with or without azacitidine in relapsed childhood acute myeloid leukemia was effective (34% complete responses), with azacytidine not improving response rates (187). Recent successful use in various adult leukemias has also been reported (188–190). Amsacrine has generally not been successful in the treatment of solid tumors, except for some responses in head-and-neck cancer, where high-dose amsacrine was a toxic but very effective drug for first-line treatment (191), with a response rate of 65%. Whereas much less cardiotoxic than the anthracyclines, pre-exposure to amsacrine is a risk factor for cardiotoxicity after anthracycline treatment for childhood cancer (192).

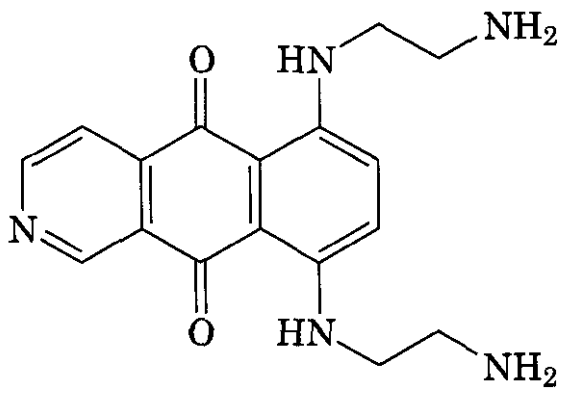
Asulacrine showed a similar mechanism of action to amsacrine, generating DNA protein cross-links and DNA breaks through inhibition of topo II (193, 194). In initial phase II trials, some drug-induced remissions were seen in non-small-cell lung cancer and breast cancer but not in colorectal and gastric cancer (195, 196). A pilot study of a trial of oral administration has been reported (197), but there are no reports of asulacrine being used clinically in combination therapy.

Mitoxantrone is used in first-line therapy for acute myelocytic leukemia (AML) (198), and along with cytosine arabinoside, is suggested as salvage therapy in AML and chronic myelocytic leukemia (CML) (199). In combination with a steroid, it is the drug of choice for palliative treatment of hormone-resistant prostate cancer (200). It is also an effective treatment for secondary progressive multiple sclerosis, but the duration of treatment is limited by cumulative cardiotoxicity (201). Again, whereas less cardiotoxic than the anthracyclines, mitoxantrone has been shown to have cumulative cardiotoxic effects (202). Mitoxantrone is genotoxic in the *in* vitro micronucleus test and in mutation assays (203) and has been reported to induce secondary cancers after use in the treatment of breast cancer (204). Resistance to mitoxantrone can develop in a number of ways: by lower expression of topo II (205), by expression of a topo II with altered DNA cleavage activity (206), by decreased drug uptake even in the absence of elevated levels of P-glycoprotein (207), and by inherent resistance to the induction of apoptosis (208). Many cells develop multifactorial resistance to mitoxantrone (209).

Topoisomerase inhibitors are also known to be tumorigenic, related to the formation of multiple DNA strand breaks. A frequent chromosomal translocation is at 11q23, where the myeloid-lymphoid leukemia (MLL) gene is located (28), but other translocations are also seen. The onset of induction of AML is shorter than with alkylating agents, with the average around 2 years and an incidence of 2–12% (210). Anthracyclines, mitoxantrone, and epipodophyllotoxins have all been shown to induce AML (211).

3.3.4 Recent Developments: Compounds with Lower Cardiotoxicity. The development of analogs of mitoxantrone has been driven largely by the requirement for lower cardiotoxicity. Two broad classes of analogs can be distinguished, and much work has been done on both. The first are close analogs of mitoxantrone, where the tricyclic chromophore has been maintained and variations occur in the side-chains or the chromophore atoms. In a study of aza analogs, Krapcho and co-workers found that the positioning of the aza group was critical, with the 2-aza derivative (43; BBR 2778) being the most potent (212). This bound less tightly to DNA but induced topo 11–mediated DNA cleavage (213). Preclinical studies showed (43) has a better therapeutic index and lower cardiotoxicity than mitoxantrone (214), and a phase I trial has been reported (215).

The second broad class are tetracyclic compounds, primarily the imidazoacridinonesand



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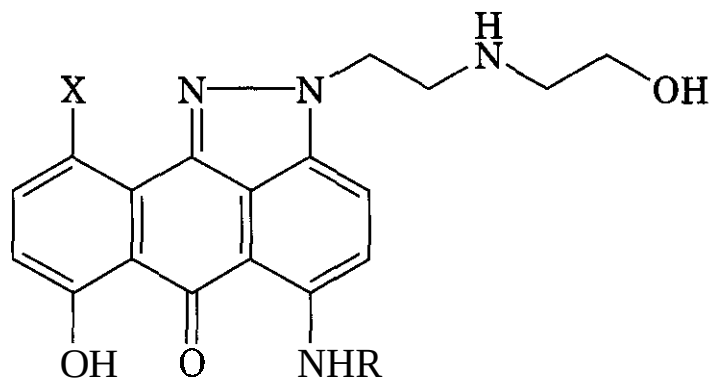
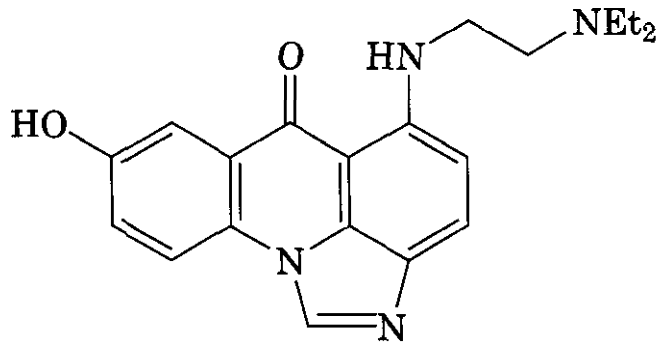


(44) X = OH, R = $(CH_2)_2NHCH_3$
(45) X = H, R = $(CH_2)_2NH(CH_2)_2OH$
(46) X = OH, R = $(CH_2)_3NH_2$

the anthrapyrazoles. Showalter and colleagues at Parke-Davis, in search of less cardiotoxic agents, developed these initially. They laid down the basic SAR, showing that activity was maximal with alkylamino side-chains at the N-2 and C-5 positions with two to three carbon spacers between proximal and distal nitrogens and showed they induced less oxygen consumption than doxorubicin in the rat liver microsomal system (216). These compounds bind very tightly to DNA by intercalation, with association constants around $2 \times 10^8 M^{-1}$ (217). They were highly active in murine leukemias and a range of human tumor xenografts (218), and three (44–46) were selected for preclinical evaluation (219). This early judgment was vindicated by the fact that all three of these later went forward to clinical trial. Teloxantrone (44) did receive a clinical trial (220) but has not been further reported on. However, losoxantrone (45) and piroxantrone (46) have been more widely studied. Piroxantrone (46) showed some responses in phase I trials (221), but this was not borne out in phase II trials (222,223). Losoxantrone (45) showed classical topo II inhibition (224), and a number of phase I trials were conducted in which the dose limiting toxicity was leucopenia; some non-cumulative cardiotoxicity was also seen (225). The major metabolites detected in humans resulted from oxidation of the hydroxymethylene side-chains to either mono- or dicarboxylic acid derivatives (226, 227). A phase II trial in hormone-refractory metastatic prostate cancer showed improvement of clinical symptoms in one-third of patients (228), and the drug is reported to be currently in phase III development (227).

Structure-activity studies on imidazoacridinones (229) identified (47) (C-1311) as a potential anticancer drug that intercalates DNA (230), inhibits the catalytic activity of topo II, and has broad-spectrum solid tumor activity (231). It is reported to be in a phase I clinical trial (232).

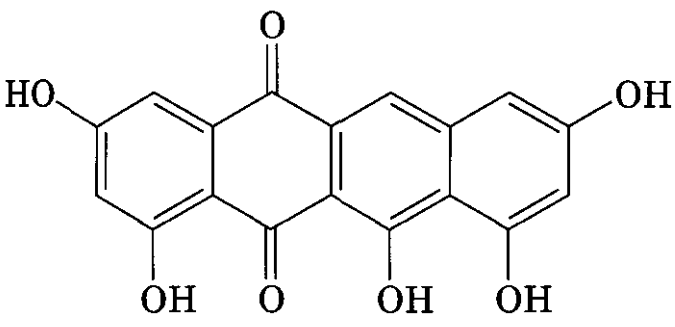


(47)

3.4 Dual Topo I/II Inhibitors

3.4.1 History. The topo I and topo II enzymes are expressed at different absolute levels in different cell types. Topo II levels are reported to be high in many breast and ovarian lines (233), whereas topo I levels are reported to be high in many colon cancer lines (234); the good clinical activity of camptothecin analogs against colon tumors has been suggested because in part of this high level of topo I expression (235). The time-course of expression of topo I and topo II also differs markedly, with topo II levels at their highest during S-phase, whereas levels of topo I remain relatively constant through the cell cycle (236). Because expression of either enzyme seems to be sufficient to support cell division, the development of resistance to topo I inhibitors is often accompanied by a concomitant rise in the level of topo II and vice versa (237,238).

Thus, one of the recent interests in topo inhibitors has been in agents capable of simultaneous inhibition of both enzymes, although relatively few compounds have been reported as dual topo 1/11 inhibitors (239). The anthraquinone saintopin (48) is a potent poison of both topo I and topo II (240) but has not been developed as a drug. The quaternary alkaloid nitidine (49)is reported (241) to be a dual poison, although more active against topo I. The related quaternary salt NK 109 (50) is described as a topo II poison, but etoposide-resistant lines with reduced topo II levels are still sensitive (242), suggesting a dual activity. Most work has been focused on the DNA intercalators DACA (51; XR-5000), intoplicine (52), and TAS 103 (53).



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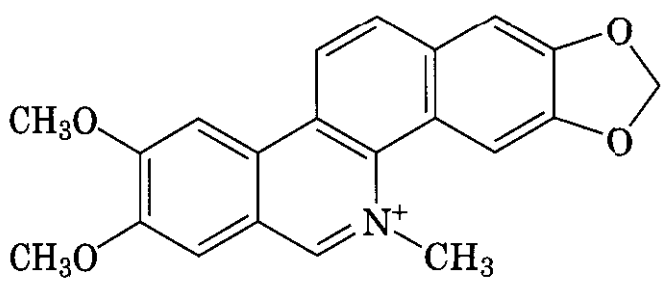

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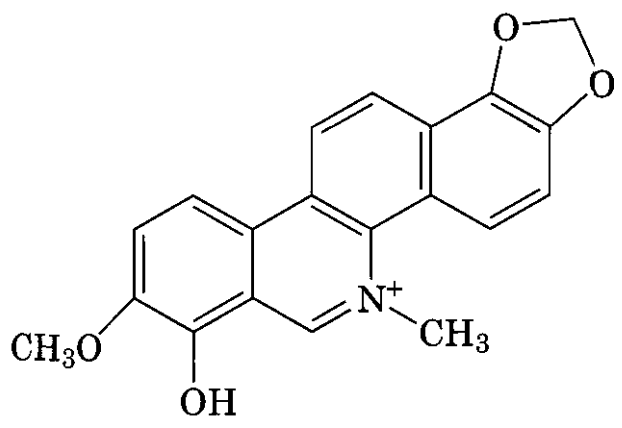

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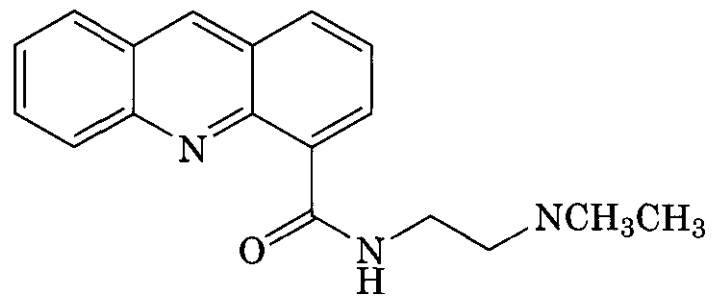

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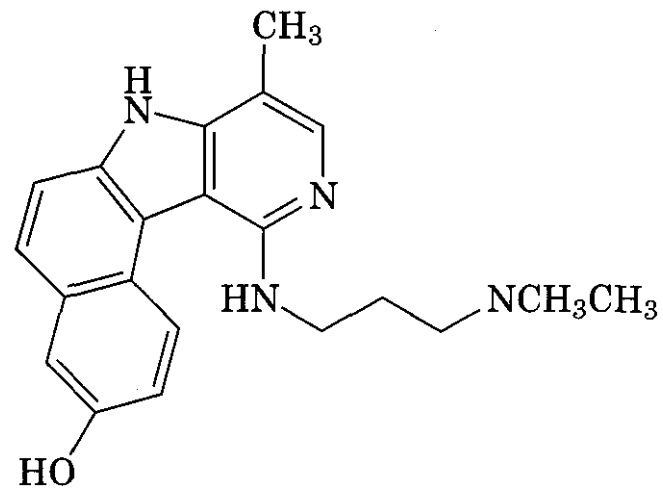

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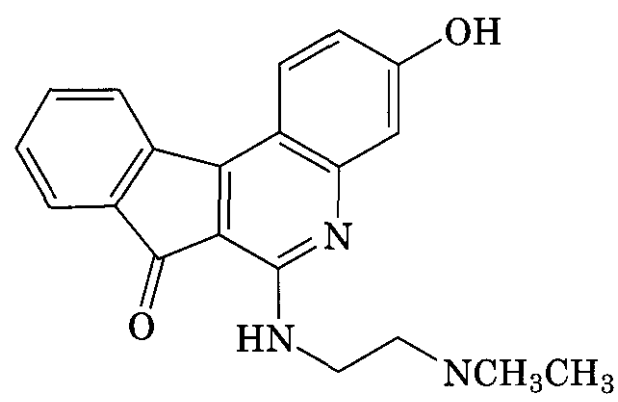


(53)

The 9-aminoacridine-4-carboxamides were first reported in 1984 as a new class of DNA-intercalating agents (243) with well-defined structure-activity relationships for both chromophore and side-chain (244). The derived acridine-4-carboxamide analog (51) (DACA) also binds to DNA by intercalation and induces DNA cleavage in the presence of either topo I or topo II enzymes, being unaffected by either P-glycoprotein–mediated multidrug resistance or "atypical" multidrug resistant caused by low topo II activity (245). DACA showed remarkable activity against multidrug resistant cells (246) and in *vivo* activity against the Lewis lung carcinoma (247), leading to clinical evaluation.

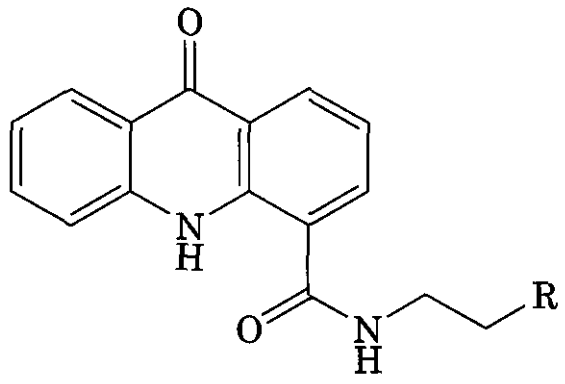
The DNA-intercalating (248) pyridoindole

intoplicine (52) is reported to also be a dual topo I/II poison (248, 249). Analogs of intoplicine that were only topo I or topo II poisons were less cytotoxic (248), suggesting the possible use of a dual poisoning ability. Intoplicine showed activity in a variety of human tumor explants in a soft agar cloning assay (250) and in transplantable mouse tumors in *vivo* (251). Phase I trials of intoplicine have been conducted (252,2531, but phase II trials have not been reported.

The indenoquinolone TAS-103 (53)is also reported to be a DNA-intercalating agent (254) and to enhance both topo I– and topo 11–mediated DNA cleavage in treated cells (255), but it is now considered that topo II is the primary cellular target (256). TAS-103 showed broad-spectrum activity against a number of cell lines, with no cross-resistance in cells with lower topo I expression and only slight cross-resistance in those where topo II was down-regulated (257). A phase I clinical trial of TAS 103 recommended a dose of 130–160 mg/m^2 for phase II trials (258), but these have not yet been reported.

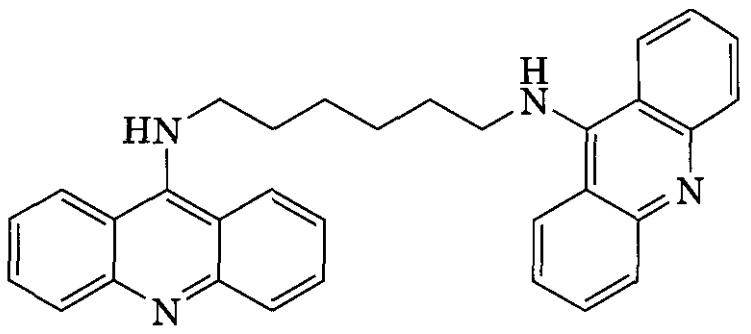
3.4.2 Mechanism and SAR. There seems to be no clear structural features predisposing to dual topo I/II activity. Raman and CD studies of intoplicine analogs suggest that the dual poisoning abilities of intoplicine are a result of its ability to simultaneously form two types of DNA complexes: a "deep intercalation mode" responsible for topo I–mediated cleavage and an "outside binding mode" responsible for topo 11–mediated cleavage (259).

3.4.3 Biological Activity and Side Effects. The primary route of metabolism of DACA is oxidation at C-9 by aldehyde oxidase to give the acridone (**54**), although oxidative demethylation of the side-chain dimethylamino group has also been observed (260). Pharmacological studies showed high binding to human al-acid glycoprotein, followed by albumin (261). In phase I clinical trials, the major urinary metabolite was the N-oxide (**54**), whereas the major plasma metabolites (262) were (54) and (**55**). The maximum tolerated dose in initial phase I trials was 750 mg/m^2 using a 3-h infusion, with the dose-limiting toxicity being arm pain of unknown cause at the infusion site (263) (avoidable using a 5-day infusion). Phase I trial reports for intoplicine noted hepatotoxicity rather than myelosuppression as the major dose-limiting toxicity (264).



(54) R = $N(O)CH_3CH_3$
(55) R = NCH_3CH_3

3.4.4 Recent Developments: bis Analogs as Dual Topo I/II Inhibitors. Because of the early SAR suggesting a positive correlation between cytotoxic potency and the strength of DNA binding and because bis-intercalation would theoretically greatly increase DNA binding, many dimeric compounds designed as bis-intercalators were evaluated as anticancer drugs (134, 265). However, the biological activities of these compounds were generally disappointing. The *bis(acridine)* (56) was considered for clinical trial (266) but had significant



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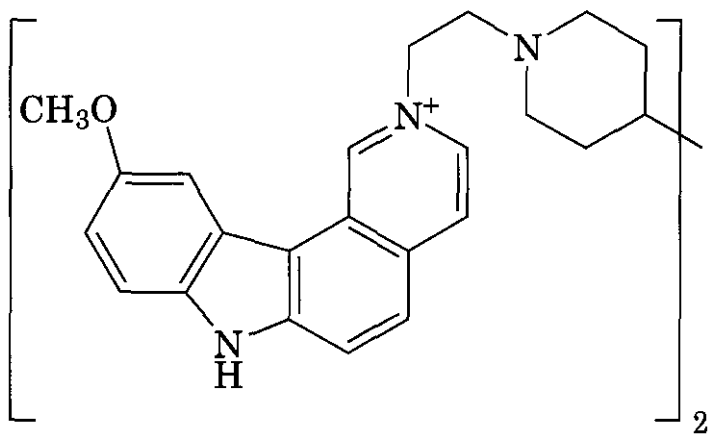
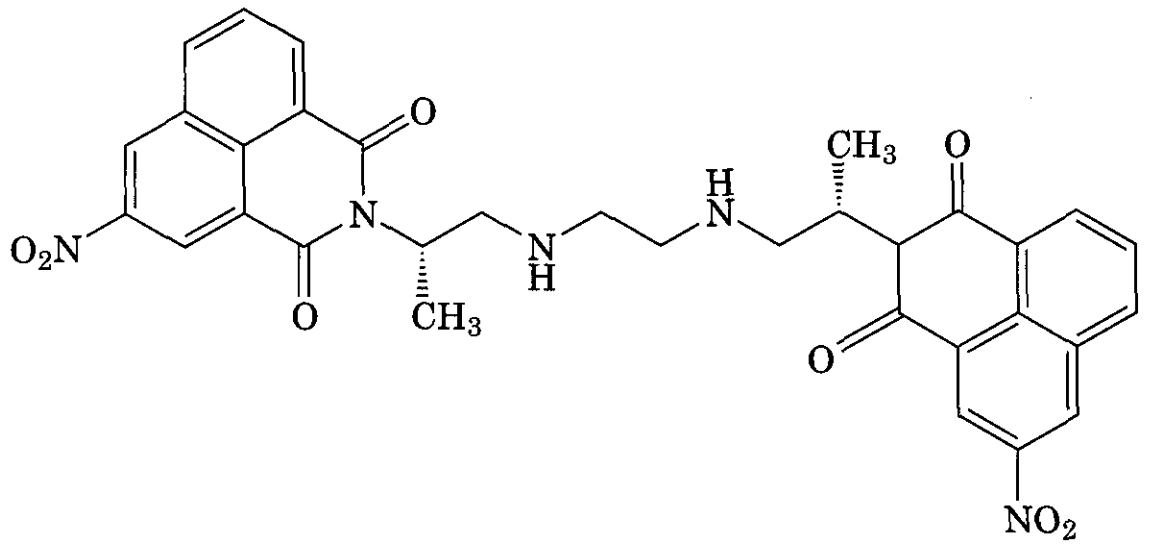


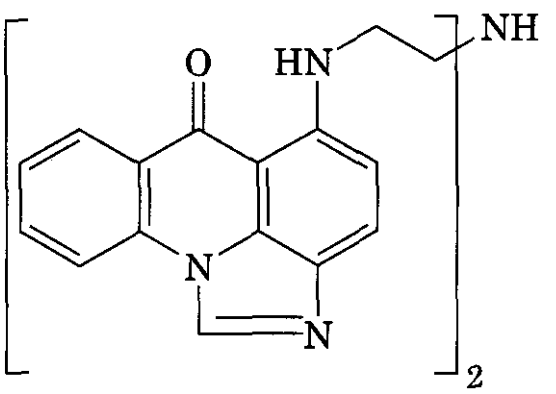
(57)



(58)

CNS toxicity, and the bis(ellipticine) analog ditercalinium (57) had unacceptable mitochondrial toxicity (267).

More recently, several series of dimers of more lipophilic chromophores have shown potent and broad-spectrum activity against a variety of human solid tumor cell lines, both in culture and as xenografts in nude mice. The bis(naphthalimide) analog DMP 840 (58)was curative in a variety of human solid tumor xenografts in nude mice (268). A series of bis(imidazoacridinones) (e.g., 59; WMC-26) showed highly selective cytotoxicity towards human colon carcinoma cells both in culture and in xenografts, although it seems that it is not a bis-intercalating agent (269). Several series of bis analogs of tri- and polycyclic carboxamides, including acridines (270) (e.g., **60**), phenazines (271) (e.g., **61**), and indenoquinolines (272) (e.g., **62**), are also potent cytotoxic agents and dual topo 1/11inhibitors. SAR studies of these compounds (270–273) show that both chromophore substitution and linker chain variations can significantly affect potency. The dicationic bis(phenazine) (63; XR5944) is of particular interest, with subnanomolar potency in a range of human cell lines (274) and active in multidrug-resistant cell lines in vitro and in *vivo* (275).

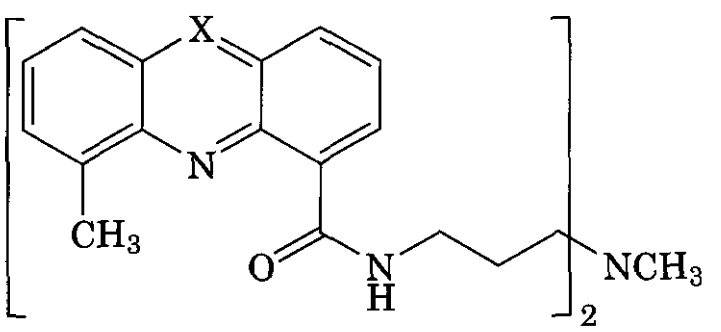


(59)

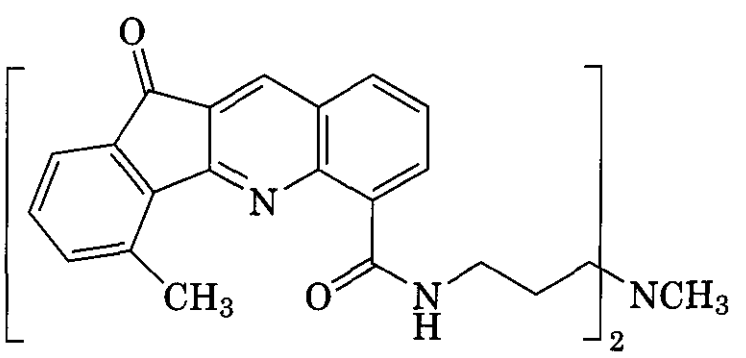
4 ANTIMETABOLITES

4.1 Introduction

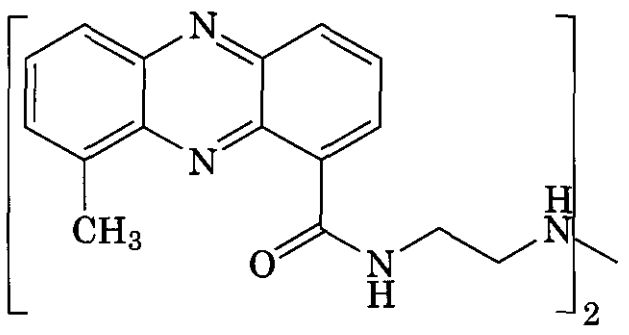
The class of compounds known broadly as antimetabolites interfere in varying ways with the synthesis of **DNA.** Along with the alkylating agents, antimetabolites such as methotrexate (**65**), Bfluorouracil (**73**), cytosine arabinoside (**74**), and 6-mercaptopurine (**76**)



(60) X = CH
(61) X = N



(62)



(63)

were some of the earliest drugs used in cancer chemotherapy.

4.2 Clinical Use of Agents

The most commonly used antimetabolites are listed in Table 2.3. These compounds are invariably used in combination with other agents in multidrug therapy regimens.

4.3 Antifolates

4.3.1 History. Antifolates interfere at various points in the process (folic acid metabolism) that provides the one-carbon unit required to convert deoxyuridine monophosphate to thymidylic acid for synthesis of the pyrimidines (Fig. 2.7). They are also key intermediates in the glycinanide ribonucleotide (GAR)-formyltransferase- and aminoimidazole carboxamide ribonucleotide (AICR)-formyltransferase-mediated construction of the purines (276). The first antifolate used clinically was aminopterin (64) and was rapidly followed by methotrexate (65), which was registered for clinical use in 1953. These "classical" (glutamate-containing) antifolates bind tightly to the enzyme dihydrofolate reductase (DHFR; Fig. 2.7). Methotrexate has been very widely used and has been extensively reviewed (277,278). A more recent classical antifolate is the 10-ethyl analog edatrexate (66). This was developed following observations that 1-alkyl analogs showed better relative uptake into tumor tissue, and edatrexate shows enhanced uptake, retention, and polyglutamate formation in tumor cells (279). Whereas edatrexate binds to DHFR similarly to methotrexate, it showed better activity in animal tumor models (280), including models resistant to methotrexate (281). Resistance to methotrexate arises in several ways, the most important of which are elevation of DHFR levels and lowering of both folate transport and polyglutamylation activities (282).

The enzyme thymidylate synthase (TS) is also intimately involved in folate metabolism, catalyzing the reductive methylation of deoxyuridine monophosphate (dUMP) to thymidylate (dTMP), a reaction in which N^5,N^{10}-methylenetetrahydrofolate is a cofactor (Fig. 2.7). Whereas the pyrimidine-binding site on

Table 2.3 **Antimetabolites Used** in **Cancer Chemotherapy**

Generic Name (Structure)	Trade Name	Originator	Chemical Class
Folic acid analogs			
methotrexate (**65**)			folate analog
edatrexate (**66**)			folate analog
raltitrexed (**68**)	Tomudex	Lilly	
permetrexed (**69**)			
trimetrexate (**70**)	NeuTrexin	US BioScience	
piritrexim (71)		Burroughs-Wellcome	
nolatrexted (**72**)	Thymitaq	Zarix	
Pyrimidine analogs			
5-fluorouracil (**73**)	Adrucil	Roche	pyrimidine
cytosine arabinoside (**74**)	Cytosar	Pharmacia & Upjohn	pyrimidine
gemcitabine (75)	Gemzar	Lilly	pyrimidine
Purine analogs			
6-mercaptopurine (**76**)	Purinethol	Burroughs-Wellcome	
6-thioguanine (**77**)	Lanvis	Glaxo-Wellcome	purine
fludarabine (**78**)	Fludara	Berlex Laboratories	purine
2'-deoxycoformycin (79)	Pentostatin	Supergen Inc	purine analog
2-chloro-2′deoxyadenosine (**80**)	Cladribine	Bedford Laboratories	purine

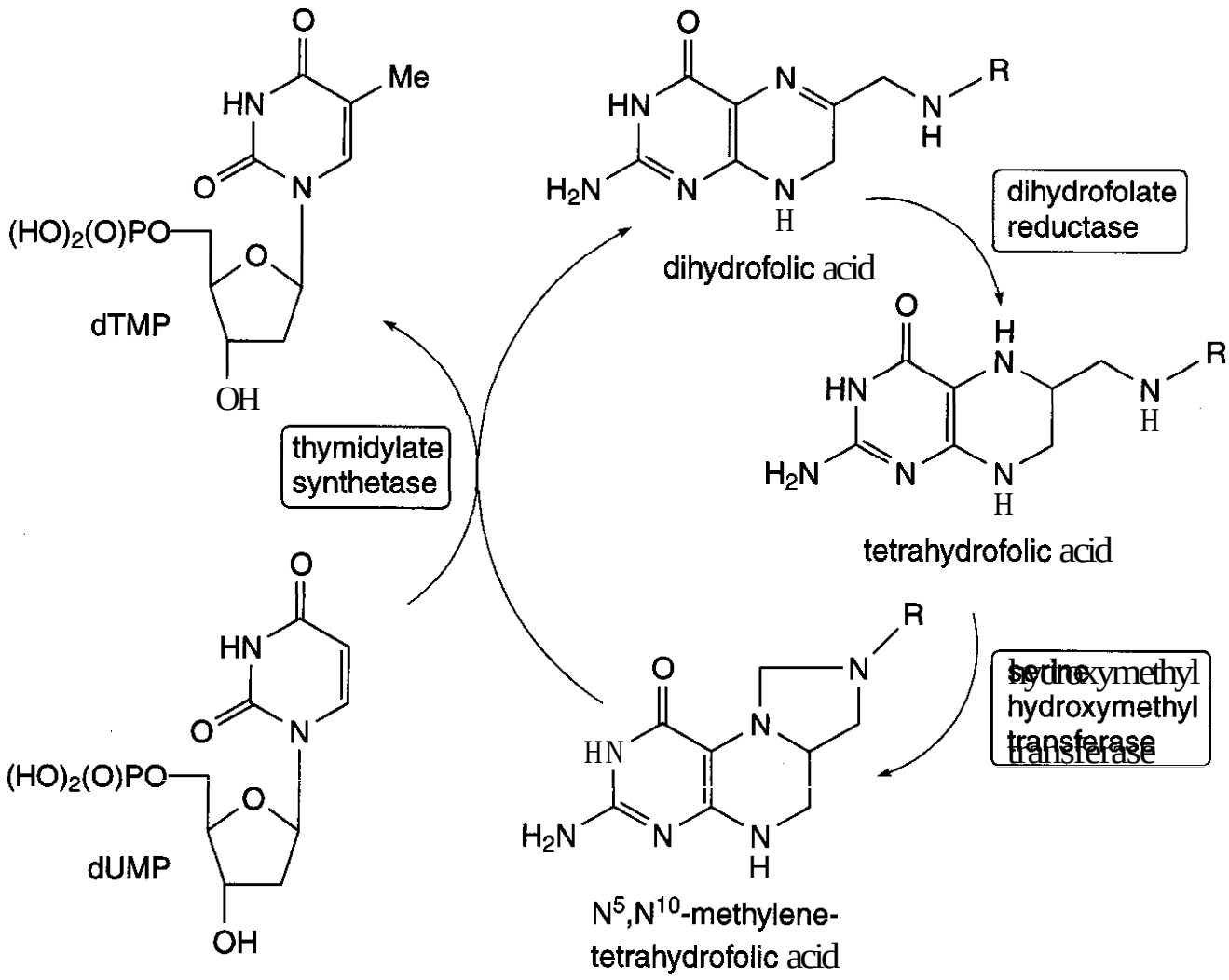
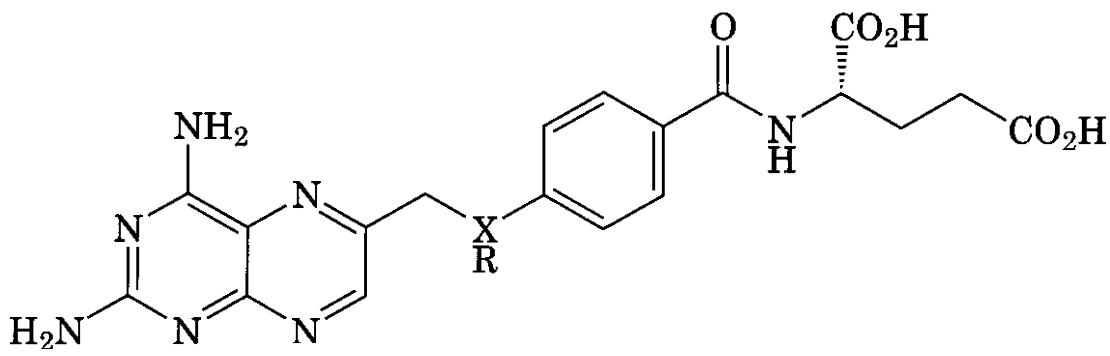


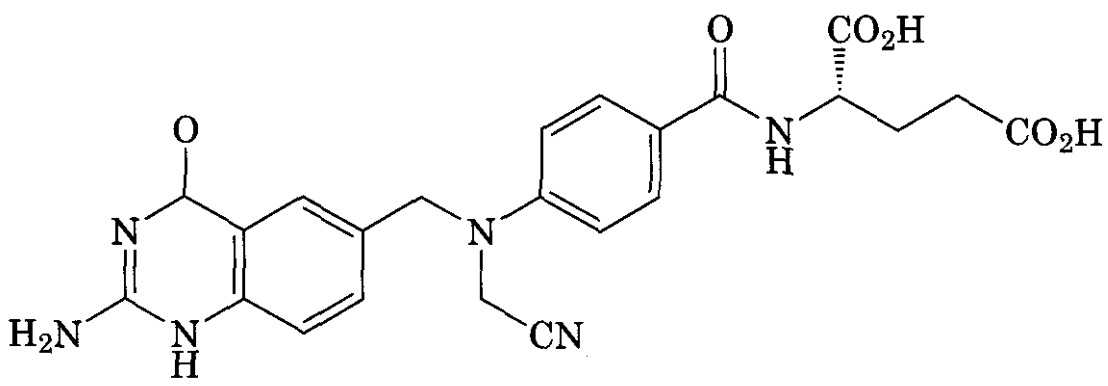
Figure 2.7. Folate biosynthesis.



(64) R = H, X = N
(65) R = CH_3, X = N
(66) R = Et, X = CH

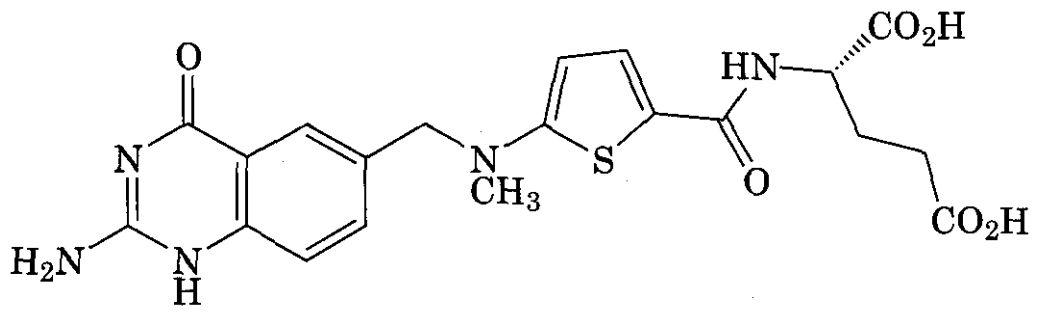
the Ts enzyme has been a major target for anticancer drugs such as **5-fluorouracil** (see Section 4.4), it also has a folate-binding site that has been a target for drug development. Methotrexate itself binds weakly to this site, and can exercise **cytotoxicity** through TS inhibition in cells that highly overexpress DHFR (283). The design of highly specific inhibitors of the folate binding site of TS led initially to the quinazoline derivative CB 3717 (67) (284, 285). This proved to be a tight-binding inhibitor of TS (K_i 4.5 n*M*), with 10-fold selectivity over DHFR, with the ability to undergo polyglutamylation in cells to metabolites that **are** more potent and more selective for TS over DHFR (286). CB 3717 showed some activity in a number of phase 1/11 clinical trials, but severe **nephrotoxicity**, caused probably by precipitation of drug in the **kidneys** (287), led to its withdrawal (288).

Raltitrexed (68; **tomudex**) is another "classical" folic acid derivative that exerts its **ther-**



(67)

apeutic effect through by inhibition of the folate site of TS (289). It is polyglutamylated in cells into metabolites that are more potent inhibitors of TS than the parent drug and are retained in cells. Raltitrexed showed activity in a number of tumor types in phase I/II trials, but a major use may be in colon cancer. Here it shows activity similar to 5-fluorouracil (response rates of 14–19%)but with lesser toxicity (290), although the results of a recent phase II/III trial question this (291).

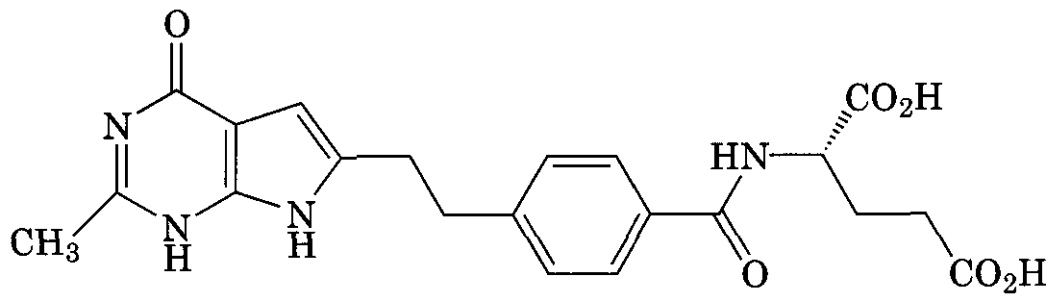


(68)

Permetrexed (69; MTA) also has TS as a major target, with DHFR and glycinamide ribonucleotide formyltransferase (GARFT) being important secondary sites of action (292). Permetrexed is an excellent substrate for FPGS, and it and its polyglutamylated metabolites are potent inhibitors for all of the above enzymes (293). Permetrexed performed well in the human tumor-cloning assay against colorectal (32% of cell lines inhibited) and non-small-cell lung cancer (25% of cell lines inhibited) (294). It showed broad antitumor activity in phase II trials with breast, colon, pancreatic, bladder, head-and-neck, *and* cervical carcinomas, and non-small-cell lung cancer, both as a single agent and in combination with agents such as gemcitabine and cisplatin, and it is in phase III evaluation (295, 296).

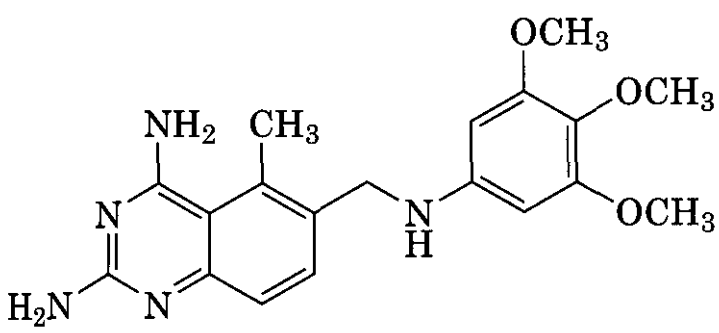
4.3.2 Mechanism and SAR. Methotrexate (**65**), introduced in 1953, and the related aminopterin (**64**) bind to DHFR, preventing transfer of the one-carbon unit from dihydrofolic acid to methylenetetrahydrofolic acid and ultimately to thymidine (Fig. 2.7). Methotrexate is taken into cells by the folate transporter and converted in cells to active polyglutamate metabolites by folylpolyglutamate synthase (297); this also has the effect of trapping the drug in cells (279). A large amount of work has been done to delineate the SAR for 2,4-diaminopteridines binding to DHFR, but no clinical successor to methotrexate as a DHFR inhibitor has yet been found among the "classical" antifolates (287), although edatrexate (66) is still in development. Because there is also a folate site on TS, these compounds have some level of binding to this as well. CB 3717 and raltitrexed were designed specifically to target TS rather than DHFR, whereas permetrexed is closer to a general folate pathway inhibitor.

4.3.3 Biological Activity and Side Effects. Methotrexate has broad-spectrum clinical activity and is still the most widely used antifolate, despite high myelosupressive activity and frequent development of resistance by various mechanisms. The newer antifolates have broadly similar toxicity profiles.



(69)

4.3.4 Recent Developments: Lipophilic Antifolates. These compounds were designed to circumvent resistance to methotrexate that arises by reduced folate uptake or reduced polyglutamylation. They are relatively lipophilic compounds, lacking a glutamate residue, that get into cells by passive diffusion. The first examples to receive clinical evaluation were trimetrexate (70) and piritrexim (71). Trimetrexate was superior to methotrexate in animal models, with activity in methotrexate-resistantlines (298)but (unlike methotrexate) is susceptible to P-glycoprotein–mediated multidrug resistance (299). Trimetrexate (70) has had extensive clinical trials and has shown activity in a number of tumors, including breast, non-small-cell lung, head-and-neck, and prostate (300), and particularly in colon cancer in conjunction with 5-fluorouracil/leucovorin (301). Piritrexim (71)was chosen for development from a range of lipid-soluble diaminoheterocyclic compounds on the basis of potent DHFR inhibition and minimal effects on histamine metabolism (302). Piritrexim is about 75% bioavailable when given orally (303), and in phase II trials showed some activity using oral dosing in bladder cancer (304). It is also more effective than methotrexate in severe psoriasis, because its lack of polyglutamylated metabolites makes it less hepatotoxic in long-term dosing (305).



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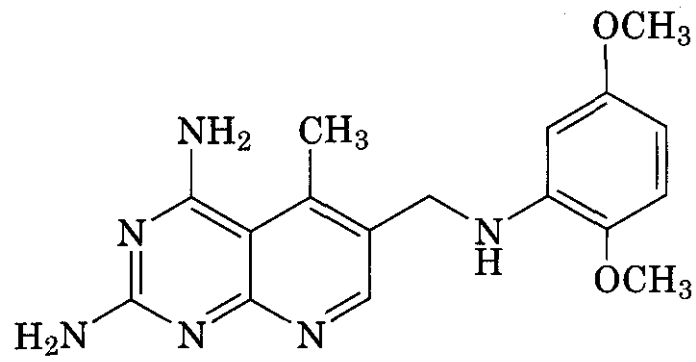
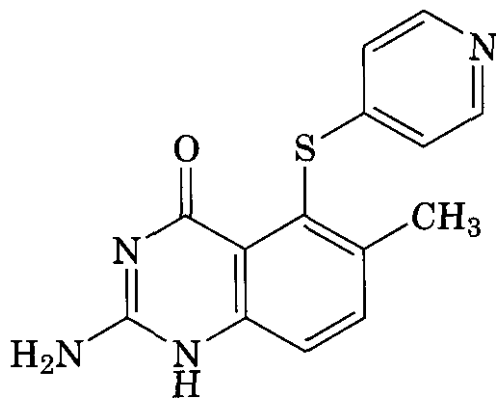


(71)

Nolatrexed (72; thymitaq) is a lipophilic folate analog designed as a TS inhibitor, using structure-based methods to maximize binding at the folate site (306). It is a potent (K_i 11 nM), non-competitive inhibitor of human TS, with modest growth-inhibitory effects (IC_{50}s 0.4–7 μM) against a wide variety of murine and human cell lines. Nolatrexed does not enter cells by the reduced folate carrier, is not polyglutamylated, and does not inhibit DHFR. The activity of the drug is abrogated by thymidine (but not hypoxanthine), and TS overexpressing cells are strongly resistant, demonstrating that the primary target is TS (307). Oral bioavailability in rats was 30–50%, and oral nolatrexed showed curative activity against both IP- and IM-implanted thymidine kinase-deficient murine L5178Y/TK-lymphomas (306). Combinations of nolatrexed and cisplatin showed synergistic activity in both 5-FU- and cisplatin-resistant ovariant and colon cancer cells (306). Modest effects were seen in phase II trials of nolatrexed in advanced hepatocellular carcinoma (307), and phase I combination studies with paclitaxel are ongoing (310).

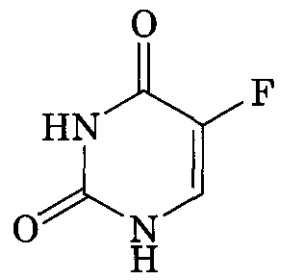
4.4 Pyrimidine Analogs

4.4.1 History. The pyrimidine analogs 5-fluorouracil (73) and cytosine arabinoside

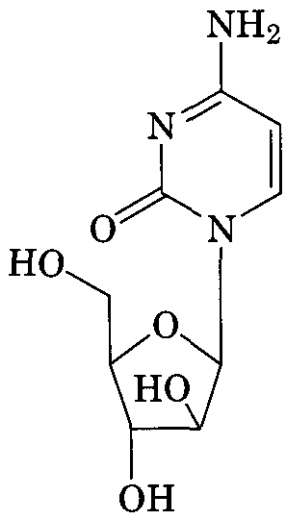


(72)

74; ara-C, cytosar) were developed from a knowledge of DNA metabolism (2) and were registered for clinical use in 1962 and 1969, respectively. A huge amount of work has gone into developing further analogs, and this has recently begun to pay off with the more recent introduction of gemcitabine (75; gemzar).

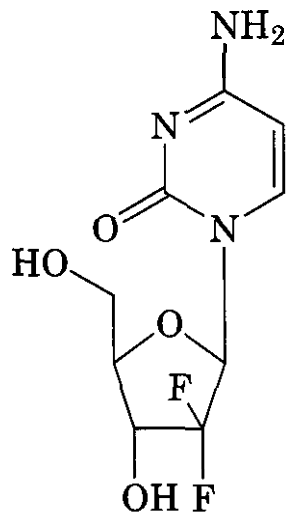


(73)



(74)

4.4.2 Mechanism and SAR. The mechanisms by which 5-fluorouracil (73) exerts its cytotoxicity have been extensively reported (311,312). It is converted in cells to the **monophosphate** 5-FdUMP, which binds initially reversibly at the **dUMP** site of the enzyme **thymidylate** synthetase (Fig. 2.7). This is followed by Michael-type attack of an SH group on the



(75)

enzyme to given an enolate-type intermediate, which reacts at the methylene moiety of N^5,N^{10}-methylenetetrahydrofolate to form a covalent drug-enzyme-cofactor ternary complex (Fig. 2.8). Because the fluorine cannot be displaced, as with the natural (**non-fluorinated**) substrates, this results in permanent poisoning of the enzyme with 1:1 stoichiometry. 5-Fluorouracil is also converted into the triphosphate **5-FdUTP**, which is incorporated into both RNA and DNA.

The mechanism of action of cytosine **arabinoside** (74) has been well reviewed (313). It acts primarily as a chain terminator during the elongation phase of DNA synthesis, incorporating into the growing chain and preventing the action of DNA polymerases **(314).** Gemcitabine also acts primarily as a chain terminator, but has additional effects, through rapid phosphorylation by deoxycytidine kinase to di- and tri-phosphate metabolites. The diphosphate inhibits ribonucleotide reductase (RR), the enzyme responsible for producing the deoxynucleotides required for DNA synthesis and repair, and the subsequent depletion of **cellular** deoxynucleotides favors **gemcitabine** triphosphate incorporation into DNA over the normal **dCTP,** in a "self-potentiating" mechanism (315). Incorporation of **gemcitabine** into the elongating DNA strand results in the halting of DNA polymerases after the addition of one more additional deoxynucleotide, in a "masked chain termination" event that seems to lock the drug into DNA, preventing proof-reading exonucleases from removing it. Gemcitabine is synergistic with cisplatin because of the triphosphate preventing chain

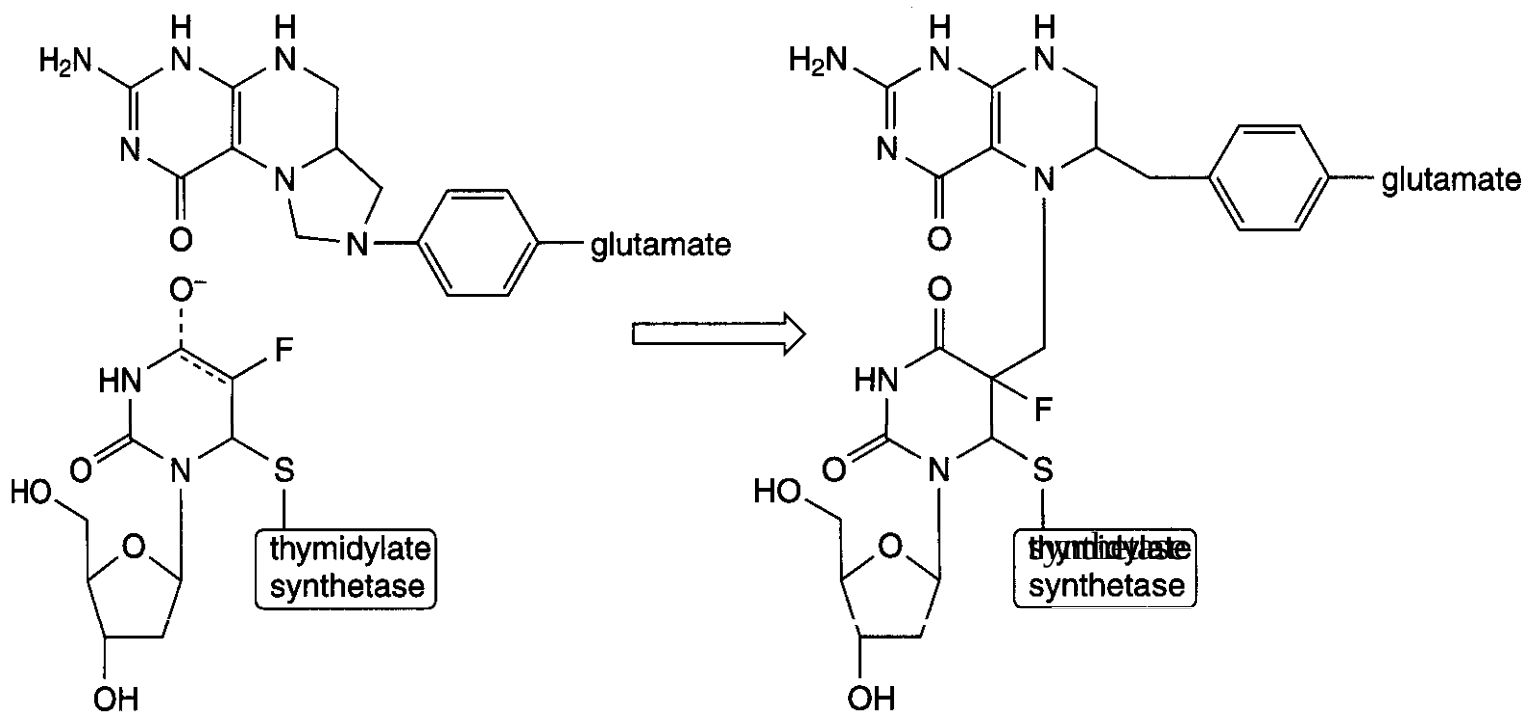


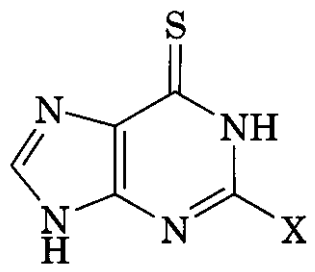
Figure 2.8. Mechanism of 5-fluorouracil inhibition of thymidylate synthetase.

elongation during the DNA resynthesis process after nucleotide excision repair of the lesions (316). The mechanism of RR inhibition by gemcitabine has been studied in E. *coli* and seems to be different to that of other 2'-substituted nucleotide inhibitors, involving inactivation of the R1 subunit (317), and overexpression of RR is a resistance mechanism for gemcitabine (318). It is also an effective radiation sensitizer, probably through depletion of dATP pools in cells (319) and can increase cellular apoptosis in irradiated cells (320).

4.4.3 Biological Activity and Side Effects. Both 5-fluorouracil and cytosine arabinoside remain widely used in combination cancer chemotherapy. 5-Fluorouracil is one of the most effective drugs against colon cancer (311). Cytosine arabinoside is effective in leukemias and lymphomas but has a very short half-life (ca. 12 min in man), because of catabolism by cytidine deaminase (321), and various non-specific prodrug forms are used (322). Gemcitabine was shown in phase I trials to be active in a number of cancers, especially in non-small-cell lung cancer, where it showed >20% responses as a single agent and up to 54% in combination with cisplatin (323). In phase II trials, it has proved active in a wide range of tumors, including non-small-cell lung cancer (>60% responses in combination with cisplatin) (324), urothelial (22–28% responses as monotherapy, 42–66% in combination with cisplatin) (325), advanced breast cancer (25.0% responses as monotherapy) (326), and metastatic bladder cancer 42–66% responses in combination with cisplatin) (327). The main adverse effects were hematological but were generally mild. A number of large phase III trials are in progress.

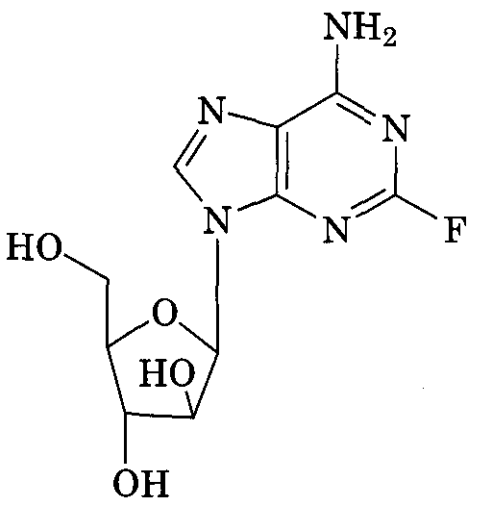
4.5 Purine Analogs

4.5.1 History. The purine analogs 6-mercaptopurine (76) and 6-thioguanine (77) were among the first anticancer drugs to be used, registered in 1953 and 1966, respectively. Later, the purine nucleoside analogs fludarabine (78) and pentostatin (79; 2'-deoxycoformycin) were registered in 1991, and cladribine (80; 2-chloro-2'-deoxyadenosine) was registered in 1992.

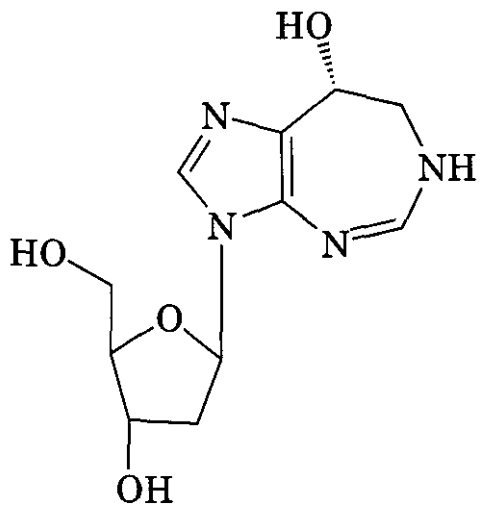


(76) X = NH_2
(77) X = N

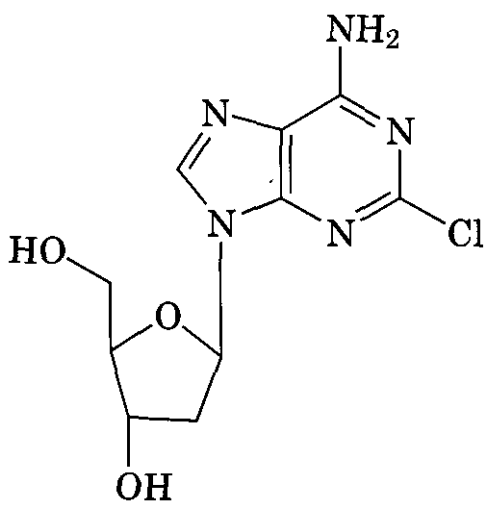
4.5.2 Mechanism and SAR. Cytosine arabinoside, fludarabine, and cladribine are taken into cells through a specific nucleoside trans-



(78)



(79)



(80)

porter protein and are phosphorylated to the mono-, di-, and triphosphates, with the first phosphorylation mainly by deoxycytidine kinase (328). The active triphosphate derivatives are incorporated into DNA, blocking polymerase function and thus DNA synthesis. Cladribine is resistant to degradation by adenosine deaminase (329) and induces apoptosis in leukemia cell lines through the Fas/Fas ligand pathway (330). It also interrupts deoxyadenosine metabolism, blocking both phosphorylation and deamination (329). Pentostatin is also converted to the triphosphate and incorporated into DNA, where it blocks polymerase function (331) but is also an extremely potent inhibitor of adenosine deaminase (K_i 2.5 $\times$ 10^{12} M^{-1}) (332).

4.5.3 Biological Activity and Side Effects. These three adenosine analogs, which are cytotoxic to both dividing and resting lymphocytes, have revolutionized the treatment of indolent lymphoid malignancies such as chronic lymphocytic leukemia, non-Hodgkin's lymphoma, cutaneous T cell lymphoma, and hairy cell leukemia. Both fludarabine and cladribine showed similar good response rates, but were cross-resistant, in refractory non-Hodgkin's lymphoma (333). Cladribine is active in hairly cell leukemia (>80% complete responses) (334), non-Hodgkin's lymphoma (89% responses) (335), refractory chronic lymphocytic leukemia (44% responses) (336), untreated chronic lymphocytic leukemia (85% response rate) (337), and cutaneous T-cell lymphomas (28% responses) (338), but it showed little activity in solid tumors.

5 TUMOR-ACTIVATED PRODRUCS

5.1 Introduction

As noted above, the majority of clinically used anticancer drugs are systemic anti-proliferative agents (cytotoxins). These kill cells by a variety of mechanisms primarily by attacking their DNA at some level (synthesis, replication, or processing). However, a large part of their selectivity for cancer cells is based on cytokinetics, in that they (to varying extents) are preferentially toxic to cycling cells. For this reason, their therapeutic efficacy is limited by the damage they also cause to proliferating normal cells such as those in the bone marrow and gut epithelia. This is especially true in the treatment of solid tumors, where cell doubling times may be very long. Whereas efforts to physically target cytotoxins to tumor tissue has not been very successful, the development of relatively nontoxic prodrug forms

Table 2.4 **Tumor-Activated Prodrugs in Clinical Trial for Cancer Chemotherapy**

Generic Name (Structure)	Trade Name	Originator	Chemical Class
Hypoxia-activated prodrugs			
tirapazamine (**82**)	Tirazone	Sanofi	benzotriazine-di-N-oxide
AQ4N (**84**)		British Technology Group	aliphatic N-oxide
porfiromycin (**86**)		Vion	aziridinylquinone
ADEPT prodrugs			
ZD 2767P (**93**)		AstraZeneca	aromatic mustard
GDEPT prodrugs			
ganciclovir (**99**)	Cytovene	Hoffmann LaRoche	
CB 1954 (**102**)		Cobra Therapeutics	dinitrophenylaziridine
Antibody-toxin conjugates			
SGN-15 (104)		Seattle Genetics	antibody/doxorubicin
Gemtuzumab ozogamycin (**105**)	Mylotarg	Wyeth-Ayerst	antibody-enediyne
SB 408075 (106)		Immunogen	antibodylmaytansinoid
KM231-DU257 (**107**)		Kyowa Hakko	antibody/cyclopropylindole

of cytotoxins, which can be selectively activated in tumor tissue, is beginning to achieve some success, and in the future, may become a major strategy.

Prodrugs can be defined broadly as agents that are transformed after administration, either by metabolism or by spontaneous chemical breakdown, to form a pharmacologically active species. Strictly speaking, agents such as cyclophosphamide (4) are prodrugs, but these undergo non-specific activation in all tissues. Of more interest are tumor-activated prodrugs that exploit various aspects of tumor physiology and other techniques to become selectively activated in tumor tissue to toxic species. The multiple criteria required of a tumor-activated prodrug has meant that these compounds, whereas sometimes using natural products such as doxorubicin as the toxins, are primarily synthetic agents. Most tumor-activated prodrugs fall under one of four categories: hypoxia-selective prodrugs (bioreductives), prodrugs for antibody-directed enzyme-prodrug therapy (ADEPT prodrugs), prodrugs for gene-directed enzyme-prodrug therapy (GDEPT prodrugs), and antibody-toxin conjugates (armed antibodies).

5.2 Clinical Use of Tumor-Activated Prodrugs

Because interest in tumor-activated prodrugs is relatively recent, only the hypoxia-selective agent tirapazamine has had extensive clinical use, and even this is still in development, although it looks likely to become the first clinically useful hypoxia-selective drug (339). The limited clinical experience with these various drugs is discussed below, in each subclass (Table 2.4).

5.3 Hypoxia-Activated Prodrugs (Bioreductives)

5.3.1 History. The imperfect neovascularization that develops in growing solid tumors results in limited and inefficient blood vessel networks and restricted and often chaotic blood flow (340). This generates chronic or diffusion hypoxia, where cells sufficiently distant from the nearest blood capillary are hypoxic for long periods, caused by the steep diffusion gradient of oxygen in tissue. The high and variable interstitial pressures caused by the growing tumor (341) can also result in transient or perfusion hypoxia, resulting from the temporary shut down of blood vessels placing sections of tissue under hypoxia for shorter periods (342). Because severe hypoxia is a common and unique property of cells in solid tumors, it is thus an important potential mechanism for the tumor-specific activation of prodrugs. This concept grew initially out of the development of radiosensitizers, drugs designed to take the place of oxygen in hypoxic tissue by oxidatively "fixing" the initial DNA radicals formed by ionizing radiation to gener-

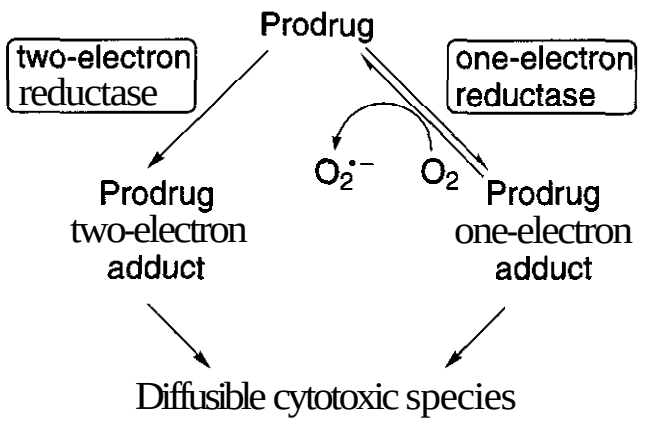
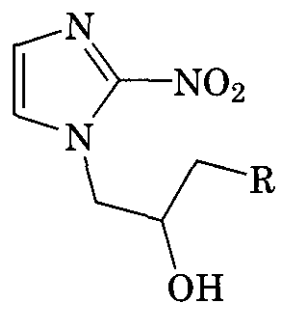


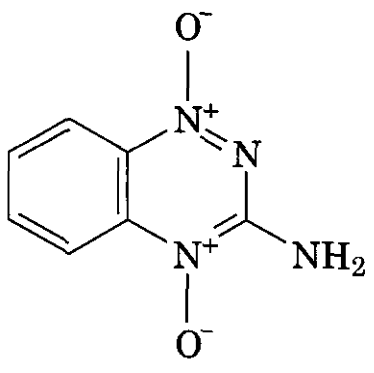
Figure 2.9. Hypoxia-activated prodrugs.

ate cytotoxic strand breaks (343). Such compounds tended to be easily reduced electron-deficient species such as misonidazole (81). In addition to their radiosensitizing properties as "oxygen-mimetics," many of these compounds were also found to have modestly higher levels (ca. 10-fold) of *cytotoxicity* in hypoxic compare with oxygenated cells in culture (344). THe mechanism of such hypoxia-selective cytotoxicity is the ability of the prodrug to the metabolized by reductive enzymes such as cytochrome P450 reductase and xanthine oxidase (345) to a transient one-electron intermediate. In normal oxygenated tissue, this is efficiently back-oxidized by molecular oxygen to the parent compound, but in hypoxic cells, it is further metabolized or spontaneously breaks down to more cytotoxic species (346) (Fig. 2.9).



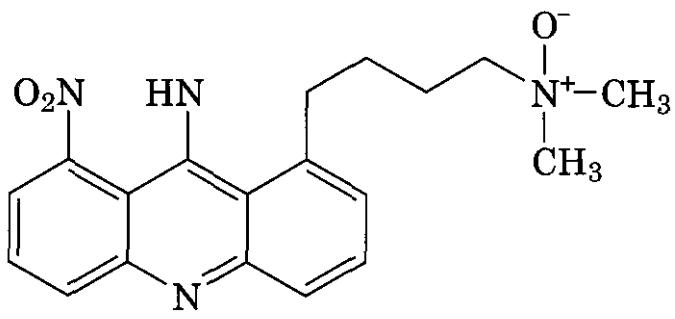
(81)

The most well-studied hypoxia-activated prodrug is the synthetic agent tirapazamine (82; 3-aminobenzotriazone-1,4-di-N-oxide). This drug was originally evaluated as an antimicrobial agent (347) but was discovered to have hypoxia-selective cytotoxicity in a screening program. It is now in widespread phase III studies (348) and may become the first clinically useful hypoxia-selective drug (339).



(82)

Aliphatic N-oxides of DNA-binding agents have also been explored as synthetic hypoxia-selective prodrugs. This was first demonstrated by the drug nitracrine-*N*-oxide (83). This is much more hypoxia-selective (>1000-fold in cell culture) (349) than the free amine itself, which also shows moderate hypoxic selectivity through reductive activation of the nitro group (350, 351). The most advanced drug of this type in development is the bis-N-oxide AQ4N (84) (352), which is due to begin phase I clinical trials shortly.



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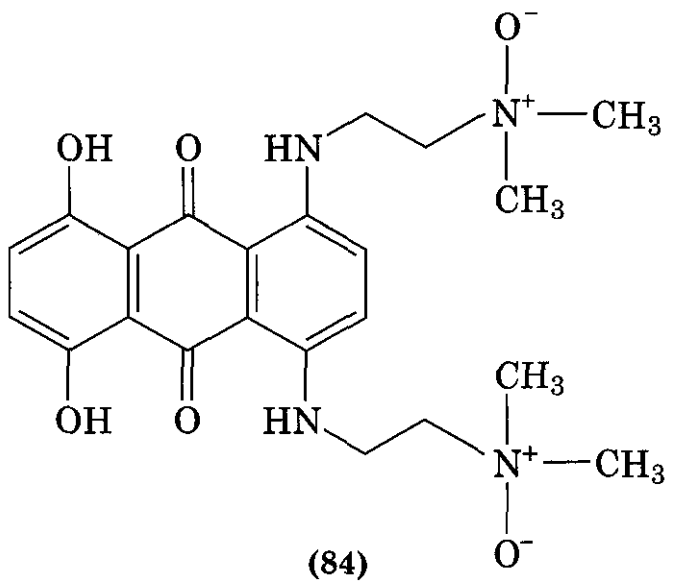
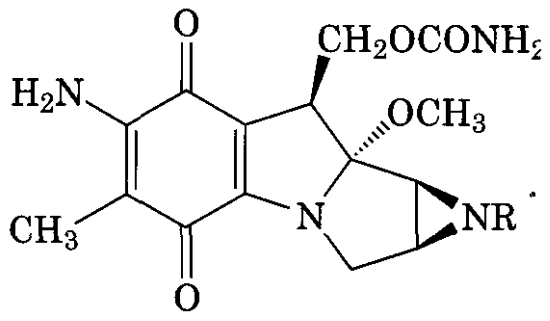


(84)

The natural product and widely used clinical agent mitomycin C (85) shows modest hypoxic selectivity (353), but this is not the main basis of its usefulness. However, its analog

porfiromycin (86) does show greater selectivity (354), and it has been developed primarily as a hypoxia-activated prodrug, to the extent of phase I clinical trials for head- and neck-cancer in combination with radiotherapy (355).



(85) R = H
(86) R = CH_3

5.3.2 Mechanism and SAR. The classes of hypoxia-activated prodrugs discussed above work by a variety of different mechanisms. Tirapazamine (82) was found to undergo enzymic one-electron reduction to a transient oxidizing nitroxyl (356) or carbon-centered radical and ultimately to the two-electron mono-N-oxide reduction product (Fig. 2.10). The transient radicals were shown to cause breaks at the C-4' ribose site of DNA, followed by the oxidation of these by oxygen or other oxidants (including tirapazamine itself) (357), through formation of a covalent adduct at the N-oxide oxygen. The main reducing enzymes responsible for the hypoxia-selective cytotoxic metabolism of tirapazamine are cytochrome P450 and cytochrome P450 reductase (345), although it is also reduced under hypoxia by aldehyde oxidase, xanthine oxidase (358), and nitric oxide synthase (359). A critical feature is that tirapazamine, although only forming a monofunctional radical, generates a high proportion of double-strand DNA breaks. This is suggested to be caused by high local radical concentrations generated by an undefined intranuclear reductase associated with DNA (339).

The aliphatic tertiary amine N-oxides of the bis-bioreductive prodrug AQ4N (84) are also reduced (to the free amines) largely by the CYP3A isozyme of nicotinamide adenine dinucleotide phosphate (NADPH):cytochrome C (P-450) reductase (360). Although this in not a one-electron process, it is still oxygen-inhibited, with a direct competition between oxygen and the drug at the enzyme site. Regeneration of the cationic side-chains of (84) allows tight binding to DNA and an ability to function as a topo II poison, similarly to the closely related drug mitoxantrone (41) (361). AQ4N is not significantly active as a single agent in most murine solid tumors *in vivo*, but it potentiates the effects of radiation therapy (which kills the oxygenated tumor cells) in a dose-dependent manner (362). Increased efficacy was also seen with combinations of AQ4N and cyclophosphamide in murine tumor models (363). AQ4N is due to begin phase I clinical trials shortly. This approach seems quite general, with compounds like DACA N-oxide (87) also showing significant hypoxic selectivity in cell culture (364). Nitracrine N-oxide (83) is an

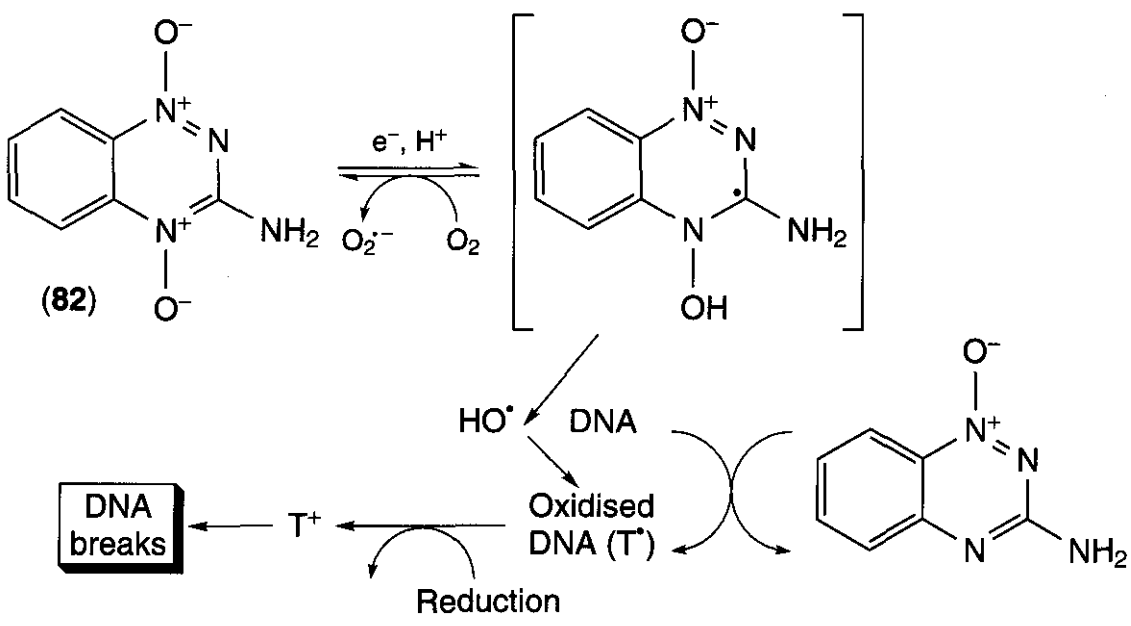


Figure 2.10. Metabolism of tirapazamine.

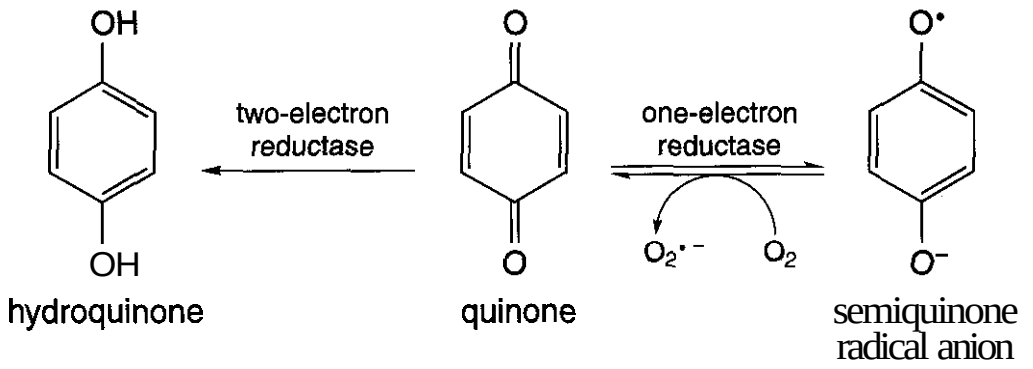
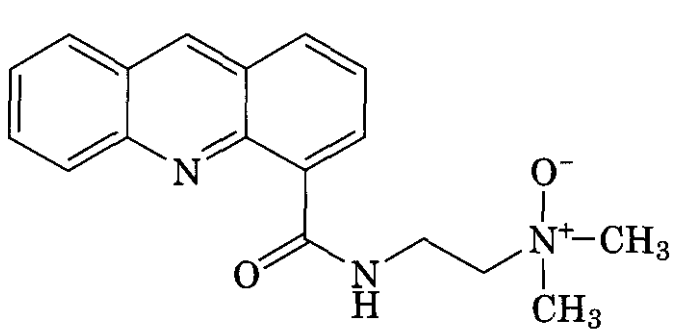


Figure 2.11. Bioreductive metabolism of quinones.

interesting example of another bis-bioreductive prodrug with two different reductive centers (nitro and N-oxide). Both centers need to be reduced for full activation, with the N-oxide demasking needing to occur before nitro reduction (365). Whereas (83) has exceptional hypoxic selectivity (>1000-fold) in cell culture (349), it shows little activity in *vivo* against the hypoxic subfraction of cells in KHT tumors (366) because of rapid metabolism. Attempted modulation of this by either lowering the reduction potential of the nitro group (366) or changing the steric environment of the *N*-oxide (367) was not useful.

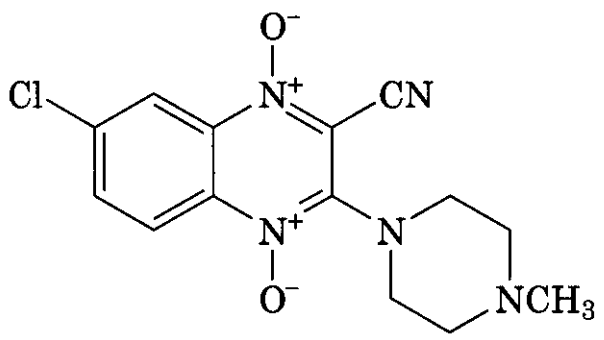


(87)

Mitomycin C (85) and porfiromycin (86) are quinones that readily undergo one-electron reduction, primarily by NADPH:cytochrome C (P-450) reductase (368), to the corresponding semiquinone radical anion that is capable of back-oxidation by molecular oxygen (Fig. 2.11). Following this, mitomycin C undergoes a well-documented fragmentation to DNA cross-linking agents that form guanine–guanine crosslinks in the major groove (369). One potential drawback to quinone-based compounds as hypoxia-activated prodrugs is that they are also often good substrates for two-electron reductases, particularly DT diaphorase (DTD; NQO1; NAD(P)H:quinone-acceptor oxidoreductase) (370).

5.3.3 Biological Activity and Side Effects. Tirapazamine shows high selective toxicity (100- to 200-fold) toward hypoxic cells in culture, but its diffusion through tissue is limited by its ready metabolism to the (non-diffusible) radical species (371). Tirapazamine has an ability to kill cells over a much wider range of oxygen concentrations (as high as 2% O_2) (372) than most other hypoxia-selective cytotoxins, so that its activation is not restricted to completely anoxic tissues (373). In animal studies, tirapazamine enhanced the effect of both single-dose (374) and fractionated (375) radiation. Combinations of tirapazamine with both cisplatin (376), cyclophosphamide (377), and other cytotoxic agents, including etoposide, bleomycin, and paclitaxel (378, 379) showed additive or greater than additive effects on both tumor cell killing and tumor growth delay. Combinations with the blood flow inhibitor 5,6-dimethyl-xanthenone-y-acetic acid (DMXAA) showed marked increases in activity in a variety of tumor models (380). Tirapazamine has had extensive clinical trials in head-and-neck cancer in conjunction with radiation (to kill oxygenated cells) (381) with encouraging results (382). Combinations with cisplatin (7) are also promising, the tirapazamine enhancing its effects, probably by delaying the repair of cisplatin-induced DNA cross-links in hypoxic cells (376). This has resulted in superior response rates compared with cisplatin alone in cervical cancer (383), mesothelioma (384), malignant melanoma (385), and particularly non-small-cell lung cancer (386). Clinical toxicities of tirapazamine include ototoxicity and muscle cramping (387). A laboratory study showed that tirapazamine caused time- and dose-dependent retinal damage in mice (388), but this does not seem to be a clinical issue.

Related quinoxalinecarbonitrile-1,4-di-*N*-oxides (e.g., **88**), where the 2-nitrogen in the benzotriazine unit of tirapazamine is replaced with a C—CN unit, are also potent and highly selective hypoxia-selective drugs (389). Structure-activity studies with these compounds show that hypoxic selectivity is retained when H or NHR replaces the 3-amino group.



(88)

5.4 Prodrugs for ADEPT

5.4.1 History. Antibody-directed enzyme-prodrug therapy (ADEPT) is an adaption of the earlier concept (390, 391) of immunotoxins. The difference is that instead of the toxin being attached to the antibody for localization on tumors, an enzyme (usually non-human) is attached and thus localized instead (392,393) (Fig. 2.12). A prodrug that can be activated efficiently and selectively by the enzyme is then administered and is catalytically activated by the localized enzyme only in the vicinity of the tumor cells. The advantage of using non-human enzymes is the enhanced ability to find prodrugs that can be selectively activated. ADEPT shares with the original immunotoxin concept the problems of limited access of the (large) antibody-enzyme conjugate to tumors and the usually heterogeneous expression of the target antigen on tumor cells. However, provided the released cytotoxin has the appropriate properties (high potency and an efficient bystander effect) it can ameliorate these problems by diffusing from the cells where it is generated to enter and kill surrounding tumor cells that may not possess prodrug activating ability. A further increase in efficacy can be achieved if the prodrug is designed to be excluded from cells until it is activated (394).

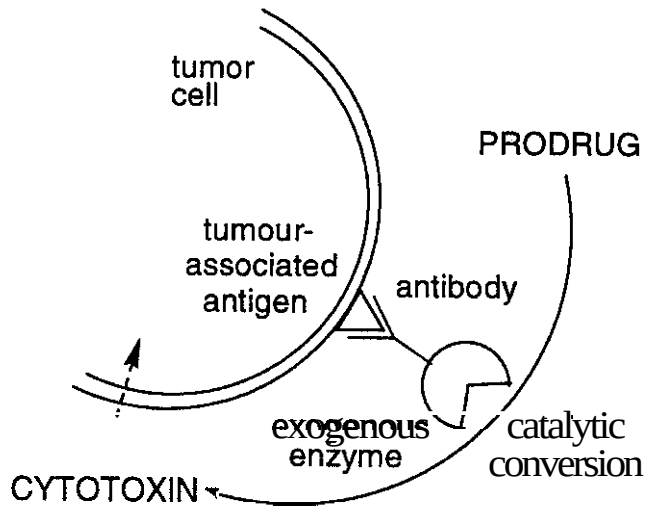
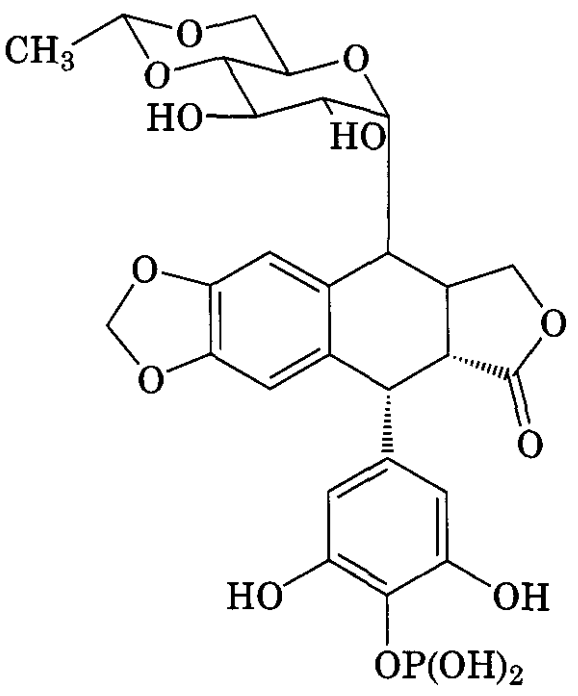


Figure 2.12. Antibody-directed enzyme-prodrug therapy (ADEPT).

5.4.2 Mechanism and SAR. The specific mechanism of action depends on the type of enzyme used to activate the prodrug. Particular requirements of the prodrug include being a selective and efficient substrate for the enzyme used. General requirements are an ability to be excluded from cells (usually achieved by high hydrophilicity and/or possession of a negative charge) until activation and the capability to then release a potent and diffusible toxin with a substantial bystander effect.

***5.4.2.1 Prodrugs* for Phosphatase Enzymes.** Phosphates have been employed as ADEPT prodrugs because both aromatic (e.g., 89; etoposide phosphate) (395) and aliphatic (e.g., 90; mitomycin phosphate) (396) examples are efficiently cleaved by alkaline phosphatases and are substantially cell-excluded. However, it proved difficult to achieve selectivity because there is an abundance of such phosphatase enzymes in human serum and other tissues, and phosphates are primarily now used directly as non-specific prodrugs; the antivascular agent (91) (combretastatin phosphate) is an example (397).

***5.4.2.2 Prodrugs* for Peptidase Enzymes.** Glutamate-type prodrugs of mustards (e.g., 92, 93) are effectively excluded from cells by the diacid side-chain and can be cleaved by the Pseudomonas-derived enzyme carboxypeptidase G2 (398). Cleavage of the amide or carbamate releases more lipophilic agent that is also activated by electron release through the aromatic ring to the mustard. The amide (92) (CMDA) was the first ADEPT prodrug evaluated clinically (399), and the carbamate pro-



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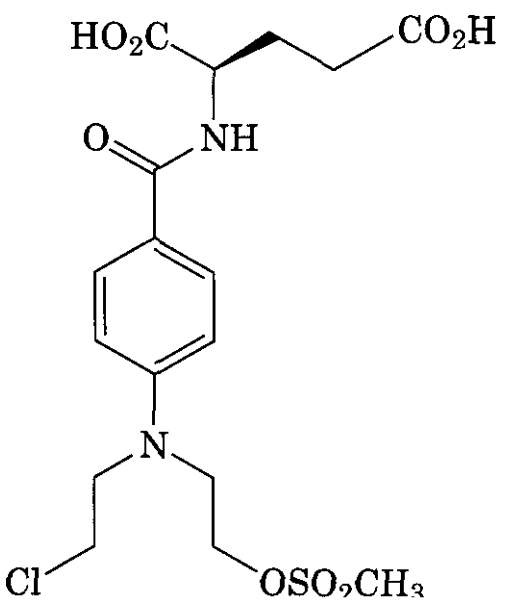

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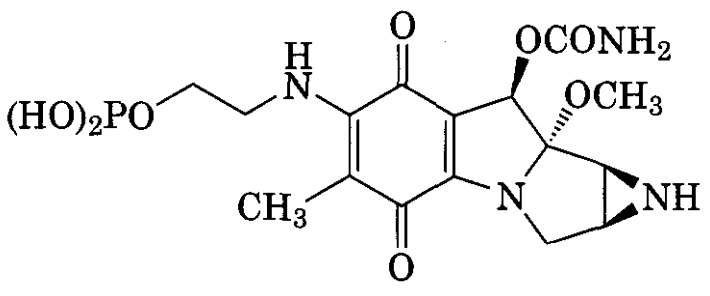

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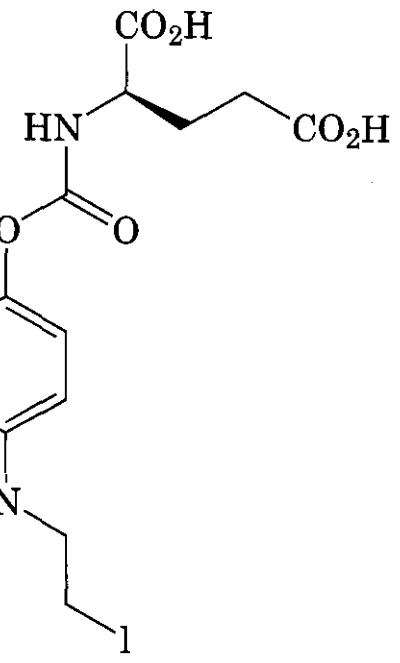
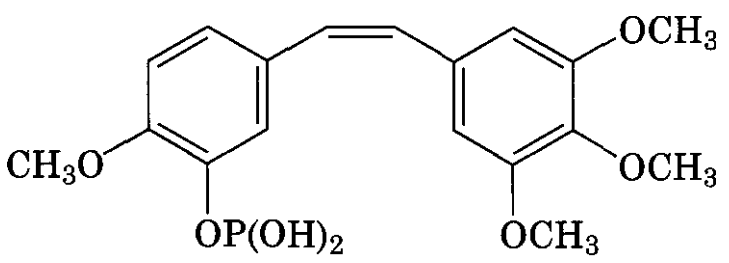




(91)

(93)

drug **(93)** (ZD 2767), releasing a more cytotoxic phenol iodomustard, is currently in phase I clinical trial **(400).**

5.4.2.3 ***Prodrugs for β-Lactamase Enzymes.*** These enzymes from Enterobacter species can selectively hydrolyze the four-membered β-lactam ring of penicillins and cephalosporins and have been used in a variety of prodrugs **(401).** Hydrolysis is followed by spontaneous fragmentation of the carbamate sidechain and the release of a variety of toxic amines (Fig. **2.13).** Carboxy and sulfoxide groups on the cephem nucleus assist with cell exclusion. Several nitrogen mustard prodrugs for β-lactamase have been evaluated. The cephem analog (94) affected cures in mice bearing xenografts of human melanoma cells if given subsequent to treatment with 96.5/bL, a mAb/β-lactamase conjugate that binds to specific surface antigens on these cells **(402).** A cephem derivative of doxorubicin **(95)** showed higher intratumoral levels of doxorubicin after treatment with the conjugate than with doxorubicin alone **(403).** However, the differential cytotoxicities between drug and prodrug in this approach are only moderate, and no such prodrugs have yet proceeded to clinical trial.

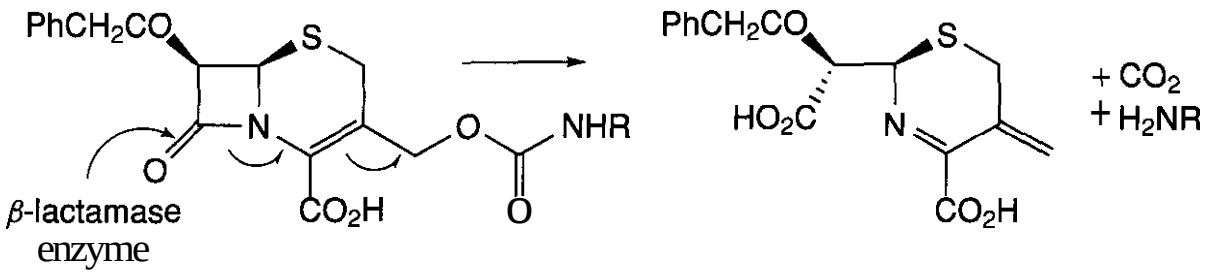
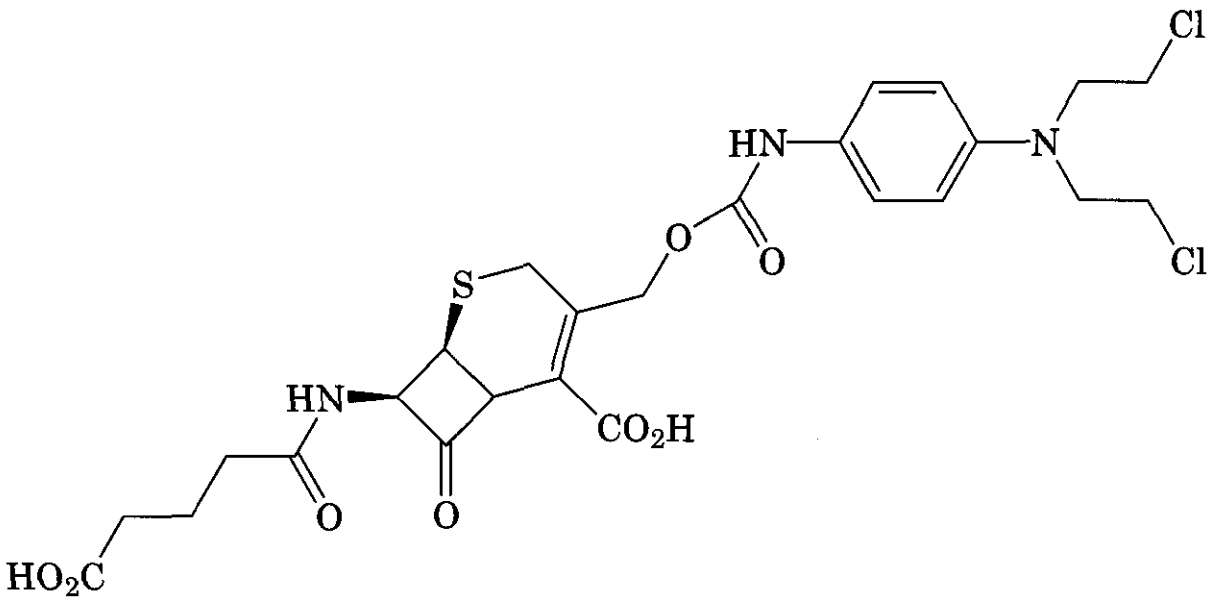


Figure 2.13. Fragmentation of β-lactamase prodrugs.



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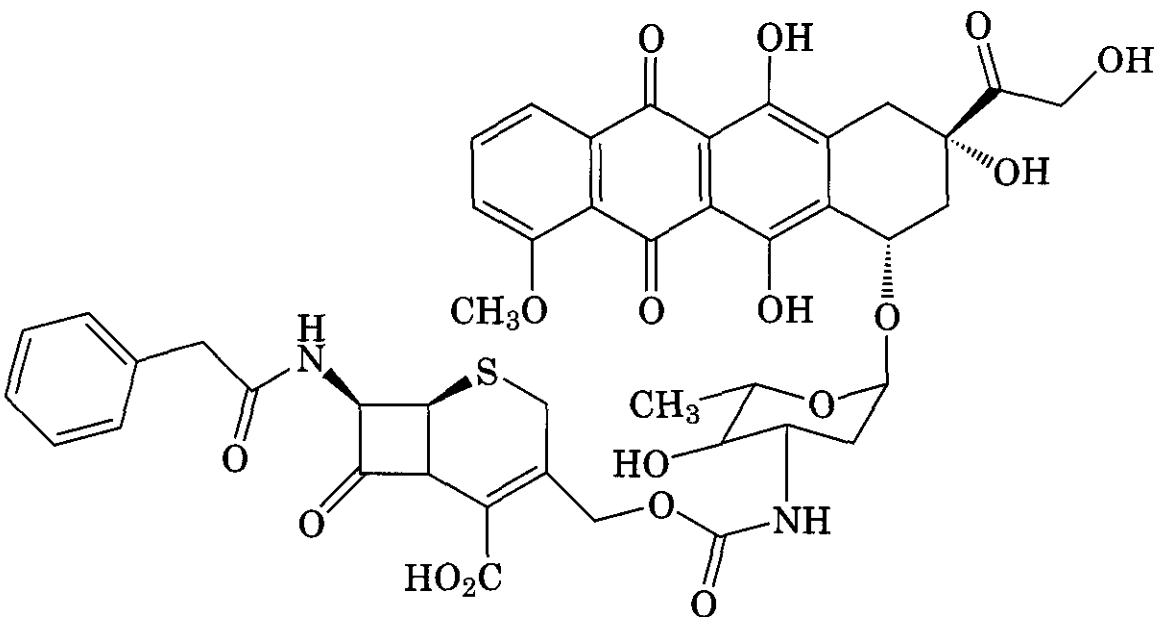
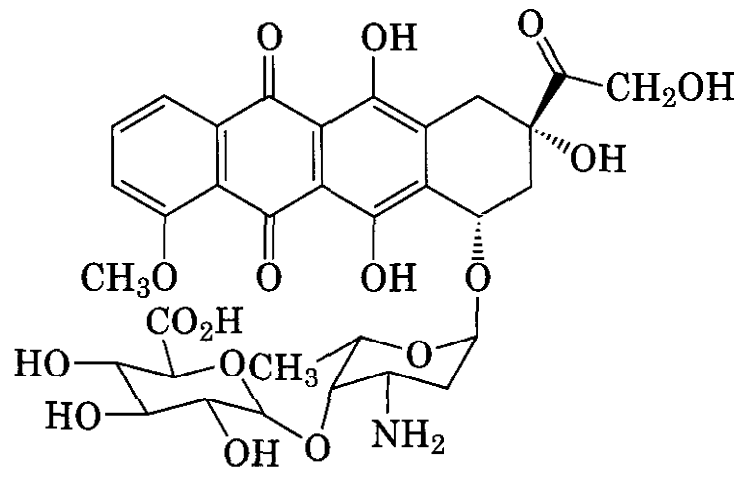


(95)

5.4.2.4 Prodrugs for Glucuronidase Enzymes. Because serum levels of β-glucuronidase (GUS) are very low (they are largely confined to lysozymes in cells), it is possible to use the human version as an activating enzyme in ADEPT, avoiding potential immunogenicity problems (404). Work with this enzyme has tended to focus on anthracycline effectors (405). The epirubicin *O*-glucuronide prodrug (96) was 100- to 1000-fold less cytotoxic than epirubicin itself *in vitro* (406), but pretreatment of antigen-positive cells with an 323/A3-GUS-E. *coli* immunoconjugate gave equivalent cytotoxicity to that of the free drug. The doxorubicin prodrug (97) used an immolative spacer unit (405), and although only 10-fold less cytotoxic than free doxorubicin, was a better substrate for the enzyme (407). The doxo-

rubicin prodrug DOX-GA3 (98) was 12-fold less toxic than doxorubicin in cells of the human ovarian cancer cell line FMa and was somewhat superior to doxorubicin against FMa xenografts in mice in conjunction with 323-A3/human β-glucuronidase conjugate (408). However, the use of such tightly DNA binding, cell cycle–specific (topo II inhibitor) effectors is not yet known.



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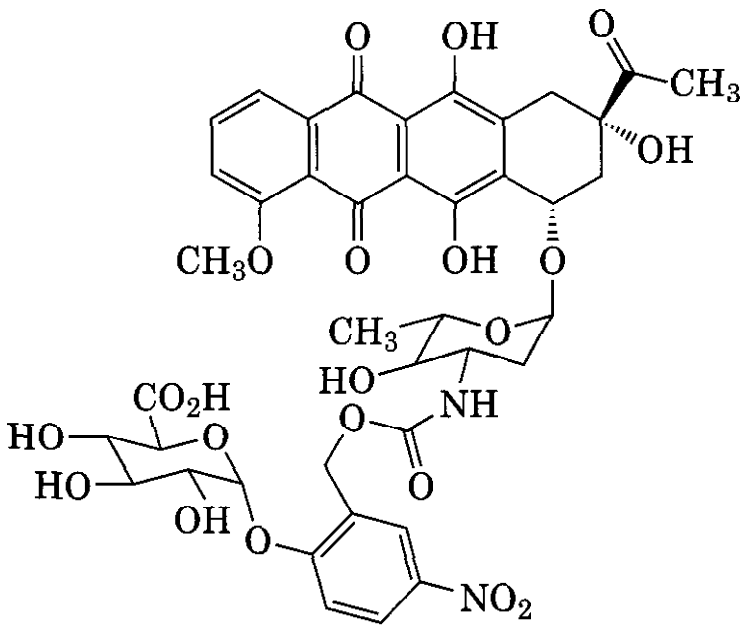


(97)

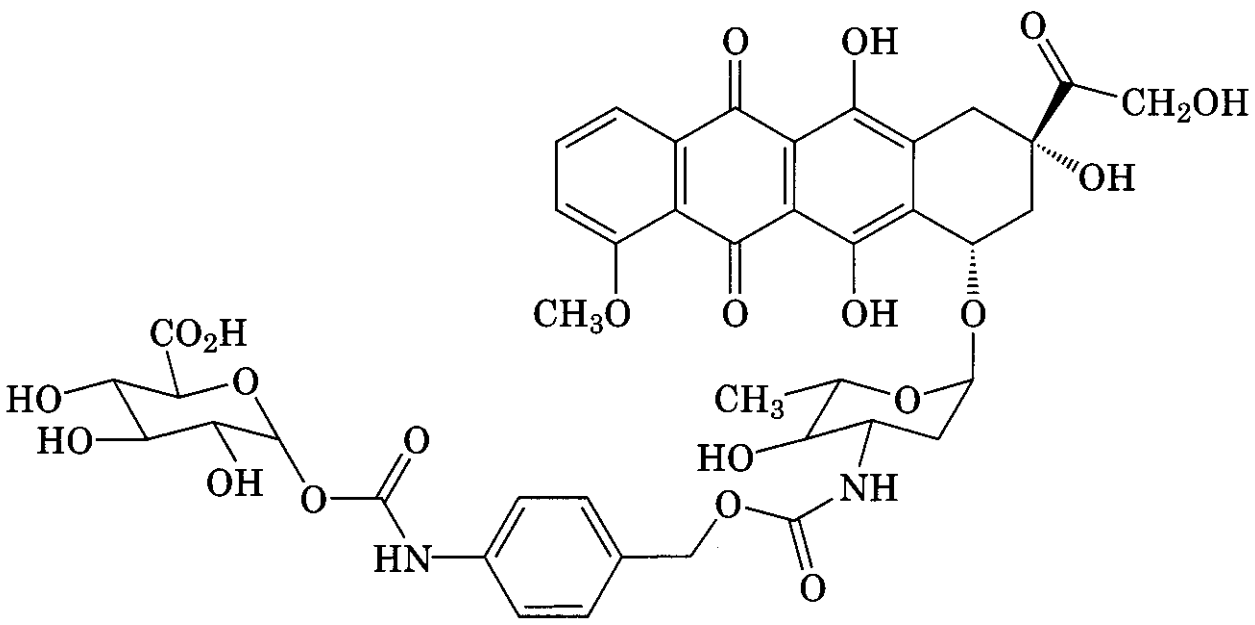
5.5 Prodrugs for CDEPT

5.5.1 **History.** The ADEPT approach is generally limited to the use of enzymes that do not require energy-producing cofactors, and it also has the likelihood of generating immune responses to the foreign proteins used. In gene-directed enzyme prodrug therapy (GDEPT), the enzyme is targeted to tumor cells by integrating the gene that produces it into the genome of the tumor cells, followed by administration of the prodrug. A small but growing proportion of the large number of "gene therapy" trials now in progress is for gene-directed enzyme-prodrug therapy, or "suicide gene therapy," although problems with systemic gene delivery remain (409). This concept theoretically retains the advantages of ADEPT in terms of selective and sufficient access of the activated drug to tumor cells and expands the class of available enzymes to those that require endogenous cofactors. However one approach to design in selectivity between prodrug and toxin is lost compared with ADEPT, because the prodrugs must be able to enter cells freely.

5.5.2 Mechanism and SAR. As with ADEPT prodrugs, the specific mechanism of action depends on the type of enzyme used to activate the prodrug. GDEPT offers a wider choice of enzymes, because those with cofactors not readily available outside cells can also be used. The protocol is also generally less immunogenic than ADEPT.

5.5.2.1 Prodrugs for Kinase Enzymes. The most widely used prodrug in GDEPT protocols is the antifungal agent ganciclovir (**99**), which is activated by the thymidine kinase enzyme from Herpes simplex virus, converting it into the monophosphate (100). This can then be converted by cellular enzymes into the toxic triphosphate, which acts as an antimetabolite. This combination has been evaluated in numerous clinical trials, primarily in gliomas by intratumoral injection (410). A limitation of the approach is the poor bystander properties of the active drug, which cannot enter cells by passive diffusion, but instead uses gap junction connections (411) that are not well developed in many types of tumors (412).

5.5.2.2 Prodrugs for Cytosine Deaminase. The yeast enzyme cytosine deaminase (413) has also been widely studied as a GDEPT system in conjunction with 5-fluorocytosine (**101**), which it converts to the thymidylate synthetase inhibitor 5-fluorouracil (**73**). This has good diffusion properties and shows better bystander effects (414). Experimental studies have focused mainly on colon cancer models for the use of this combination, because clinically 5-fluorouracil is one of the most effective



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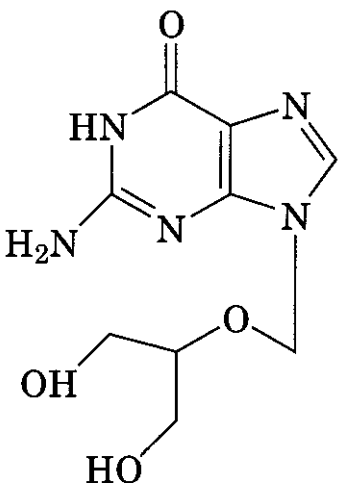

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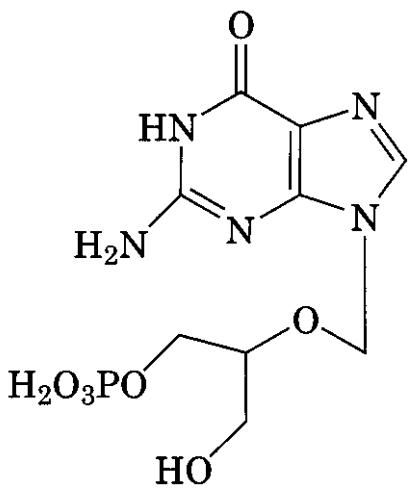

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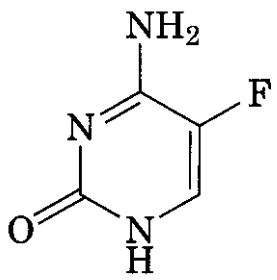
drugs for colon cancer. Possible drawbacks include the relatively low potency of 73, coupled with its pronounced cell cycle selectivity (415), and no clinical trials of the protocol have yet been reported.



(101)

5.5.2.3 Prodrugs for Oxidative Enzymes. The cytochrome P450 enzymes that non-specifically activate the clinical agent cyclophosphamide (4) in the liver to the active species phosphoramide mustard (see Section 2.3.2 and Fig. 2.2) have also been employed in a GDEPT protocol with cyclophosphamide. Treatment of sc 9L gliosarcoma tumors transduced with various isozymes, especially CYP2B6 or CYP2C18-Met, with (4)gave large enhancements over the normal liver P450-dependent antitumor effect seen with control 9L tumors (growth delays of 25–50 days compared with 5–6 days), with no apparent increase in host toxicity (416).

5.5.2.4 Prodrugs for Reductase Enzymes. The dinitrophenylaziridine (102102; CB 1954) is activated by the aerobic nitroreductase (NTR) from E. *coli* (417), in conjunction with NADH or NADPH, to a mixture of hydroxylamines (Fig. 2.14). The 4-hydroxylamine (103) is then further metabolized by cellular enzymes to DNA cross-linking species. CB 1954 shows high selectivity (up to 1000-fold) for a variety of cell lines transduced

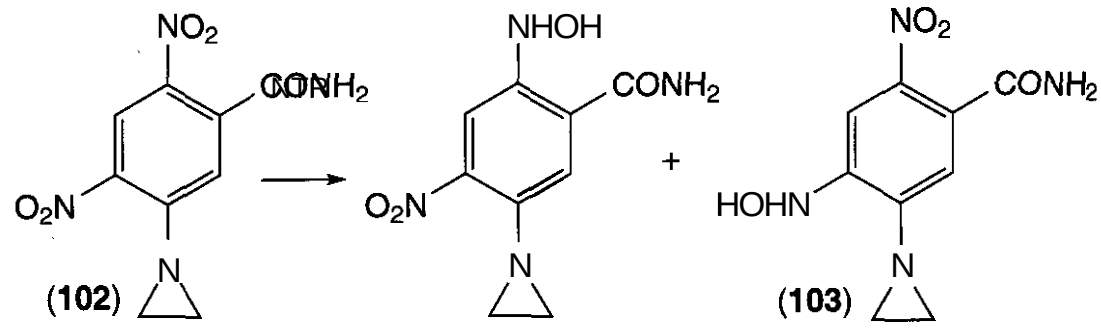


Figure 2.14. Metabolism of CB 1954 by E. *coli* NTR.

with the enzyme over the corresponding wild-type cell lines (418). It is now in clinical trial in conjunction with NTR, using a GDEPT protocol (419).

5.6 Antibody-Toxin Conjugates

5.6.1 History. This is a direct development of the "immunotoxin" approach, again exploiting the fact that many types of tumor cells present characteristic tumor-associated antigens on their surface (391). Despite much work, this approach has not been particularly successful until recently, with a combination of the availability of more resurgence of interest. The hypothesis is that conjugation of toxic drugs to the antibodies deactivate the drug (by limiting diffusional access to cells) without changing the selectivity of binding of the antibody. This allows it to locate on (antigen-bearing) tumor cells, internalize, and release the toxin (often through an acid-labile linker) when it is taken up into acidic endosomes (Fig. 2.15).

5.6.2 Mechanism and SAR. A wide variety of antibodies, linkers, and toxins are currently being explored in this approach. Doxorubicin continues to be widely used as a toxin because

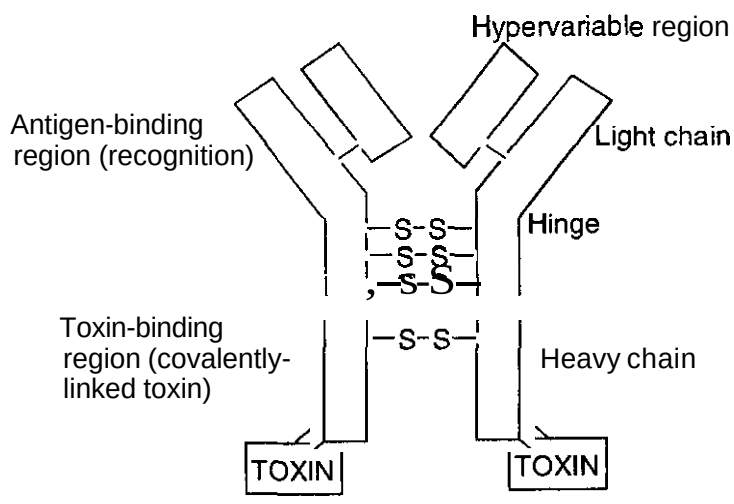
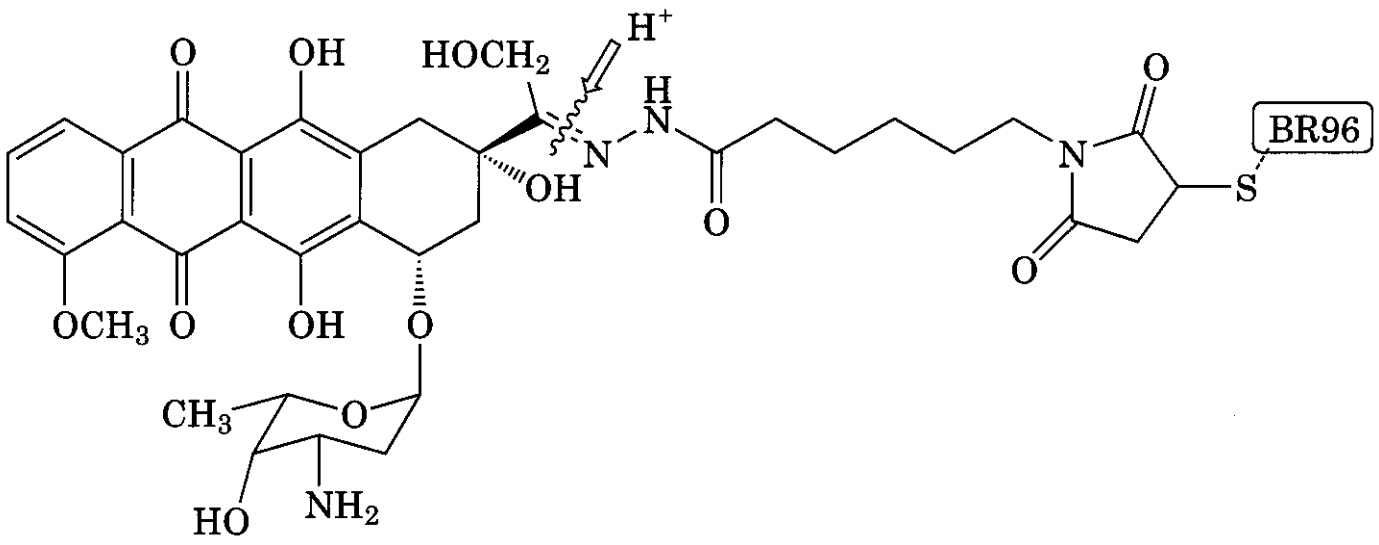


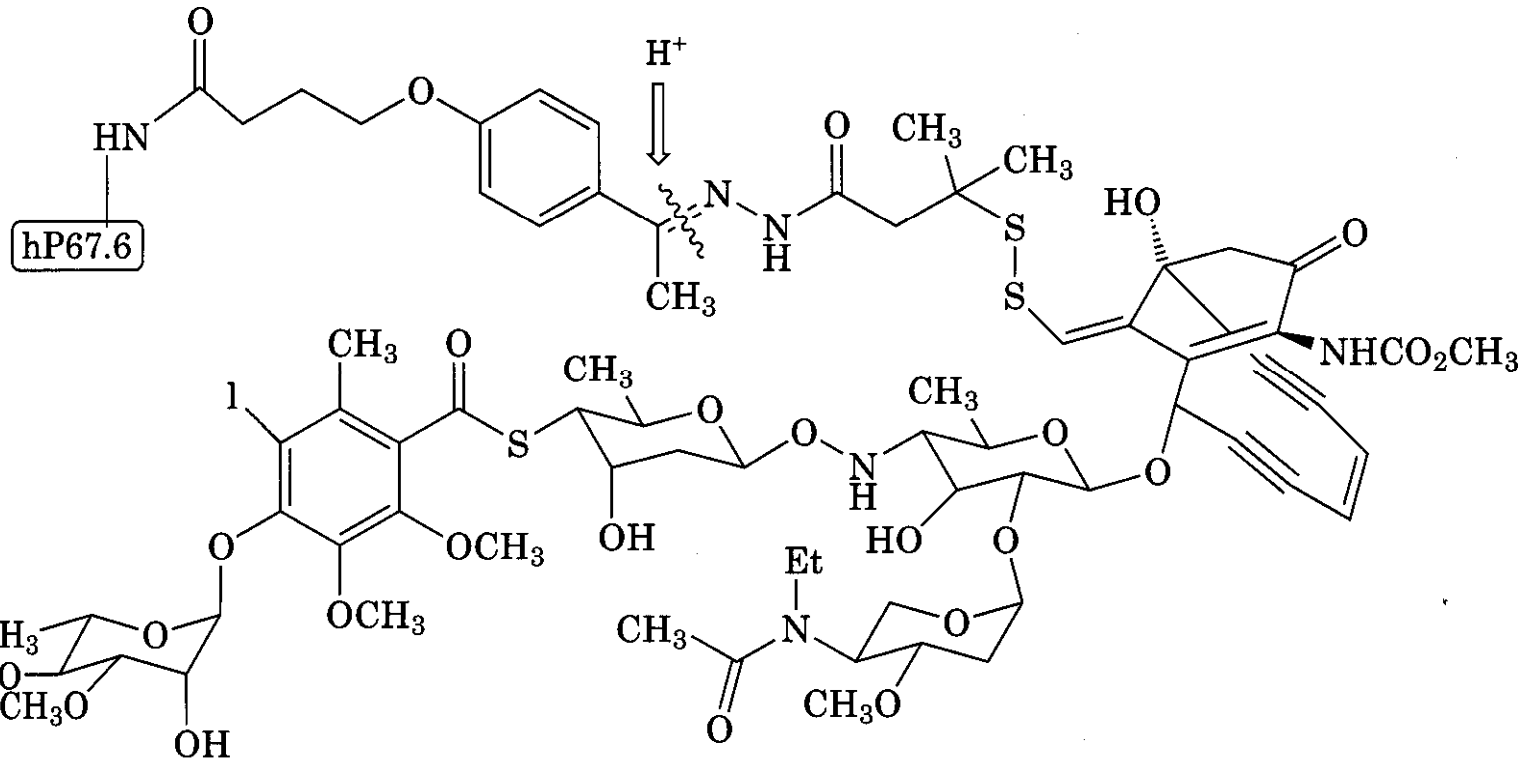
Figure 2.15. Schematic of toxin-armed antibody.

it is so well characterized, although it is not exceptionally potent. The most advanced doxorubicin-containing conjugate is SGN-15 (**104**), in which an average of eight molecules of doxorubicin are linked through an acid-labile hydrazone link, through the C-14 carbonyl, to the chimeric mAb BR96, which binds to a modified Le^y antigen on tumor cells. The major route of breakdown of (104) in *vitro* has been shown to be acid-catalyzed hydrazone hydrolysis, as designed (420). SGN-15 induced cures of established subcutaneous human colon carcinomas in athymic mice and rats (421), where free doxorubicin at its maximum-tolerated doses were ineffective. A recent phase I clinical trial of SGN-15 in patients with metastatic colon and breast cancers expressing the Le^y antigen determined the optimal dose to be 700 mg/m^2 (equivalent to 19 mg/m^2 of doxorubicin), with only mild toxicity (422).

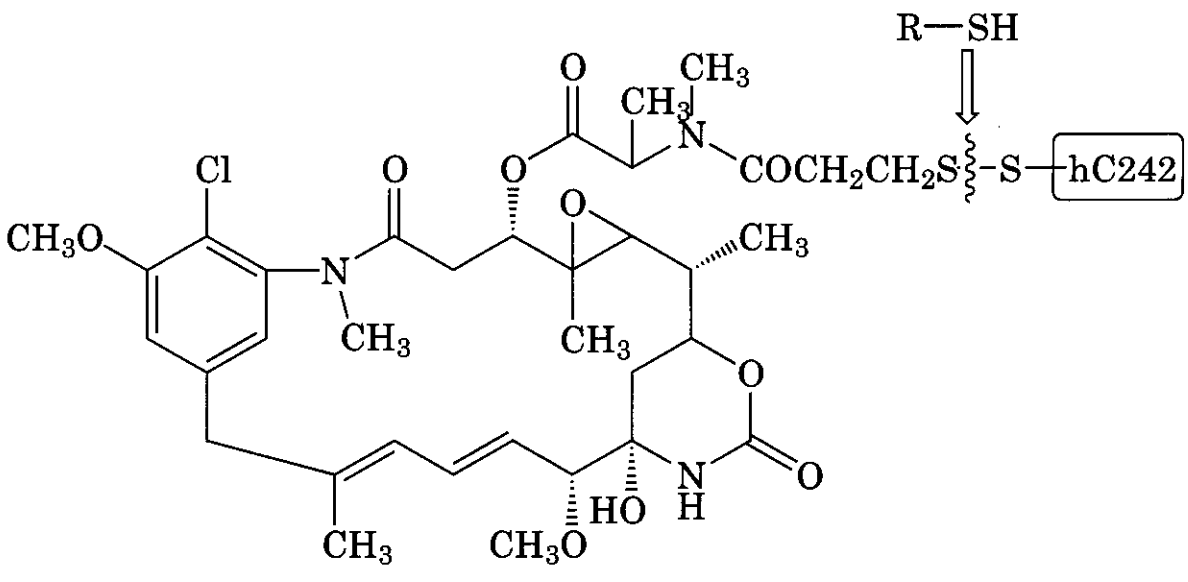
Conjugates of the extremely potent calicheamicin-type DNA cleaving agents have been under development for some time (423, 424). The conjugate (**105**) (gemtuzumab ozogamicin; mylotarg) was the first antibody armed with a small-molecule cytotoxin to reach clinical trial. Mylotarg has an average of four to five calicheamicin molecules linked through an acid-labile hydrazone linker, through a sterically-hindered disulfide, to a humanized hP67.6 IG1-based antibody that recognizes the CD33 antigen on normal and leukemic myeloid progenitor cells (425). Cleavage of the linker in the low pH endosomic environment in cells is followed by intramolecular cyclization to generate the transient benzenoid diradical that results in DNA double-strand cleavage (426). In phase II studies in relapsed AML patients, an overall 30% response rate was seen (427), with delayed hepatotoxicity as a possible side effect (428). Conjugate (105) has also been reported to be active clinically in brc/abl-positive CML (429).


O
OH
HOCH2
H+
H
N
N
OH
O
N
O
O
S
BR96
OCH3
O
OH
O
CH3
O
HO
NH2

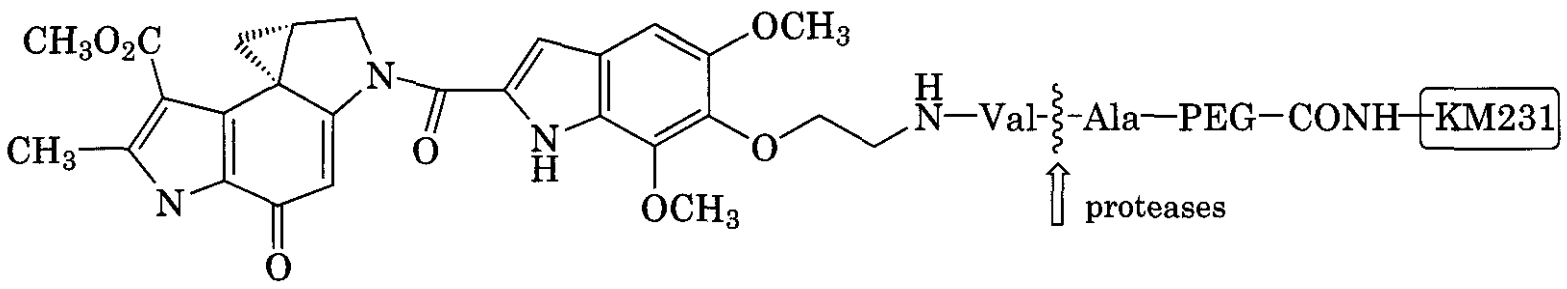
(104)


O
HN
hP67.6
O
H+
N
N
H
O
CH3
CH3
CH3
S
S
HO
O
NHCO2CH3
CH3
O
CH3
O
CH3
O
S
O
O
N
H
O
O
OCH3
OH
HO
Et
CH3
N
O
CH3O
OCH3
CH3
O
HO
CH3O
OH
CH3
N
O
CH3O

(105)


R—SH
O
CH3
CH3
N
COCH2CH2S
S
hC242
Cl
O
O
O
CH3O
CH3
N
CH3
CH3
O
N
H
O
HO
CH3
CH3O

(106)



(107)

The conjugate SB 408075 (106) employs the very potent synthetic maytansinoid-type tubulin inhibitor DM1 (430), with an average of four molecules of the toxin attached by a disulfide link to hC242, an antibody to a mucin-like glycoprotein on colorectal cancer cells. This conjugate affected cures in mice bearing large COLO205 human colon tumor xenografts (431) and is reported to be in phase II clinical trials.

Finally, members of the class of very cytotoxic DNA minor groove alkylators exemplified by the natural products CC-1065 and duocarmycin (83) have also been used to arm antibodies. As discussed in Section 2.5, these DNA minor groove alkylators were also evaluated clinically in their own right, but proved too toxic. Conjugate (107) (KM231-DU257) contains an average of two molecules of the duocarmycin analog DU257, linked through a PEGylated dipeptide (HO_2C-Val-Ala-NH_2) to an M231 antibody that targets the sLe^a antigen (432). The PEGylated linker prolongs plasma half-life, and the Val-Ala link is cleaved by tumor proteases to primarily release the DU257-Val conjugate, which has similar potency to DU257 itself.

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CHAPTER THREE

Antitumor Natural Products

LESTER A. MITSCHER
APURBA DUTTA
Department of Medicinal Chemistry
Kansas University
Lawrence, Kansas

Contents

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1 INTRODUCTION

Cancer is now believed to be the number one cause of premature death in industrialized nations. The market for anticancer agents was estimated at about US$10 billion in 1997 and continues to escalate. Because of the need and the value of these drugs, many laboratories are intensively investigating the chemistry and biology of novel anticancer agents. Major advances have been made in understanding the nature and vulnerability of cancerous cells, resulting in development of novel screens and approaches. For the present, however, cytotoxic agents, many of natural origin, are the mainstays of anticancer chemotherapy.

A wide array of complex terrestrial and marine natural products possesses antitumor activity **(1–5).** A few of these were in folkloric use in fairly ancient times, whereas many have been discovered very recently as the result of directed screening programs. In earlier times screening was principally carried out against P388 and L1210 (murine leukemia models), but now there is greater emphasis on slower growing solid tumors. Antitumor natural products possess some of the most intricate structures of any compounds finding medicinal use today, and most are so toxic that each patient must be carefully titrated with them. Even with this care, patients still find the side effects attendant on their use hard to bear.

The question of why these substances occur in nature is endlessly debated. Many believe that they are defensive secretions that allow the organisms that produce them to survive in a hostile world. Others believe that they represent growth regulators that allow organized and controlled growth of cells and that they are not particularly toxic in the quantities normally found in the producing cells. It is not easy to resolve such arguments, but the point remains beyond dispute that such compounds are widespread, are easily detected, and that individual plants or animals have evolved widely disparate structural solutions to whatever needs these compounds actually fulfill.

These agents are collectively the most complex nonpolymeric organic medicinal agents in present use. At the time of their discovery, elucidation of their chemical structures frequently pushed the limits of chemical science. Unraveling their molecular modes of action in many cases revealed previously unsuspected complexities in cellular growth regulation and biochemistry. Successful synthesis of these and related compounds has greatly enriched our synthetic capabilities and a number of these syntheses have become classics of the art. Learning how to administer them safely to patients required the highest level of clinical expertise.

It is also interesting to contemplate their structural diversity from a biosynthetic standpoint. Starting with fairly ordinary monomeric units, complex enzymic pathways ultimately produced these cytostatic/cytotoxic agents without at the same time poisoning the microorganism or plant producing them. It is not credible to suppose that the organisms produced these substances as a gift to humanity. Each of these products represents such a finely crafted idiosyncratic design that one wonders why so many different organisms came up with such different solutions, given that the starting materials are basically similar. One might have guessed that fewer general solutions would have developed over biological time if one wishes to believe that they serve a role in regulating the growth of .the producing organisms. In any event, mankind is fortunate that their activity spectrum is broad enough for us to use.

The toxicity of these agents is not particularly surprising, in that the screens employed in their discovery have historically depended on lethality to cells as an endpoint (6–8). It has historically been considered that rapidly growing cells, including cancer cells in particular, have a greater appetite for nutrients than more quiescent cells and so are selectively intoxicated on a kinetic basis. Thus the safety margin toward untransformed cells is not great. Furthermore, comparatively slow growing tumors are particularly hard to treat with such agents. Host cells that have a high growth fraction are also killed. Thus the usual constellation of side affects [alopecia, gastrointestinal (GI) ulceration, fertility impairment, immune suppression, blood dyscrasias, etc.] is relatively unavoidable. Very recently, synthetic agents able to interfere with aberrant cytokine-mediated growth signals

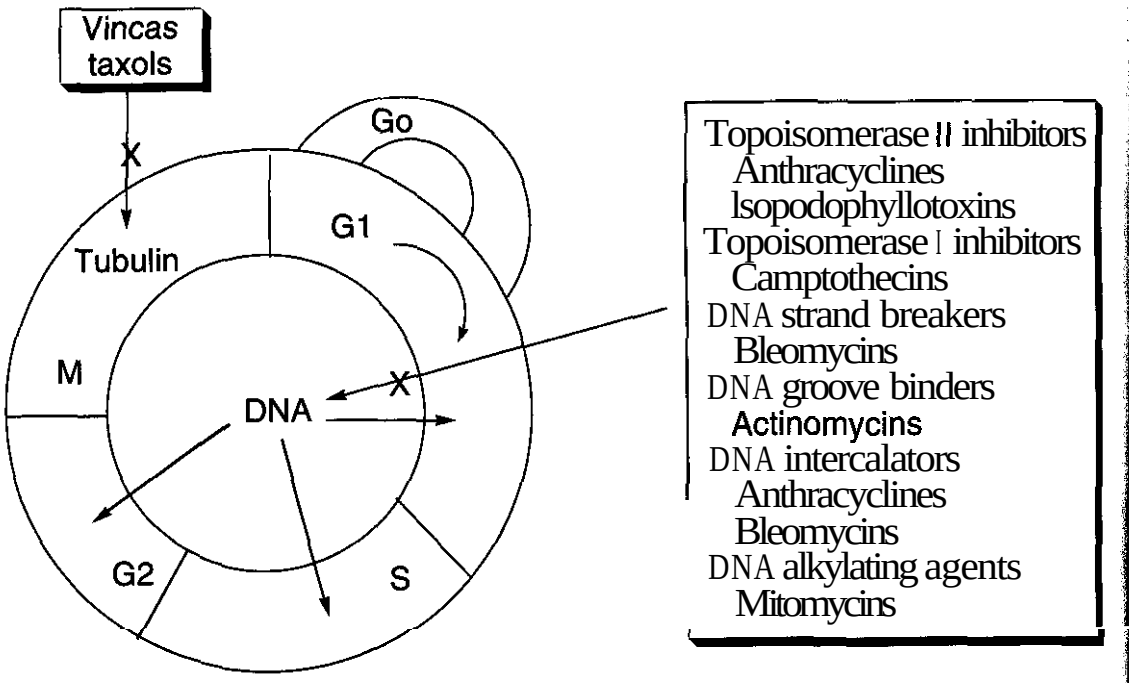


Figure 3.1. Synopsis of molecular modes of action of various prominent antitumor natural products.

have begun to appear on the market. Gleevec is the first commercial success embodying this approach and its antitumor application is comparatively nontoxic. It seems likely that natural products can be found sharing these characteristics. If so, a new era of natural product chemotherapeutic agents with minimal toxicity to normal cells will dawn.

The results of one of the principal screening methods in present use is collected in the National Cancer Institute database that was established in 1990 (6). This is based on comparative potency against 60 different human cancer cell lines grown in tissue culture. More than 70,000 compounds have been put through this screen and the data for each are presented in graphical form. From this, insights into mechanism of action and mode of resistance can be drawn (9). Many other tests are in present use, including screens for signal transduction inhibitors, antiangiogenesis, cell-cycle inhibition, exploitation of functional genomics, immunotherapeutics, vaccines, and chemoprevention. Much inventive biology is coming forward and exciting days appear to lie ahead.

The natural agents presently in use can be conveniently classified according to their molecular modes of action as follows:

1. Drugs attacking DNA
   - Dactinomycin
   - Bleomycin
   - Mitomycin
   - Plicamycin (mithramycin)
2. Drugs inhibiting enzymes that process DNA
   - Anthracyclines (daunorubicin, doxorubicin, epirubicin, idarubicin, valrubicin)
   - Camptothecins (topotecan, irinotecan)
   - Isopodophyllotoxins (etoposide, teniposide)
3. Drugs interfering with tubulin polymerization/depolymerization
   - Taxus diterpenes (docetaxel, paclitaxel/taxol)
   - Vinca dimeric alkaloids (vinblastine, vincristine, vinorelbine)

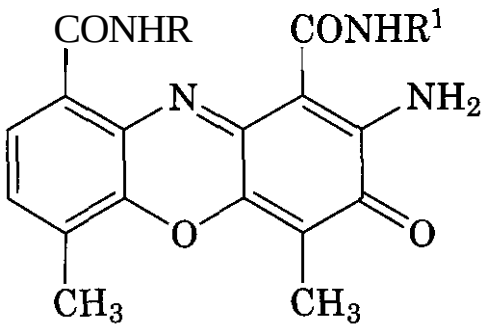
Figure 3.1 illustrates in summary form the various points of attack of prominent natural antitumor agents on growing cells. One notes that DNA or tubulin in one way or another (either by direct attack or by interference with enzymes processing these important cellular macromolecules) is the primary target of all of these agents and that most phases of the cell cycle are involved, especially when mixtures ("cocktails") are employed.

Added biological detail of the properties and applications of these naturally occurring natural products can be found in *The AHFS Drug Information 2001* book (10) and in *Goodman and Gilman's Pharmacological Basis of* Therapeutics (11).

2 DRUGS ATTACKING DNA

2.1 Dactinomycin (Cosmegen)

2.1.1 Introduction. The actinomycins are a family of yellow-red peptide-containing antitumor antibiotics produced by fermentation of various *Streptomyces* and *Micromonospora* species. The first members of the actinomycin family were discovered in the early 1940s in the hopes of finding nontoxic antibacterial antibiotics in fermentations of soil microorganisms (12), although in the actinomycin case this ambition was dashed by their high toxicity. Somewhat later (since about 1958) this was compensated for by the discovery that the toxicity to rapidly growing cells could be useful in cancer chemotherapy. One should note, however, that later discoveries demonstrated that the potencies against microbes and against tumors do not parallel well. At the present time about seven different complexes of actinomycins have been identified, each differing from the others primarily by the various amino acids constituting the two cyclic depsipentapeptide side chains pendant from the common phenoxazinone chromophore [called actinocin (1)]. When the two cyclic peptide side chains are identical, these agents are referred to as isoactinomycins (R = R′). When they are different from each other, they are known as anisoactinomycins (R ≠ R'). Of the 20 or so natural actinomycins and a much larger number of synthetic and biosynthetic analogs, actinomycin D [(2), from which the generic name dactinomycin is derived] is the most prominent medicinally. A useful trivial nomenclatural system has also grown up. In this system, dactinomycin is referred to as Val-2-AM and other analogs are named by the position and identity of the amino acids that are exchanged. Actinomycin C (cactinomycin-3) is thus known as Ile-2-AM.

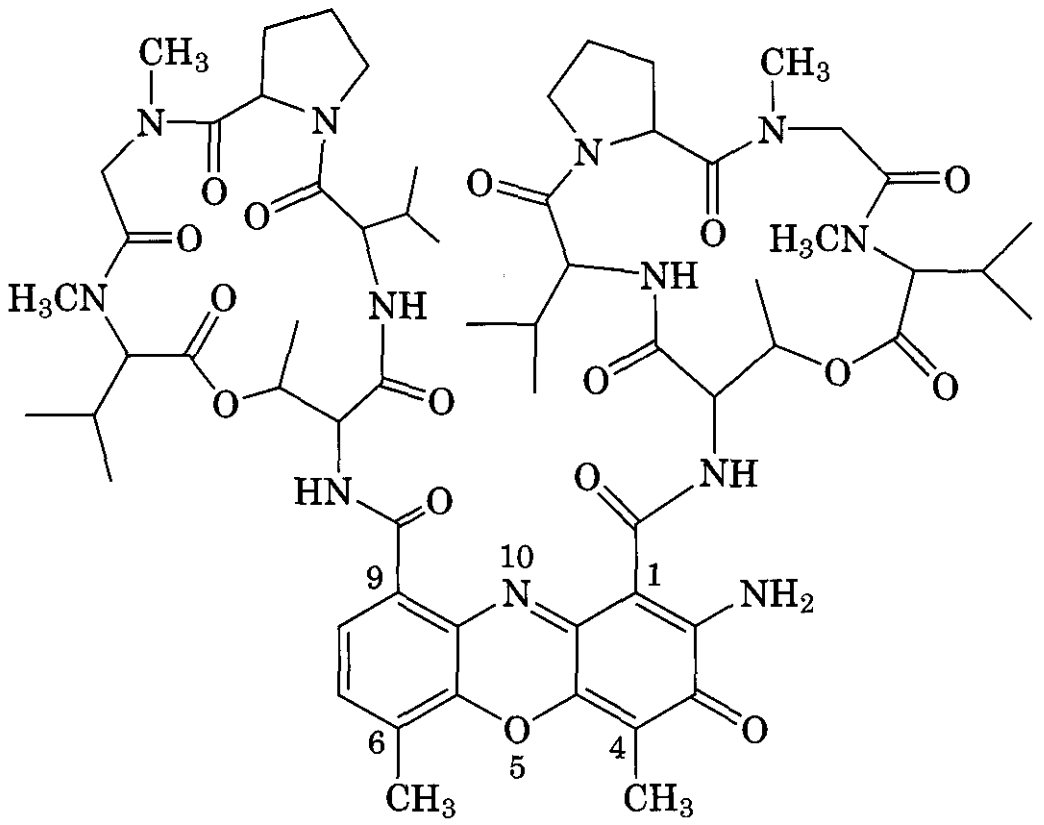


(1)

2.1.2 Medicinal Uses. As noted in the summarizing table, dactinomycin is used medicinally by intravenous (i.v.) injection for the treatment of Wilm's tumor, rhabdomyosarcoma, metastatic and nonmetastatic choriocarcinoma, nonseminomatous testicular carcinoma, Ewing's sarcoma, nonmetastatic Ewing's sarcoma, and sarcoma botryoides. The usual dose is 10–15 μg/kg i.v. for 5 days. If no serious symptoms develop from this, additional treatments are given at 2- to 4-week intervals. Other treatment schedules have also been used. The drug is often combined with vincristine and cyclophosphamide in a cocktail to enhance the cure rate (13).

2.1.3 Contraindications and Side Effects. Dactinomycin is contraindicated in the presence of chicken pox or herpes zoster, wherein administration may result in severe exacerbation, occasionally including death. The drug is extremely corrosive in soft tissues, so extravasation can lead to severe tissue damage (14). To avoid this the drug is usually injected into infusion tubing rather than being injected directly into veins. When combined with radiation therapy, exaggerated skin reactions can occur as can an increase in GI toxicity and bone marrow problems. Secondary tumors can be observed in some cases that can be attributed to the drug. Dactinomycin is carcinogenic and mutagenic in animal studies and malformations in animal fetuses have also been observed. Nausea and vomiting are common along with renal, hepatic, and bone marrow function abnormalities. The usual alopecia, skin eruptions, GI ulcerations, proctitis, anemia, and other blood dyscrasias, esophagitis, anorexia, malaise, fatigue, and fever, for example, are also observed. Clearly this is a very toxic drug.

2.1.4 Pharmacokinetic Features. Dactinomycin is not very available after oral administration, so it is primarily administered by injection. About 2 h after i.v. administration very little circulating dactinomycin can be detected in blood. It is primarily excreted in the



(2)

bile and the urine. It does not pass the blood-brain barrier. Dactinomycin is only slightly metabolized. Despite these factors it has a half-life of about 36 h. Persistence is largely accounted for by tight binding of the drug to DNA in nucleated cells (15–17).

2.1.5 Medicinal Chemical Transformations. Total synthesis of dactinomycin has been accomplished but this has not proved as yet to be of practical value (see Fig. 3.2). Analogs can be assembled from appropriately substituted benzenoid analogs. The overall strategy commonly involves construction of the external aromatic rings, attachment of the depsipeptide side-chain precursors, oxidative generation of the actinocin ring system, and functional group transformations to complete the synthesis (18–26).

Semisynthetic side-chain analogs of the actinomycins are prepared by removal of the depsipentapeptide side chains and their replacement by synthetic moieties. Analogs with altered peptide side chains are also prepared by directed biosynthetic manipulation of the fermentations. The synthetic replacement has been done in a combinatorial mode as well (27). Replacement of the normal side chains by simple amines leads to inactive products. Most of the other side-chain variations have led to compounds with reduced *in vivo* potency. None of those few analogs where this is not true has been commercialized.

Some chemical alterations in the chromophoric phenoxazinone moiety have also been accomplished. After considerable work it has emerged that the C-2 and the C-7 positions can be substituted with retention of signifi-

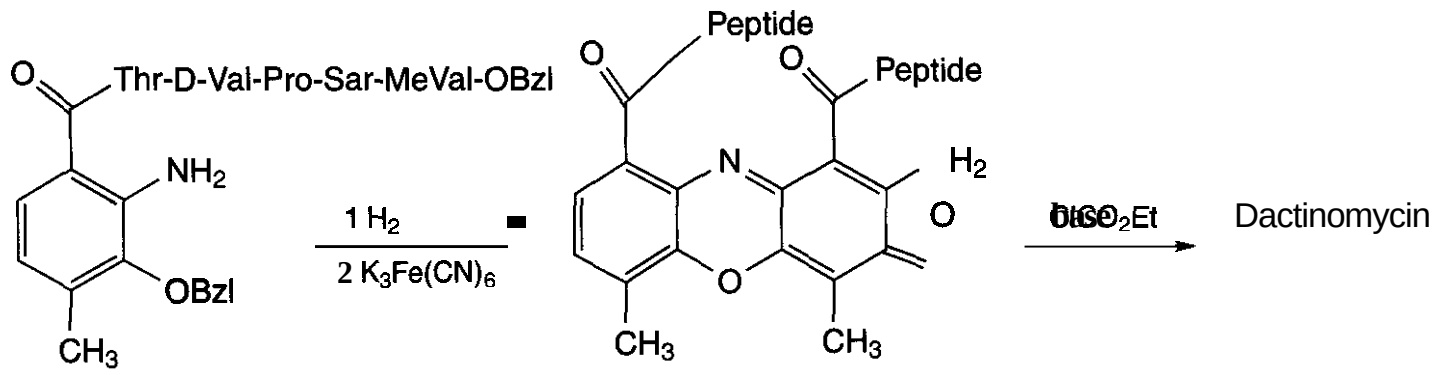


Figure 3.2. Synthesis of dactinomycin.

cant activity. Among some of the useful reactions leading to testable analogs are a series of addition/elimination reactions, starting with careful alkali hydrolysis to produce the C-2 OH analog. This can be converted by thionyl chloride treatment to the C-2 Cl analog. This in turn can be reacted with a variety of amines to produce alkylated C-2 amino substances (28). Catalytic reduction of the 2-C1 analog results in the protio analog, which is inactive. The C-2 chloro analog can be halogenated with chlorine or bromine to produce the C-2 chloro-C7 chloro or bromo analogs. These in turn can be solvolyzed to the C-2 amino-C7 halo analogs (29–32). Nitration and hydroxylation at the C-7 position can be accomplished but require prior protection with pyruvate. After nitration or oxidation to the quinone imine- and reduction, careful alkaline hydrolysis of the blocking pyruvate moiety leads to the desired analogs (28, 29, 33). The C-7 OH analog can be converted to the allyl ether and this can be epoxidized to produce an analog that not only can intercalate by virtue of its aromatic rings but can also alkylate DNA. The nitrogen analog of the epoxide (aziridinylmethylene) can be prepared by a somewhat different route. Hydrogenation of this last opens the aziridine ring to produce the primary amine (34–36). (See Fig. 3.3.)

The central chromophoric ring can also be modified to, for example, the phenazine (37, 38) analogs and to oxazinone and oxazole ring analogs (39, 40). These products have not become important (41).

In sum, these studies demonstrate that the side-chains are important determinants of activity as is the basic chromophoric three-ring system. Peripheral adornments are tolerated but not superior (42–48). Considering the putative molecular mode of action described below, this definition of the pharmacophore is not surprising. This definition of the pharmacophore is schematically represented in Fig. 3.4, where the pharmacologically successful transformations that take place are represented by the boxes.

2.1.6 Molecular Mode of Action. The flat three-ring fused aromatic portion of dactinomycin intercalates into double-helical DNA between the stacked bases (preferring guanine-cytosine pairs), whereas the attached cyclic peptide side chains of the drug bind into the minor grooves, thus further anchoring the complex (49–58). These combined interactions produce a tight and long-lasting binding. This model is supported by extensive X-ray studies with model nucleotides. As with other intercalating drugs, this interaction stretches the DNA and interferes with DNA transcription into RNA by RNA polymerase. The interference with the functioning of DNA-dependent RNA polymerase by dactinomycin is much stronger than the interference with DNA polymerases themselves. The consequences of intercalation are believed to be responsible for the antitumor action and most of the toxicity of dactinomycin. Some strand breaks are also reported. These broken products are believed to result from redox reactions of the quinonelike central chromophoric ring (57). Although relatively non-cell-cycle specific, dactinomycin's action is particularly prominent in the G-1 phase. The cytotoxic action of dactinomycin on rapidly proliferating cells is pronounced, resulting not only in antitumor activity but also in severe toxicities to certain host organs. Figure 3.5 illustrates the intercalation and minor-groove binding of dactinomycins.

Resistance to dactinomycin is primarily attributable to drug export through overexpression of P-glycoprotein and to alterations in tumor cell differentiation mechanisms (58–63).

2.1.7 Biosynthesis. The actinomycins are biosynthesized starting with tryptamine (see Fig. 3.6). This passes through kynurenine to 3-hydroxyanthranilic acid then to 4-methyl-3-hydroxyanthranilic acid. To this last the peptide side chains are added. Oxidative dimerization then results in completion of the phenoxazinone ring chromophore. This process is rather similar to that used in total chemical synthesis of dactinomycin. The unusual amino acids in the side chains provide strong evidence for very significant post-translational modifications. The various D-amino acids are converted from the L-stereoisomers and, in the case of dactinomycin, sarcosine is N-methylated (64). By varying the amino acid composition of the medium, a variety of actinomycin analogs can be made by directed fermentation (65, 66).

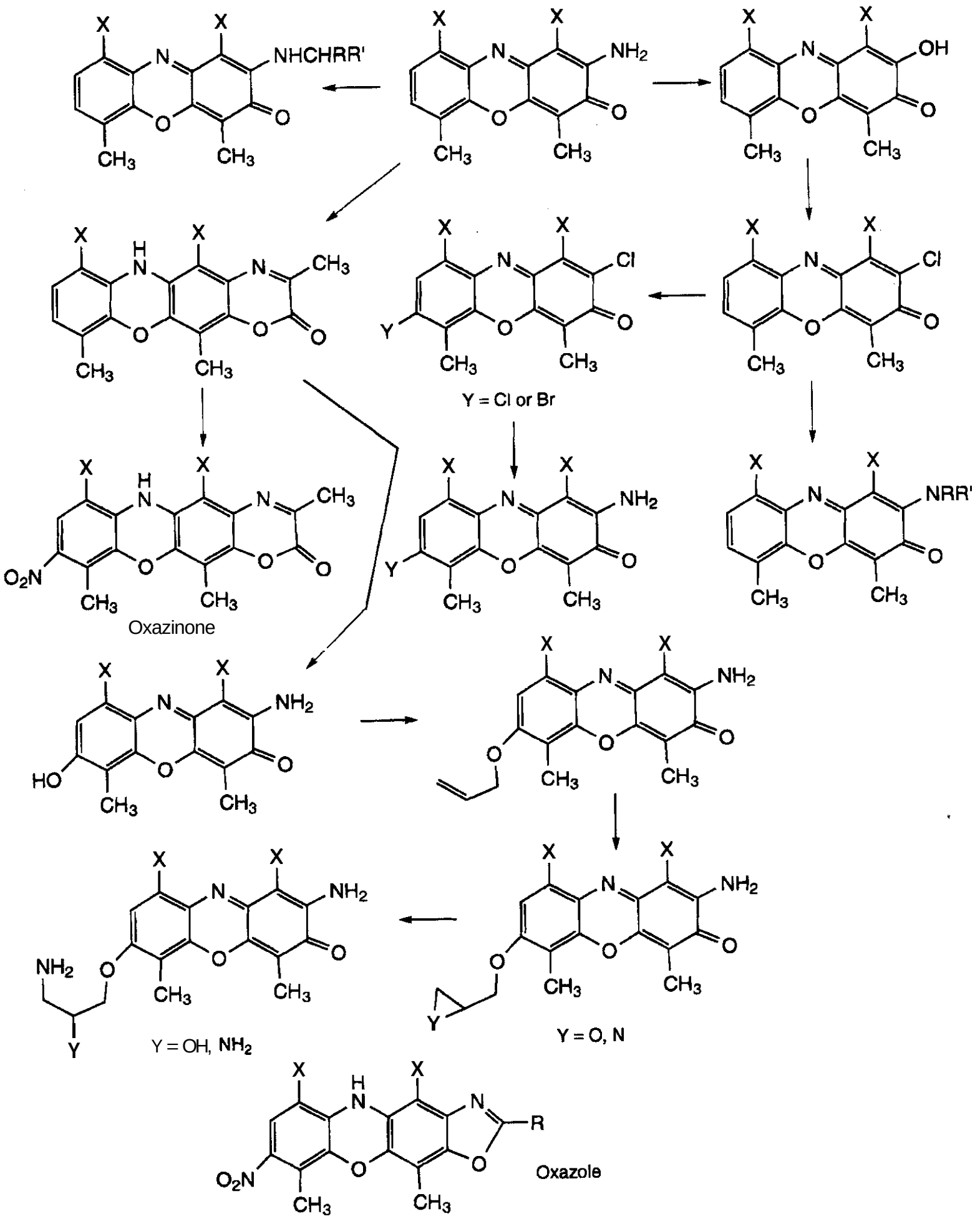
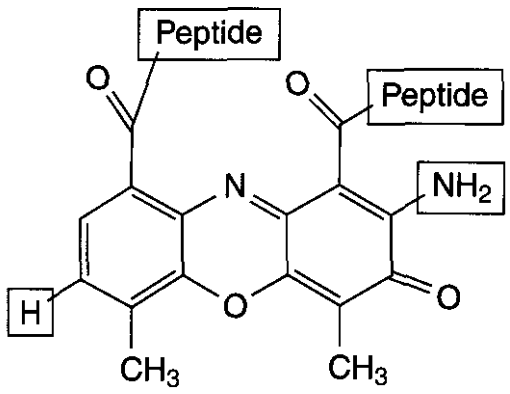


Figure 3.3. Synthesis of dactinomycin analogs.



The "boxed" functional groups can be changed with retention of significant biological activity. Not all such changes, however, are successful.

Figure 3.4. Synopsis of pharmacologically successful transformations of actinomycins.

The chemistry of actinomycins has been the subject of a number of detailed reviews (67–71).

2.2 Bleomycin (Blenoxane)

2.2.1 Introduction. Bleomycin sulfate is a mixture of cytotoxic water-soluble basic glycopeptide antibiotics isolated by the Umezawa group from fermentation broths of *Streptomyces verticillus*. The commercial form consists of cuprous chelates primarily of bleomycins A-2 (3) and B-2 (4). Subsequently, many analogs have been isolated by various groups and been given various names. Among these are the pepleomycins (5), phleomycins, (11) cleomycins, (12) tallysomycins, (13), and zorbamycins (14).

2.2.2 Medicinal Uses. Bleomycin is used intramuscularly (i.m.), subcutaneously (s.c.), i.v., or intrapleurally, often in combination with other antibiotics, for the clinical treatment of squamous cell carcinomas, Hodgkin's disease, testicular and ovarian carcinoma, and malignant pleural effusion. It is also instilled into the bladder for bladder cancer so that less generalized side effects are obtained. It is often coadministered with a variety of other antitumor agents to enhance its antitumor efficacy. One advantage that bleomycin has in such combinations is that it possesses little bone marrow toxicity and is not very immune suppressant, so it is compatible therapeutically with other agents (72–75).

2.2.3 Contraindications and Side Effects. Bleomycin is contraindicated when idiosyncratic or hypersensitive reactions are observed. Immediate or delayed reactions resembling anaphylaxis occur in about 1% of lymphoma patients. Because of the possibility of anaphylaxis, it is wise to treat lymphoma patients with 2 units or less for the first two doses. If no acute reaction occurs, then the normal administration schedule can be followed.

The most severe toxicity of bleomycin is pulmonary fibrosis and is more common with higher doses. This toxicity is observed in about 10% of patients and is difficult to anticipate, hard to detect in its early stages, and in about 10% of those affected it progresses to fatal lung compromise (76–78). Renal damage occurs occasionally and further decreases the rate of excretion of the drug. In rats, bleomycin has been observed to be tumorigenic. In pregnant females, fetal damage can result.

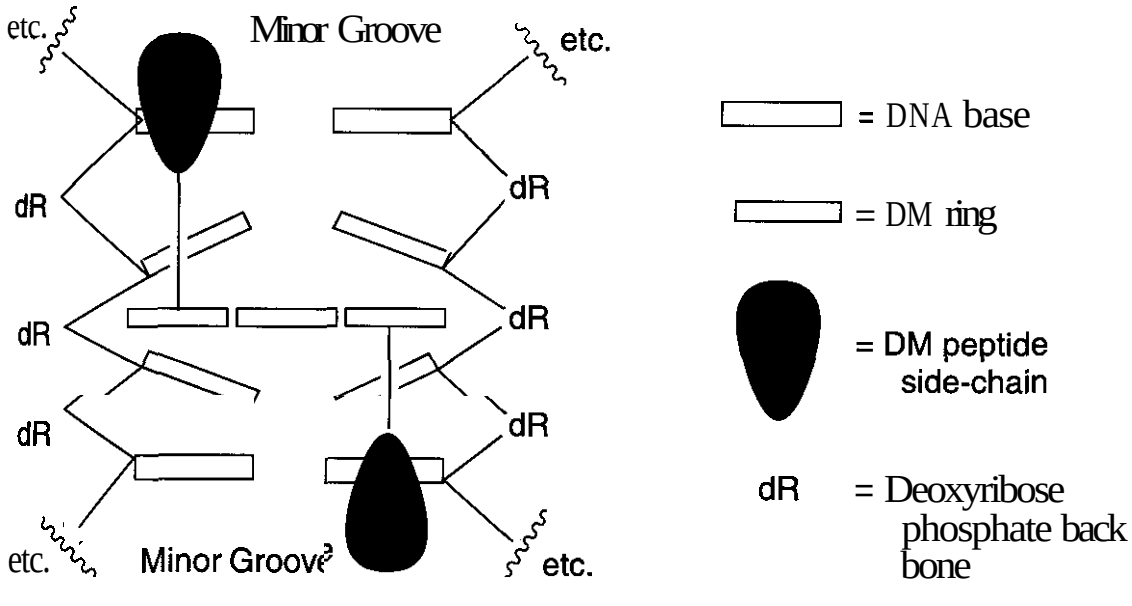


Figure 3.5. Cartoon of intercalation and minor groove binding of dactinomycins.

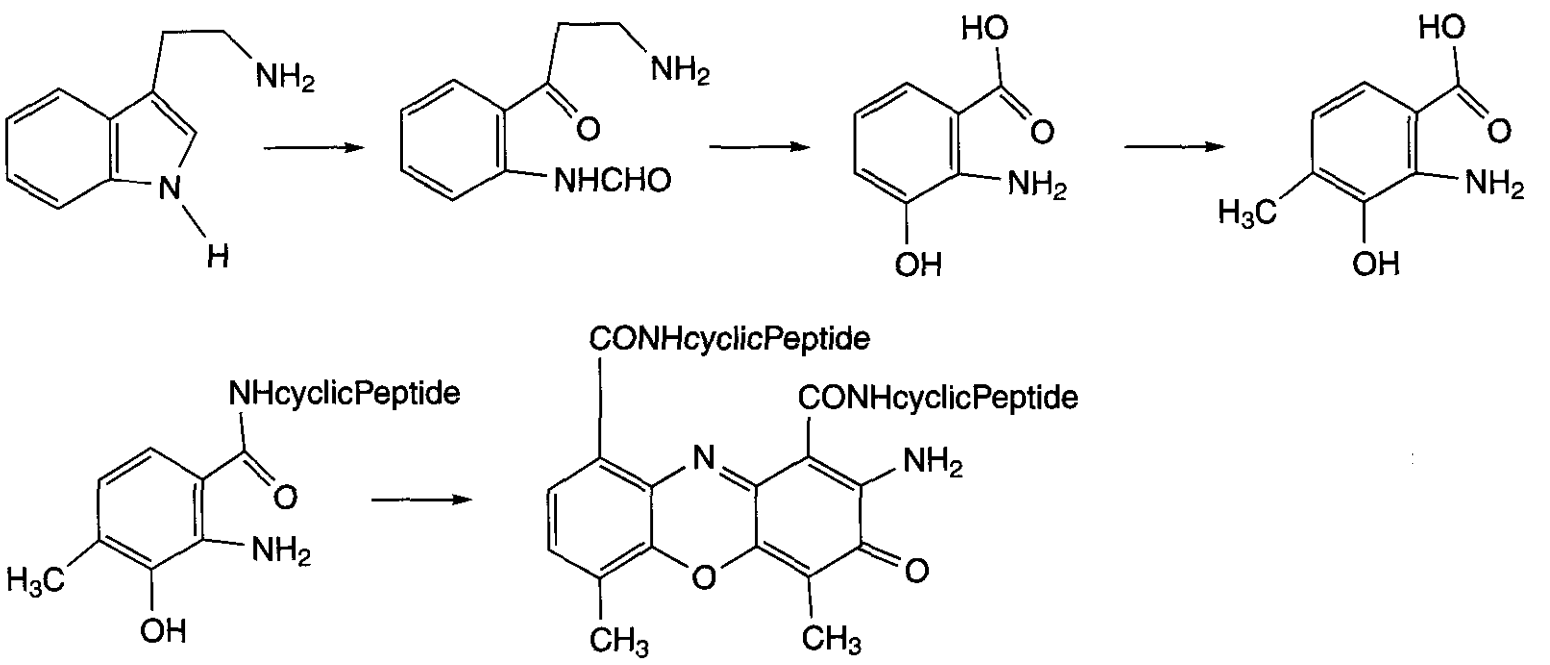


Figure 3.6. Biosynthesis of dactinomycin.

Skin and mucous membrane damage, hair loss, rash, and itching, for example, are not uncommon and may require discontinuation of the drug. In addition, the common constellation of fever, nausea, chills, vomiting, anorexia, weight loss, pain at the tumor site, and phlebitis are seen.

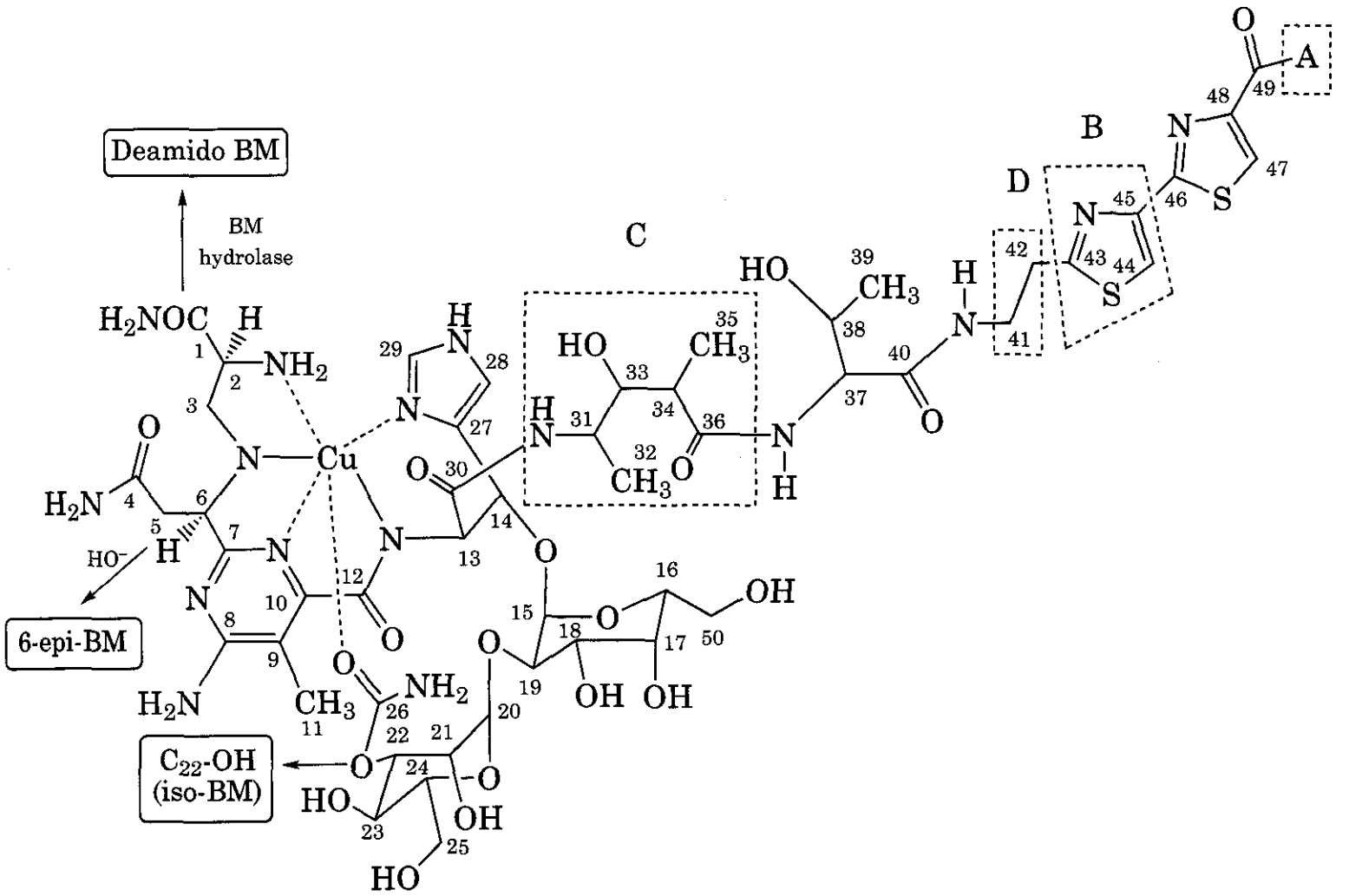
Coadministration with digoxin and phenytoin may lead to a decrease in blood levels.

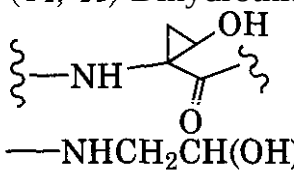
The side effects of bleomycin generally do not reinforce the toxicities of other antitumor agents, so it is often used in anticancer cocktails.

2.2.4 Pharmacokinetic Features. When injected i.v., bleomycin is rapidly distributed and has a half-life of 10–20 min. Intramuscular injections peak in 30–60 min, although the peak levels are less than about one-third those obtained i . ~The overall half-life of bleomycin is about 3 h. Skin and lungs accumulate particularly high concentrations of the drug, in part because these are apparently the only tissues that do not rapidly deactivate it by enzymatic hydrolysis. It does not cross the blood-brain barrier efficiently because of its size and polarity. About 60–70% of the administered dose is recoverable as active bleomycin in the urine. Excretion is progressively delayed when the kidneys are damaged, so the doses are reduced by reference to creatinine levels (79).

2.2.5 Medicinal Chemistry. The essential central core of bleomycin provides a chelating environment for transition metals, especially Cu(I) and Fe(II) (3). The branched glycopeptide side chain is less essential for activity and appears to serve in facilitating passage across cell membranes and to assist in oxygen binding. Removal of the sugars and the oxygen to which they are attached produces molecules that are fully active but distinct from bleomycin itself. The dipeptide unit is a linker arm but contributes key hydrogen bonding and perhaps other binding interactions that intensify activity and produce degrees of base specificity to the cleavages. The bithiazole unit and its pendant terminal cation are important in DNA targeting of the drug. These contributions were uncovered by the chemical synthesis of analogs that could not readily have been prepared by degradation of bleomycin itself or by directed biosynthesis.

Partial chemical synthesis, with or without the aid of enzymes, has also produced a variety of analogs through modifications of this peripheral side-chain array (80–91). Bleomycin is a conglomerate molecule built up from a collection of unusual subunits. Most of these were prepared independently by synthesis, in preparation for ultimate assembly into bleomycin itself or its analogs. The terminal bithiazole and its pendant amides are the portion of the molecule that binds to DNA. For the purpose of making analogs, the charged dimethylsulfonium group is monodemethylated through the agency of heat. The resulting compound is then cleaved to bleomycinic acid (6) by use of cyanogen bromide followed by mild alkaline treatment. Some soil



(3) A = —NH$(CH_2)_3\overset{+}{S}(CH_3)_2X^-$
(4) A = —NH$(CH_2)_4$NHC(NH)NH_2
(5) A = —NH$(CH_2)_3$NH$(CH_2)_2$—2-pyridyl
(6) A = —OH
(7) A = —NH$(CH_2)_3$S(O)CH_3
(8) A = —NH$(CH_2)_3$NH$(CH_2)_4NH_2$
(9) A = —NH$(CH_2)_3$NH$(CH_2)_4$NH$(CH_2)_3NH_2$
(10) A = —NH_2
(11) A = as in **(3)**; B = (44, 45) Dihydrothiazole
(12) A = as in **(3)**; C = 
(13) A = as in **(3)**; C = —NHCH$_2$CH(OH)CH(CH$_3$)CO— ;
D = —CH(β-O-4-amino-4,6-dideoxy-L-tallose)—CH(OH)—
(14) C_{50} = CH_3; A = as in **(3)**; B = as in **(11)**;
C = —NHCH$_2$(CH$_2$OH)CH(OH)CH(CH$_3$)CO— ;

microorganisms possess **acylagmatine amidohydrolase** capable of converting bleomycin to **bleomycinic** acid. Bleomycinic acid is then converted to the desired **amides** by use of water-soluble **carbodimide** chemistry. Whereas the chemical method is capable of producing greater structural variation, in practice the semisynthetic method has proved more convenient.

Although bleomycin and its analogs have also been totally synthesized in various laboratories, the processes are too complex to be of commercial value (92–95).

The phleomycins **(11)** are related in that one of the thiazole rings has been reduced to its **C-44,45-dihydro** analog. The phleomycins have substantial antitumor activity but are too nephrotoxic for clinical use. The **cliomycins** (12), tallysomycins (13), zorbamycins (**14**), zorbonamycins, platomycins, and **victo**mycins are also structurally related to the bleomycins. None of these various alternative substances has displaced the bleomycin complex from the market, even though many possess significant antitumor properties. The

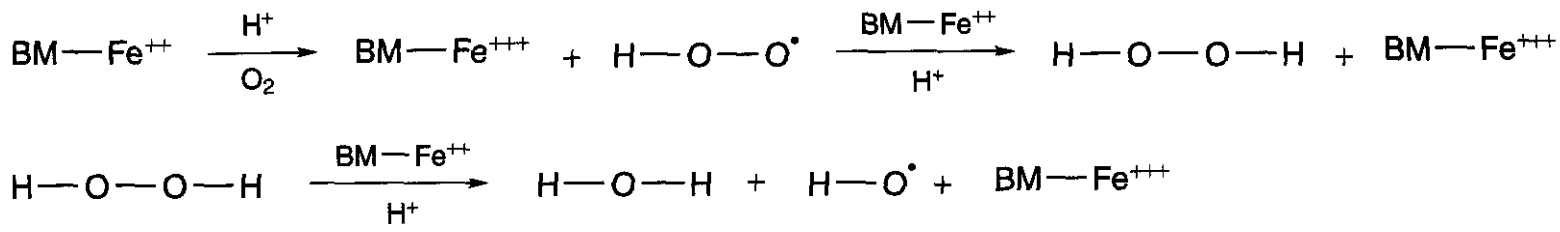


Figure 3.7. Generation of reactive oxygen species by transition metal chelates of bleomycins.

specific potencies and toxicities vary widely with structural variations.

In the presence of a mild base, metal-free bleomycin isomerizes to isobleomycin through an 0-to-0 acyl migration of the carbamoyl moiety from position 22 to 23 of the mannosyl group. Copper (II) bleomycin, under the same conditions, slowly isomerizes at its masked aspartamine moiety attached to the pyrimidine substituent (at C-6). This isomer is substantially less active than bleomycin itself.

Bleomycin chelates with various transition metals, the most relevant of which are iron (II) and copper (I), to form the corresponding complexes. The iron complex binds oxygen and becomes oxidized, producing the hydroxyl radical and the hydroperoxyl radical. This is schematically illustrated in Fig. 3.7. The bithiazole moiety intercalates into DNA and the complex is stabilized by electrostatic attractions between the sulfonium or ammonium side chains with the phosphate backbone of DNA This fixes the drug at DNA, whereupon the reactive oxygen species generated by its transition-metal complex breaks the DNA molecule at the sugar backbone, thus releasing purine and pyrimidine bases. This important reaction is illustrated in Fig. 3.8. Specific details of this complex interaction are still emerging.

Given that the biological action of bleomycin depends collectively on its ability to intercalate, to stabilize the intercalation complex by electrostatic forces, and to complex transition metals capable of generating oxygen radicals, the pharmacophore is distributed through the molecule. Acceptable variations involve substitution of various groups onto bleomycinic acid and a variety of other comparatively trivial changes such as partial reduction of the thiazole moieties and alterations of the amino acids near the bleomycinic acid carboxyl group.

Recently, efforts have been directed to the synthesis of various macromolecular conjugates of bleomycin, in an attempt to produce tissue selectivity and, perhaps, reduce lung toxicity. Some of these agents retain very significant nucleic acid clastogenicity **in** *vitro* (96).

2.2.6 Biosynthesis. Many analogs of bleomycin have been prepared by directed biosynthesis through appropriate media supplementation (97–100). Approximately 10 naturally occurring bleomycins have been reported (3–4, 7–10, etc.). These differ from one another by possessing a variety of different diamino analogs in place of the sulfoniumamino side chain attached to C-49 of bleomycinic acid **(6).** In addition, directed biosynthetic methods involving media supplementation with suitable precursors have produced approximately 21 others, which also consist of a variety of diamino analogs in which the C-49 moiety has been replaced. Thus the biosynthesis of bleomycinic acid is relatively tightly controlled, although the amide synthase that puts on the various side chains is not very specific in its substrate tastes.

2.2.7 Molecular Mode of Action and Resistance. The precise molecular mode of action of bleomycin is incompletely understood because it has numerous actions in test systems. The bleomycins are known to bind preferentially to the minor groove of DNA, although the specific details of this host-guest interaction are still elusive. The cytotoxicity of the bleomycins is enhanced when a DNA-binding region is present and the specific nature of the DNA-binding moiety can convey sequence specificity. The nucleic acid-cleaving capacity is metal ion and oxygen dependent and it is believed that the complexes generate reactive oxygen species that are responsible for the single- and double-strand nucleic acid cleavages observed (see Fig. 3.8). This DNA destruction is generally believed to account for its cytotoxicity

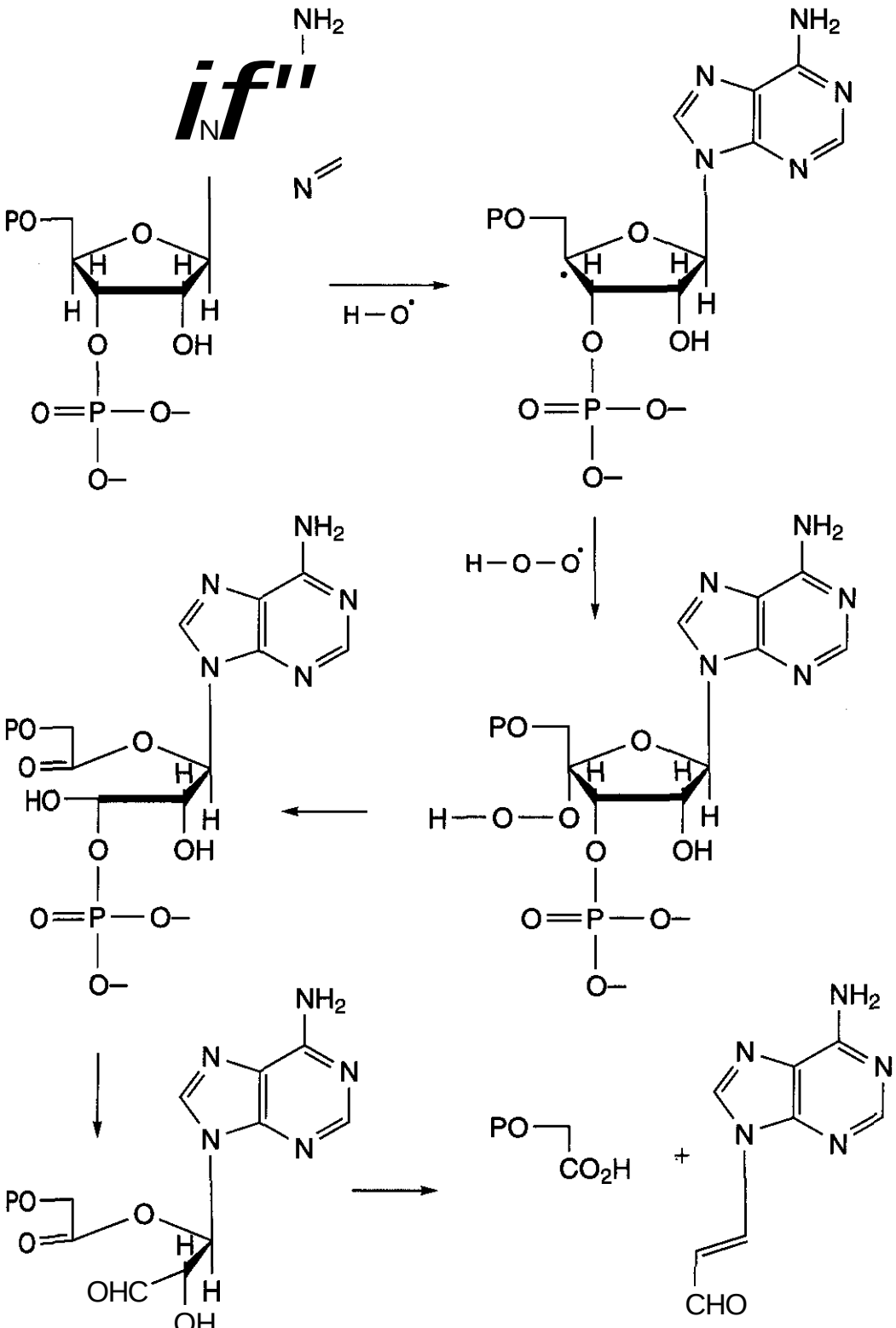


Figure 3.8. DNA backbone cleavage catalyzed by bleomycins.

(101–104). Interestingly, in the absence of DNA, bleomycin is also capable of destroying itself instead, presumably through the action of the same reactive oxygen species (105). A number of artificial analogs have been prepared to explore the contribution of various molecular features of these drugs and to exploit these features. Some of these products include agents that are inert by themselves but that enhance the cytotoxicity of bleomycin fragments when attached craftily to them. These agents usually contain aromatic moieties and have the capacity to have a cationic moiety as well. Bleomycin is known to generate oxygen-based free radicals when chelated to certain metal ions, notably ferrous iron and copper. When chelated to ferric iron, a reducing agent adds an electron to convert the complex to ferrous iron. This, in turn, transfers an electron to oxygen, producing either the superoxide radical or the hydroxide radical (see Fig. 3.7). These radicals attack ribosyl moieties in DNA and RNA, leading to nucleic acid fragmentation and subsequent interference with their biosynthesis. This action is believed to be primary in the cytotoxic action of bleomycin. Bleomycin's action is cell cycle specific, causing major damage in the *G-2* and less in the M phase.

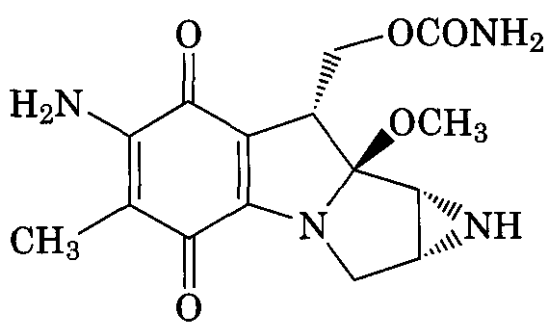
Resistance to bleomycin occurs primarily through the action of bleomycin hydrolase, which attacks metal-free bleomycin at the C-4 carboxamide moiety to produce deamidobleomycin (106). This last produces radicals at a much lower frequency than that of bleomycin itself. This causes a much lower cleavage of DNA and removes the majority of the antitumor action of bleomycin. In support of this idea, resistant cells usually possess a higher concentration of bleomycin hydrolase than do sensitive cells. The hydrolase is present in normal tissues, particularly in the liver. Interestingly, recent evidence implicates this enzyme in the formation of amyloid precursor protein characteristic of Alzheimer's disease (107). Other experts implicate enhanced DNA repair capacity or decreased cellular uptake as contributory to resistance.

2.2.8 Recent Developments and Things to Come. Considering bleomycin's particular ability to destroy DNA and RNA molecules, there is comparatively little likelihood that molecular manipulation of bleomycin will soon produce a nontoxic version of the drug.

The chemical properties of the bleomycins have been reviewed recently (104,108–113).

2.3 Mitomycin (Mutamycin)

2.3.1 Introduction. Mitomycin C (**15**) was discovered initially at the Kitasato Institute (114) and at the Kyowa Hakko Kogyo labora-



(15)

tories in Japan, as a metabolite of *Streptomyces caespitosus* (115), and elsewhere (116). A number of analogs have been discovered at several other places. These drugs are a group of blue aziridine-containing quinones, of which mitomycin C is the most important from a clinical perspective. Mitomycin A and porphiromycin also belong to this group but have not been marketed. Mitomycins apparently were the first of the useful bioreductively activated DNA alkylating agents to be discovered. Literally thousands of alkylating agents, notably the α,β-unsaturated sesquiterpene lactones of the Compositae, have been found in nature, and an enormous effort has been expended in their synthesis and evaluation without notable success. The contrasting success of the mitomycins seems to derive from the finding that they are relatively inert until bioreductively activated, so they show greater biological selectivity compared with that of many other naturally occurring alkylating agents.

2.3.2 Clinical Use. Mitomycin is administered i.v. in combinations of antitumor agents for treatment of disseminated adenocarcinoma of the stomach, colon, or pancreas, or for treatment of other tumors where other drugs have failed (117–120).

2.3.3 Contraindications and Side Effects. It is contraindicated in cases of hypersensitivity or idiosyncratic responses to the drug or where there are preexisting blood dyscrasias. The drug can cause a serious cumulative bone marrow suppression, notably thrombocytopenia and leukopenia (121, 122), that can contribute to the development of overwhelming infectious disease. This requires reducing dosages. Irreversible renal failure as a consequence of hemolytic uremic syndrome is also possible (121). Occasionally adult respiratory distress syndrome has also been seen. When extravasation is seen during administration, cellulitis, ulceration, and sloughing of tissue may be the consequence (123,124). The drug is known to be tumorigenic in rodents. Its safety in pregnancy is unclear and teratogenicity is seen in rodent studies. Other side effects include fever, anorexia, nausea, vomiting, headache, blurred vision, confusion, drowsiness, syncope, fatigue, edema, thrombophlebitis, hematemesis, diarrhea, and pain. It is not clear that all of these are related to the use of mitomycin or whether they are at least partly the consequence of other agents in antitumor cocktails.

2.3.4 Pharmacokinetics. Mitomycin is poorly absorbed orally and is rapidly cleared when injected i.v., with a serum half-life of about 30–90 min after a bolus dose of 30 mg. Metabolism takes place primarily in the liver and is saturable. As a consequence of the saturability, the amount of free drug in the urine increases with increasing doses. Only about 10% of an average administered dose is excreted unchanged in the urine and the bile because extensive metabolism takes place. The drug is distributed widely in the tissues, with the exception of the brain, where very little penetrates (125–128).

Because mitomycin C is activated as an antitumor agent by reduction, significant effort has been expended on trying to decide whether DT-diaphorase activity correlates well with antitumor activity in vivo. This is as yet imperfectly resolved but the correlation appears to be poor. Other studies suggest that NADPH:cytochrome P450 reductase (a quinone reductase) contributes strongly under some circumstances.

Inactivation and activation occur by metabolism and/or by conjugation, and a number of metabolites, principally 2,7-diaminomitosene, have been identified (129–131). The ratio between inactivation and activation is partially a function of whether DNA intercepts the reduced species before it is quenched by some other molecular species.

2.3.5 Medicinal Chemistry. Much exploration of the chemistry of the mitomycins has been carried out accompanied by excellent reviews in the literature (132–134). Total chemical syntheses of mitomycins A and C have been achieved, but these are not practical for production purposes (135–137). More than a thousand analogs have been prepared by semisynthesis but none of these agents has succeeded in replacing mitomycin C itself. Generally, it has been found that mitomycin C analogs are less toxic than mitomycin A derivatives. Most modifications have been achieved at the N-la, C-7, C-6, and C-10 positions. The C-7 position is particularly conveniently altered through addition/elimination sequences, and some of these agents have received extensive evaluation. It is noted that the C-6 and C-7 positions play only an indirect role in the activation of the ring system, so substitutions there might be regarded as primarily significant in altering the pharmacokinetic properties of the mitomycins. It has been found quite recently, however, that the participation of the C-7 substituent in activation by thiols differs significantly when C-7 bears a methoxyl group (the mitomycin A series) compared to the activation when C-7 bears an amino group (the mitomycin C series). Indeed, thiols activate the methoxy analogs but not the amino analogs. Mechanistically, both series arrive at the same bisalkylating species in vivo but through different routes. This may help rationalize why mitomycin A is both more potent and more cardiotoxic than mitomycin C (138). The results of a comparison of physicochemical properties and biological activity of the mitomycins led to the conclusion that potency correlates with uptake, as influenced primarily by log P, and also with the redox potential (E1/2) (139).

The metabolism of mitomycin C in vivo primarily leads through reduction and loss of methanol to a dihydromitosene end product. Interception by DNA, on the other hand, leads to alkylation of the latter instead (138, 139).

2.3.6 Molecular Mode of Action and Resistance. Mitomycin C undergoes enzymatic reductive activation to produce reactive species capable of bisalkylation and crosslinking of DNA, resulting in inhibition of DNA biosynthesis (140–142). This effect is particularly prominent at guanine-cytosine pairs. The reductive activation of mitomycin C makes it particularly useful in anaerobic portions of tumor masses that have a generally reducing environment. Mitomycin is also capable of causing single-strand breaks in DNA molecules.

The apparent chemical mechanism by which mitomycin is reductively alkylated to a bisalkylating agent is illustrated in Fig. 3.9. The process is initiated by a quinone reduction followed by elimination of methanol, opening of the aziridine ring, conjugate addition of DNA, ejection of the carbamate function, and further addition of DNA.

The bisalkylation of DNA can be either intrastrand or interstrand, as illustrated in Fig. 3.10.

Resistance is attributed to failure of reduction (143), to premature reoxidation (143,

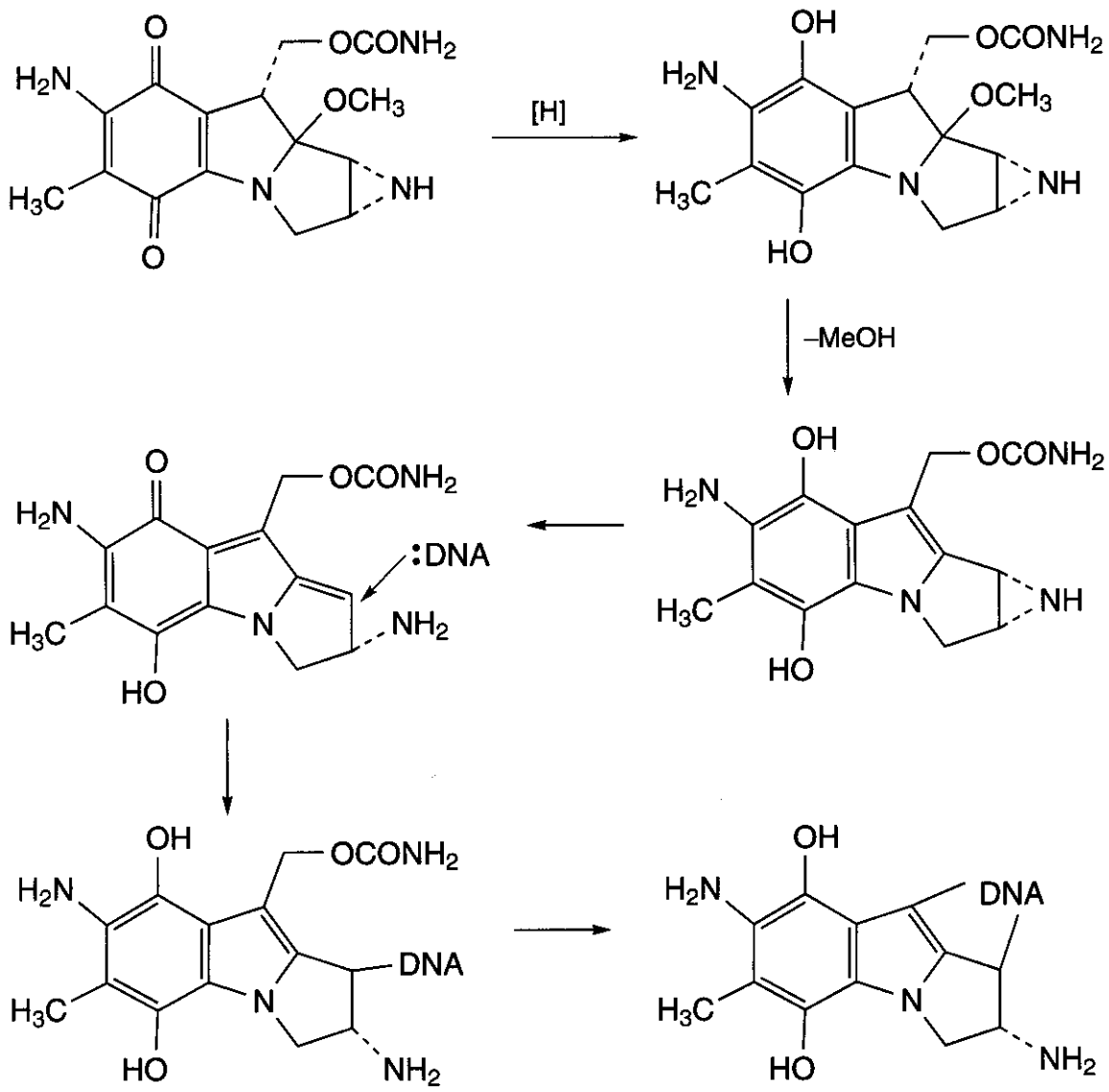


Figure 3.9. Reductive activation and bisalkylation of DNA by mitomycin C.

144), binding to a drug-intercepting protein that also has oxidase activity (145), and to P-glycoprotein–mediated efflux from cancer cells (146, 147).

2.3.7 Medicinal Chemistry. The pharmacologically successful chemical transformations of mitomycin are schematically summarized in Fig. 3.11.

The chemistry and pharmacological actions of the mitomycins have been reviewed (132–134, 148).

2.4 Plicamycin (Formerly Mithramycin; Mithracin)

2.4.1 introduction. Plicamycin (**16**), produced by fermentation of Streptomyces *plicatus* and *S. argillaceus*, was isolated in 1953 (149). It is a member of the aureolic acid family of glycosylated polyketides, which also includes chromomycins, chromocyclomycins, olivomycins, and UCH9. It was subsequently

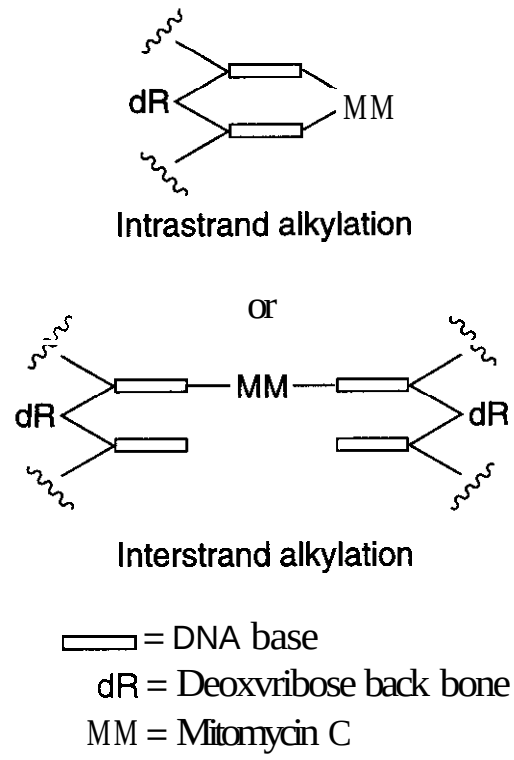
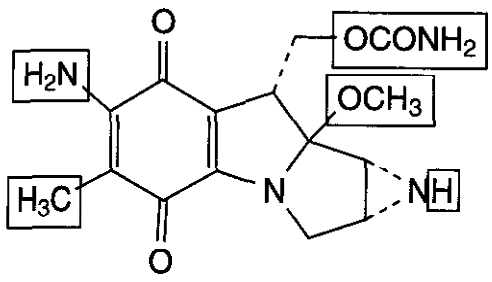


Figure 3.10. Interstrand and intrastrand alkylation of DNA by bioreductively activated mitomycin C.



The "boxed" functional groups can be changed with retention of significant biological activity. Not all such changes, however, are successful.

Figure 3.11. Pharmacologically successful modifications of mitomycin C.

found to be identical to mithramycin, a fermentation product of *S. argillaceus* and *S. tanashiensis*.

2.4.2 Clinical Uses. Plicamycin is highly toxic but is nevertheless administered i.v. for treatment of testicular tumors (150–153). In lower doses it is used for treatment of hypercalcemia and hypercalciuria associated with advanced cancer, particularly involving Paget's bone disease (154–157).

2.4.3 Contraindications and Side Effects. Severe thrombocytopenia, hemorrhagic tendency, and death can be encountered with the use of plicamycin (158, 159). Renal impairment, mutagenicity, and interference with fertility are also known to occur with the use of plicamycin. Anorexia, nausea, vomiting, diarrhea and stomatitis, fever, drowsiness, weakness, lethargy, malaise, headache, depression phlebitis, facial flushing, skin rash, hepatotoxicity, and electrolyte disturbances (decrease in serum calcium, potassium, and phosphate levels) are also encountered.

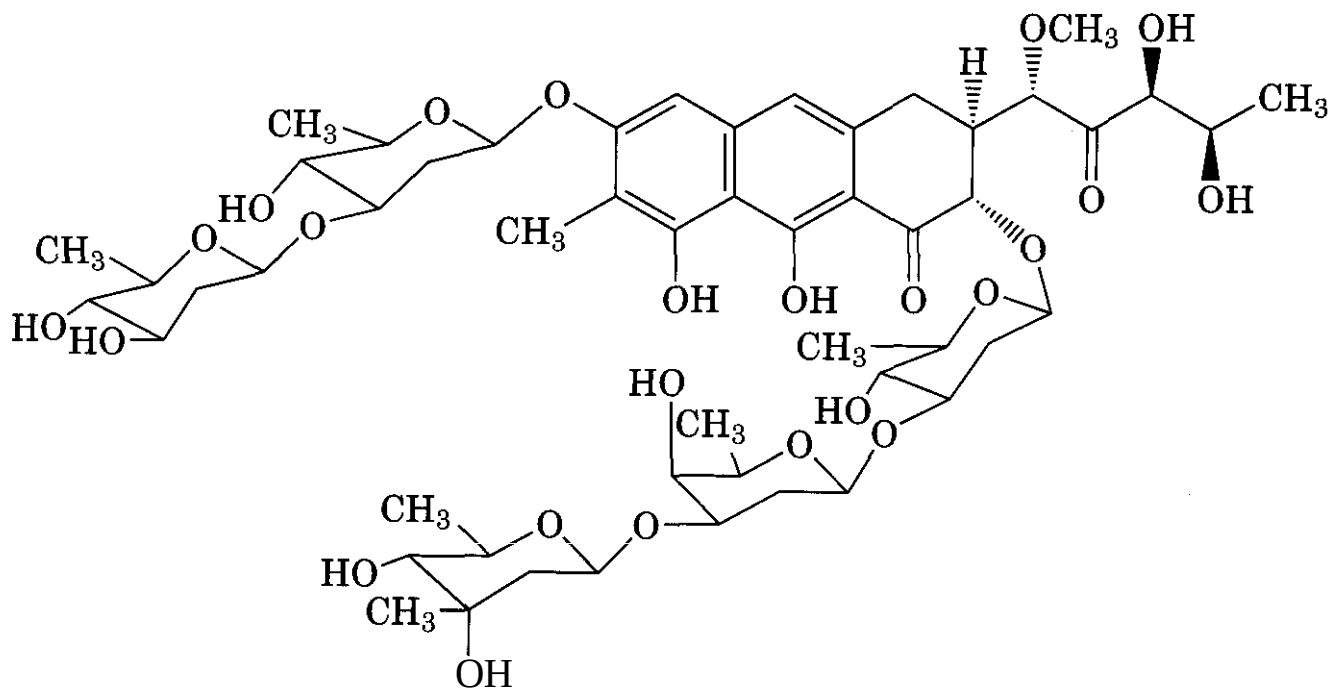
Plicamycin is contraindicated with coagulation disorders, thrombocytopenia, thrombocytopathy, impairment of bone marrow function, and in pregnancy.

The toxic reactions of plicamycin are much less severe and frequent in the lower dosages employed to lower calcium ion levels.

2.4.4 Pharmacokinetics. Plicamycin is given i.v., whereupon a complex excretion pattern ensues, with a half-life of approximately 11 h having been reported (160).

2.4.5 Mode of Action and Resistance. The exact mechanism of action of plicamycin is elusive but it is known to intercalate into DNA, favoring G-C base pairs, resulting in the inhibition of enzymes that process DNA (161–163). Plicamycin also interferes in the biosynthesis of RNA (163). The effect of plicamycin is enhanced in the presence of divalent metal ions such as magnesium (II). Its hypocalcemic action is unrelated to this but is rather mediated by interference with the function of vitamin D in some unclear manner (164). Plicamycin also acts on osteoclasts and blocks the action of parathyroid hormone (165, 166).

Resistance to plicamycin involves efflux



(16)

through the action of P-glycoprotein (167), although recent publications suggest that plicamycin has the capacity to suppress *MDR* 1 gene expression in vitro, thereby modulating multidrug resistance (168).

2.4.6 Medicinal Chemistry. The chemistry of plicamycin and its analogs has been reviewed (169). For a long time there was considerable confusion about the precise chemical structure of plicamycin (mostly with respect to the number and arrangement of the sugars) but this has now apparently been resolved by careful NMR studies (170).

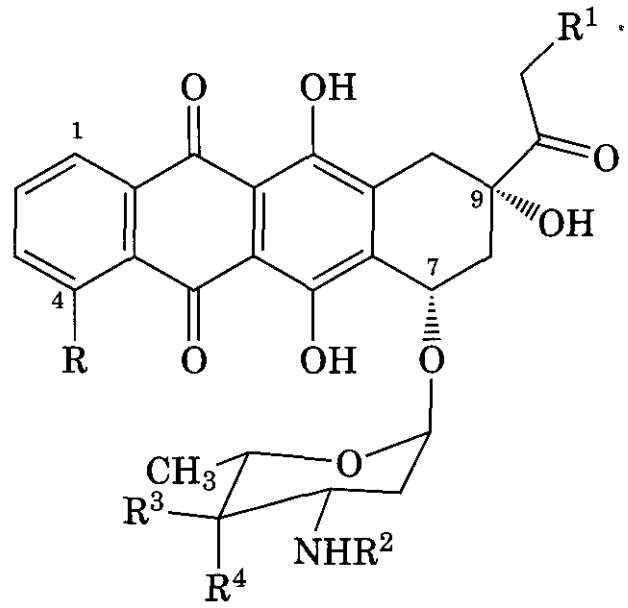
The sugars must be present in plicamycin for successful DNA binding and magnesium ion also promotes the interaction.

2.4.7 Biosynthesis. Biosynthesis of the aureolic acid group of antitumor antibiotics begins with condensation of 10 acetyl units to produce a formal polyketide that, on condensation, produces a tetracyclic intermediate whose structure and that of the subsequent intermediates is reminiscent of those involved in tetracycline biosynthesis (171). After the formation of premithramycinone, a rather complex sequence of reactions ensues, as illustrated in Fig. 3.12. A sequence of methylations and glycosylations lead to premithramycin A3. Of particular interest in the remaining sequence is an oxidative ring scission and decarboxylation, which leads to the final tricyclic ring system. This is followed by oxidation level adjustment, producing plicamycin itself, or to one of the other members of this class, depending on the specifics of the biosynthetic intermediates (172, 173). Omission of the key C-7 methylation step leads, for example, through a parallel pathway to the formation of 7-demethylmithramycin (174).

3 DRUGS INHIBITING ENZYMES THAT PROCESS DNA

3.1 Anthracyclines

The anthracyclines are an important class of streptomycete-derived tetracyclic glycosidic and intercalating red quinone-based drugs. None of the first generation of this widespread class of natural products became clinically prominent. The structures of some of these chemically interesting compounds, generally named as rhodomycins, including pyrromycin, musettamycin, and marcellomycin (whose names will please opera buffs), are given in Fig. 3.13. Those anthracyclines of clinical value were discovered initially in the Pharmitalia Laboratories in Italy and subsequently in a number of other places (175, 176). The first of the clinically useful group was the *Streptomyces* peucetius metabolite, daunorubicin (18). This was followed by its hydroxylated analog doxorubicin (17), a metabolite of S. *peuceteus* var. *caesius*. Many synthetic anthracyclines resulted from intense study in many laboratories. These synthetic methods led to a number of marketed products, including daunomycin's desmethoxy analog idarubicin (20) and doxorubicin's diastereomer epirubicin (19) (177, 178), and the bisacylated product of doxorubicin, valrubicin (21). Daunomycin and idarubicin are primarily used for the treatment of acute leukemia, and epirubicin is used for solid tumors, but doxorubicin is used for a much wider range of cancers.



Compd.	R	R^1	R^2	R^3	R^4
(17)	OCH_3	OH	H	H	OH
(18)	OCH_3	H	H	H	OH
(19)	OCH_3	OH	H	OH	H
(20)	H	H	H	H	OH
(21)	OCH_3	OCOBu	$COCF_3$	H	OH

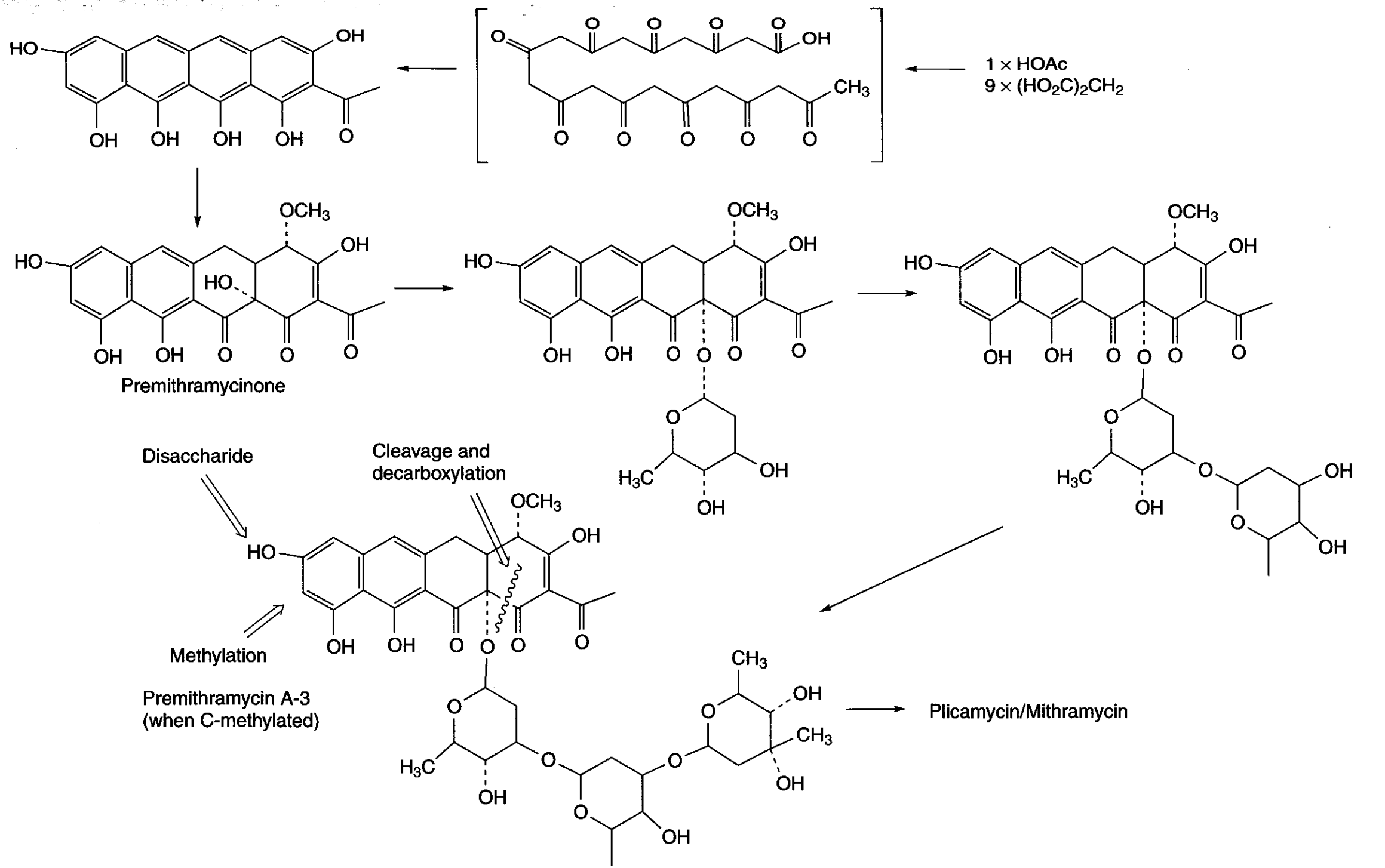


Figure 3.12. Biosynthesis of plicamycin.

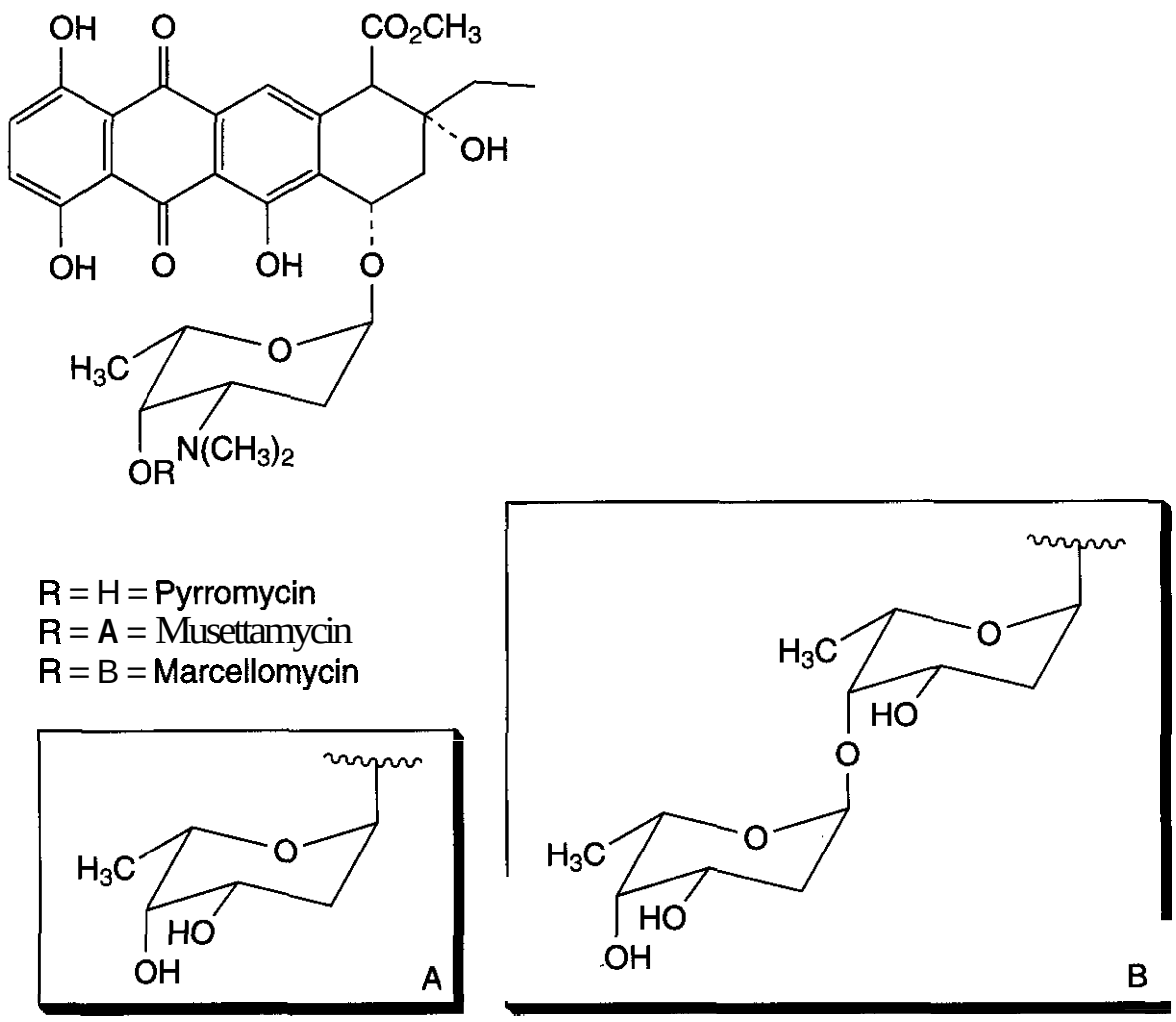


Figure 3.13. Structures of some unmarketed **anthracyclines**.

3.1.1 Daunorubicin (Daunomycin; Cerubidine, Rubidomycin; 18)

3.1.1.1 Therapeutic Uses. Daunorubicin is used in combination with other agents by i.v. infusion for treatment of acute myelogenous and lymphocytic leukemias (179–181).

3.1.1.2 Side Effects and Contraindications. It is not generally used i.m. or s.c. because of the severe tissue damage that may accompany extravasation (182). It is contraindicated when hypersensitivity reactions are present. Among the side effects that are encountered are severe cumulative myocardial toxicity that can include acute congestive heart failure after cumulative doses above 400–550 mg/m^2 of body surface in adults and less in infants (183), severe myelosuppression (hemorrhage, superinfections), bone marrow suppression, secondary leukemia, renal/hepatic failure, carcinogenesis, mutagenesis, teratogenicity, and fertility impairment. The cardiomyopathy is characteristic of the anthracycline class and can occur long after therapy is concluded (184, 185). The highly colored nature of the drug can lead to urine discoloration that alarms the patient because of drug excretion. In addition, alopecia, rash, contact dermatitis, urticaria, nausea, vomiting, mucositis, diarrhea, abdominal pain, fever, chills, and (occasionally) anaphylaxis are observable. When given along with cyclophosphamide, its cardiotoxicity is enhanced and enhanced toxicity is seen when given concurrently with methotrexate.

3.1.1.3 Pharmacokinetics. On i.v. administration the drug is rapidly distributed into tissues but does not enter the central nervous system. Rapid liver reduction to daunomycinol is seen followed by hydrolytic or reductive loss of the sugar along with the oxygen atom with which it is attached to the ring system. These two reactions also can take place before reduction. Demethylation of the 0-methyl ether moiety also occurs followed by sulfation or glucuronidation of the resulting phenolic OH. These and other transformation products have lesser bioactivity (186). Patients with decreased liver function should receive smaller doses because they are not able to detoxify the drug effectively. The half-life is about 8.5 h and about 25% of the active drug is found in the urine along with about 40% in the bile (187). Liposomally encased daunorubicin ci-

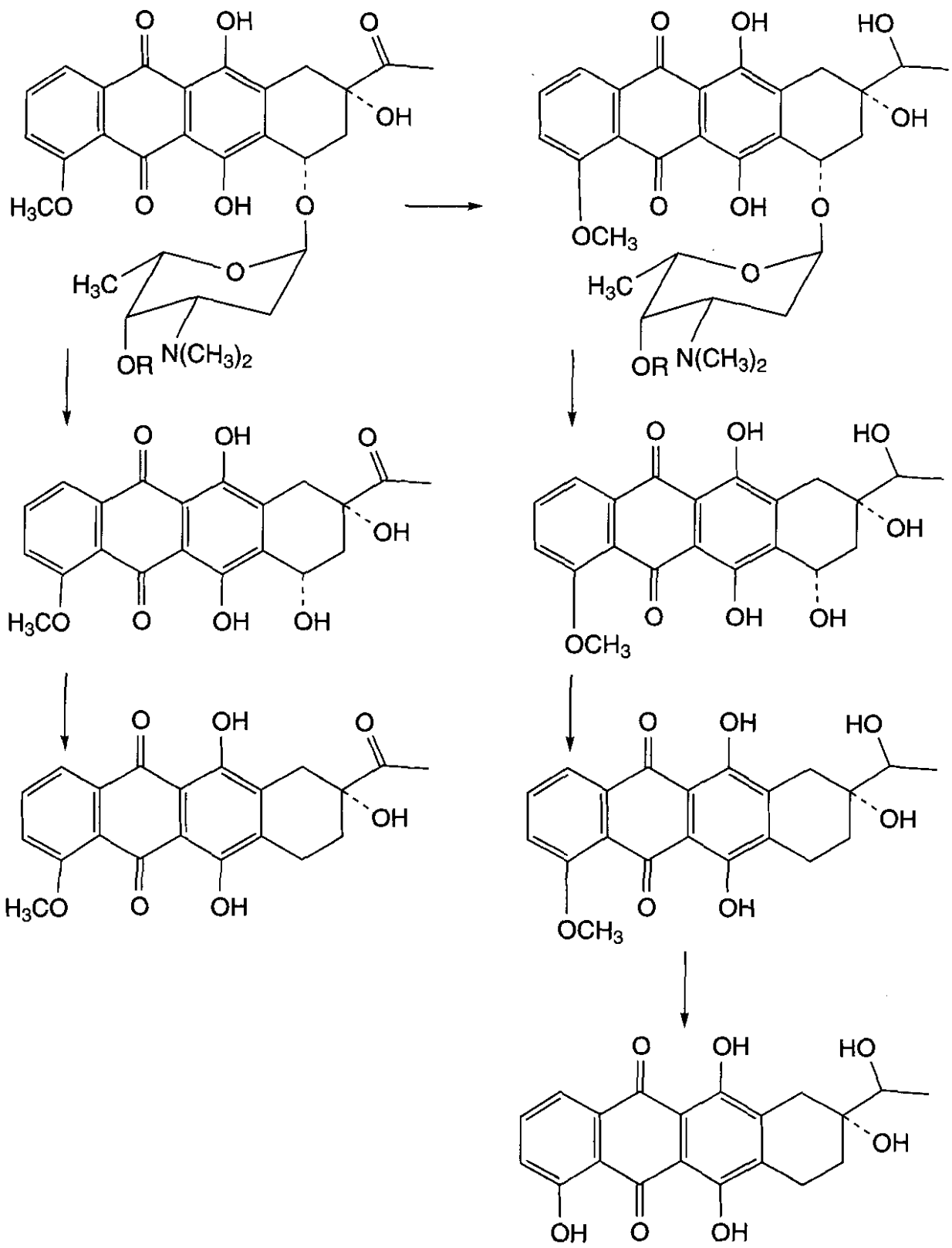


Figure 3.14. Metabolism of daunorubicin.

trate shows greater selectivity for solid tumors and is translocated in the lymph (180). Nuclear binding of anthracyclines is sufficiently strongto complicate the excretion pattern and to determine the tissue distribution of these agents (188). Different tissues bind doxorubicin in direct proportion to their **DNA** content. The metabolism of daunorubicin is illustrated in Fig. 3.14.

3.1.1.4 Mechanism of Action and Resistance. The mode of action of daunorubicin and the other clinically useful anthracyclines is multiple. Authorities differ with respect to which is the most significant but most attribute this to inhibition of the mammalian topoisomerase II, essential for shaping **DNA,** so that it can function and be processed (189). The drug also intercalates into DNA, inhibits **DNA** and **RNA** polymerases, and also causes free-radical single- and double-strand damage to **DNA** (190). These drugs are, therefore, also mutagenic and carcinogenic. Free-radical (reactive oxygen species) generation is promoted by the interaction of these drugs with P450

(191) and with iron, which they chelate (192). The reactive oxygen species that they can generate also cause severe damage to membranes and this may contribute not only to their antitumor efficacy but also to the cardiomyopathy that they cause (193).

Resistance to daunorubicin and the other anthracyclines is attributed to efflux mediated by P-glycoprotein, whose expression is amplified in response to their use (187, 194–196). A number of other mechanisms have been advanced as contributory such as use of other export mechanisms, increased endogenous antioxidant mechanisms, and decreased action of mammalian topoisomerase II (197).

3.1.2 ***Doxorubicin.*** Doxorubicin [adriamycin, rubex; (**17**)] is a hydroxylated analog of daunorubicin but finds much wider anticancer use.

3.1.2.1 Therapeutic Uses. Doxorubicin is given i.v. by rapid infusion for the treatment of disseminated neoplastic conditions such as acute lymphoblastic leukemia, acute myeloblastic leukemia, Wilms' tumor, neuroblastoma, soft tissue and bone sarcomas, breast carcinoma, ovarian carcinoma, transitional cell bladder carcinoma, thyroid carcinoma, Hodgkin's and non-Hodgkin's lymphomas, bronchogenic carcinoma, and gastric carcinoma.

3.1.2.2 Side Effects and Contraindications. Doxorubicin is contraindicated in patients with preexisting severe myelosuppression consequent either to other antitumor treatments or to radiotherapy. It is also contraindicated when hypersensitivity to anthracyclines is present or when significant previous doses of other anthracyclines have been administered, given that their doses coaccumulate toward congestive heart failure.

Side effects are generally similar to those seen with daunorubicin (which see), with particular reference to cumulative drug-related congestive heart failure, extravasation problems, myelosuppression, and hepatic damage.

3.1.2.3 Pharmacokinetics. As with daunorubicin, the tissue distribution of doxorubicin is strongly influenced by the cellular content of DNA in various parts of the body (188). Metabolites of doxorubicin are its aglycone, its deoxyaglycone, doxorubicinol and its deoxyaglycone, and demethyldeoxyadriamycinol aglycone as its 4-*O*-β-glucuronide and 0-sulfides. Thus carbonyl reduction is the main metabolic reaction and this is followed by various hydrolytic and reductive losses of the sugar, O-demethylation, and various conjugative reactions (198). These reactions quite parallel the findings with those of doxorubicin.

3.1.2.4 Molecular Mode of Action and Resistance. The manifold cytotoxic actions of doxorubicin on cells are qualitatively the same as those of daunorubicin. Likewise, the resistance mechanisms, especially those involving P-glycoprotein expulsion, are closely similar. Interestingly, expulsion is significantly lessened by liposome encapsulation (199).

3.1.3 ***Epirubicin.*** Epirubicin (Ellence, (19) is a C-4'-diastereoisomer of doxorubicin given by i.v. infusion as an adjunct to the use of other agents for the treatment of breast cancer, when axillary node tumor involvement is seen after breast removal surgery (200). The toxicities of epirubicin are analogous to those described above for daunorubicin and doxorubicin (which see). Particular note should be paid to drug-related cumulative congestive heart failure, extravasation problems, myelosuppression, and hepatic damage.

3.1.4 Valrubicin. Valrubicin (21) and idarubicin (20) are also anthracyclines that have seen significant clinical use (201). Idarubicin differs from doxorubicin in lacking the methoxy group in the chromophore and has an epimeric hydroxyl group in the sugar (202). This molecule is comparatively lipophilic, resulting in increased cellular uptake (the cellular concentrations exceed 100 times those achieved in plasma) (203) and strong serum protein binding (204). Extensive extrahepatic metabolism to the 13-dihydro analog occurs (205).

Valrubicin is the valeric ester trifluoroacetic amide of doxorubicin (206–208). It is instilled into the bladder through a urethral catheter after bladder drainage and is voided after 2 h (209). It is highly toxic on contact with tissues but its means of administration limits systemic exposure. Its local adverse reactions are usually comparatively mild and resolve in about 24 h. Evidence indicates that

the valeryl ester moiety is removed enzymically in *vivo* before exerting its cytotoxic effect (210, 211).

3.1.4.1 Biosynthesis. The anthracyclines are polyketides, as can be readily discerned from their structures. Doxorubicin is produced from daunorubicin by a late hydroxylation step that is genetically unstable. As a consequence, it is apparently produced commercially by an efficient chemical transformation instead of by fermentation (212).

3.1.4.2 Medicinal Chemistry, Many hundreds of analogs have been prepared either by chemical transformation of the natural products themselves or by total synthesis. As a result a reasonable understanding of their structure-activity relationships is at hand (213–218).

The impressive anticancer activity and clinical potential of the anthracyclines resulted in intensive research toward total synthesis and structural modification studies of these compounds (175,176,219,220). From a structure-activity relationship (SAR) viewpoint, the anthracycline structural core can be divided into three major components: (1) Ring D, the alicyclic moiety bearing the two-carbon side-chain group and the tertiary hydroxy group at C-9, concomitantly having a chiral secondary hydroxy group at C-7, which in turn is connected to the aminosugar unit; (2) the aminosugar residue, attached to the C-7 hydroxy group through an a-glycosidic linkage; (3) the anthraquinone chromophore, consisting of a quinone and a hydroquinone moiety on adjacent rings. The C-13 and C-14 positions of the various anthracyclines are obvious functional sites for derivatization. Thus, the 13-keto functionality has been subjected to reduction, deoxygenation, hydrazide formation, and so forth, without adversely affecting the bioactivity. Similarly, incorporation of various ester and ether functionalities at C-14, through initial halide formation and subsequent displacement of the halogen with nucleophiles, was found to be a useful approach in modulating the activity of the parent anthracyclines. However, homologation of the C-9 alkyl chain or introduction of amine functionalities at C-14 is detrimental to activity. Additionally, formation of 9,10-anhydro or the 9-deoxy analogs results in decreased activity. Interestingly, the natural stereochemical configurations at C-7 and C-9 were found to be an important contributor to bioactivity, wherein it has been proposed that H-bonding between the two cis-oxygen functionalities at these positions stabilizes the preferred half-chair conformation of the D-ring.

The amino sugar residue of the various anthracyclines is an essential requirement for bioactivity. Among the various SAR studies involving the carbohydrate core, it has been seen that attachment of this moiety to the anthracycline nucleus through an α-anomeric bond is necessary for optimum activity. Conversion of the C-3' amine group to the corresponding dimethylamino or morpholino functionalities confers improved activity; however, acylation of the amine (the exception being trifluoroacetyl) or its replacement with a hydroxy group results in loss of activity. Interestingly, conversion of the C-4' hydroxy group to its corresponding methyl ether, C-4' epimerization, or deoxygenation has a negligible effect on bioactivity. In more recent studies, novel disaccharide analogs of doxorubicin and idarubicin have been found to exhibit impressive antitumor activity (221).

The anthraquinone chromophore is an important structural feature of the anthracyclines. The various oxygenated functionalities present in this fragment have been the focus of considerable synthetic activity in search of analogs with improved activity. Thus, the phenolic hydroxy groups present in this core were found to undergo ready acylation and alkylation under standard reaction conditions. It has been shown that, 0-methylation of the C-6 or C-11 phenolic groups results in analogs with markedly reduced activity, whereas C-4 modifications such as demethylation and deoxygenation do not affect bioactivity. Interestingly, a serendipitous transformation of the C-5 carbonyl to the corresponding imino functionality resulted in an analog that retained activity and was found to be significantly less cardiotoxic than the parent compound.

3.1.4.3 Biosynthesis. The proposed biogenesis of the anthracyclines invokes the involvement of a polyketide synthon. In studies involving various blocked mutants of anthracycline-producing Streptomyces and utilization of ^{14}C-labeled acetate and propionate pre-

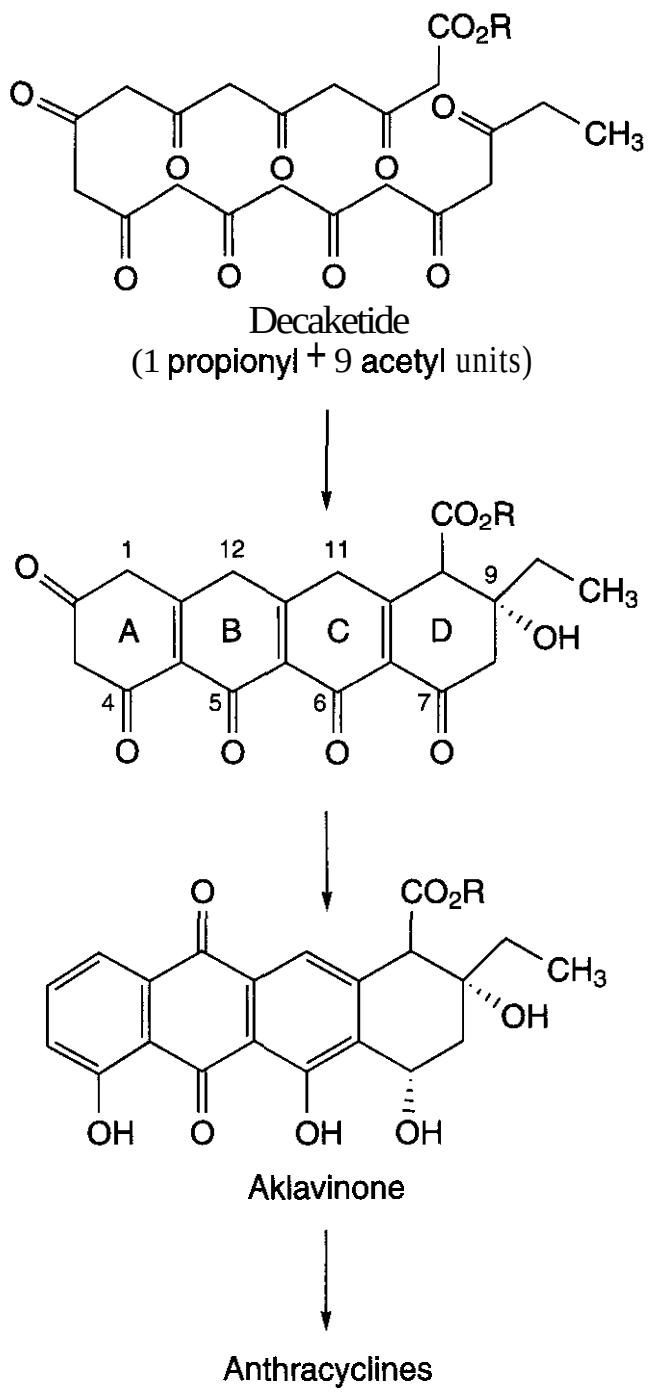


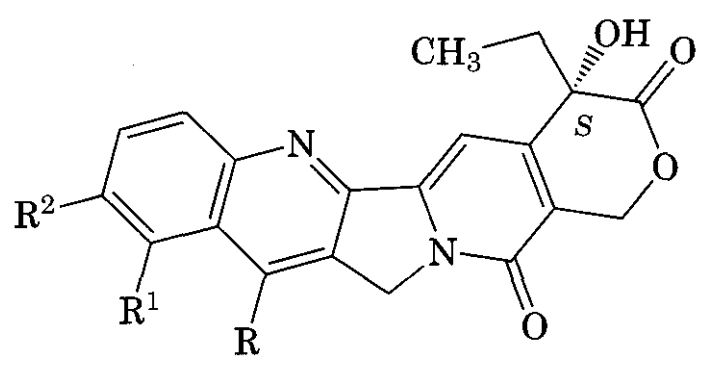
Figure 3.15. Proposed biosynthetic pathway leading to anthracyclines.

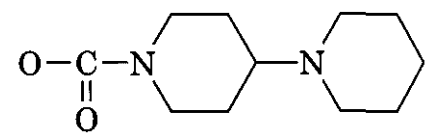
cursors, it has been shown that there are two biosynthetic pathways responsible for the formation of the polyketide fragment. Daunomycinone, pyrromycinone, and related aglycones are derived from a polyketide synthon having one propionate and nine acetate units, whereas deviant members such as steffimycinone and nogalanol are obtained from a 10-acetate polyketide unit. Thus, a "head-to-tail" condensation of the decaketide chain forms the parent tetracyclic core and the C-9 quaternary center of the anthracyclines. A sequence of biotransformations involving C-2 and C-7 carbonyl reduction, dehydration (C-2/C-3), enolization/aromatization, and B-ring oxidation leads to aklavinone. Further oxidation, decarboxylation (for some class of compounds), and glycosidation finally result in the corresponding bioactive glycosides. See Fig. 3.15 for a schematic illustration of the proposed biosynthetic pathway leading to anthracyclines.

3.1.4.4 Recent Developments and Things to Come. Reviews of this topic are available (203,210, 222).

3.2 Camptothecins

Camptothecin (22) was discovered almost at the same time (1966) as was taxol and by the same research group (223). It is present in the extractives of the Chinese tree Camptotheca *acuminata* (growing in California) and has subsequently been found to be abundant in the extractives of Mappia foetida, a weed that grows prolifically in the Western Ghats of India. Despite its early promise in laboratory and rodent studies, it was disappointing in clinical studies because of severe toxicity and so it has not found clinical use by itself, but serves as the inspiration for the preparation of its clinical descendants prepared both by partial and total chemical synthesis methods. Camptothecin itself is very insoluble. This made early evaluation difficult. Tests were performed on its sodium salt (prepared by hydrolysis of the lactone ring) but clinical trials of this salt had to be discontinued because of severe, unpredictable hemorrhagic cystitis, even though some patients with gastric and colon cancers were responding to the drug. A quiet period followed. Much later came a re-



Compd.	R	R^1	R^2
(22)	H	H	H
(23)	H	$CH_2N(CH_3)_2$	OH
(24)	Et	H	

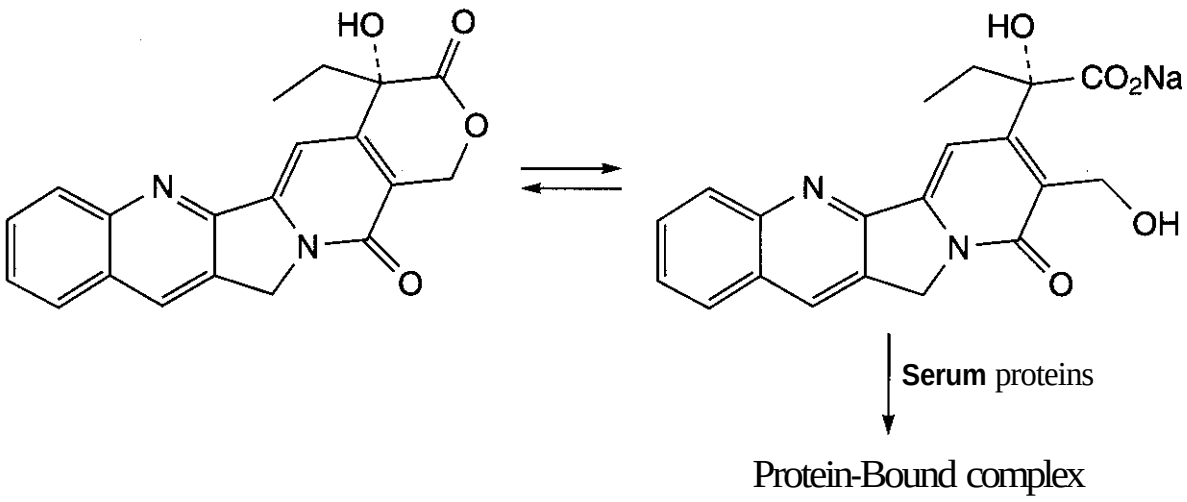


Figure 3.16. Hydrolysis of camptothecin analogs.

surgence of interest because of the discovery that the drug works by inhibiting nuclear mammalian topoisomerase I, a novel mechanism of action among contemporary antitumor agents (224). Topoisomerase I is a ubiquitous enzyme essential for changing the twisting number of DNA molecules (relaxing supercoils) so that they can be transcribed and repaired. The levels of topoisomerase I are often raised in tumor cells. Topoisomerase I exerts its action by making transient single-strand breaks in duplex DNA, rotating the molecule, and resealing again. Camptothecin and its analogs form a ternary complex with the cut DNA and topoisomerase I, which prevents progression or regression. The cut DNA is unavailable to the cell, so that it is stranded in the S-phase of the cell cycle and the DNA is degraded, thus leading to cell death.

Camptothecin itself is very water insoluble, thus impeding its use by injection. Furthermore, it is quite unstable in the body because of ease of hydrolysis of the lactone ring under physiological conditions, to produce the highly toxic acid analog (Fig. 3.16). The ring-opened form is also highly serum protein bound, helping to account for its comparatively poor activity *in vivo*. This high level of binding also displaces the equilibrium further in the direction of the undesirable ring-opened acid form. These factors apparently are less limiting in mice, producing a significant species difference in behavior. This raised the level of disappointment when, despite favorable animal studies, the drug performed poorly in the clinic. Many analogs were subsequently prepared by total synthesis and by conversions of camptothecin itself. The more promising of these newer analogs are much more soluble in water and less serum protein bound, helping them to overcome some of the defects of camptothecin itself.

Metabolism. Hydrolysis to the less-active and toxic ring-opened lactone occurs readily *in vivo* under physiological conditions (Fig. 3.16). Further, the lactone binds to serum proteins approximately 200 times more than does camptothecin itself. By mass action, this shifts the equilibrium toward ring opening. The lactone-opened analogs are significantly more water soluble than the lactone forms but are generally rather less active.

3.2.1 Irinotecan (CPT-11)

3.2.1.1 Clinical Uses. Irinotecan (24) is an analog hydroxylated in the quinoline ring and further converted to an amine-bearing prodrug carbamate linker. It is given by i.v. infusion, often in combination with 5-FU and leucovorin (which combination is particularly toxic) for the treatment of metastatic carcinoma of the colon or rectum (225). Irinotecan and its metabolites are much less serum protein bound than topotecan and have a somewhat longer half-life in serum. Irinotecan, however, is poorly orally bioavailable and is also subject to a significant first-pass metabolism.

3.2.2 Topotecan

3.2.2.1 Clinical Uses. Topotecan (23) is used for ovarian (226,227) and small-cell lung cancers (228–235). Topotecan is rapidly me-

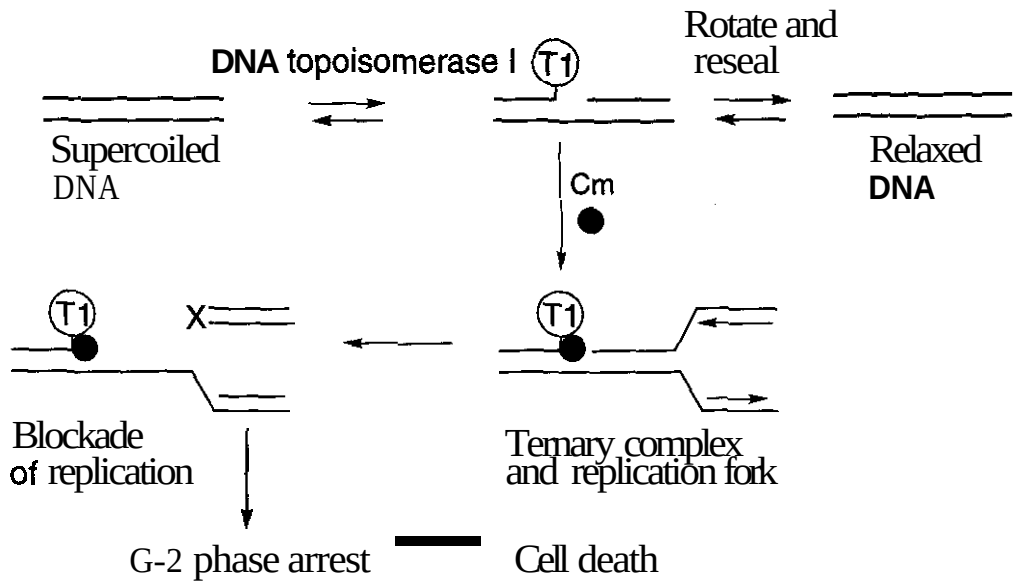


Figure 3.17. Schematic of camptothecin molecular mode of action.

tabolized by hydrolysis and the majority of the drug (75–80%) is hydrolyzed in the plasma, with a half-life of a couple of hours. *N*-Demethyltopotecan and its glucuronide are found to a lesser extent.

3.2.2.2 Contraindications and Side Effects. Extravasation of the camptothecin-derived drugs leads to tissue damage, and the drugs are strongly emetic and neutropenia is common. Hypersensitivity to irinotecan is observed and is a contraindication. Diarrhea occurs by various mechanisms. Prior exposure to pelvic/abdominal irradiation enhances the risk of severe myelosuppression and deaths have been observed attributed to consequent infections (236). Orthostatic hypertension, vasodilation, insomnia, dizziness, alopecia, rash, anorexia, constipation, dyspepsia, anemia, weight loss, dehydration, colitis/ileus, renal function, and fertility impairment are also seen with irinotecan but are generally considered to be mild (237).

3.2.2.3 Pharmacokinetic Features. The pharmacokinetic features of topotecan are very complex. The drug is subject to alteration by esterases and the products are variously glucuronidated as well as oxidized by CYP 3A4, so is subject to a number of possible drug-drug interactions (238). After oral administration, about 30–40% of the drug is bioavailable (239–241). After i.v. dosage of prodrug irinotecan, rapid metabolic conversion by hydrolysis of the carbamoyl moiety to an active phenolic metabolite (SN-38) occurs as a result of the action of liver carboxylesterase; this is followed by glucuronidation to a metabolite that is much less potent. Metabolite SN-38 is about 1000-fold more active than irinotecan itself and accounts for the bulk of the antitumor activity of the drug. Fortunately, SN-38 has much less affinity toward serum proteins and this shifts the equilibrium toward retention of the active lactone form. Irinotecan is also converted in part to a metabolite in which the piperazine ring is oxidatively opened to produce an acid analog (presumably through its lactam) (242). About 11–20% of active irinotecan is excreted in the urine but the majority of the drug and its metabolites are excreted in the bile. There appears to be a significant patient-to-patient variation in ability to metabolize irinotecan (236).

3.2.2.4 Molecular Mode of Action and Resistance. The camptothecins are inhibitors of the action of mammalian topoisomerase I. The normal function of this essential enzyme is to produce temporary single-strand breaks by which the topography of DNA can be altered, so that the molecule can be processed. In the presence of camptothecin and its analogs a ternary complex forms (camptothecin analogs + DNA + enzyme) that results in single-strand breaks that cannot be resealed and this leads to defective DNA. In particular, when the replicating fork of DNA reaches the cleavable complex generated by camptothecin derivatives, irreversible strand breaks result, causing a failure in DNA processing, thus causing the cells to die. The camptothecins are thus S-phase poisons (Fig. 3.17). The specific molecular details are still obscure, however (237).

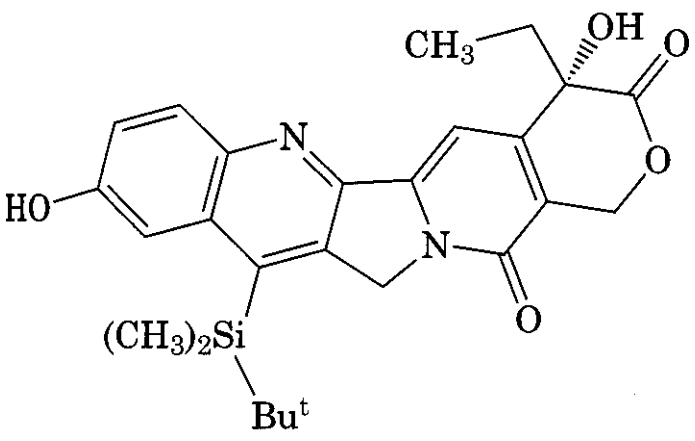
Resistance to the camptothecins is believed to result in part from excretion mediated by

P-glycoprotein and MRP-3 (multidrug resistance-associated protein) mechanisms (237, 243), although a number of other biological effects are seen in *in vitro* studies (244–247). Their meaning in clinical cases is as yet unclear. In some resistant cells, reduced levels of hydrolases capable of cleaving irinotecan to SN-38 seem to contribute. Another mode of resistance involves decreases in content and potency of topoisomerase I (237, 248,249).

3.2.2.5 Medicinal Chemistry. Several papers and reviews on the various total synthesis and analog studies of camptothecin and related molecules have been published (250–254).

The objectives of much of this work are clear. Drawbacks of camptothecin that have to be overcome are its poor water solubility, ease of hydrolytic lactone opening to the undesirable acid form, high serum protein binding, and the reversibility of its drug-target interaction. The solubility problem has been approached, interestingly, in quite opposite directions. Some groups have sought to increase water solubility and others to make the molecules even more lipophilic. Each approach has worked significantly.

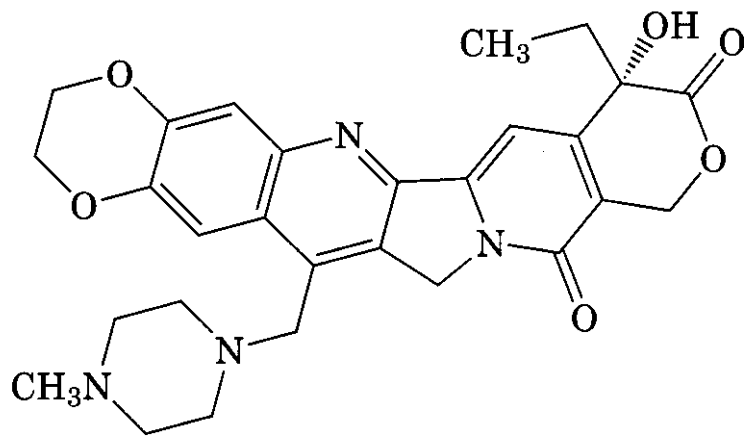
After considerable effort it was discovered that placing substituents at the C-9 and C-11 positions considerably decreased serum protein binding, even with the lactone-ring opened analogs, and yet this did not interfere with antitumor activity. Among the analogs that have received clinical examination but have not (yet) been marketed are lurotecan (26), 9-nitro and 9-aminocamptothecin (255), and DX-8951f (256). A number of other analogs stand out from the many that have been made. Among these are the hexacyclic 1,4-oxazines (257), Ring E homocamptothecins, 7-cyanocamptothecins (258), and the silatecans (25)(259). The latter are structurally unusual, in that very few candidate drugs contain silicon atoms. Furthermore, the best analogs are quite lipid soluble and, despite this, display superior stability in human blood and decreased albumin binding combined with significant potency. A summary of camptothecin SARs is illustrated in Fig. 3.18.



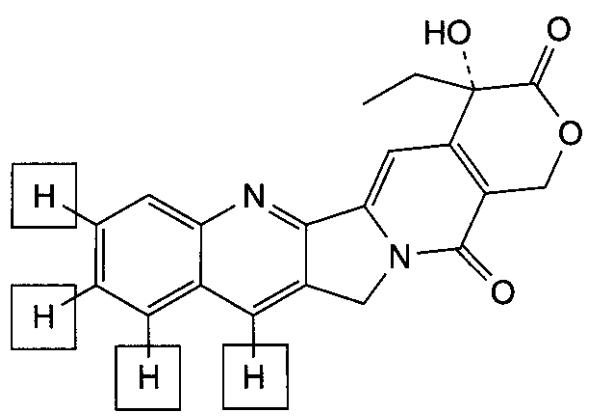
(25)

Much effort has been expended also to enhance the water solubility of the camptothecins by inventive formulations. A number of prodrugs have also been made in attempts to enhance stability and water solubility. Among these are the C-16 esters, such as the butyrates and propionates, some sugar-containing molecules, and the C-11 carbamates, of which irinotecan is the most successful to date.

Topotecan is likewise hydroxylated in the quinoline ring but with a dimethylamino-



(26)



The "boxed" functional groups can be changed with retention of significant biological activity. Not all such changes, however, are successful

Figure 3.18. Summary of camptothecin structure-activity relationships.

methylene moiety adjacent. It is administered i.v. for the treatment of ovarian and small-cell lung cancer. As with the other camptothecins, topotecan undergoes a reversible pH-dependent hydrolysis of its lactone moiety. It is the lactone form that is pharmacologically active. The drug has a complex excretion pattern, with a terminal half-life of about 2–3 h, and about 30% of the drug appears in the urine. Kidney damage decreases the excretion of the drug. Binding of topotecan to serum proteins is about 35%. The clinical side effects of topotecan are similar to those of irinotecan.

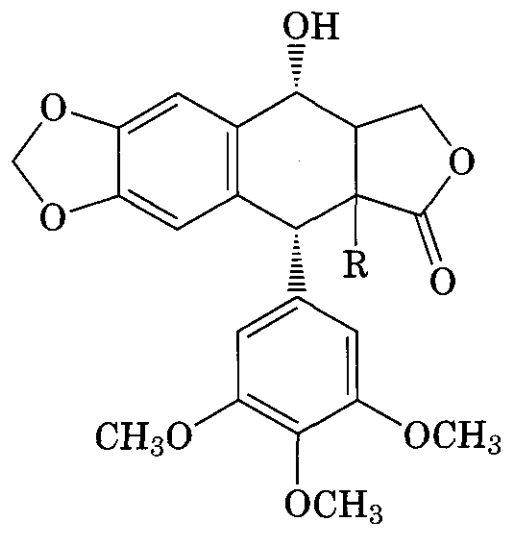
The chiral center of the carnptothecins is *S*; the R-enantiomers are much less (10- to 100-fold) potent.

3.2.2.6 Quantitative Structure-Activity Relationships (QSARs). Many synthetic camptothecin analogs have been prepared in attempts to stabilize the active lactone form and to enhance water solubility. A QSAR correlation has been published based on the NCI database information for 167 camptothecin analogs. The key functions that emerged from this are the presence and comparative positions of the E-ring hydroxyl and lactone carbonyl and the D-ring carbonyl (260).

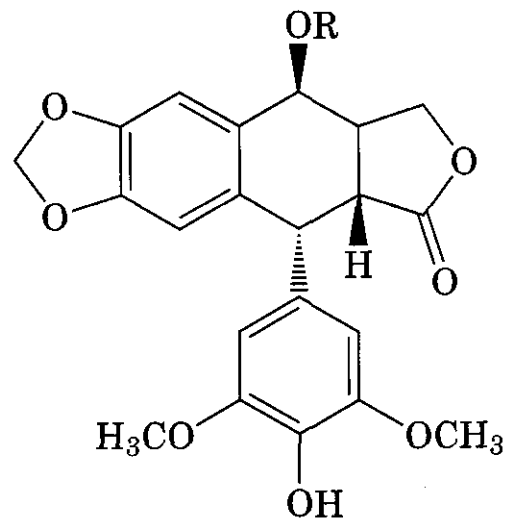
3.2.2.7 Recent Developments and Things to Come. Topoisomerase I inhibition is a popular area of contemporary research and a number of analogs are in various stages of preclinical and clinical workup. It seems likely that the immediate future will see the emergence of additional agents in this class (261–264).

3.3 Isopodophyllotoxins

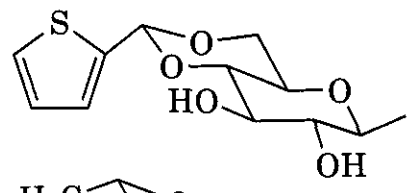
The lignan podophyllotoxin (27) is an ancient folk remedy (classically used for treatment of gout) found in the May apple, *Podophyllum peltatum* (265,266). Interestingly podophyllotoxin binds to tubulin at a site distinct from that occupied by taxol and the vinca bases, although its molecular mode of action does not involve this in any obvious way, and modern clinical interest lies in its isomers instead. The isopodophyllotoxins are semisynthetic analogs resulting from acid-catalyzed reaction with suitably protected sugars followed by additional transformations. This results in attachment of the sugars to the ring system, with opposite stereochemistry to podophyllotoxin itself. Etoposide



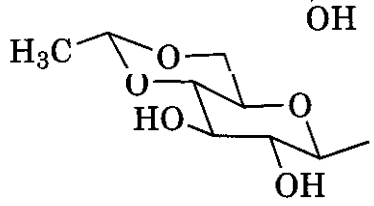
(27) R = β-H
(30) R = α-H



(28) R =



(29) R =



(28) and teniposide (29) are the most prominent analogs so produced and these possess a different mode of action than that of podophyllotoxin (267)! Another diastereoisomer, picropodophyllotoxin (30), is produced by epimerization of podophyllotoxin at the lactone ring but it has not led to interesting analogs.

3.3.1 Etoposide

3.3.1.1 Therapeutic Uses. Etoposide is injected for the treatment of refractory testicu-

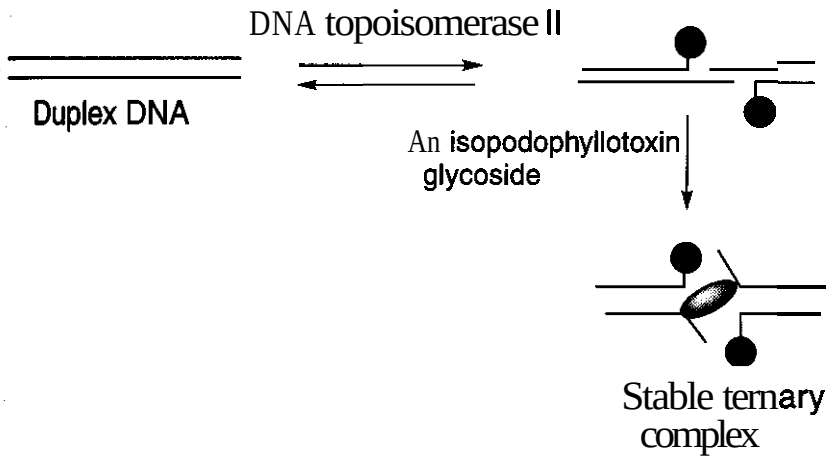


Figure 3.19. Illustration of the formation of a ternary complex between DNA, DNA topoisomerase II, and an isopodophyllotoxin glycoside.

lar tumors (268), small-cell lung cancer, and other less thoroughly established tumor regimes (269–271).

3.3.1.2 Side Effects and Contraindications. Hypersensitivity to etoposide or to the cremophor EL vehicle are contraindications (272, 273). Myelosuppression, alopecia, nausea, vomiting, anorexia, diarrhea, hepatic damage, leukopenia, and thrombocytopenia are among other side effects. In a small number of patients treated with etoposide, a therapy-related leukemia results (274,275).

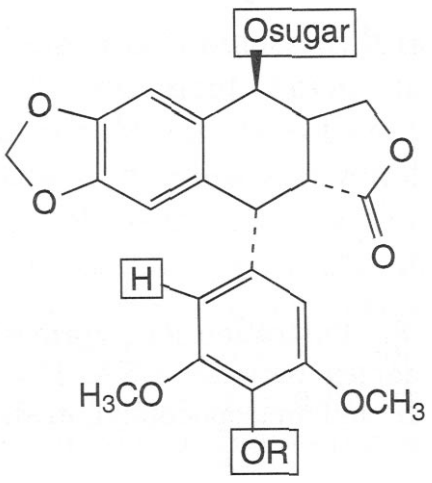
3.3.1.3 Pharmacokinetics. The drug is given by slow i.v. infusion and also can be given orally. About half of the administered dose is bioavailable and follows a biphasic elimination kinetic profile after infusion (276). Oxidative de-0-methylation is seen as a result of the action of human cytochrome P450 3A4 (277). Hydrolysis of the lactone is seenand conjugates are excreted in the urine (278). About half of the administered dose is excreted unchanged in the urine. Etoposide binds 76–96% to serum proteins and is displaced therefrom by bilirubin, so liver damage can require reduction in dosage (279, 280). The drug does not effectively pass the blood-brain barrier (281). The drug distributes best into small bowel, prostate, thyroid, bladder, spleen, and testicle but does not stay in the body for extended times after cessation of treatment (282).

3.3.1.4 Mode of Action and Resistance. Etoposide administration causes DNA single- and double-strand breaks and DNA-protein links. This effect appears to be based on inhibition of topoisomerase II (283,284). Furthermore, it is not an intercalator nor does it bind directly to DNA in the absence of the enzyme. Its action is most prominent in the late S or early *G-2* cell-cycle phases; thus cells do not enter the M-phase. The details of the interaction between topoisomerase II, DNA, and the isopodophyllotoxins are still emerging. Human toposiomerase II is a homodimericenzyme responsible for manipulating DNA supercoiling, chromosomal condensation/decondensation, and unlinking of intertwined daughter chromosomes. These steps require energy gained by hydrolysis of ATP. Etoposide (and teniposide) act by stabilizing the covalent topoisomerase 11-DNA intermediate and this stabilized ternary complex containing enzyme-cleaved DNA acts as a cellular poison. Figure 3.19 illustrates the formation of a ternary complex between DNA, DNA topoisomerase II, and an epipodophyllotoxin glycoside. During one topoisomerase II catalytic cycle, two ATP atoms are hydrolyzed. Etoposide and teniposide inhibit release of the ADP resulting from hydrolysis of the first ATP in a manner yet to be determined precisely, although the net result is that the ATPase activity of the enzyme is inhibited and resealing is prevented (285). Resistance takes the form of P-glycoprotein-related efflux (286), decreased expression and biosynthesis of topoisomerase II (287), or mutations in human topoisomerase IIα (288) or *p53* tumor-suppressor gene (289).

3.3.1.5 Medicinal Chemistry (290, 291). Although etoposide is widely used, it is inconveniently water insoluble. A water-soluble prodrug, etopophos, has been introduced. This agent is rapidly and extensively converted back to etoposide after injection (267,292).

3.3.2 Teniposide

3.3.2.1 Therapeutic Uses. Teniposide is injected for the treatment of acute nonlymphocytic leukemia, Hodgkin's disease and other



The "boxed" functional groups can be changed with retention of significant biological activity. Not all changes, however, are successful.

Figure **3.20.** Summary of isopodophyllotoxin glycoside structure-activity relationships.

lymphomas, Kaposi's sarcoma, neuroblastoma, and other less thoroughly validated tumor situations. Unfortunately, in a number of children treated for leukemia, later development of a teniposide-generated leukemia may occur (267).

3.3.2.2 Pharmacokinetics. The drug is given by slow i.v. infusion and also can be given orally. About half of the administered dose is bioavailable and follows a biphasic elimination kinetic profile after infusion. The drug does not efficiently pass the blood-brain barrier. Its tissue distribution and persistence are similar to those of etoposide (282). Hydrolysis of the lactone is seen and conjugates are excreted in the urine after oxidative demethylation, more so than with etoposide (277).

3.3.2.3 Mode of Action and Resistance. Teniposide administration causes DNA single- and double-strand breaks and DNA-protein links. This effect appears to be based on inhibition of topoisomerase II because the drug is not an intercalator nor does it bind to DNA. Its action is most prominent in the late S or early G-2 cell-cycle phases; thus cells do not enter the M-phase. Resistance takes the form of P-glycoprotein–related efflux, decreased biosynthesis of topoisomerase II, or mutations in *p53* tumor-suppressor gene (293).

3.3.2.4 Structure-Acfivity Relationships. Figure 3.20 illustrates the comparatively limited information relating to isopodophyllotoxin glycosides.

Detailed reviews of the properties of the isopodophyllotoxins are available (294–297).

4 DRUGS INTERFERING WITH TUBULIN POLYMERIZATION/DEPOLYMERIZATION

Microtubules provide a sort of cytoskeleton for cells so that they can maintain their shapes. They also form a sort of "rails," along which the chromosomes move during mitosis. These microtubules are constructed by the controlled polymerization of monomeric tubulin proteins of which there are two types, a and β. Figure 3.21 illustrates this process. The dimeric vinca alkaloids interfere with polymerization, thus preventing cell division by preventing the formation of new microtubules. The taxus alkaloids, on the other hand, promote the polymerization into new microtubules but stabilize these and prevent their remodeling. This prevents cell growth and repair. These mechanisms are compatible with the modes of action of other antitumor agents, thereby allowing for synergy when combined with these substances in cocktails.

4.1 Taxus Diterpenes

4.1.1 Paclitaxel/Taxol. Taxol (**31**), a diterpene ester, was discovered initially as a minor component in the bark of the Pacific yew at approximately the same time as camptothecin was found by the same group in quite another source

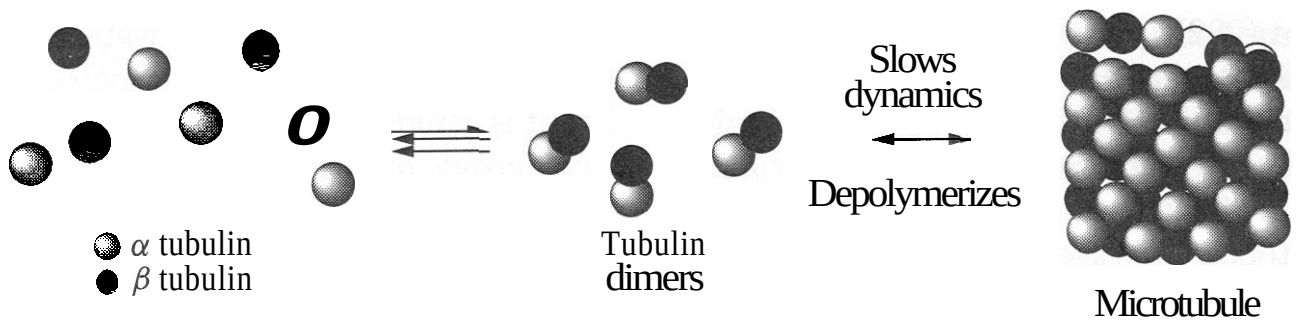
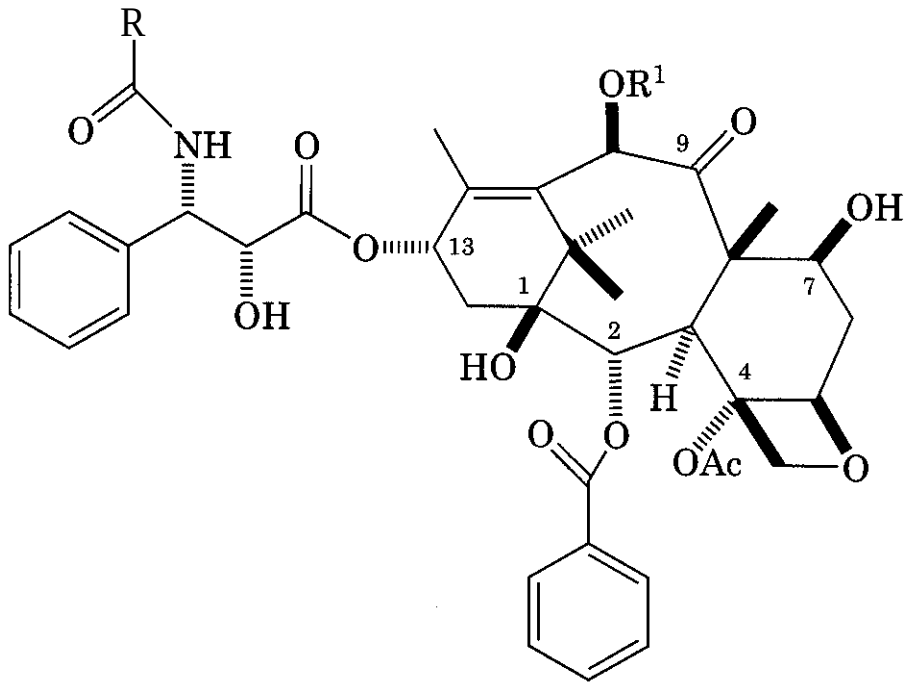


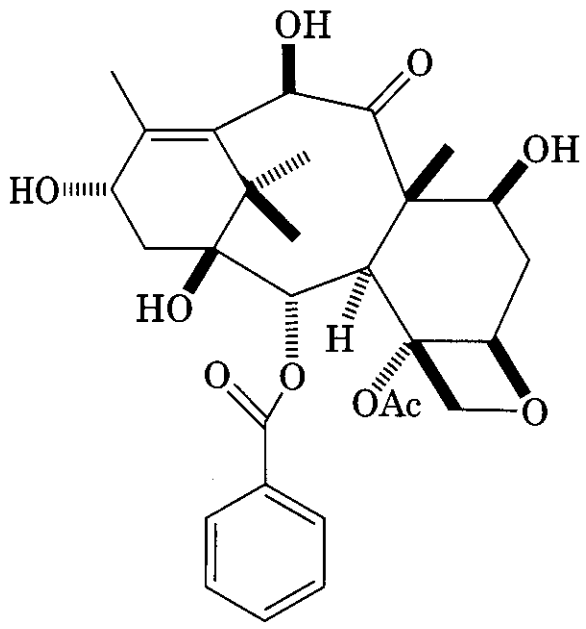
Figure 3.21. Tubulin polymerization to microtubules and their disassembly.



(31) R = Ph; $R^1 = COCH_3$
(32) R = ^tBuO; R^1 = H

(223,298). It took a great many years for taxol to come to the clinic because its initial performance in tumor-bearing mice was comparatively unimpressive. The progress to market was accelerated materially by the discovery of a novel (at the time) molecular mode of action. Taxol stimulates the formation of microtubules from tubulin and stabilizes this polymer, which stops cells from dividing (299–301).

After an enormous effort, semisynthesis from 10-deacetylbaccatin III (**33**), itself available in quantity from the much more renewable needles of various abundant yew species, proved economical and also allowed the synthesis of many analogs, of which docetaxel



(33)

(32) is the most prominent (302). Many partial and total syntheses of taxol have been reported but none of these has as yet proved to be practical. Despite a long tradition allowing the discoverer to name an important compound, taxol was renamed paclitaxel for commercial purposes by the CRADA winner, Bristol-Myers Squibb.

4.1.1.1 ***Clinical Uses.*** Taxol is widely used in combinations for the therapy of refractory ovarian, breast, lung, esophageal, bladder, and head and neck cancers.

4.1.1.2 ***Side Effects and Contraindications.*** Taxol is a very toxic drug and it must be used with care. Bone marrow suppression (neutropenia) is a major dose-limiting side effect (303). A few patients develop severe cardiac conduction abnormalities (304). Patients with liver abnormalities may be especially sensitive to taxol. Fertility impairment and mutagenesis is seen in experimental animals, so taxol should only be given to pregnant patients with special care. Hypersensitivity is not uncommon and is often associated with the solvent in which this especially water insoluble drug must be administered (cremophor EL, a polyethoxylated castor oil) (305). Peripheral neuropathy occurs frequently and requires reduced dosage. In contrast to many other antitumor agents, extravasation, although causing discomfort and local pathologies, does not generally lead to severe necrosis. Gastro-

intestinal distress (diarrhea, fever, anemia, mucositis, nausea, and vomiting), alopecia, edema, and opportunistic infections are also reported by many patients. Paclitaxel is metabolized by the P450 system, so coadministration of drugs requiring processing by CYP2C8 and CYP3A4 requires caution. This statement is also true of docetaxol (306).

4.1.1.3 Pharmacokinetics. The drug is generally given by long-time (3 or 24 h) infusion at 3-week intervals or short-time (1h) infusions at weekly intervals and is heavily protein bound (90–98%).Attempts to infuse the drug over very long times (96 h) have been made but involve significant practical limitations. The drug is excreted after a biphasic mode, with an initial rapid serum decline as the drug is distributed to the tissues and the overflow excreted. Return from peripheral tissues is slow and accounts for the second part of the excretion curve. The excretion half-life is fairly long (– 13–55 h) (307–309). Extensive clearance other than by urine takes place, given that only 1–13% of the drug is found in the urine. Metabolism is primarily oxidative, with the main metabolite being the 6α-hydroxy analog and lesser amounts of the 3′-parahydroxybenzamide and the 6α-hydroxy, 3'-parahydroxybenzamide analogs being detected (310–312).

4.1.1.4 Molecular Mode of Action and Resistance. Taxol binds to the p-tubulin component and stimulates the formation of microtubules. These, however, do not break down, so the cell is unable to repair and to undergo mitosis (301). Resistant cells in culture are often seen to produce P-glycoprotein to excrete the drug (313), and also to have mutations in the β-tubulin component (314,315). Whether this is responsible for clinical resistance is still being studied. Overexpression of the *ErbB2* gene occurs fairly often in breast tumors and this leads to overproduction of a transmembrane growth factor receptor belonging to the ErbB receptor tyrosine kinase subfamily. Cells with this characteristic have reduced responsiveness to taxol (316). Other growth factor anomalies involving, for example, EGFRviii and HER-2 are also seen in some cell lines (317).

4.1.2 **Docetaxel/Taxotere.** Docetaxel (32) is a semisynthetic analog of taxol prepared by a variety of chemical means, starting with the more abundant 10-deacetylbaccatin III (33) (302). It has found a significant place in anticancer chemotherapy but is still a significantly toxic drug that must be used with care.

4.1.2.1 Clinical Applications. Docetaxel is administered by i.v. infusion for the treatment of breast cancer, non-small-cell lung cancer and a variety of other less well established antitumor indications (318).

4.1.2.2 Side Effects and Contraindications. Many of the adverse effects of docetaxel are similar to those of taxol itself. The drug, however, is administered in polysorbate 80 rather than cremophor, so allergy is more commonly to the drug itself and can be severe. Poor liver function greatly enhances patient sensitivity to docetaxel. Severe fluid retention can also be observed. Patients are often administered corticoids before being exposed to docetaxel to assist in their tolerance of the drug. Myelotoxicity is potentially severe, so blood cell counts should be monitored. The toxicity of docetaxel is exaggerated when liver disease is present (319).

4.1.2.3 Pharmacokinetics. In contrast to paclitaxel, docetaxel has linear pharmacokinetics at the doses used in the clinic. As with paclitaxel, metabolism takes place in the liver through cytochrome P450 enzymic oxidation and the metabolites are excreted primarily in the bile. The involvement of P450 3A4 and 3A5 requires care in coadministering drugs that are also metabolized by these common enzymes (320). The metabolites are generally less toxic and less potent than docetaxel itself (321).

4.1.2.4 Mode of Action and Resistance. See paclitaxel.

4.1.2.5 Chemical Transformations. Although several total syntheses of taxol have been achieved during the last few years, low overall yields and high costs preclude them from being of commercial importance. Fortunately, isolation of the two taxol biosynthetic precursors, baccatin III and 10-deacetyl baccatin III, initially from the regenerable needles of the yew species T. baccata and subsequent development of highly efficient semisynthesis of taxol and taxotere from the above precursors have apparently solved the present supply problem of these precious drugs. Moreover, the semisynthetic routes have also provided means to carry out extensive SAR studies and

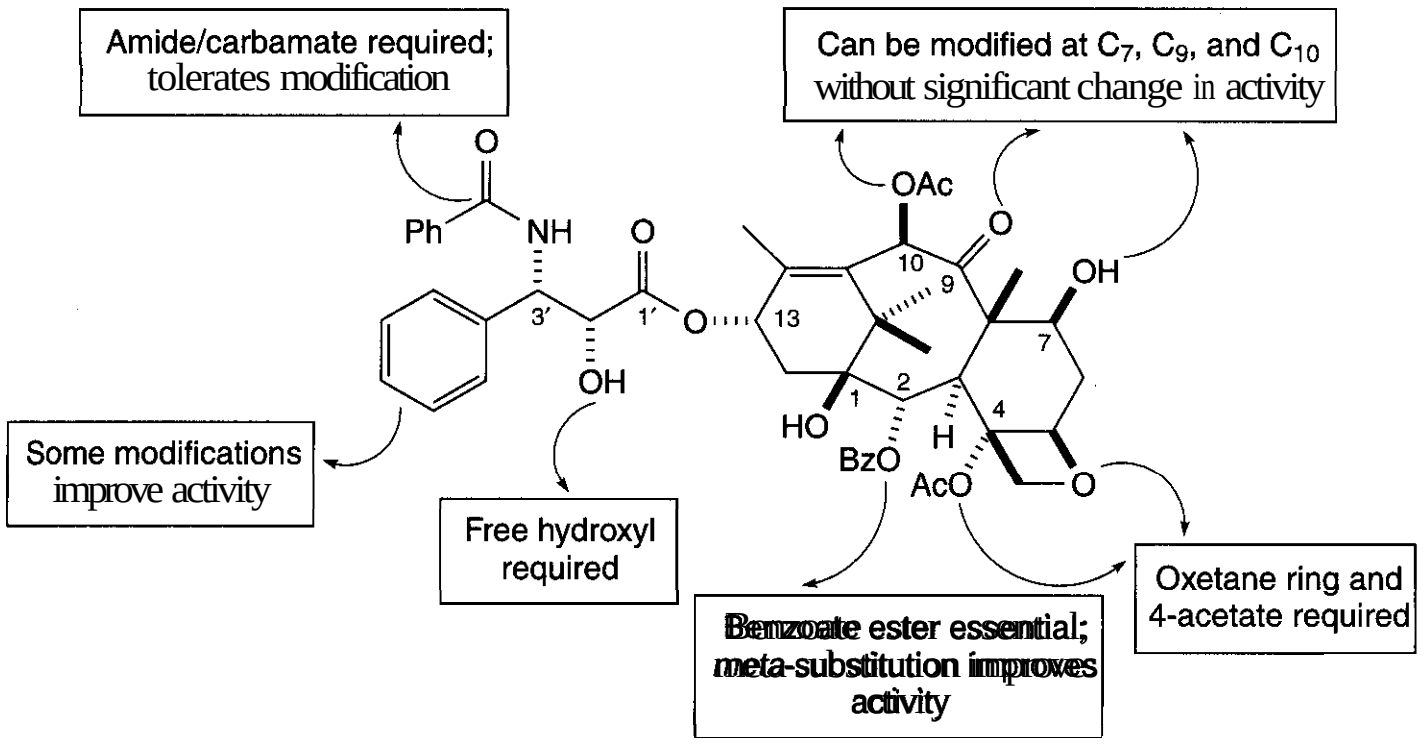


Figure 3.22. Structure-activity relationships of the taxol series.

consequent access to a large number of taxol analogs. The SAR studies on taxol have demonstrated that the C-3 phenylisoserinate side chain is an essential component for bioactivity, wherein limited modifications can be carried out at the 3'-phenyl and the 3'-N-benzoyl sites toward attenuating activity. Similarly, the presence of an intact oxetane ring in the diterpenoid core appears to be essential for bioactivity. Additionally, although the oxygen-bearing functionalities at C-7, C-9, and C-10 allow various modifications, an acetoxy at C-4 and an aroyloxy group at C-2 are indispensable for optimum activity. Interestingly, A-ring-contracted taxol analogs (A-nortaxol) were found to retain tubulin assembly activity, albeit with significantly diminished cytotoxicity (322–325).

The SARs of the taxol series are summarized in Fig. 3.22.

4.1.2.6 Biosynthesis. Taxol is one of the structurally more complex members of the diterpene family, characterized by the presence of the unusual taxane ring system. The initial steps in taxol biosynthesis involve the cyclization of geranylgeranyl diphosphate to taxa-4(5),11(12)diene, forming the taxane core structure. Subsequent cytochrome P450-mediated hydroxylation at C-5 of the olefin is followed by several other cytochrome P450-dependent oxygenations at C-1, C-2, C-4, C-7, C-9, C-10, and C-13 (the precise order of these regiospecific oxidations, however, is not yet known) and CoA-dependent acylations of the taxane core, en route to taxol (326). Biosynthetically, the N-benzoyl phenylisoserine side chain has been shown to originate from phenylalanine and its further elaboration involves a late-stage esterification at C-13 of an advanced baccatin III intermediate (327).

The biosynthetic pathway between geranylgeranyl diphosphate and taxol remains to be fully elucidated but apparently passes through taxa-4(5),11(12)-diene and taxa-4(20),11(12)-diene-5α-ol, as shown in Fig. 3.23.

4.1.2.7 Things to Come. Recent interest has developed about the properties of the epothilones. These apparently bind to tubulin at approximately the taxol site but resistance by P-glycoprotein expulsion is apparently not significant with these fermentation products and they are active against a number of taxol-resistant cell lines. Elutherobin is another natural product binding to the taxane-binding site but this agent is cross-resistant with taxol (328). The clinical future of these agents is as yet uncertain and they have inspired much synthetic and biochemical attention.

4.2 Dimeric Vinca Alkaloids

The dimeric indole-indoline alkaloids were initially isolated from the Madagascan periwinkle, Catharanthus rosea (formerly named Vinca rosea). The plant was originally investi-

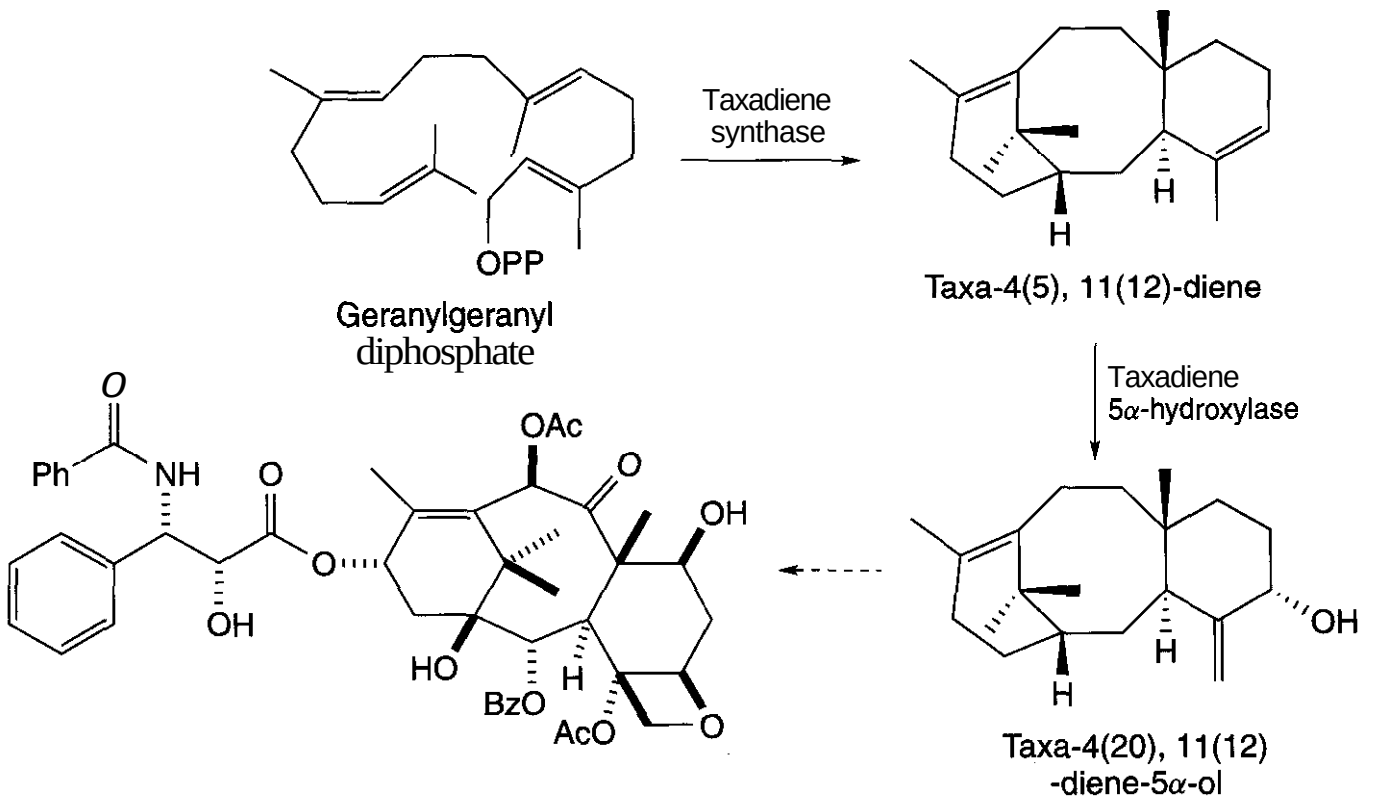


Figure 3.23. Biosynthesis of taxol.

gated as a follow-up to folkloric reports of hypoglycemic activity, and it was hoped to be of value in treating diabetes mellitus. This did not prove to be true but, during the investigation of extracts, certain fractions produced granulocytopenia and bone marrow suppression in animals. The active alkaloids were isolated from a matrix of indole alkaloids and were found to be active antileukemic agents against P-1534 cells. Development for human use followed after extensive experimentation. Four of these unsymmetrical dimeric alkaloids ultimately found use as antitumor agents. The best agents contain C-linked vindoline and 16β-carbomethoxy velbanamine units. Apparently minor structural differences between the alkaloids led to major differences in potency and utility (329). Because of their relative scarcity and medicinal value, these dimers have been attractive synthetic targets and a rich synthetic and biosynthetic literature has grown up around them. Inspection of their structures readily leads to the inference that they are the products of unsymmetrical free-radical coupling. After much work, two groups, those of Potier in France (330, 331) and of Kutney in Canada (332), succeeded in stereoselective dimerization. Treatment of the abundant alkaloid catharanthine as its N-oxide with trifluoroacetic anhydride leads to a fragmentation into an enamine that can be intercepted by vindoline, another comparatively abundant alkaloid, and the product reduced by sodium borohydride. Under low temperature conditions the condensation is stereospecific in the desired manner. This is believed to reflect a concerted interaction. When the reaction is run at higher temperatures, a mixture of diastereomers is produced instead. This is believed to be the result of a stepwise condensation. Variation of this chemistry leads to the formation of useful synthetic analogs and interconversions into natural analogs as well. Figure 3.24 illustrates the partial chemical synthesis of vinca dimers with the natural stereochemistry.

It is interesting to note that dolastatin 10, a marine natural product with exceptional antitumor properties, also binds near to the vinca alkaloid binding domain and inhibits tubulin polymerization (333,334).

4.2.1 Vinblastine (Velban)

4.2.1.1 Medicinal Uses. Vinblastine sulfate (34) is given i.v. with great care, to avoid damaging extravasation (123), for the treatment of metastatic testicular tumors (usually in combination with bleomycin and cisplatin). Various lymphomas also may respond. It has only limited neurotoxicity, thus enhancing its utility.

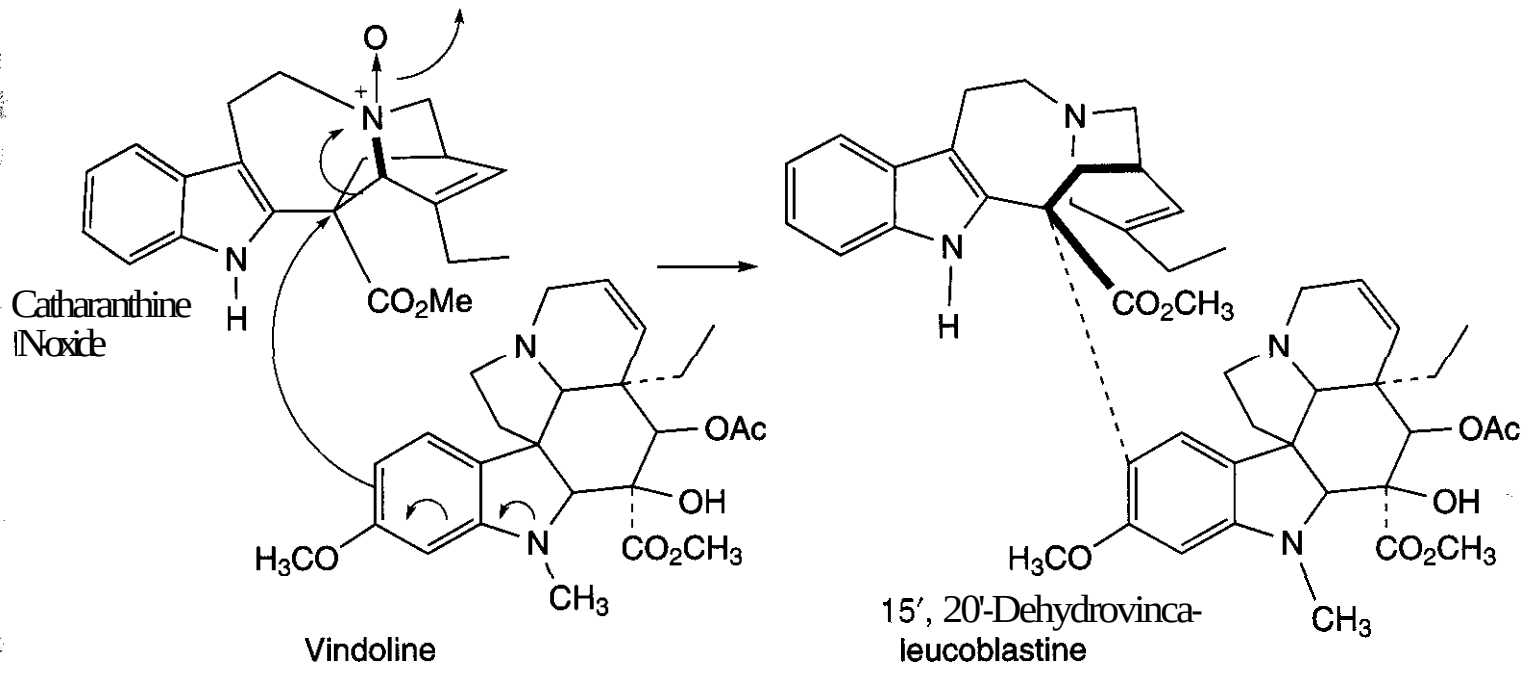
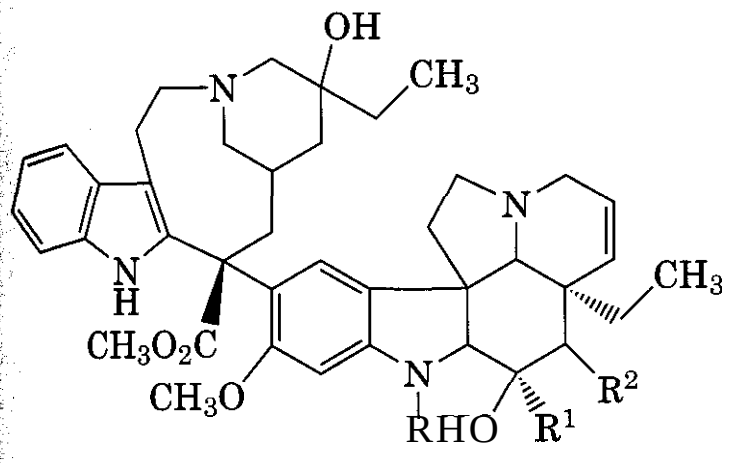


Figure 3.24. Partial chemical synthesis of vinca dimers with the natural stereochemistry.



Compd.	R	R^1	R^2
(34)	CH_3	CO_2CH_3	$OCOCH_3$
(35)	CHO	CO_2CH_3	$OCOCH_3$
(36)	CH_3	$CONH_2$	OH

4.2.1.2 Side Effects and Contraindications. Vinblastine causes severe tissue necrosis upon extravasation. Mild neurotoxicity and myelosuppression occur and these effects should be monitored to prevent significant toxicity to the patient. The other side effects of vinblastine are common to antitumor agents (alopecia, ulceration, nausea, etc.).

4.2.1.3 Pharmacokinetic Features. Vinblastine is extensively metabolized in the liver and the metabolites are excreted as conjugates in the bile. About 15% of the drug is found unchanged in the urine (335, 336). Oxidative degradation of the catharanthus alkaloids occurs in part catalyzed by the action of myeloperoxidase. Cleavage occurs between C-20′ and C-21′ and is structurally facilitated by the presence of a C-20′ hydroxyl moiety (337). Peroxidase and ceruloplasmin also catalyze oxidative transformations of vinblastine (338,339).

4.2.1.4 Medicinal Chemical Transformations. Hydrolysis of the acetyl group at C-4 of vinblastine abolishes its antileukemic activity. Furthermore, acetylation of the free hydroxyl groups also inactivates the molecule. The dimeric structure is required as is the stereochemistry of the point of attachment. Hydrogenation of the olefinic linkage and reduction to the carbinol also greatly diminish potency. Thus the antileukemic activity is substantially dependent on the specific structural groups present in the molecule.

4.2.1.5 Molecular Mode of Action and Resistance. Vinblastine blocks cells in the M-phase. It binds to the β-subunit of tubulin in its dimer in a one-to-one complex, thus preventing its polymerization into microtubules. The binding site is near to, but different from, that of colchicines but similar to that of maytansine and rhizoxin (although the consequences of binding of the latter are different from those of vinca binding). Nontubulin oligomers form from the component parts as a consequence, and preformed tubulin depolymerizes and the complex with vinblastine crystallizes (340). Failure to produce functional microtubules prevents proper chromosome formation and thus prevents cell divi-

sion. The blocked cells then die (become apoptotic). Other cellular processes dependent on microtubules are also interfered with, although the blockade of chromosome formation is regarded as central to their action (341). Resistance mainly takes the form of elaboration of P-glycoproteins that export vinblastine, and this cross-resistance is broad enough to include the other vinca alkaloids and other antitumor agents as well (342). Resistance is also attributed to alterations in the tubulin subunits (343).

4.2.2 Vincristine (Oncovin, Vincasar PFS)

4.2.2.1 Medical Uses. Vincristine (35) *is* a common component of antitumor cocktails used in treating acute lymphoblastic leukemia and solid tumors of youngsters and in adult lymphoma. It is commonly used with corticosteroids. Study of its use in the form of liposomes has also been carried out (344). Its use produces limited myelosuppression, so it is an attractive component in cocktails. The reduced myelotoxicity may be attributable to oxidative degradation of the drug by myeloperoxidase, a heme-centered peroxidase enzyme present in acute myeloblastic leukemia but not in acute lymphoblastic leukemia (345,346).

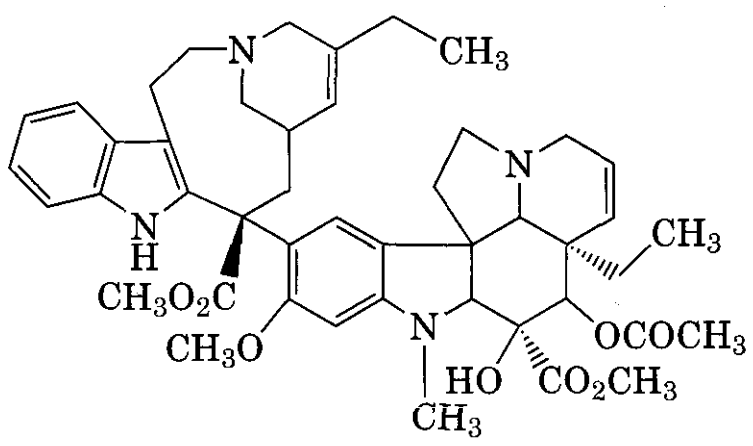
4.2.2.2 Pharmacokinetic Features. Vincristine is extensively metabolized in the liver and the metabolites are excreted as conjugates in the bile. About 15% of the drug is found unchanged in the urine.

4.2.2.3 Side Effects and Contraindications. Vincristine causes severe tissue necrosis upon extravasation (123). Neurotoxicity is a significant potential problem with vincristine and is often treated in part by reducing the dose of the drug (347). Myelosuppression also occurs but to a lesser extent and this effect should be monitored to prevent significant toxicity to the patient. Gout can occur with vincristine administration and can be controlled by use of allopurinol. The other side effects of vinblastine are common to antitumor agents (alopecia, ulceration, nausea, diarrhea, etc.).

4.2.2.4 Resistance. Resistance to vincristine is mediated in part by export resulting from the multidrug resistance protein and, interestingly, is characterized by cotransport with reduced glutathione (348).

4.2.3 Vinorelbine (Navelbine)

4.2.3.1 Medicinal Uses. Vinorelbine (37) is used against non-small-cell lung cancer and



(37)

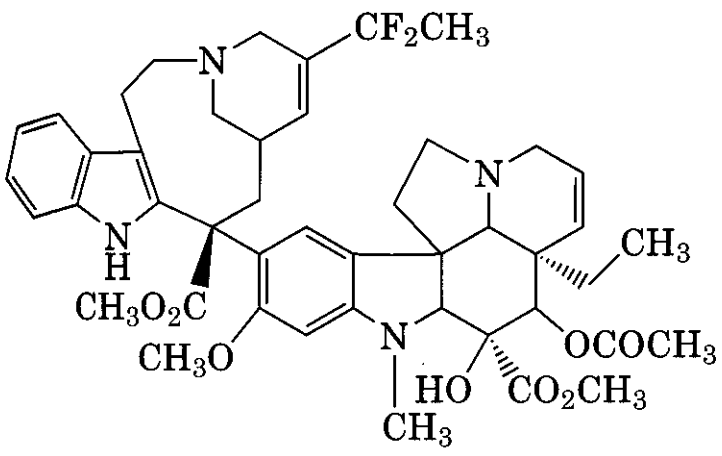
against breast cancer (349–353). It appears to be intermediate in its neurotoxicity and myelosuppression compared to that of the other vinca antitumor agents (354).

4.2.3.2 Pharmacokinetic Features. Vinorelbine is extensively metabolized in the liver and the metabolites are excreted as conjugates in the bile. About 15% of the drug is found unchanged in the urine.

4.2.3.3 Side Effects and Contraindications. Vinorelbine causes severe tissue necrosis upon extravasation as well as phlebitis (355). Prior i.v. administration of cimetidine partially avoids this. Mild neurotoxicity and myelosuppression occur and these effects should be monitored to prevent significant toxicity to the patient. Its most notable toxic side effect appears to be granulocytopenia. The other side effects of vinorelbine are common to antitumor agents (alopecia, ulceration, nausea, etc.).

4.2.3.4 Things to Come. Vindisine (36) is an analog prepared from vinblastine (34). Its antitumor spectrum, however, is more closely similar to that of vincristine. Clinical studies show activity against acute leukemia; lung cancer; breast carcinoma; squamous cell carcinoma of the esophagous, head, and neck; Hodgkin's disease; and non-Hodgkin's lymphomas. Its toxicities include myelosuppression and neurotoxicity. Despite these promising findings, it has yet to be introduced into the clinic (356).

Vinflunine (38) is a dimeric alkaloid, containing two gem-fluorine atoms, prepared by a mechanistically interesting process using super acidic reactants on vinorelbine. This com-



(38)

pound has improved antitumor potency in a variety of model tumor systems, shows less drug resistance (357), and has entered clinical trials (358, 359).

Some additional reviews of this topic are available (328, 360–362).

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CHAPTER FOUR

Radiosensitizers and Radioprotective Agents

EDWARD A. BUMP
DRAXIMAGE Inc.
Kirkland, Quebec, Canada

STEPHEN J. HOFFMAN
Allos Therapeutics, Inc.
Westminster, Colorado

WILLIAM O. FOYE
Massachusetts College of Pharmacy and Health Sciences
Boston, Massachusetts

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1 PROTECTIVE AGENTS AGAINST IONIZING RADIATION

1.1 Introduction

The protective action of certain substances against the damaging effects of ionizing radiation was first noted in 1942 (but not published until 1949) by Dale, Gray, and Meredith. A decrease in the inactivation of two enzymes by X-rays was observed upon addition of several substances, including colloidal sulfur and thiourea, to aqueous preparations of the enzymes (1) Radioprotective effects for a bacteriophage were observed by Latarjet and Ephrati in 1948, using cysteine, cystine, glutathione, thioglycolic acid, and tryptophan (2). Radioprotection of mice against X-rays was achieved shortly thereafter in three different laboratories, in Belgium, the United States, and Britain, by use of cyanide (3), cysteine (4), and thiourea (5), respectively. These protective effects were attributed at the time to inhibition of, or reaction with, cellular enzymes. The importance of sulfur-containing molecules for radioprotection was thus demonstrated from the very earliest experiments with living systems, although the reasons for selection of sulfur compounds were not clear.

The importance of the mercapto (or thiol) function was demonstrated in 1951 by Bacq et al. (6), a Belgian physiologist, who removed the carboxyl group of cysteine and obtained 2-mercaptoethylamine (MEA, or cysteamine; $NH_2CH_2CH_2SH$), which proved to be a much stronger protective agent in mice than any previously tested. The presence of the amino group was also considered essential for good radioprotection, and most of the mercaptans and other sulfur-containing molecules, later synthesized, also contained an amino or other basic function. MEA and its derivatives, particularly those having greater lipophilic character, are still regarded as the most potent of the whole-body radioprotective agents.

Since 1952 other types of structures with radioprotective activity have been found, including a number of physiologically important agents, notably serotonin, but none has yet exceeded the amino alkyl mercaptans in effectiveness on a molar basis. Various explana-

tions have been put forward for the protective activity of the thiols, but it was not until more knowledge was available regarding the radicals generated by ionizing radiation, and their effect on DNA, that present concepts became established.

Research attempts to explain the action of chemical radiation protectors have involved the use not only of mammals but also of plants, bacteria, distinct types of nonmammalian cells, and even some synthetic plastics affected by ionizing radiation. This discussion attempts to categorize the various types of chemical structures that afford some protection against the deleterious effects of ionizing radiation in mammals and to describe the most widely held mechanistic theories of radioprotection.

1.2 Radiation Damage

Biologic effects of radiation have been reviewed in detail by a number of authors, including von Sonntag (7), Pizzarello and Witcofski (8), Casarett (9), Okada (10), and Dertinger and Jung (11). Ionizing radiation can have three types of biological effect: perturbation of cellular regulation, mutation, or cell death. Most of the research on radioprotection has focused on cell death and consequences at the level of tissue injury and death of the organism. In mammals, death can result from damage to the blood-forming organs, gastrointestinal system, or central nervous system, depending on radiation dose. Hematopoietic death from bleeding, infection, or anemia is the endpoint that was used in most of the early studies on radioprotection, and follows 7–30 days after exposure to a potentially lethal single dose (~400 rads) in mice. These whole-body effects of relatively high doses of ionizing radiation are most easily explained in terms of depletion of stem cells by cell killing that in turn is most directly explained by DNA damage that occurs at the time of irradiation. However, the mechanisms of some other radiation effects, such as carcinogenesis, teratogenesis, and delayed vascular injury, could include altered cellular regulation and secondary DNA damage. The full spectrum of biologically relevant mechanisms of radiation injury has not been fully elucidated. Many mechanisms of molecular damage that have been elucidated may not be relevant to biological damage. It is therefore difficult to classify radioprotectors according to mechanism. They are organized in this discussion mostly according to chemical features, although it should be kept in mind that structurally similar compounds could be acting by completely different mechanisms, and even a single compound may protect by different mechanisms depending on the model system used for the study.

Absorption of radiation energy by biological molecules has been considered to be either direct or indirect (12–14), although they both can result in the same kind of damage to a target molecule. Direct action involves absorption of radiation energy by a target molecule, such as DNA. The absorbed energy is sufficient to cause the ejection of an electron from an atom of the target molecule (hence the term *ionizing* radiation), leaving the target molecule with an unpaired electron: that is, converting it into a free radical. Indirect action involves the absorption of radiation energy by a molecule (such as H_2O) other than the target molecule, and subsequent transfer of the energy to the target molecules by reaction of radiolytically produced nontarget free radical with the target molecule. In either case, the result is a target molecule free radical. Subsequent reactions of the target molecule free radical can result in permanent chemical alteration, leading to a biological consequence. Reaction of the target molecule free radical with a hydrogen donor, such as a thiol, can restore the lost electron, thus restoring the target molecule (13). Repair usually refers to an enzymatic process, whereas the term *restoration* is the preferred usage to describe this type of chemical radioprotection.

Another mechanism of radioprotection is to scavenge the nontarget free radicals produced by radiation before they can react with the target molecule. The most important diffusible free radical involved in the indirect effect is the hydroxyl radical, formed by radiolysis of water. Hydroxyl-radical scavenging can be the most effective mechanism of radioprotection of target molecules in dilute solution. However, radioprotection of mammalian cells by hydroxyl-radical scavenging is difficult to achieve because these highly reactive mole-

cules will react with cellular constituents very rapidly at the high solute concentrations that exist in cells, and a very high concentration of hydroxyl-radical scavenger is required to intercept the hydroxyl radicals before this happens (7).

1.3 Antiradiation Testing

Much of the early testing of radioprotective agents employed either X-rays or y-rays from an external source. Animals for initial in *vivo* testing most often have been mice or rats; guinea pigs have been used less frequently. Large animal radioprotector testing, with dogs or monkeys, has been limited to the more effective compounds; as determined from screening with mice or rats. Further information on this testing, through the use of 30-day survival [lethal dose for 50% survival at 30 days ($LD_{50/30}$)] as a criterion for protection, may be found in texts devoted to radiobiology (15, 16) from that era, the 1960s.

Various physiological effects may be observed, depending on the dose and type of radiation, as well as on the type of animal used. In theory, the appearance of any observable symptom of radiation damage may be used as the basis of a testing procedure but, historically, lethality has generally been the criterion for protection. Sufficient numbers of animals must be employed for statistical significance, and in the case of mice irradiated with a lethal dose of X- or y-rays, the endpoint for survival is generally taken to be survival for 30 days after irradiation. Testing results are expressed most commonly as the percentage survival for the observation period compared to the survival of control animals. Another method of expression of test data is in terms of the dose reduction factor (DRF, which is the ratio of the radiation dose causing an effect such as LD_{50} in the treated animals or cells to the dose causing the same effect in the unprotected animals or cells). Recently, radioprotection studies have been most frequently carried out in cell culture (17). Particular mechanisms of protection may be more effective with regard to certain endpoints (17). For example, protection against mutagenesis has been observed at lower concentrations of amino thiols than are required for protection against cell killing.

The dose of protective agent employed is usually the maximum tolerated dose (MTD), that is, the dose causing no deleterious effects. In a drug-screening program, candidate compounds are usually tested at their MTD level by use of a radiation dose that is lethal to all control animals in 30 days. The time interval between administration of the drug and irradiation of the animals is usually 15–30 min for intraperitoneal dosing and 30–60 min for oral dosing. Drugs believed to act by inducing hypoxia or other metabolic changes usually must be administered several hours before irradiation. The rate of irradiation in screening programs has commonly been 50–250 rads/min. At lower dose rates, the time for maximum effectiveness of the radioprotector can be exceeded before the total radiation dose is administered. In addition, repair processes could become significant before the irradiation is complete. Chronic radiation studies have been carried out with repeated administration of protector, but results have been less decisive (18).

Several concepts should be kept in mind with regard to testing results of radiation protectors (1). Because many compounds have been tested at the maximum tolerated dose, the best protectors are not necessarily the most active on a molar basis, but rather the most effective at a given level of toxicity (2). Although many of these compounds are as active as the parent compound, some are activated metabolically, or may act indirectly by inducing an endogenous protective mechanism.

Other testing procedures used to a lesser extent include the inhibition of bacterial or plant growth and the prevention of depolymerization of polymethacrylate or polystyrene (19) or of DNA (20). Other test procedures for evaluation of radioprotectors have included the plaque-forming ability of coliphage T (21), effect on Eh potential (22), inhibition of the formation of peroxides of unsaturated lipids or 13-carotene (22), inhibition of chemiluminescence of γ-irradiated mouse tissue homogenates (23), and use of spleen colony counts (24).

1.4 Protective Compounds

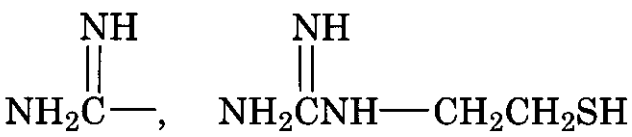
The more extensively investigated compounds have been discussed in books by Thomson (16); Bacq (25, 27); Balubukha (26); Nygaard

and Simic (27); Livesey, Reed, and Adamson (28); and Bump and Malaker (17). A catalog of compounds tested for radiation protection up to 1963 was compiled by Huber and Spode (29). Extensive reviews on protective agents since 1963 have been written by Melching and Streffer (30), Overman and Jackson (31), Romantsev (32), Foye (33), Klayman and Copeland (34), Yashunskii and Kovtun (35), and Bump and Brown (36). Reports of two international symposia on radioprotective and radiosensitizing agents have been published by Paoletti and Vertua (37) and by Moroson and Quintiliani (38). A series of symposia on radiation modifiers has also been held (39). Chapters on radioprotective agents have appeared in *Annual Reports in Medicinal Chemistry* in 1966 and 1967 (40) and in 1968 and 1970 (41), and in *Military Radiobiology in 1987* (42).

In the following discussion of structure-activity relationships, results on radioprotection of mice are compared unless otherwise stated. Relevant details concerning radiation dose, compound dose, route of administration, or strain of test animal, variations of which can alter results significantly, may be found in the original references.

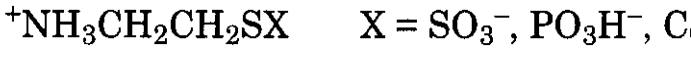
1.4.1 Thiols and Thiol Derivatives. 2-Mercaptoethylamine (MEA, cysteamine) and its derivatives have constituted the most effective class of radioprotective compounds. Since the initial discoveries of the protective action in mice of cysteine by Patt et al. (4) and its decarboxylated derivative, MEA, by Bacq et al. (6), hundreds of derivatives and analogs of the mercaptoethylamine structure have been synthesized and tested for radioprotective activity. In the United States, the Walter Reed Army Institute of Research (WRAIR) funded a large synthetic program and developed a screening procedure for compounds mainly of this type, during the period from 1959 to 1986. A compilation of the compounds tested in this program was made by T. R. Sweeney of the WRAIR in 1979. Many European countries also supported research programs on the development of radioprotective compounds and have been joined more recently by China and India. Other types of agents have been found with protective activities, but sulfur-containing molecules have been by far the most numerous.

Several structural requirements for activity in this series have become established. The presence of a free thiol group, or a thiol derivative that can be converted to a free thiol *in vivo*, is essential for activity. The presence of a basic function [amino, amidino, or guanidino (43)] (1) located two or three carbon atoms



(1)

distant from the thiol group is favorable for the best activity. Activity for these basic thiols drops off drastically with more than a three-carbon distance (44). The benefit arising from the basic group has not yet been explained, however. Several acyl thiol derivatives (2),

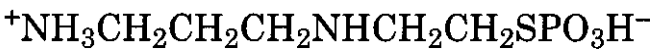


(2)

such as the thiosulfuric acid (45), phosphorothioic acid (46), and trithiocarbonic acid (47), most likely liberate free thiol in the animal.

Some radioprotectors may act by releasing endogenous nonprotein thiols normally bound by disulfide linkages with serum or interstitial proteins. An increase in tissue nonprotein thiol levels results after administration of the thiosulfate and phosphorothioate of MEA (48).

Alkylation of the nitrogen of MEA causes loss of activity in some cases, but has also resulted in some of the most potent of the MEA derivatives, of which WR2721 (3) is the best



(3)

known. The *N*-β-phenethyl and *N*-β-thienylethyl derivatives have good activity (49). Dialkylation of the nitrogen of MEA usually results in some loss of activity. Whereas the *N,N*-dimethyl and *N,N*-diethyl derivatives re-

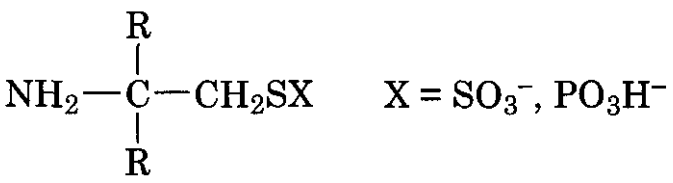
tain much of the activity of the MEA, the *N,N*-dipropyl and *N,N*-diisobutyl derivatives retain a little activity, and the di-n-butyl derivative is inactive (44). *N,N'*-Polymethylene bridging of the MEA structure provides compounds (4)

$$XS(CH_2)_2NH(CH_2)_nNH(CH_2)_2SX$$

(4)

that are active, where X is PO_3H_2 and n is 3 or 4, but inactive where X is SO_3H (50).

Alkylation of the carbon atoms of the MEA structure has given varied results. Active compounds have been found among C-monoalkyl derivatives, with 2-aminopropan-1-thiol having moderate activity and 1-aminopropan-2-thiol having good activity (51, 52). Whereas α,α-dialkyl-β-aminoethanethiols are inactive (53, 54), some β,β-dialkyl-β-aminoethane thiosulfates and phosphorothioates (5) have



(5)

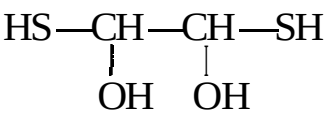
substantial activity (55). 2-Amino-1-pentanethiol and 2-amino-3-methyl-1-butanethiol also have good activity (55). C-Trialkyl derivatives of MEA (54), *sec*-mercaptoalkylamines (56), and 2-mercapto-2-phenethylamine (57) are inactive. Generally, the presence of a phenyl group in the MEA structure blocks activity (58). α,α-Dimethyl-2-aminoethanethiol, derived from penicillamine, is not protective but has radiosensitizing activity (59).

Alkylation or arylation of the mercapto group generally results in loss of activity. The *S*-benzyl derivative of MEA has some activity, probably resulting from in uiuo debenzylation (60).

Attempts to determine whether the stereochemical structure of the aminoalkanethiols is important have revealed that a given stereoisomer may provide greater radioprotection than others. A small difference in activity was found for the cis and trans isomers of 2-aminocyclohexane-1-thiol (61). The cis forms of 2-mercaptocyclobutylamine and 2-mercaptocyclobutyl-N-methylamine have higher radioprotective activities in mice than those of the trans forms. No correlation could be found between protective activity and ability to protect against either induction of DNA single-strand breaks or inactivation of proliferative capacity of hamster cells in vitro (62), however. On the other hand, the trans forms were less toxic and somewhat more effective in competing for free radicals in DNA. The D and L isomers of 2-aminobutylisothiuronium bromide have been separated, and the ***D*** isomer was twice as active in mice as the L isomer (63). The optical isomers of dithiothreitol show a greater difference in protective ability, the ***Dg*** isomer protecting 50% of mice exposed to 650 rads, whereas the ***Lg*** isomer afforded no protection (64). The ***Dg*** isomer was also less toxic.

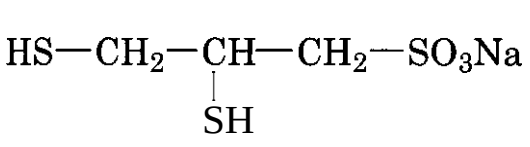
Other functional groups in the MEA structure have generally caused diminution or loss of protective ability. The presence of a carboxyl group frequently causes lower activity; cysteine, for instance, has the same dose reduction factor (1.7) in mice as that of MEA or MEG, but a much larger dose is required (65). This may be explained by the charge, or Z value, of the RSH molecule, which determines the concentration of thiol in the immediate vicinity of DNA, which has been shown to be in agreement with scavenging and chemical-repair reactions (66). The negative charge of a carboxyl group would thus be repelled by negative charges on DNA and prevent close accumulation of the thiol, necessary for DNA protection and repair.

N-Monosubstituted derivatives of MEA containing thioureide or sulfone substituents are inactive, although sulfonic acid zwitterions, $HS(CH_2)_2NH_2^+(CH_2)_3SO_3^-$, are strongly protective (67). The presence of hydroxyl often favors activity [e.g., *L*(+)-3-amino-4-mercapto-1-butanol gives good protection to mice (68)]. An additional thiol group diminished activity in a series of 2-alkyl-2-amino-1,3-propanedithiols, which showed little activity in mice (67). Dithiothreitol (Cleland's reagent, **6**)



(6)

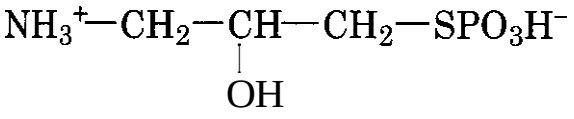
has protective ability (64), and Carmack et al. (64) found no protection from the oxidized dithiane form. Its protective activity *in vivo* may be attributable to release of other nonprotein thiols from mixed disulfides. Also, this may reflect a requirement for a suitable redox potential. Dithiothreitol is so readily reduced that it quickly becomes oxidized in biological systems. It is a good protector *in vitro,* under conditions where it remains reduced. *N*-Carbamoyl ethyl derivatives of the phosphorothioate of MEA, however, had high protective activity (69). Sodium 2,3-dimercaptopropane sulfonate (Unithiol, 7), which was studied in



(7)

Russia, was claimed to be more protective and less toxic than MEA (70).

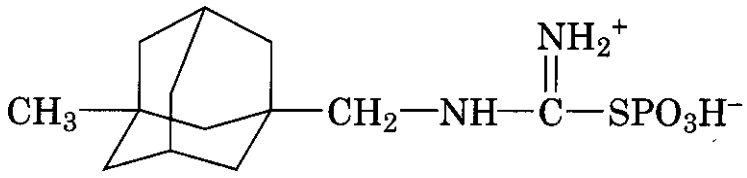
S-Acylation of the MEA structure has provided some very active compounds, particularly where zwitterions have resulted. The thiosulfate (Bunte salt) (45), phosphorothioate (46), and trithiocarbonate (47) of MEA, all of which form zwitterions, have protective activities comparable to that of MEA. Corresponding zwitterions of mercaptoethylguanidine (MEG) also give protection to mice corresponding to that of MEG (47, 71). Of these S-acyl derivatives, the phosphorothioates have been particularly effective; S-(3-amino-2-hydroxypropyl)phos-phorothioate (8) and S-(2-aminopropyl)-phosphorothioate



(8)

have DRF values in mice of 2.16 and 1.86, respectively, compared to a DRF value of 1.84 for MEA (72). S-[2-(3-Aminopropylamino) ethyl]-phosphorothioate (3), better known as WR2721, from the screening program at the Walter Reed Army Institute of Research (73), has high antiradiation activity and has been studied in numerous investigations. 3-Aminopropylphosphorothioate (71), however, and N-substituted derivatives of 2-aminoethylphosphorothioate are essentially inactive (71). Numerous publications concerning the synthesis and screening activities of the amino thiol derivatives submitted to the Walter Reed Army Institute of Research, leading to the selection of WR2721 as the most effective compound resulting from this screening program, have not been included here.

A comparison of the relative activities and toxicities of thiols with the corresponding thiosulfates showed the thiosulfates to be less toxic and comparable in activities (52, 74). In a series of 2-N-alkylaminoethanethiols,consisting of 66 compounds, the thiosulfates were generally superior to the corresponding thiols, disulfides, or thiazolidines (74), given intraperitoneally (i.p.) to mice. Another comparison of the relative effectiveness of thiols with the common mercapto-covering groups, the disulfide, thiosulfate, and phosphorothioate, was made with a series of 84 derivatives of 2-mercaptoacetamidine (75). Although generalities were not evident, by the i.p. route, the (3,5-dimethyl-1-adamantyl) methyl phosphorothioate (9) was the most effective compound. Perorally, the disulfides appeared to be superior, the most effective compound of which was the 1-adamantyl-methyl disulfide. In a series of N-heterocyclicaminoethyl disulfides and aminoethiosulfuric acids, the thiosulfates were generally more active and less toxic than the disulfides, administered either i.p. or perorally (76). The most effective compound of this series was 2-(2-quinoxali-



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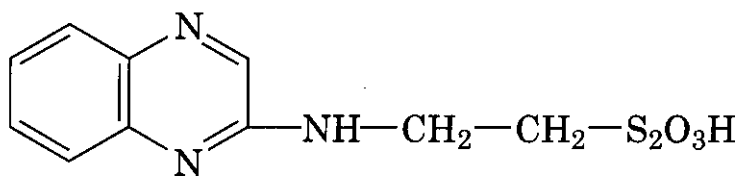
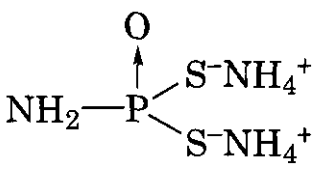


(10)

nylamino)ethanethiosulfate (10) It is believed that the phosphorothioate group aids in cellular transport (77).

Two inorganic phosphorothioates, diammonium amidophosphorothioate (11) and diammonium thioamidodiphosphate (12) gave



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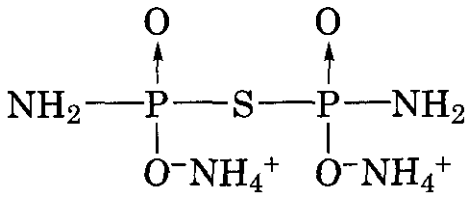
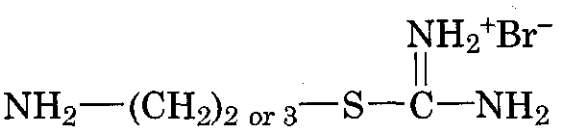


(12)

DRF values, respectively, of 2.30 and 2.16 at relatively low doses (72). Alkylation of the arnidophosphorothioate lowered or eliminated activity, however (78).

In a series of straight-chain aliphatic thioesters of MEA, the best protection was found with the acetyl and octanoyl derivatives (79); the benzoyl ester was essentially inactive. N-Acetyl and *N,S*-diacetyl MEA showed minimal activity (80). In a series of hemimercaptals of MEA derived from glycolic acid, the most active protected mice at one-half the LD_{50} dose, with activity comparable to that of MEA (81).

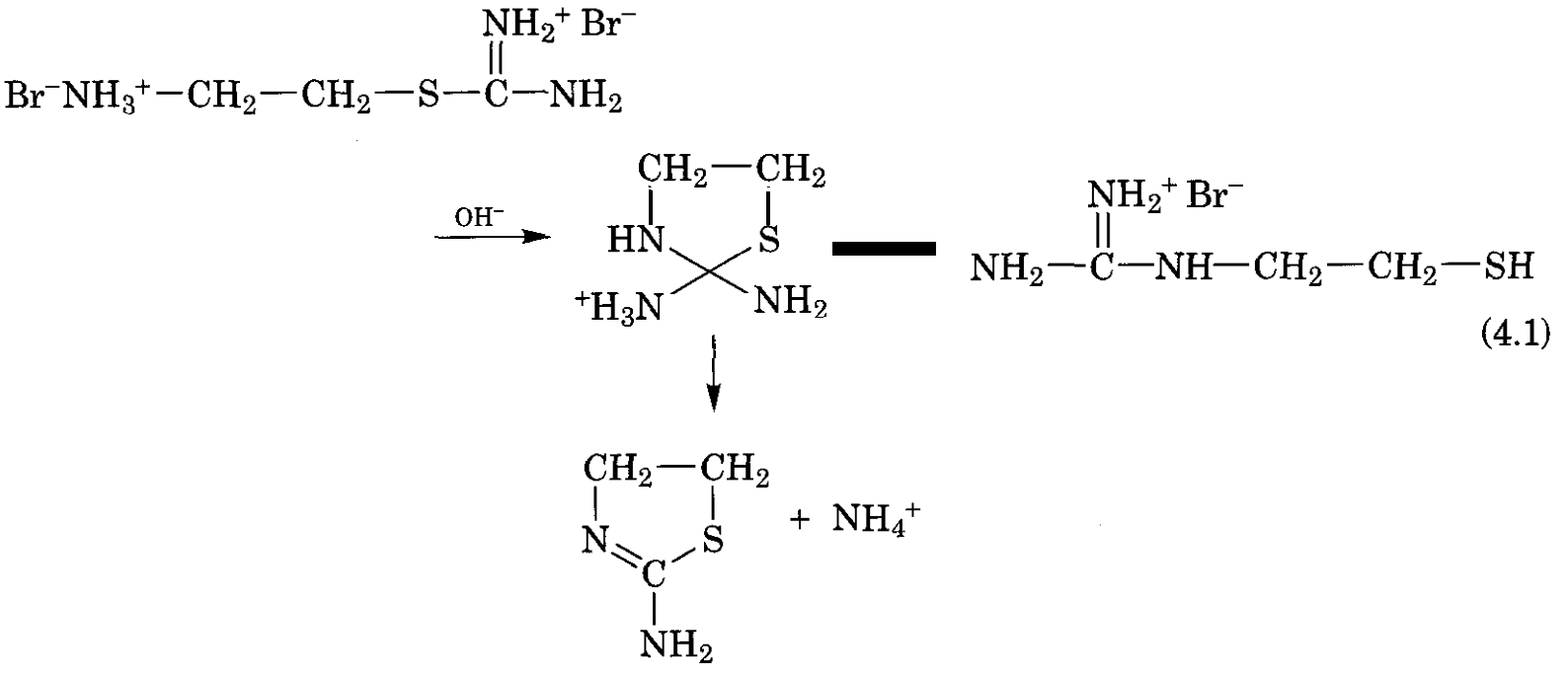
Other basic functional groups can replace the amino group in the MEA structure to provide protective thiols. The inclusion of the guanidino group has provided very active compounds, notably 2-mercaptoethylguanidine (MEG, 1) and 2-mercaptopropylguanidine (MPG) (80). Solutions of these compounds were obtained by alkaline rearrangement of the aminoalkylisothiuronium (AET or APT; 13) salts. When these compounds are em-



(13)

ployed for radiation protection tests, the hydrobromides of the aminoalkylisothiuronium bromides are rearranged in neutral or alkaline media. This rearrangement has been termed "intratransguanylation." Thus, AET or APT yields solutions of MEG or MPG (Equation 4.1). These compounds are usually not isolated by this procedure, but they may be isolated as the sulfates (82) or the trithiocarbonate esters (47).

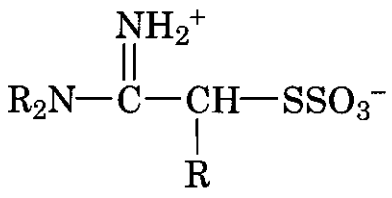
Although AET is not subject to air oxidation, as are most thiols, it is affected by moisture, resulting in conversion to 2-amino-2-thiazoline. The disulfide, bis(2-guanidinoethyl) disulfide (GED), is readily prepared, however, and is relatively stable. With more than three carbon atoms between the amino and isothiuronium functions, rearrangement



(4.1)

does not readily occur, and the isothiuronium salts give little protection. 2-Aminobutylthiopseudourea dihydrobromide, however, requires about one-fourth the molar quantity of AET for comparable protection in mice (63).

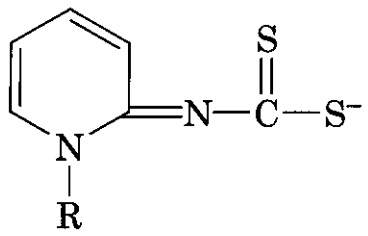
Replacement of the amino group by amidino has also resulted in compounds with good protective activity, particularly with Bunte salts of *α*-mercaptoacetamidines (**14**)



(14)

(83). Among the most effective of the amidinoalkylthiosulfuric acids are several terpene derivatives, including the bornyl (84). Other amidines related to MEA and MEG have been effective; 3,3′-dithiobis(propionamidine) (85) and propionamidines containing isothiuronium groups (86), for instance, have good activity.

Use of strongly basic nitrogen heterocycles having pK_a values of 10–12.5 has also provided protective compounds having the dithiocarbamate group as the sulfur-containing function. Reaction of imino-*N*-alkyl pyridines, pyrimidines (87), quinaldines, and acridines (88) with carbon disulfide gave imino-N-carbodithioates (15) having moderate protective effects in mice.

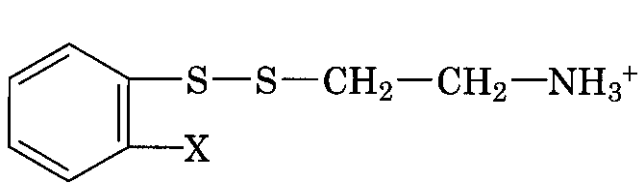


(15)

Substitution of the hydrazino group for amino has not provided many active compounds. Protection of mice has been reported for *N*,*N*′-bis(mercaptoacetyl) hydrazine (89), as well as for *N*-acetylthioglycolic hydrazide, $HSCH_2CONHNHCOCH_3$, and its disulfide (90).

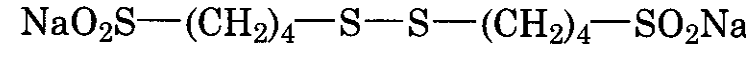
Oxidation of the thiol group of the MEA structure has provided products with radioprotective properties, particularly with the disulfides. The disulfides of MEA (cystamine) and MEG (GED) are as active as the parent thiols, although GED is more toxic than MEG (91). The argument has been advanced that the thiol is the active form of these compounds, given that some in vitro systems protected by MEA are not protected by cystamine (92), and the reduction of cystamine to MEA during irradiation of mice has been observed (93, 94). In the case of GED, appreciable amounts of this disulfide were found in vivo after administration of either MEG or GED (95). In theory, disulfides should be as effective as thiols because they are readily reduced to thiols intracellularly. The converse is also true, in that thiols are easily oxidized in vivo and therefore lose activity.

Cystine is nonprotective in mammals, probably because of its inability to penetrate some cellular membranes (93). Mixed disulfides of MEA have provided good protection, particularly those derived from o-substituted mercaptobenzenes, where zwitterions are formed with carboxyl, sulfonyl, or sulfinyl anions (16) (96). It is possible, however, that in



(16) X = CO_2^-, SO_3^-, SO_2^-

vivo the unsymmetrical disulfides are disproportionating to the two symmetrical disulfides, giving rise to cystamine. Mixed disulfides containing N-decyl MEA are also effective (97), as is the mixed disulfide of thiolacetic acid and N-acetyl MEA (98). Disulfides lacking basic groups have generally been found inactive, although a bis(butanesulfinate) disulfide (**17**), derived from (18) by disproportionation, was highly active (99). The



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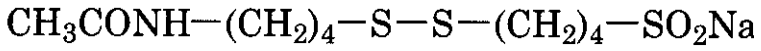
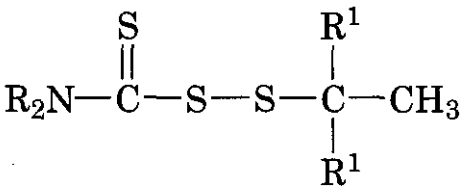


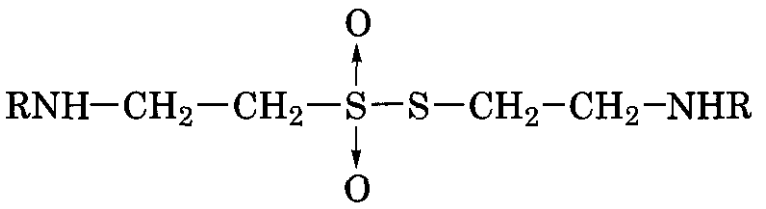
(18)

bis(butanesulfinate) trisulfide was also very active, protecting 100% of mice against a lethal dose of radiation. Two thiocarbamoyl disulfides (19; R = H, CH_3) also gave good protection (100).



(19)

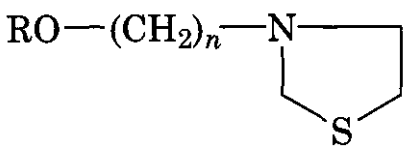
Higher oxidation states of the sulfur in MEA and MEG molecules have been obtained, and some protective activity has been found with these derivatives. The thiolsulfinates of both MEA (101) and MEG (102) have been prepared, as well as the corresponding thiolsulfonates (**20**) (103).



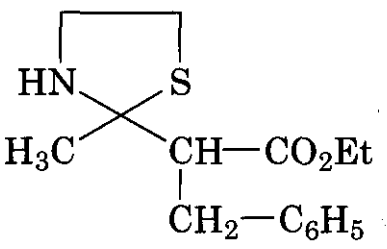
(20) R = H, $C(=NH_2^+)-NH_2$

Protective activity has been reported for both the thiolsulfonate (103) and the thiolsulfinate of MEG (104), as well as for the thiolsulfonates of N-acetyl and N-decyl MEA. Taurine and hypotaurine (the SO_3H and SO_2H derivatives, respectively, of MEA), both metabolites of MEA in mice (105), provide essentially no protection (27).

Thiazolidines have been prepared from MEA or its N-substituted derivatives by reaction with aldehydes and ketones. A number of thiazolidines have shown good protective activity in mice, which has been attributed to ring opening in *vivo* to give the amino thiols (106). N-Substituted thiazolidines having oxy or thio cycloalkyl, aryl, or heterocyclic alkyl groups (21) have good activity (107). Thiazolidine-4-carboxylic acid, derived from cysteine, affords 40% protection to rats (108). Thiazolidines with particularly good activity are 2-propylthiazolidine (106), the 2-(3-phenyl propionate ester) derivative (22) (109), and the



(21) R = cycloalkyl, aryl or heterocyclyl



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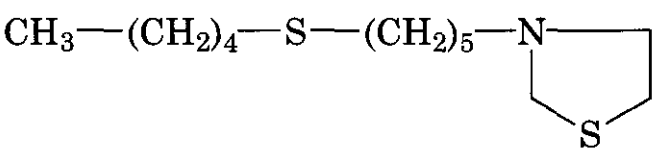
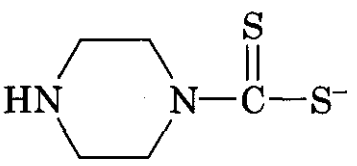


(23)

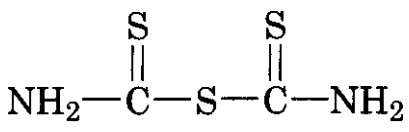
N-pentylthiopentyl derivative (23) (110). The latter compound was active orally. 2-Aminothiazoline, which is derived from AET at pH 2.5, has protective activity (111); it is probably converted to N-carbamyl cysteamine at pH 9.5 (112). 2-Mercaptothiazoline has been found active in two laboratories (16, 60); others have found it inactive (111, 113).

1.4.2 Other Sulfur-Containing Compounds. A number of dithiocarbarnates have significant radioprotective effects, although the order of activity is less than that of MEA and its derivatives. The simplest compounds of this type, either with the nitrogen unsubstituted or bearing small alkyl groups, up to n-butyl, have shown the most activity (114, 115). 2-Methylpiperazinedithioformate (**24**), however, provides protection more nearly comparable to that of MEA (116). The mechanism by which the dithiocarbamates protect is believed to differ from that of the aminothiols. Xanthates have not been found protective (117).



(24)

The related thiocarbamyl derivatives (**25**) and (**26**) have been reported to provide good protection (118).



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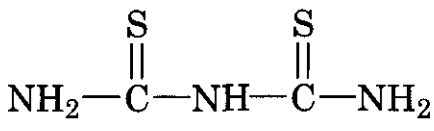

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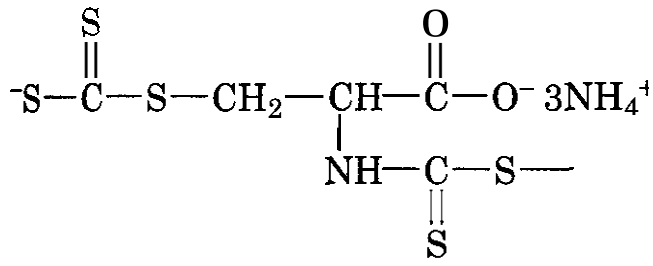
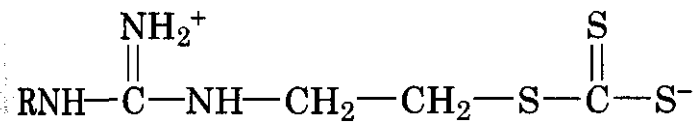
Reaction of cysteine with carbon disulfide gives the trithiocarbonate dithiocarbamate (27) (119), which is equivalent in protective



(27)

activity in mice to that of MEG but is only one-third as toxic. A metabolism study in mice showed the dithiocarbamate group to be appreciably stable *in vivo,* but the trithiocarbonate to be unstable (120). Trithiocarbonates of MEG and MPG and several derivatives of MEG (28) also provided good protection to mice against a lethal dose of X-radiation (47).



(28)

Thioureas and cyclic thioureas have shown only marginal or no protection. Thiourea itself protects mice only in massive doses (1800–2500 mg/kg) (5). S-Alkylisothioureas, with alkyls up to n-butyl, have shown moderate protective effects (121). Dithiooxamide is nonprotective, but symmetrical *N,N'*-dialkyldithiooxamides provide some protection (113). 1,5-Diphenylthiocarbohydrazide and several derivatives have fair activity (122).

Simple, nonbasic thiols have no value as radiation protectors. Conflicting results have been reported for the dithiol BAL as well as for thioctic acid (27). 2,3-Dithiosuccinic acid is protective in mice vs. 700R (123), but most other dithiols are inactive, with the exception of dithiothreitol (64). 2-Mercaptoethanol protects bacteria (124) but not mice (125); it has also been found to be radiosensitizing (126).

Other sulfur-containing compounds with significant radioprotective ability include dimethyl sulfoxide (127) when given in large doses; other sulfoxides afford little or no protection. Large doses are required because the mechanism of protection may be hydroxyl-radical scavenging rather than hydrogen atom donation to radicals or DNA. Organic thiosulfates, other than those that liberate MEA or an active derivative of MEA, have generally failed to protect. Inorganic thiosulfate is a good protector of macromolecules *in vitro* or of the mucopolysaccharides of connective tissue *in vivo* (128); it does not protect animal cells, however, because of its inability to penetrate. Sodium cysteinethiosulfate (**29**), derived from

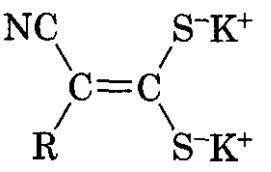
(29)

the cleavage of cystine with thiosulfate ion, has good activity, being protective of the intestines and kidneys of mice (129). The related *S*-sulfocysteine, having one less sulfur atom, is almost devoid of activity (130).

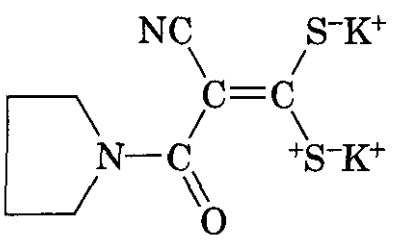
Mercapto acids have shown little protection, with the exception of thioglycolic acid, which is slightly protective, inactive, or sensitizing, depending on the system tested (27). The β-aminoethyl amide of thioglycolic acid, $HSCH_2CONHCH_2CH_2NH_2$, has good activity (81), however.

Monothio acids and their derivatives are generally inactive, although several dithio acid dianions, obtained by condensation of carbon disulfide with cyanomethylene compounds (**30**) show some protection of mice (131). The most active of this series is the di-

thio acid derived from 2-cyanoacryloylpyrrolidide (**31**), which provides 80% protection to mice against a lethal dose of y-radiation (76). Dithio esters derived from pyridinium dithioacetic acid betaine (32) also show some protec-



(30) R = CN, CO_2Et, C_6H_5CO



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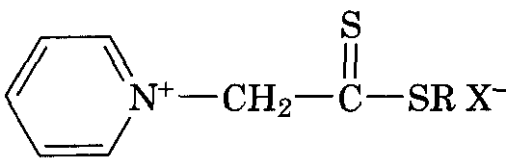
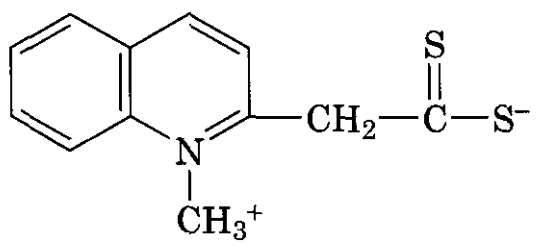


(32)

tive activity in both mice and bacteria (132). Quinolinium-2-dithioacetic acid zwitterions (33)provided good protection to mice, and the more soluble bis(methylthio) and methylthio amino derivatives (34) had slightly better effects (133). The corresponding pyridinium compounds had equally good activities, and both



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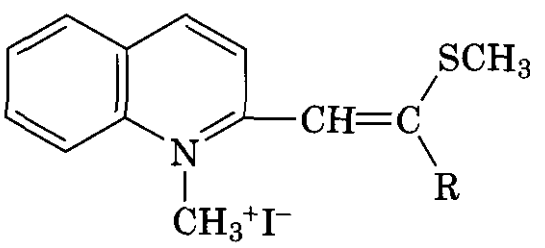


(34) R = SCH_3, NR_2

the quinolinium and pyridinium compounds were protective at much lower dosage levels (2–18 mg/kg) than those for the aminothiols (150–600 mg/kg). Replacing the aromatic function with aliphatic in these compounds, yielding 3-amino-2-phenyldithiopropenoate esters, $R_2NCH = C(C_6H_5)CS_2CH_3$, resulted in only fair or poor protection (134).

Thiols that occur naturally have not been found appreciably protective in animals, with the exception of glutathione (135), which has moderate activity. Pantoyl taurine apparently has some activity (136). Bacq (25) presented arguments that make it appear unlikely that coenzyme A is involved in radioprotection.

Selenium compounds have generally been ineffective in animal tests. Selenium analogs of the well-known radioprotectors, 2-aminoethaneselenol, 2-aminoethaneselenosulfuric acid (52), and 2-aminoethylselenopseudourea (137) are much more toxic than their sulfur analogs and are nonprotective. Sodium selenate (138) has been reported to be effective in rats administered postirradiation. Some selenium-containing heterocycles (e.g., selenoxanthene, selenoxanthone, and selenochromone) have been described as effective in rats (139). The investigation of organic selenium compounds as potential antiradiation agents has been reviewed by Klayman (140).

1.4.3 Metabolic Inhibitors. Cyanide ion has been found radioprotective in several laboratories (3,1411, but it must be administered immediately before irradiation because of its rapid detoxification (17). It has a number of biological properties in common with thiols, such as reduction of disulfide linkages and inhibition of copper-containing enzymes, but unlike thiols, it also inactivates cytochrome C oxidase, which controls oxygen consumption in mammals. Among other enzyme inhibitors, azide (142), hydroxylamine (143), and 3-amino-1,2,4-triazole (144) are weak protectors. The latter two compounds are inhibitors of catalase, but no relation between this effect and radioprotection was apparent.

Several organic nitriles show radioprotective effects; the most effective may be hydroxyacetonitrile (113). Fluoroacetate is protective (145) when sufficient time is allowed before irradiation for its conversion to fluo-

rocitrate, an inhibitor of citrate metabolism. Other thiol group- or enzyme-inhibiting agents, such as iodoacetic acid, malonic acid, mercurials, and arsenicals have no protective ability, but some of these agents have radiosensitizing effects.

1.4.4 Agents Involving Metal Ions. A number of metal-binding agents are radioprotective and are also known to inhibit enzymes. Some metal complexes imitate the action of enzymes, such as copper complexes, which catalyze the decomposition of peroxides (146). In this aspect, the copper complex of 3,5-diisopropylsalicylate has shown activity against γ-radiation (147), presumably by mimicking the action of superoxide dismutase.

Copper complexes of the radioprotective pyridinium and quinolinium dithioacetic acid derivatives (133) also are able to mimic the action of superoxide dismutase (148). These effects may play a role in radiation protection.

Metal-binding agents already discussed include the dithiocarbamates as well as the aminothiols (149). EDTA protects mice only in very large doses (150), probably because very little EDTA enters the cells. 8-Hydroxyquinoline (oxine) is too toxic for animal studies, but was found highly protective in a polymer system (125). Other common metal-binding agents, such as *N*-nitroso-*N*-phenylhydroxylamine and nitrilotriacetate, show appreciable protection (151). Derivatives of 1,5-diphenylthiocarbohydrazide, avid metal binders, protect mice, rats, and dogs (122).

Some metal complexes have been tested and found to afford some protection. Iron complexes of polyamines (152) are active, as well as zinc complexes of MEA and MEG, the copper and iron complexes showing little or no activity (149). Copper complexes of diethyldithiocarbamate and dithiooxamide, however, give less protection than that of the uncomplexed ligands (125). Complexes of chlorophyllin, with Co, Mg, Mn, and V, are radioprotective in mice (153). Zinc aspartate has shown some protective properties (154).

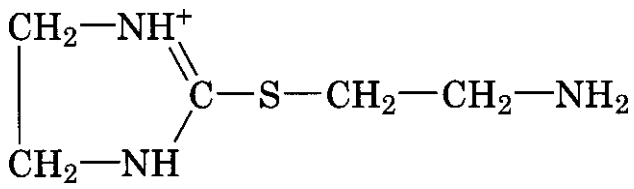
1.4.5 Hydroxyl-Containing Compounds. Significant protection in animals by hydroxyl-containing compounds, including ethanol, was at first considered the result of antioxidant properties. Glycerol, however, is protective in mice as well as in other systems (5, 150). Phenols are protective in polymethacrylate tests (125), but many of them are too toxic for animal tests. The catecholamines provide some protection, possibly by lowering oxygen tension in the cells (27). A compound that increased catecholamine levels in irradiated rats, 1-acetylhydrazinylthiophenylformamidine, protected against a half-lethal dose of radiation (600 R) (154). The protective effects of gallic acid esters are attributed to inhibition of chain oxidation processes induced by radiation (155). Arachidoyl derivatives of pyrogallol and the naphthols have shown some protective activity (156). Ionol (2,6-di-*t*-butyl-4-methylphenol), injected after irradiation, prolongs the life of mice and alleviates intestinal damage (157).

Organic acids provide little or no protection, but the polycarboxylic acids, pyromellitic and benzenepentacarboxylic, are protective in mice (158). These polyionic substances are believed to protect by causing hypoxia from osmotic effects, rather than by chelating calcium ion, which also is believed to have an effect on radiation damage.

In a series of S-2-(3-aminopropyl-amino) alkylphosphorothioates, which are effective protectors in mice when given orally, the presence of hydroxyl groups in the alkyl chain generally lowers effectiveness for oral administration, but still allows good protection by i.p. injection (159).

1.4.6 Heterocyclic Compounds. Several relatively simple heterocyclic compounds provide significant protective activity in mice. In a series of imidazoles, imidazole itself, benzimidazole, and 1-naphthylmethylimidazole were the most effective compounds (160). Related imidazolidine-5-thiones were also protective. The cyclic analogs of AET, 2-aminoethyl- (35), and 2-aminopropylthioimidazoline, are moderately protective (161).

Of a large number of amine oxides tested for radiation protection, quinoxaline 1,4-di-*N*-oxide (36) (believed to act in part by radical trapping) was the most effective (162). It is protective in mice but radiosensitizing in the dog (163). A more recent N-oxide, 2,2,6,6-tetramethyl-4-hydroxypiperidine-N-oxide (Tem-



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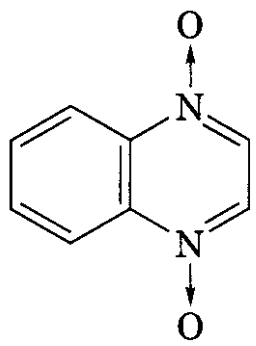
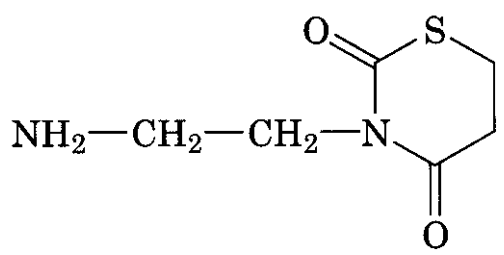


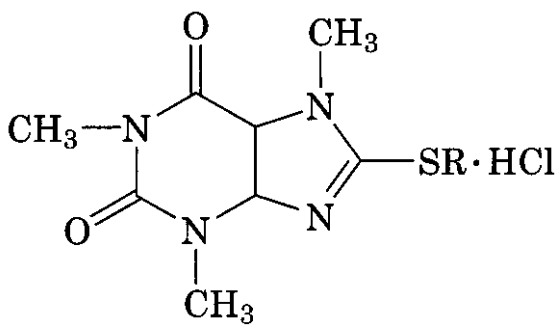
(36)

pol) and its reduced hydroxylamine are radioprotective in mice without lasting adverse effects (164). 3,5-Diamino-1,2,4-thiadiazole (165) and 3-(β-amino-ethyl)-1,3-thiazane-2,4-dione (166) (37)have some protective activity.



(37)

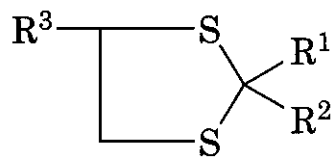
Arninoethyl and aminomethyl purines and pyrimidines gave one-third as much protection in mice as MEA (167). 8-Mercaptocaffeine and its *S*-β-aminoethyl and *S*-β-hydroxyethyl derivatives **(38)**had protective activity in mice



(38) R = H, $CH_2CH_2NH_2$, CH_2CH_2OH

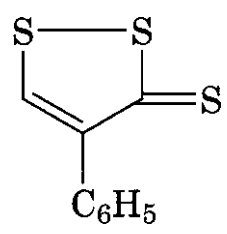
similar to that of cystamine (168). These compounds also enhance hemopoiesis and decrease blood loss in irradiated animals.

Good protection was provided by 6-acyl-2,3-dimethyl-4,7-dimethoxybenzofurans (169), and fair protection was observed for several 2-dialkyl-1,3-oxathiolanes (170). In a large series of 1,3-dithiolanes tested, moderate protection was shown by 1,3-dithiolane itself and by its 2- and 4-methyl derivatives (171) (39). Although



(39)

aryl 1,2-dithiole-3-thiones are known to raise glutathione levels in cells (172), 4-phenyl-1,2-dithiole-3-thione (40) and its S-methyl iodide



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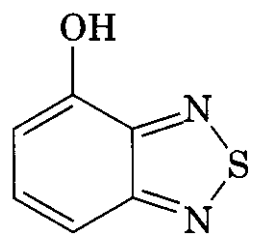
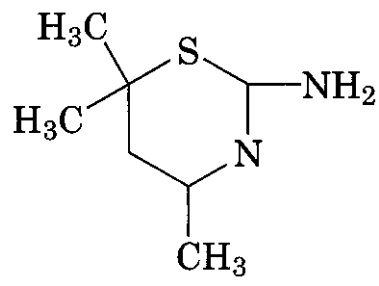
showed no activity in mice against a lethal dose of y-radiation (134).

In a series of 2,1,3-benzothiadiazoles, the 4-hydroxy derivative (41) had the best protec-



(41)

tive effect in mice (173), either i.p. or orally. Several aminothiazines, including 2-amino-4,6,6-trimethyl-1,3-thiazine (**42**), increased survival time in mice (174), without liberation of



(42)

a thiol group. 2-Pyridinemethanethiol, structurally related to cysteamine, showed good protective potency in mice, but 2-pyrazinemethanethiol was inactive (175). The three isomeric mercaptopyridines also were active.

1.4.7 Physiologically Active Substances. A number of familiar physiologic agents exert some radiation protection, which is generally of a lower order of activity than that provided by the amino thiols. Many of these agents are believed to be radioprotective by virtue of their ability to lower oxygen tension in the cells or to depress whole-body metabolism. Serotonin [5-hydroxytryptamine (5-HTT)] has been reported equal in activity to MEA (136). It is effective at a dose well below the toxic level (113). It has been most often used as the creatinine sulfate salt. Its activity has been attributed to its vasoconstrictor effect leading to hypoxia in radiosensitive tissues (176). Psilocybine (4-hydroxy-N,N-dimethyltryptamine), however, a more potent vasoconstrictor than serotonin, is inactive, suggesting that this effect may not be of major importance (177). There is evidence of central nervous system involvement in the activity of serotonin (178).

5-Hydroxytryptophan is comparable in activity to serotonin (179), and the 5-methyl ether of serotonin (mexamine) is also as effective, although higher alkyl ethers are ineffective (180). Numerous indole derivatives have been prepared as potential protectors, including 5-acetylindole, which has some activity (181), but none has exceeded serotonin or mexamine in potency. A series of acyl derivatives of 5-methoxytryptamine showed good protective effects in mice against a lethal dose of X-rays, the hexanoic and octanoic amides being the most potent (182). A synergistic radioprotective effect results from a combination of AET, ATP, and serotonin in mice and rats (183).

Central nervous system depressants have only small or moderate effects as radiation protectors. Chlorpromazine has been extensively studied, but exerts only a slight effect, which is most pronounced when administered 4.5 h before irradiation, when a state of hypothermia exists (184). Chlorprothixene is also most effective when body temperature and metabolism are depressed (185). Reserpine is effective when given 12–24 h before irradiation (186), possibly by release of serotonin and catecholamines (187).

Central nervous system stimulants generally are nonprotective. An exception is the magnesium complex of pemoline (2-imino-5-phenyl-4-oxazolidinone), which provides moderate protection to mice against 750 R (188). Of 21 analogs of imipramine, three showed significant activity (189).

The different classes of autonomic drugs provide some radiation protection; the causative factor is believed to be production of hypoxia by various mechanisms. Epinephrine provides some protection (190), but norepinephrine, which decreases oxygen tension in the spleen much less than does epinephrine, gives very little protection to mice (191). The cholinomimetic compounds arecoline, tremorine, and oxytremorine are also protective to a small extent in mice (192).

p-Aminopropiophenone (PAPP) apparently protects by induction of tissue hypoxia (193). It has been used in relatively small quantities in combination with other protective agents, such as MEA and AET (194,195). The radioprotection afforded by PAPP is abolished by increased oxygen pressure during irradiation (196). PAPP and its ethylene ketal also gave good protection orally to mice against X-rays (197). p-Aminobenzophenone also provided good protection in this test. Monothio and dithio ketals of this compound gave little or no protection (198).

Physiological changes can probably account for the radioprotective action of some substances. Urethane (199), estrogens (200), and colchicine (201) can stimulate blood cell production by damaging bone marrow. If irradiation is carried out while there is an increased leucocyte/lymphocyte ratio in the blood, so that a greater percentage of more radioresistant cells are present, enhanced survival may result. The effect of colchicine may also be attributable to inhibition of mitosis, but there is evidence against this supposition (201). Colchicine is protective only when administered 2 or 3 days before irradiation, by which time mitotic inhibition has ceased. Urethane and the estrogens are similar in that they must be given a day or more before irra-

diation. The proestrogen tri-*p*-amisylchloroethylene is effective when given 5–30 days before irradiation (202). Other inhibitors of mitosis, however, can enhance survival; these include demecolcine (Colcemid), sodium arsenite, epinephrine, cortisone, and typhoid paratyphoid vaccine (203). Tranquilizers and other psychotropic drugs possess only moderate radioprotective activities. These compounds probably are active by depression of whole-body metabolism through diminished oxygen uptake (35).

Procaine (204) and several derivatives of procaineamide, particularly the p-nitro derivative (205), have shown appreciable protective activity. 4-Hydroxybutyric acid and 6-phosphonogluconolactone, substances that stimulate turnover of $NADP \cdot H_2$, a physiologic reducing agent, provide protection to mice (206). An antihistamine, thenaldine, affords moderate protection (204). Alloxan protects both mice (207) and the pancreatic ultrastructure of dogs (208).

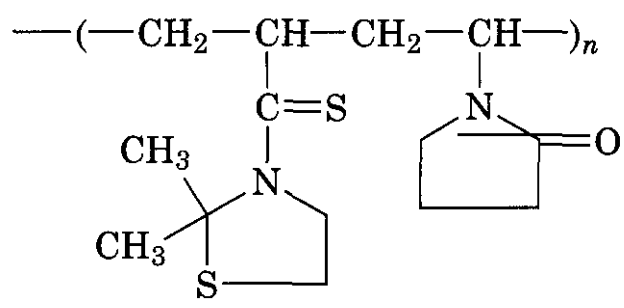
1.4.8 Metabolites and Naturally Occurring Compounds. A variety of compounds in these categories have been examined for radiation protection, but few effective protectants have been found. Some polysaccharides, such as dextran (209), those extracted from typhoid and proteus organisms (210), and a lipopolysaccharide from S. *abortus* (211), provide some protection for mice, possibly by inducing phagocytosis. Bacterial endotoxins, which are lipopolysaccarides of molecular weight around 1,000,000, show relatively good protective properties in both normal (200) and germ-free mice (212). Typhoid/paratyphoid vaccine shows similar protective properties (213).

Vitamins and coenzymes are not appreciably protective. Pyridoxal phosphate, however, has a moderate effect (214), which may be connected with a repair rather than a protective process (215). Several thiol-containing derivatives of vitamin B_6, including 5-mercaptopyridoxine (216), are also protective. Some of the naturally occurring pyrimidine bases and nucleotides (217), including ATP (218), have an effect in mitigating radiation damage, but their value may be ascribed to postirradiation repair. Protection from RNA, DNA, and derivatives has been claimed, but their effects are more likely attributable to repair processes (218–220). A protamine-ATP combination provides good protection to rats (221).

Among the commonly used antibiotics, the tetracyclines have shown the most favorable effects on survival rates of mice (222); this is believed to be the result of an increase in metabolic activity. A gallate-tannin complex (223) was active probably because of its antioxidant effect. 5,7-Dihydroxyisoflavones are effective when administered to mice percutaneously but not intraperitoneally (224), presumably because of protection of the capillaries. *O*-β-Hydroxyethylrutoside is also protective in mice, possibly by strengthening vascular walls and reducing bacterial invasion of the bloodstream (225). The radioprotective effect of rutin and other flavonoids has been controversial.

A series of extracts from the Chinese drug plants *Carthamus tinctorius, Sargentodoxae cuneata, Paeonia lactiflora, Salvia miltiorrizha,* and *Ligusticum chuanxiong* have shown significant protection in mice versus 7.5–8.0 Gy of γ-radiation (226). Their protective properties are believed to be related to their inhibitory effects on radiation-induced platelet hypercoagulation in the capillaries, which prevents excessive bleeding. Active constituents in the Ligusticum drug extract have been found to be harman alkaloids, including 1-(5-hydroxymethyl-2-furyl)-9 *H*-pyrido-[3,4-*b*]indole (227). Acetylsalicylic acid also provides moderate protection at this radiation dose.

1.4.9 Polymeric Substances. A synthetic polymer prepared from *N*-vinylpyrrolidone and *S*-vinyl-(2,2-dimethylthiazolidyl)-*N*-monothiol carbamate (43) was found protective in mice, possibly by liberation of thiol groups (228). Other copolymers containing isothiouronium



(43)

salts, thiosulfates, and dithiocarbamate groups give appreciable protection when administered 24–48 h before irradiation (229). Polyinosinic-polycytidylic acid increases survival of mice, possibly by increasing the stem cell fraction in blood-forming tissues (230). Both poly(vinyl sulfate) (231) and heparin (232), a sulfated mucopolysaccharide, increase survival rates, possibly by affecting deoxyribonuclease activity.

1.4.10 Miscellaneous Substances. In a series of diethylsulfides and diethylsulfoxides, bis[2-*n*-butyrylamino)ethyl]-sulfoxide showed the greatest radioprotectant activity (233). Among 20 benzonitriles evaluated as radioprotectants in mice, 3,5-dinitrobenzonitrile showed significant activity (234). A series of N-substituted 1-*m*-hydroxyphenyl-2-aminoethanols, which are derivatives of phenylephrine, gave significant radioprotective effects (235). Cinnamonitriles and hydrocinnamonitriles produced survival rates of 43–58% in three derivatives of the former, the p-MeO, p-NO_2, and 2-chloro-p-MeO, after lethal doses of radiation (236). The S-phosphate of 5-mercaptomethylcytosine exhibited protective activity at a dose of 6 Gy (237). Radioprotective effects of two halogenated 1,3-perhydrothiazines have been described (238).

Radioprotective effects of isobombycol and its enanthate and cinnamate in mice have been reported (239). In a series of substituted anilines tested in mice versus a near-lethal dose of 6 mV photons, compounds with electronegative groups in the meta or para positions gave good protection to mice (240). No correlations between protective activity and a variety of molecular parameters could be found, however. Schiff bases of salicylaldehyde, 5-chlorosalicylaldehyde, and benzaldehyde with anilines reduced toxicity of the parent amines, but gave erratic protective results (241). The highest protection was observed for mixtures of p-aminopropiophenone with its Schiff bases or with the Schiff base of 1-(*p*-aminophenyl)-1-propanol.

1.5 Mechanisms of Protective Action

The manner in which mammalian cells are protected from the damaging effects of ionizing radiation is not known in complete detail, although evidence is accumulating for several postulated pathways of radioprotection. Protection by means of radical trapping or antioxidant action, which can be demonstrated for simple systems, such as polymers, may be operative in animal cells as well. It is also probable that other mechanisms are more important in protection of cells, and possibly more than one mode of protection may be operative for a given type of agent. A number of the physiological agents that have been observed to be radioprotectors are believed to protect by producing various levels of anoxia; the evidence for this has been discussed (27).

1.5.1 Protection by Anoxia or Hypoxia. Protection by producing a state of cellular anoxia or hypoxia is based on the phenomenon of the "oxygen effect," the increase by two- to threefold of the damaging effects of radiation attributed to the presence of oxygen. A number of radioprotective drugs possess the physiological function of producing anoxia or severe hypoxia in various tissues; these include the catecholamines, histamine, choline esters, p-aminopropiophenone, morphine, ethyl alcohol, and nitrite. Other physiological effects, however, may contribute to their ability to protect, particularly with serotonin. Although the powerful protection afforded by this compound is not completely explained, a correlation between vasoconstrictive effects and radioprotection was found for a series of indolamines (242). Increasing the amount of oxygen available to radiosensitive tissues reversed the radioprotective effect of serotonin, histamine, and epinephrine, but caused much less reduction of protection by MEA or cysteine (243). The amino thiols, notably cysteine, MEA, and AET, can decrease oxygen consumption in the cells (244), but no appreciable hypoxia exists during the protective period (245). Enzymatic oxidation of radioprotective thiols has been reported (246), although the extent to which the resulting oxygen depletion contributes to radioprotection is uncertain.

A series of reports (reviewed in Ref. 247) provided evidence that WR2721 and its dephosphorylated thiol WR1065 have a major effect through their ability to cause local tissue hypoxia. The thiol also rapidly depletes the oxygen content of mammalian cell suspen-

sions in culture (248). However, these results are put in question by the lack of adequate procedures to measure the oxygen concentration at critical sites for radiation protection (249).

1.5.2 Inhibition **of** Free-Radical Processes. Mechanisms of protection involving "free-radical scavenging" are based on the assumption that the free radicals resulting from radiolysis of water are the main cause of radiation damage to the cells. Radioprotectors then would react with these radicals, such as H', $HO^{\bullet}$, and $HO_2^{\bullet -}$, and prevent them from damaging biologically important molecules. This concept received support when a correlation was found between the protective action of about 100 substances in two systems: an aerated aqueous solution of polymethacrylate and the mouse (125). It is probable that radical scavenging is the primary event in the prevention of radiolytic fragmentation of the polymer (250), but it is probably not of equal importance in the cell. Radioprotection of mammalian cells by the $HO^{\bullet}$ scavenger dimethylsulfoxide requires concentrations of the order of 1 M, and the degree of protection is insensitive to oxygen concentration (251). Radioprotection of mammalian cells by thiols can be achieved at concentrations of the order of 1–10 m*M* but this radioprotection can be reversed by exposure to molecular oxygen, indicating that it is not the result of HO' scavenging (251). Diffusible radical scavenging could be radioprotective if mechanisms other than double-strand lesion formation are involved. In that case, it is more likely that less-reactive radicals than $HO^{\bullet}$ would be involved, given that lower concentrations of scavenger could be effective. Cytoplasmic irradiation (precluding direct damage to DNA) can be mutagenic, and there are indications that radical scavenging could be protective against this effect (252).

Reaction of sulfhydryl compounds with free radicals formed on macromolecules is considered more likely than reaction with HO', to account for radioprotection in mammalian cells. Reaction rates with such radicals were measured for several radiation protectors: MEA, AET, APT, thiourea, cysteine, glutathione, propyl gallate, and diethyldithiocarbamate (253). The fastest rates were observed for diethyldithiocarbamate, MEA, and cysteine. Cysteine and glutathione were found to accept electrons from irradiated proteins, whereas cystine and some nonsulfur compounds did not (254).

A number of antioxidant phenols, pyridines, and gallic acid esters are believed to be effective by virtue of their antioxidant action. A direct relation between radical inhibitory action and radiation protection has been observed (255). Protection by aliphatic alcohols, including glycerol, generally requires large concentrations for maximum protection (1–3 *M*) in cultured mammalian cells (256), but protective effects of radical scavengers have been found at much lower concentrations in bovine erythrocytes (257) and the erythrocyte membrane (258).

1.5.3 Mixed Disulfide Hypothesis. This hypothesis of Eldjarn and Pihl (259) proposed that radioprotective thiols form mixed disulfides with thiol groups of proteins. The mixed disulfides provide protection to the protein thiols either by interfering with indirect radiation damage from radiolysis products of water or by facilitating energy transfer from the directly damaged protein to the administered thiol. Some arguments with this hypothesis have arisen; many thiols do not protect, and most thiols are capable of forming mixed disulfides (260). However, equilibrium constants for mixed disulfide formation are high for protective thiols but low for poor protectors (261).

In their original hypothesis, Eldjarn and Pihl proposed that the mixed disulfide bond would be cleaved by radical scavenging, but subsequent studies with protein solutions indicated that this may not be the case (262). Disulfide formation may also protect by moderating radiation-induced rearrangements (263). Radical scavenging may be an important function of the mixed disulfides (264), but mixed disulfide formation may also be a precursor for the liberation of cellular thiols, to be discussed under Section 1.5.4.

Another argument against this hypothesis is that many proteins are not damaged seriously by a dose of radiation that is lethal to mammals (265). Also, the nucleic acids, important target molecules of the cell nucleus, do

not contain thiol or disulfide groups. The nuclear proteins involved in cell division have been proposed, however, as likely sites for mixed disulfide formation (266). RNA polymerase is particularly implicated for this process (267). Also, in favor of this hypothesis, ^{35}S-MEA was found mainly bound to protein at the time of maximum protection (268). Certain enzymes, containing essential sulfhydryl groups, were found to be protected from X-ray damage by mixed disulfide formation (269).

1.5.4 Biochemical Shock. A number of biochemical and physiological disturbances take place in the cells after administration of thiols, and realization of the full extent of the cellular changes produced led to the postulation of the "biochemical shock" hypothesis of Bacq (270) and others. This states that protective thiols undergo mixed disulfide formations in the cells, leading to a series of disturbances including decreased oxygen consumption, decreased carbohydrate utilization, and mitotic delay by temporary inhibition of DNA and RNA synthesis, along with cardiovascular, endocrine, and permeability changes. The mitotic delay allows time for repair processes to restore normal nucleic acid synthesis.

Other metabolic effects observed after thiol administration include hypotension, hypothermia, and hypoxia (271). An increase in serotonin level has also been noted in rats after injection of amino thiols (272). Release of endogenous thiols is another metabolic effect of the radioprotective thiols. This has been caused not only by amino thiols but also by serotonin and hypoxia-causing compounds, as well as by the anoxic state (273). This increase in cellular thiol content is often 30- to 40-fold greater than the amount of thiol supplied by the protective agent. Protective effects of the amino thiols in Ehrlich ascites (274) and other tumor cells (275), as well as in mice (276), show direct correlations with the levels of nonprotein thiols. The natural radiosensitivity of mice was related to the concentration of thiol groups in the blood-forming tissues of the spleen (277), and development of radioresistance in cells was attributed to increased concentration of non-protein-bound thiols (278). Radioresistance in some tumor cells was believed to be attributed to protein thiol content (279), although the level of hypoxia in tumor cells is also a factor affecting radiation sensitivity (280).

Further evidence of the importance of cellular thiol levels in radioprotection is found in the following situations. Protection of the chromosomal apparatus in Ehrlich ascites cells by MEA was associated with the increase in nonprotein thiol levels (281). Both MEA and cystamine increased plasma, liver, and spleen concentrations of free thiols and disulfides (282). Radioresistance of bacterial cells was believed to be the result of a repair system dependent on the thiol content of the cells (283). Revesz and coworkers considered that glutathione was a principal endogenous radioprotector released by administered thiols (284), but more recent research showed that only a small fraction of low molecular weight protein-bound thiols was identified as glutathione (285, 286).

1.5.5 Control of DNA Breakdown. The ability of the disulfides of the radioprotective amino thiols to bind reversibly to DNA, RNA, and nucleoproteins has been postulated as a result of *in vitro* studies (287). This, according to Brown (288), can result in two restorative effects: first, the loose ends of the helix resulting from single-strand rupture are held in place, so that shortening or alteration of the chain is prevented; and second, the replication rate of DNA is decreased or halted, so that repair can take place before radiation-induced alterations are replicated. This binding, together with either radical scavenging (289) or repair by proton donation, provides a possible route of protection of the nucleic acids by the amino thiols. It requires that the disulfide of the amino thiol be present for binding, and it also could explain why more than a three-carbon distance between amino and thiol functions leads to a sharp drop in protective ability. Portions of the DNA helix unprotected by histone have been found to accommodate an aliphatic chain of approximately 10 atoms; consequently, a disulfide with two or three carbons between the amino and disulfide functions would fit this exposed portion of the helix. Other strongly protective derivatives of MEA and MEG, such as the thiosulfate, phosphorothioate, trithiocarbonate, or acylthio-

esters, readily undergo disulfide formation. Arguments against this hypothesis include the fact that not all of the exposed areas of DNA are the same size. Also, in a series of protective disulfides and thiosulfates of MEA with N-heterocyclic substituents, the thiosulfates showed no binding ability for DNA, and whereas the disulfides have appreciable binding constants, there was no correlation between binding ability and protective activity (290).

Protective effects for DNA have been shown by thiourea and propyl gallate, as well as by cysteine and cystamine, apparently through antioxidant activity (291). Another proposed explanation for the protection of DNA by the amino thiols is that MEA renders cell membranes more resistant to radiation damage. Localization of repair enzymes and nucleases on the nuclear membrane makes it possible that radiation damage to the nuclear membrane could result in irreversible damage to DNA by nucleases, and interference with repair of DNA (292).

Other observations regarding the temporary inhibition of nucleoprotein synthesis by thiol protectors have been reported. Temporary inhibition of nuclear RNA synthesis in the radiosensitive tissue of rat thymus was found along with inhibition of thymidine phosphorylation for a short period (292). Radiosensitizers, such as penicillamine and β-mercaptoethanol, inhibited thymidine phosphorylation for a longer period. Some evidence for mixed disulfide formation with proteins (e.g., thymidine kinase) was also found. Inhibition of DNA synthesis in rat thymus, spleen, and regenerating liver by MEA and AET was believed to arise from a delay in the synthesis of relevant enzymes, nuclear RNA polymerase, and thymidine phosphorylating kinases (294). Although MEA decreases the frequency of radiation-induced single-strand breaks in DNA of mammalian cells (295), this was not considered to be the lesion responsible for the killing of E. *coli* cells by y-radiation (296).

1.5.6 Metabolic Effects. Alteration of cellular metabolism can affect radiosensitivity either by changing oxygen concentration, and thus altering the extent of initial DNA damage, or by altering cell cycle progression, thus allowing greater or lesser repair of potentially lethal damage. Even in cases where it can be demonstreated that a radioprotector can act by a purely radiochemical mechanism, it is possible that it could also act by altering metabolism (297) and the extent to which one mechanism dominates may be difficult to determine. Although a state of hypoxia considered sufficient to provide radiation protection is not brought about by most radioprotectors, some effect on oxygen availability and oxidation-reduction potential of the cells does result after their administration. A relation was observed between the duration of respiration inhibition and the radioprotective effect of cystaphos, the phosphorothioate of MEA (298). Several phosphorothioates were also found to induce vasodilation in the spleen, resulting in altered blood supply to the body, and decreasing tissue oxygen tensions (299). Aminoethyl and aminopropyl thiosulfates also decreased the oxidation-reduction potential in body tissues of rats and mice (300). They also increased serotonin and histamine levels, and decreased peroxide levels. Several heterocyclic compounds, including aryl derivatives of triazoline-2,5-dithione, decreased the oxygen tension in rat spleen, liver, and muscle (301); a correlation was observed between the decrease in oxygen tension and radioprotective effects of the compounds.

Radioprotective and radiosensitizing effects of various compounds have been related to an oxygen effect. A theory has been developed consisting of an "oxygen fixation hypothesis" (302), in which target free radicals react either with radical-reducing species, resulting in restoration, or with radical-oxidizing species, resulting in fixation of radical damage to a potentially lethal form. MEA and other thiols protect by adding to the pool of radical-reducing species, resulting in enhanced repair of free-radical damage. Electron-affinic compounds radiosensitize by adding to the pool of radical-oxidizingspecies, enhancing free-radical damage; N-ethylmaleimide has a similar effect. Metal ions, however, do not alter sensitivity to radiation inactivation of bovine carbonic anhydrase by oxidizing radicals, but do exert a protective effect against inactivation by reducing radicals (303).

An explanation of the protective effects of ethanol, and other hydroxy compounds, arose from the observation that ethanol adds to thymine under y-irradiation (304). This prevents formation of thymine dimers, deleterious to the structure of DNA. It also explains the radiation resistance of bacterial spores, and protection of bacteria in glucose medium, where hydroxy compounds are in adequate supply to add to thymine.

Other cellular effects produced by the amino thiols may be involved in the complex process of radiation protection. Release of enzymes is one such effect, and various enzyme releases have been observed in rat plasma after introduction of either MEA or 5-mercaptopyridoxine, or by a state of hypoxia (305). Treatment with two nonprotective thiols, 2-mercaptoethanol and 4-mercaptopyridoxine, did not affect the plasma enzyme levels. The liberation of cellular thiols, discussed earlier, may be attributed to enzyme liberation, at least in part. Mixed disulfide formation may be a factor in this release, as suggested by the biochemical shock hypothesis.

The radioprotective thiols protect the erythropoietic system of animals, ^{59}Fe uptake being used as the test for protection (306). MEA, AET, penicillamine, and 2-mercaptoethanol all inhibit phosphorylation of thymidine in rat thymus and spleen (307). This effect of the two protective agents MEA and AET is reversible, whereas that of the two sensitizing compounds is irreversible.

Addition of MEA to mitochondria first accelerates then slows respiration. A decrease in ATP synthesis was also noted, both in mitochondria and rat thymus nuclei, thus diminishing both respiration and phosphorylation coupling (308). Mixed disulfide formation is believed to be involved.

Bacq and Alexander proposed that a significant contribution to the radiobiological effects of ionizing radiation is attributed to cell membrane damage (15). The effect of X-rays on the permeability of Ehrlich ascites tumor cell membranes has since been studied by measuring loss of potassium from the cells (309). The radiosensitizing effects of Synkavit and excess oxygen were demonstrated by a marked loss of potassium from the irradiated cells, whereas the protective effects of MEA and 2-amino-3-methylbutanthiol prevented this loss. Also, blocking of cell-surface amino and probably thiol groups with citraconic anhydride, dimethylmaleic anhydride, and diacetyl also modified radiation damage to the cell membrane. It was suggested that radioprotection may depend on a combination with cell-surface protein groups, which determine the surface charge and maintain the integrity of the cell membrane.

Radiation-protective effects were sought in a screening program involving more than a thousand kinds of Chinese herbs (310). Some of them raised the survival rate of dogs irradiated with a lethal dose of γ-rays by 30–40%. The ability to protect the hemopoietic and immune systems was believed to be the mechanism of protection.

Another class of radioprotectors, the nitroxides, are membrane permeable, stable free radicals, and protect both cell cultures and mice from radiation-induced cytotoxicity. A water-soluble nitroxide, Tempol, protected hamster cells from superoxide and other peroxides, and protected mice against an LD_{50} dose of radiation (311). Potential mechanisms of protection include oxidation of reduced transition metals, superoxide dismutase-like activity, and scavenging of oxygen- and carbon-based free radicals.

Cytokines have been found to protect cells from the damaging effects of ionizing radiation. Interleukin 1 and tumor necrosis factor alpha (TNF-a) protect mice from lethal doses of radiation, given before irradiation. At lower doses of radiation, hemopoietic growth factors, interleukins 1, -4, and -6, TNF-a, interferon, and leukemia inhibitory factor promote recovery when administered after radiation, possibly by initiating autocrine/paracrine recovery and repair pathways (312).

Radioprotection by leukotrienes, especially with regard to hematopoietic stem cells, has been reviewed (313). *In vivo* radiation protection by prostaglandins and related compounds of the arachidonic acid cascade has also been noted (314). The role of mast cell mediators in radiation injury and protection has been discussed (315). Immunomodulators, either microbial agents (e.g., glucan) or recombinant cytokines can enhance hematopoietic and functional cell recovery after irradiation (316).

DNA-binding ligands, such as the **bibenzimidazoles** (317) and modulators of DNA repair and lesion **fixation**, such as **2-deoxy-D-glucose** (318) have provided protection against radiation-induced cytogenetic damage. Also, calcium ion channel blockers. such as **diltiazem** (319) have increased survival rates of irradiated animals, alone or in combination with zinc aspartate (320). A postirradiation increase in calcium influx in mouse spleen lymphocytes (321) and an increase in cytosolic calcium in rat thymocytes, leading to apoptosis and necrosis, has been observed (322). Calcium ion influx activates a **significant** number of enzymes, including endonuclease (323), and Ca^{2+}/Mg^{2+}-**dependent** endonuclease mediates fragmentation of DNA. Lipid **peroxidation** is also reduced by calcium channel blockers, thus decreasing damage to cell membranes (324).

1.5.7 Use of Radioprotective Agents in Radiotherapy and Chemotherapy of Cancers. The use of radioprotective agents to augment the effects of either radiotherapy or chemotherapy of various types of cancer has been investigated for 30 years or more, but until recently little positive benefit was observed. It is now clear that selective concentrations of protective agents can be realized in normal tissues, and that tumors are protected to a lesser extent. Favorable timing schedules for administering protective agents and anticancer therapies have played a significant role in the moderate success so far realized with **radioprotector** adjuvant treatments.

MEA gave favorable results when used in conjunction with X-rays and **cyclophosphamide** in rats with **Geren's** carcinoma (325). Cystamine decreased chromosomal aberrations in peripheral blood lymphocytes in uterine cancer patients (326). Although **AET** was found to penetrate normal and cancerous tissue of mice to the same extent, it prolonged the life spans of mice bearing **ascites** tumor cells (327). Favorable effects on irradiation of mice with Ehrlich carcinoma were reported for **AET** and DL-trans-2-amino-cyclohexan**thiol** (328). A combination of **AET**, serotonin, cysteine, and glutathione was definitely favorable to the survival of mice with Landschutz **ascites** tumors treated with 6000 R (329).

Differential distributions of MEA released from its phosphorothioate and thiosulfate in various tissues have been found (330); the phosphorothioate of MEA had a lower concentration in sarcoma **M-1** than in the organs of mice (331). The phosphorothioate of MEA also diminished symptoms of radiation sickness in human patients undergoing radiation therapy for breast cancer (332). Also, in cancer patients, 2-mercapto-propionylglycine decreased the severity of lymphopenia and decreased the number of chromosome aberrations after irradiation (333). MEA, AET, **1-cysteine**, and **1-cysteinyl-D-glucose** restored the mitotic index in X-irradiated rats bearing Yoshida sarcoma (334); 5-fluorouracil was **radiosensitizing** in these experiments.

More recently, **WR2721** (Amifostine, **Ethyol**) has exhibited activity as a **chemoprotector**. The compound requires activation by dephosphorylation to produce the free thiol. This process is catalyzed by capillary alkaline phosphatase, close to the desired site of protection. The neutral pH of normal tissues, compared with the slightly acidic pH of tumors, favors selective activation. Amifostine was able to reduce DNA platination when **preincubated** with cisplatin, but the effect was much weaker when given postincubation (335). Arnifostine can also reduce the **myelosuppression** produced by cyclophosphamide, the combination of cyclophosphamide and **cisplatin**, and possibly carboplatin (336). The favorable effects noted with cisplatin therapy have been termed ***cisplatin rescue*** (337). Protection of hematopoietic stem cells from irradiation by amino thiols, synthetic **polysaccharides**, vitamins, and cytokines has been discussed (338). Possible mechanisms by which amifostine may protect normal **hematopoietic** stem cells from chemotherapeutic agents have been proposed, including direct binding to the alkylating agents, cisplatin and cyclophosphamide, or acceleration of the recovery of hematopoietic stem cells exposed to high doses of radiation (339).

Other radioprotective agents have shown promise as selective protective agents for normal tissue; dexrazoxane and mesna have been cited (340). Reviews on the effects of **amifostine** in protection of normal bone marrow

stem cells (341) and as modulator of cisplatin- and carboplatin-induced side effects (342) have been published.

2 RADIOSENSITIZERS

2.1 Introduction

Radiosensitizers were developed for use in cancer therapy (37–39, 343). It is important for this purpose that they have a differential effect on the tumor vs. the normal tissue. Otherwise, nothing would be gained over increasing the dose of radiation. The relative increase in antitumor efficacy compared to normal tissue toxicity is referred to ***as therapeutic gain.*** Many of the compounds or strategies listed in this chapter have yet to be proven useful in cancer therapy, but are of mechanistic interest.

Two particular strategies have a clear rationale for selective radiosensitization of tumor cells and are currently in clinical use:

1. Sensitization of hypoxic cells by oxygen-mimetic drugs (344), or agents that alter oxygen delivery, because hypoxia occurs often in tumors but not in normal tissues, and about three times more radiation is required to kill hypoxic cells than oxygenated cells.
2. Incorporation of radiosensitizing thymidine analogs into DNA (345) because this will occur only in proliferating cells, and in some cases (e.g., brain tumors), the normal tissue surrounding the tumor is composed primarily of nonproliferating cells.

Strategies that take advantage of signal transduction differences between cancer cells and normal cells represent an exciting new class of radiosensitizers, although these strategies are still in the early stages of development.

Radiosensitizers are traditionally defined as agents that do not have a therapeutic effect of their own, but act to enhance the therapeutic effect of radiation. However, the term does apply to some antitumor agents, such as low dose cisplatin (346–349) and hyperthermia (350, 352), which appear to enhance radiation damage in a truly synergistic manner. Otherwise, the combined use of radiation and antitumor agents is referred to as ***combined modality therapy,*** and relies on nonoverlapping normal tissue toxicities or on attacking different tumor cell populations to achieve a therapeutic gain. Combined modality therapy, although not discussed in this chapter, was reviewed recently by Phillips (353).

2.2 Radiosensitization by Alteration of Energy Absorption

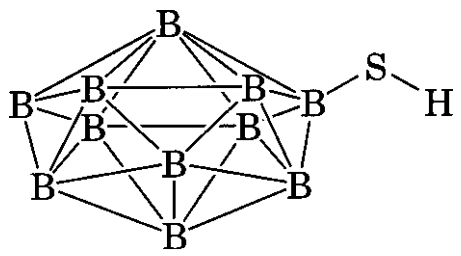
The probability of ionization is essentially proportional to the number of electrons in the target molecule, regardless of chemical composition for the types of ionizing radiation that are normally used in radiation therapy (high energy photons or electrons). However, preferential energy absorption by particular elements can occur with certain energies or types of radiation. Two such cases are boron neutron capture and k-edge absorption of photons by atoms of high atomic weight.

2.2.1 Boron Neutron Capture Therapy (BNCT). Certain isotopes, such as ^{10}B, capture low energy (thermal) neutrons very efficiently. This property is expressed as the thermal neutron cross section, in units of barns. The thermal neutron cross section for ^{10}B is 3837 barns. As early as 1936 (354), it was suggested that preferential incorporation of ^{10}B into tumors could be a useful strategy for selectively radiosensitizing tumor cells. ^{10}B itself is not radioactive, but neutron capture by ^{10}B is followed by radioactive decay of the resulting ^{11}B nucleus. ^{11}B splits into ^{4}He nuclei (alpha particles) and ^{7}Li ions, both of which are densely ionizing [i.e., high linear energy transfer (LET)], and consequently very cytotoxic. The ranges of these particles are such that their energy is deposited within one cell diameter of the neutron-capture event. These characteristics make ^{10}B a very desirable isotope for neutron-capture therapy, even though other isotopes have higher thermal neutron capture cross sections.

There are two challenges to implementation of this strategy. One is to deliver thermal neutrons to the tumor, given that low energy neutrons do not travel very far in tissue. The

other is to design drugs that will selectively deliver ^{10}B to tumors. The first challenge is being met with advances in instrumentation, such that BNCT is now regarded as a more realistic possibility than in the past (355). Neutrons of a sufficiently high energy to penetrate tissue (epithermal neutrons) are used in such a way that they become thermal neutrons at the depth corresponding to the tumor.

It is estimated that 10 parts per million (ppm) ^{10}B would be enough to increase cytotoxicity twofold over that seen with neutrons alone (355). BSH ($Na_2B_{12}H_{11}SH$; 44) was one



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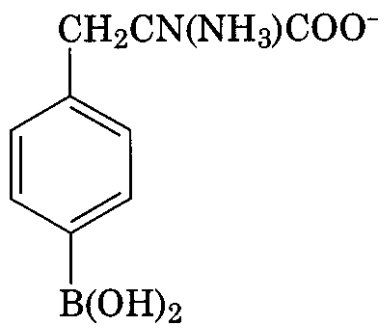
of the first compounds synthesized for this purpose (356). BSH and its disulfide, BSSB, are reported to accumulate in animal tumors, but the clinical biodistribution results from patients with brain tumors have been variable and unpredictable (357). In animal studies, very high tumor to normal brain tissue ratios were achieved, but there was no evidence of a therapeutic gain, suggesting that normal brain injury may be more related to the dose than to the vasculature (358). Clinical studies of brain tumor BNCT with sodium tetraborate and BSH did not result in improved survival and there was evidence of increased normal brain injury, consistent with preferential damage to the vasculature (359). Other types of BNCT agents are currently under development. Encouraging results were obtained with BPA (*p*-boronophenylalanine, 45) in the treatment of melanoma (360). ^{157}Gd has been proposed as another isotope that could be useful in neutron-capture therapy (361). Although BNCT is a promising idea, the selective delivery of atoms of high neutron cross section (such as boron) to the target cells is still a limiting factor.



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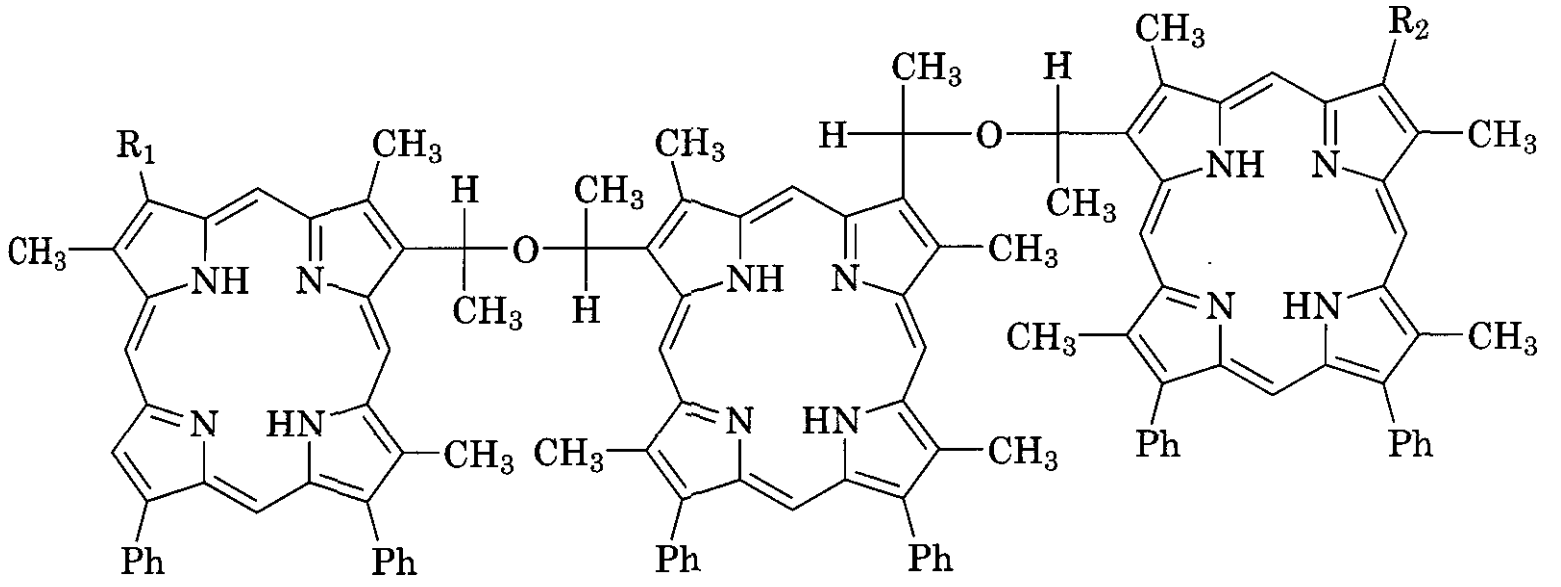
2.2.2 k-Edge Absorption and Photoactivation of Elements of High Atomic Number. The probability of absorption of a photon is highest when the energy of the photon is close to the binding energy of an electron in the target molecule (362). This effect is particularly noticeable for k-shell electrons of elements of high atomic number. A strategy for radiosensitization based on this effect is to incorporate atoms of high atomic number into DNA (363). The k-shell binding energy is characteristic for each element, and irradiation at energies just above this k-edge characteristic energy will result in selective absorption by a particular element. Iodine is particularly attractive for this purpose because: (1) the optimal energy of the activating photon is in a range that is reasonably achievable; (2) iodine is easily incorporated into compounds that can be delivered to tumors; and (3) photoactivation can occur, further enhancing the biological effect. Photoactivation is a process whereby inner shell electrons are ejected from an atom as a consequence of photon absorption, and cascading outer shell electrons fill the successively vacated orbitals, resulting in the emission of multiple low energy X-ray photons and electrons (364).

2.2.3 Photodynamic Therapy. Photodynamic therapy (PDT) consists of administration of a photosensitive compound and illumination of the tumor with visible light. Recent advances in light-delivery technology have provided methods for selective and thorough illumination of the tumor (365), although light delivery continues to be the principal limitation of this therapeutic approach.

PDT has been found to be effective in the treatment of several types of solid tumors in humans. Most of the clinical experience has been with hematoporphyrin derivative (HPD, 46) or porfimer sodium (Photofrin), which is a derivative of HPD (366). A problem with these



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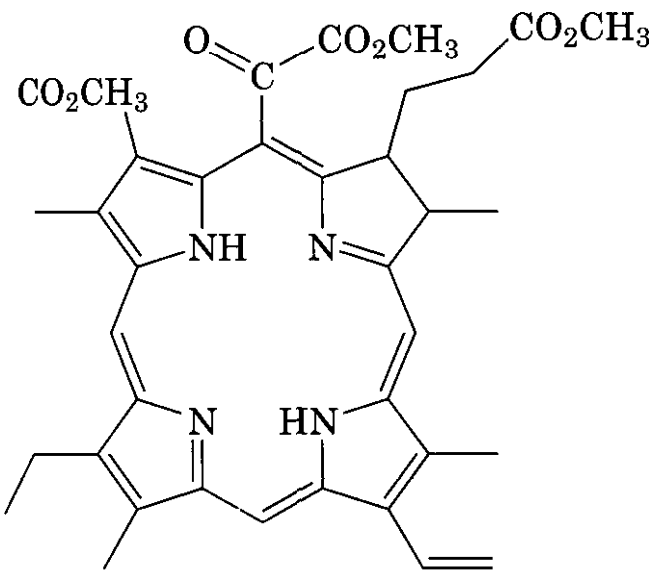
compounds is photosensitization of skin that persists for 6–8 weeks, (367). *meta-tetra*(Hydroxyphenyl)chlorin (m-THPC) (368–370) has a shorter plasma half-life and a higher tumor/normal tissue ratio, and is currently in Phase 1/11 trials. 8-Aminolaevulinic acid (ALA) has been found to be effective clinically by topical application (371,372) and in animal studies by systemic administration (373). ALA is converted in vivo to protoporphyrin IX, which is the active photosensitizer. Other tetrapyrroles that are being considered for PDT include purpurins (47) (374) and phthalocya-



(47)

nines (375, 376). Cationic dyes have been investigated (377), given that there is evidence that a common feature of many types of tumor cells is the ability to concentrate moderately lipophilic cationic dyes, attributed to differences in mitochondria1 membrane potential (378).

The tumoricidal mechanism of PDT has two components: direct tumor cell killing and damage to the vasculature leading to tumor necrosis. The number of tumor cells from excised murine tumors that produce colonies in vitro decreases markedly with time between treatment and excision (379, 380), in support of the importance of damage to the vasculature. In contrast, tumor cell killing by ionizing radiation is evident when tumors are excised immediately after irradiation and plated for clonogenic assay (381). Hypoxia induced by PDT can be exploited by concurrent treatment with drugs that are metabolized under hypoxia to toxic species (380, 382).

2.3 Alteration of the Primary Radiolytic Products

Ionization of water is the most common consequence of irradiation of biological systems because they are composed mostly of water. Figure 4.1 shows the relative yields of products of water radiolysis (383). These reactive species differ considerably in their chemical properties. For example, $H^{\bullet}$ is a reducing radical and $HO^{\bullet}$ is highly oxidizing. A potential approach to radiosensitization is to convert the initial radiolytic products into more reactive or more selective species. This can be accomplished by including a substance that can react with the primary radiolytic products before they have a chance to react with other

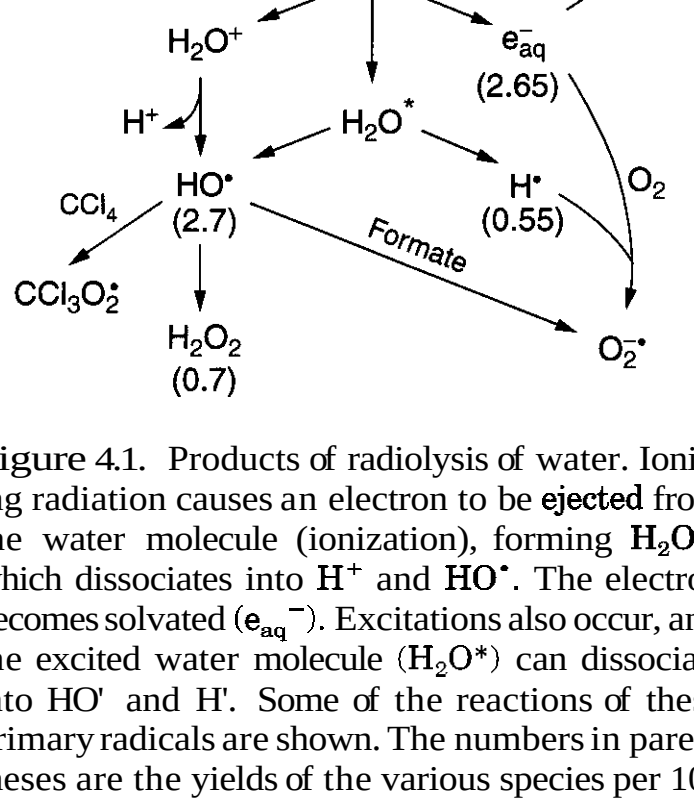


Figure 4.1. Products of radiolysis of water. Ionizing radiation causes an electron to be ejected from the water molecule (ionization), forming H_2O^+, which dissociates into H^+ and $HO^•$. The electron becomes solvated (e_{aq}^-). Excitations also occur, and the excited water molecule (H_2O^*) can dissociate into HO' and H'. Some of the reactions of these primary radicals are shown. The numbers in parentheses are the yields of the various species per 100 eV (G-values) (433).

molecules. For example, O_2 reacts readily with the aqueous electron (e_{aq}^-) and $H^•$ to convert them to $O_2^{•-}$ (Fig. 4.1). N_2O, on the other hand, can react with e_{aq}^- to convert it to HO' (Fig. 4.1). Hyperbaric H_2 can be used to convert $HO^•$ radicals into $H^•$. Isopropyl alcohol can be used to scavenge $HO^•$ and $H^•$, leaving the aqueous electron.

Such methods have been used to demonstrate differences in reactivity of these radiolytic products toward model biological targets, such as DNA (383). Studies with bacteria and mammalian cells have yielded variable results (384, 385), possibly because the modifiers can act by multiple mechanisms (385). In general, such studies have been consistent with lack of toxicity of the reducing radicals, suggesting that $HO^•$ is the only diffusible primary radical that contributes to toxicity. One explanation is that multiple attacks on DNA must occur within a very small volume to produce a cytotoxic lesion (386). Only radicals with the reactivity of $HO^•$ can contribute to these clustered lesions because other radicals diffuse away from the critical volume before reacting with DNA (387). For example, the conversion of e_{aq}^- to $HO^•$ by N_2O more than 10^{-9} s after irradiation should not affect cytotoxicity because the $HO^•$ would be formed outside the critical volume (387).

A variation on this theme is to use modifiers that react with products of water radiolysis, to produce a species that is more selective in reacting with cellular targets (388) (Fig. 4.1). For example, Hiller et al. (389) found that the effectiveness of ionizing radiation in inactivating bacteriophage T_2 is five times greater if $HO^•$ is converted to $CCl_3O_2^•$ by inclusion of CCl_4 in the medium, whereas it is decreased by a factor of 20 by conversion of $HO^•$ to $O_2^{•-}$ by inclusion of formate. Similarly, uric acid can enhance radiation damage to alcohol dehydrogenase (in dilute solution), even though it protects lactate dehydrogenase (390). The reaction of HO' scavengers with HO' results in the formation of a more selective radical that may be more effective in damaging a specific cellular target. Dimethylsulfoxide (DMSO) reacts with HO' to form $H_3C^•$, or in the presence of oxygen, $CH_3OO^•$. These are relatively nonreactive radicals, and DMSO is therefore considered to be an HO' scavenger. DMSO protects against radiation-induced DNA damage. However, DMSO does not protect against $HO^•$-initiated membrane damage, whereas other $HO^•$ scavengers do (391).

2.4 Radiosensitization by Reaction with DNA Radicals

The principal mechanism of cell killing by ionizing radiation is the formation of clustered DNA lesions (386,392,393) by a combination of direct ionizations in the DNA molecule (the direct effect) and reaction of DNA with free radicals produced in the vicinity of DNA (the indirect effect). The reactions that produce the DNA radicals that are the precursors of these clustered lesions are complete within nanoseconds (387). However, the chemical reactions of these free radicals that result in damage fixation are not complete until 10 ms after irradiation (394), and there is an opportunity to alter the outcome of these reactions (Fig. 4.2). Damage fixation is a process that renders the damage nonrestorable by chemical protectors.

Reaction of DNA radicals with molecular oxygen results in damage fixation. This reaction occurs in competition with the restorative

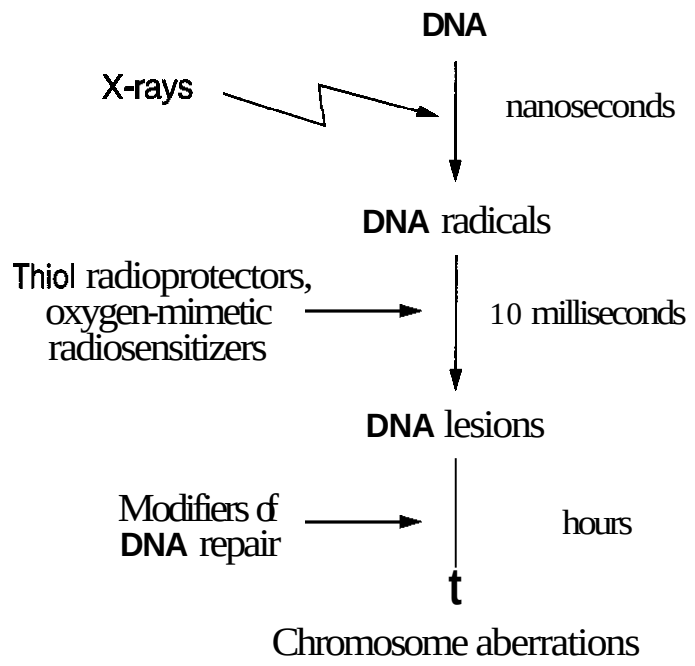
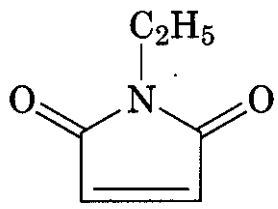


Figure 4.2. Time frame for radiochemical events. In living cells, **DNA** radicals are formed within nanoseconds by a combination of direct ionization of DNA and reaction of **DNA** with HO' produced by the radiolysis of water. **Protectors** and sensitizers that modify initial lesion formation act in a millisecond time frame by reaction with these **DNA** radicals. Biological processes that **can** lead to repair or misrepair of the lesions take place over a period of hours.

reaction of DNA radicals with endogenous protectors (395). In experiments using chemical model systems (dilute solutions of macromolecules), sensitization by oxygen was observed only when radioprotectors were also present (395,396). Thus, damage fixation will occur **without** a sensitizer (by internal bond rearrangement) if protective reactions are not fast enough. Either an increase in the concentration of oxygen-mimetic sensitizer or a decrease in the concentration of endogenous protector can result in radiosensitization.

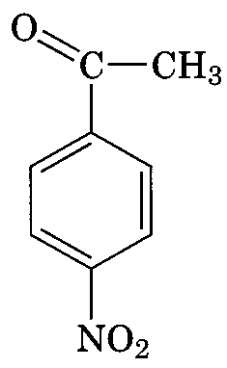
The chemical property of oxygen that is the basis for oxygen mimetic radiosensitization is the one-electron **redox** potential, or electron **affinity** (397). Electron affinity correlates with **hypoxic** cell radiosensitization, for agents that sensitize by this mechanism, over a range of different chemical structures, with some exceptions (397, 398). Radiosensitization by an oxygen-mimetic mechanism occurs within milliseconds of irradiation, and techniques have been developed to distinguish between this mechanism and other mechanisms of sensitization by determining the time frame for the effect (399). For example, a rapid-mix experiment was used to show that *N*-ethylmaleimide (48) can sensitize by an oxygen-mimetic mechanism, even though it can also sensitize



(48)

by reacting with cellular thiols (399). Whillans and Hunt (400) used a rapid-mix experiment to demonstrate that radiosensitization by misonidazole (53) does not occur if misonidazole is mixed with **hypoxic** cells more than 10 ms after irradiation. Some of the first compounds that were found to be **radiosensitizers**, such as *N*-ethylmaleimide (401) and menadione (402), are electron **affinic**, but were too toxic, too complicated mechanistically, or not sufficiently effective to be thoroughly developed for clinical use.

Attention in recent years has been concentrated on two general classes of **electron-affinic** radiosensitizers: quinones and **nitroimidazoles** (403). Highly electron **affinic** agents are effective in vitro, but not in vivo, presumably because they are so reactive they are depleted before they reach the target cells. The first nitro compounds that were found to be effective **electron-affinic** sensitizers in *vitro*, *p*-nitro-acetophenone (49) (404), p-nitro-3-dimethylamino-propiophenone (50) (405), and **nitrofurazone** (51) (406), were too toxic and



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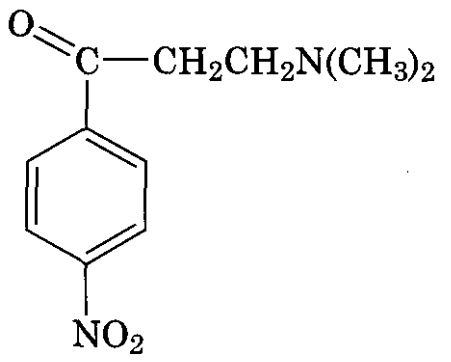
too metabolically unstable to be useful in vivo. Similarly, many **quinones** are effective in *vitro* but have been disappointing in *vivo* (407).



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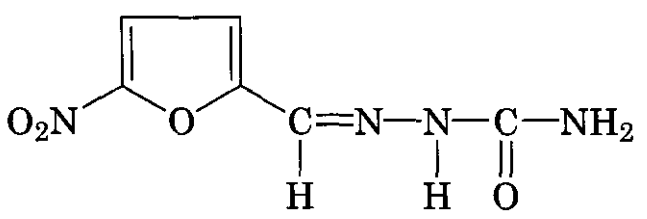
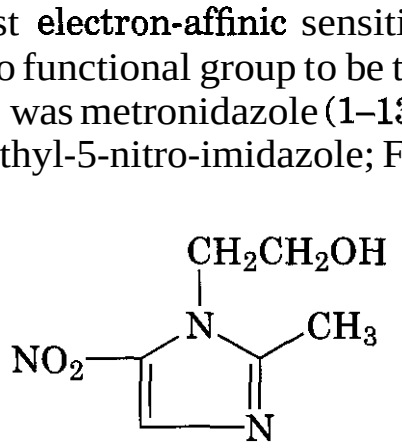


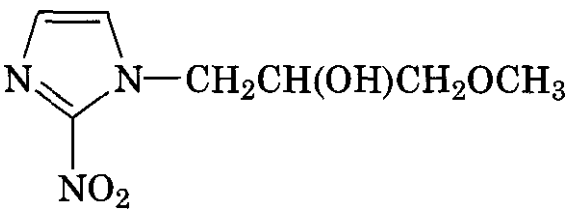
(51)

The first electron-affinic sensitizer based on the nitro functional group to be tested clinically (407) was metronidazole (1–13-hydroxyethyl-2-methyl-5-nitro-imidazole; Flagyl, **52**),



(52)

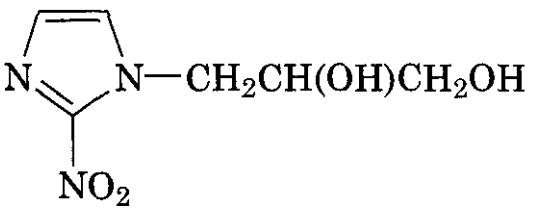
a 5-nitroimidazole that was already in clinical use as an antitrichimonal agent. The trial, conducted with patients with glioblastoma multiforme using nonstandard fractionation, was positive, in that the median survival for the sensitizer group (7 months) was superior to the median survival for the controls (3 months). However, the long-term survival of the sensitizer group was not superior to that of historical controls given standard fractionated radiotherapy. The 2-nitroimidazole, misonidazole (**53**), was tested more extensively



(53)

in clinical trials. Only 5 of 33 trials showed some possible benefit (408). The most promising result came from a large randomized trial of patients with pharyngeal cancer (409), with an overall disease-free survival of 46% for the misonidazole group vs. 26% for the controls.

The dose of misonidazole that can be administered is limited by peripheral neuropathy (410). In an effort to reduce this side effect, less lipophilic 2-nitroimidazoles were synthesized (411, 462). Desmethylmisonidazole (54) and etanidazole (**55**) are less neurotoxic than



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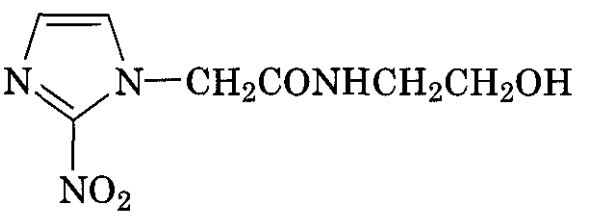

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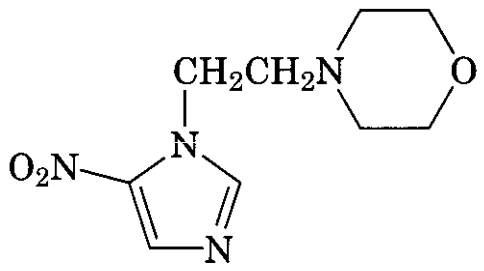
misonidazole, in keeping with their lower lipophilicity. In a phase I clinical trial (413), it was determined that 30% more desmethylmisonidazole than misonidazole could be administered, but this compound was not tested further. Etanidazole can be administered at about 4 times the dose of misonidazole (414), and peripheral neuropathy can be almost completely avoided by determination of individual patient pharmacokinetics and adjustment of the dosage accordingly (415). Efficacy data for etanidazole are not yet available, although several trials are nearing completion (415–418).

Nimorazole (56) (419), a 5-nitroimidazole, is less effective on a molar basis than the 2-nitroimidazoles, but its dose-limiting toxicity is different. The dose-limiting toxicity for nimorazole is nausea and vomiting, whereas it is peripheral neuropathy for the 2-nitroimidazoles. The toxicity of nimorazole is not cumulative, and it can therefore be given with each radiation fraction. A phase III trial of nimora-



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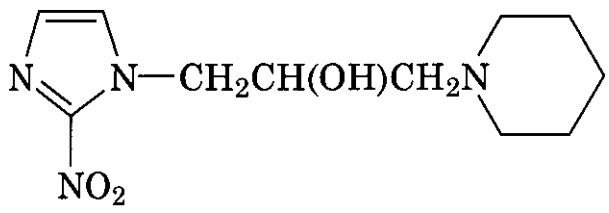
zole with 422 patients with squamous cell cancer of the larynx and pharynx (420, 421) showed an improvement in local control.

Pimonidazole (Ro 03–8799) (57) is a 2-ni-



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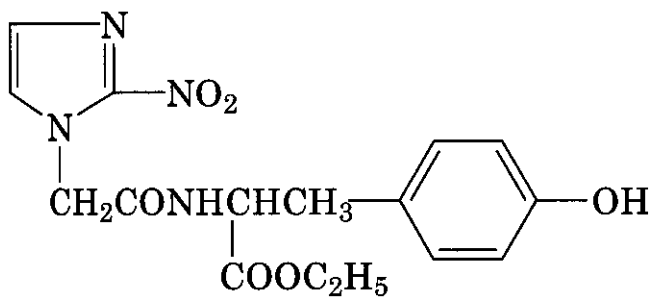
troimidazole with a basic side chain that has been found to accumulate in the acidic environment of tumors (422). Although pimonidazole is a better sensitizer than misonidazole in *vitro*, it is less effective than misonidazole in animal models, and clinical trials have indicated that it may be detrimental in combination with radiation therapy (423). A possible explanation for this adverse effect is suggested by a finding that pimonidazole can decrease blood perfusion in tumors (424). The idea of enhancing tumor uptake by attaching a basic side chain may still have merit, however (425–477).

Several new electron-affinic drugs have been developed, but have not yet been extensively tested. A series of 2-nitroimidazole derivatives were synthesized by attaching various amino acids at the 1-position through an amide bond (58) (428). Results with a series of nitroimidazoles with an acetohydroxamate



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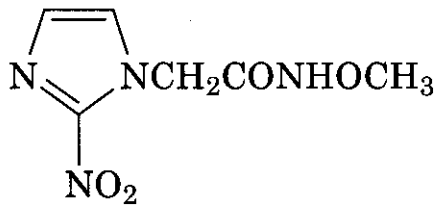
moiety on the side chain, of which KIN-804 (59) (429) and KIH-802 (60) (430) appear most promising, suggest that this functional



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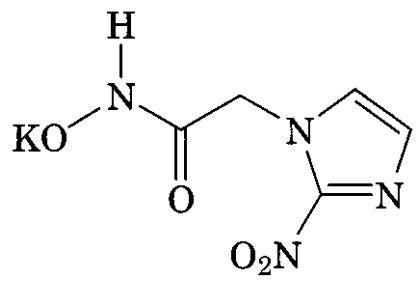

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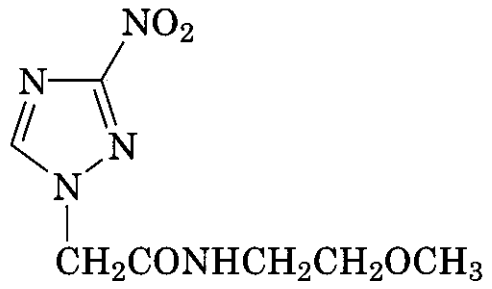
group may be useful for radiosensitizer design. AK-2123 (61)is a 3-nitrotriazole that is less toxic than misonidazole and is now in clinical trials (431, 432).

NLP-1 (5-[3-(2-nitro-1-imidazoy1)-propyll-phenanthridinium bromide, 62) was synthe-



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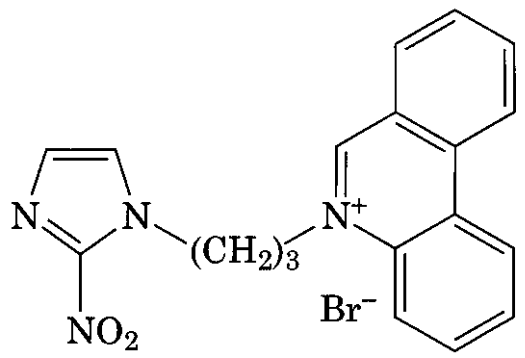

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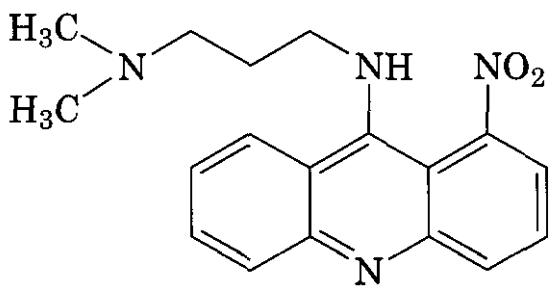
sized with the rationale of targeting the nitroimidazole to DNA through the intercalation of the phenanthridine ring (433). Nitracrine

[1-nitro-9-(dimethylaminopropyamino)acridine; (**63**)] is a DNA intercalating antitumor agent. Under conditions that minimize metabolism and cytotoxicity, nitracrine is a selective radiosensitizer of hypoxic cells, with an efficiency at least as great as expected from its electron affinity (434).



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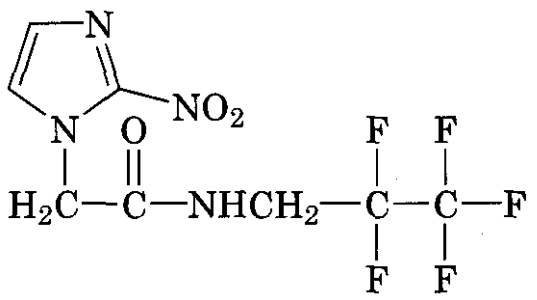
2.5 Additional Applications for Electron-Affinic Drugs in Cancer Therapy

The electron-affinity of hypoxic cell radiosensitizers confers additional biological properties to this class of drugs. They are readily reduced by cellular enzymes to reactive species that can cause biochemical alterations and cytotoxicity. The reversal of the first one-electron reduction, by reaction with oxygen, provides a mechanism for selectively affecting hypoxic cells. This biochemistry has been exploited for detection of hypoxia, additional radiosensitization, chemosensitization, and tumoricidal activity. Following this theme, new classes of drugs have been developed to optimize these mechanisms. Drugs that are selectively reduced under hypoxia include nitroaromatics, quinones, N-oxides, and transition-metal complexes.

2.5.1 Binding of Nitroimidazoles to Hypoxic Cells: Use in Detection of Hypoxia. Nitroimidazoles are reduced under hypoxic conditions to intermediates that bind to cellular macromolecules. This mechanism has been exploited in the detection of tumor hypoxia (435). Early studies made use of ^{14}C-labeled misonidazole and autoradiography, and served to demonstrate that hypoxia does occur in patients' tumors (436). Another approach has been to use nitroimidazole derivatives that can be detected immunohistochemically (437–450). Fluorinated nitroimidazoles have been synthesized for use in noninvasive detection of hypoxia (439–443). EF5 [2-(2-nitro-1*H*-imidazol-1-yl)-*N*-(2,2,3,3,3-pentafluorpropyl)acetamide, (**64**)] is a pentafluorinated



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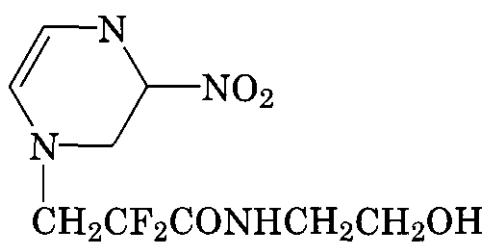
derivative of etanidazole that has been used for immunohistochemical analysis of hypoxia and is suitable for noninvasive imaging (440). KU-2285 (65) is a fluorinated etanidazole de-



(65)

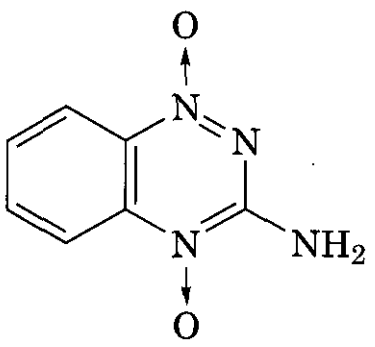
rivative that has a higher sensitizer efficiency than that of etanidazole and is in clinical trials as a hypoxic cell radiosensitizer (441). Pimonidazole (442) and IAZGP [iodinated-β-D-azomycin-galactopyranoside (443)] have also shown promise as hypoxia-imaging agents.

2.5.2 Additional Sensitization by Hypoxic Metabolism of Nitroimidazoles. Oxygen-mimetic radiosensitization by nitroimidazoles depends only on the intracellular concentration of the nitroimidazole at the time of irradiation. However, these same compounds are metabolized under hypoxic conditions to reactive intermediates that can cause radiosensitization by another mechanism. This additional sensitization is called the ***preincubation effect*** (444,445). The effect is primarily a decrease in the shoulder of the radiation survival curve and is obtained even if the cells are reoxygenated before irradiation. Chemotherapeutic drug cytotoxicity is also enhanced by hypoxic preincubation with nitroimidazoles (446). The biochemical mechanism of the preincubation

effect has not been identified. One contribution to this effect is depletion of glutathione (447), but this cannot explain the full effect, and does not explain radiosensitization of cells that are reoxygenated before irradiation. When glutathione was depleted to a similar extent with another agent in a study by Taylor et al. that used Chinese hamster ovary cells (448), sensitization to melphalan was only a fraction of that observed with hypoxic preincubation with misonidazole.

2.5.3 **Bioreductive Drugs.** Hypoxic incubation of cells with nitroimidazoles can kill cells without the need for a second agent (444). The concentrations of 2-nitroimidazole radiosensitizers required to kill tumor cells are too high to allow their use as cytotoxic agents in the clinic. However, the chemistry involved is common to a class of agents, called bioreductive drugs, that have shown promise in the treatment of cancer, and represent a new drug design opportunity. Bioreductive drugs are drugs that are reduced under hypoxia to cytotoxic species. Because radiation spares hypoxic cells, the combined use of bioreductive drugs and radiation should provide a therapeutic benefit, even if the effects are strictly additive.

Tirapazamine (SR-4233; WIN 59075; 3-amino-1,2,4-benzotriazine 1,4-dioxide; 66)



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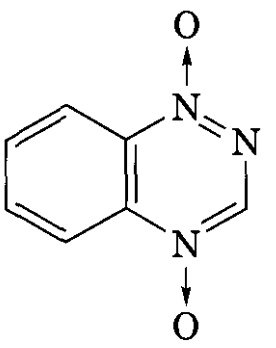
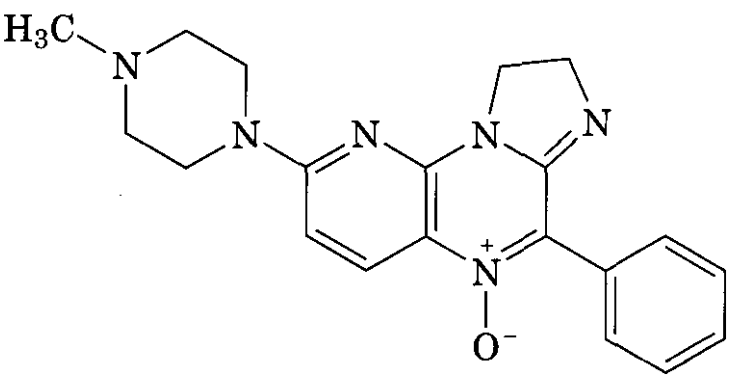
selectively kills and radiosensitizes hypoxic mammalian cells *in vitro* and murine tumor *in vivo* (449,450). The selective killing of hypoxic cells by tirapazamine is attributed to its one-electron reduction to a reactive intermediate that produces DNA damage. Radiosensitization appears to be by a preincubation effect, given that hypoxic preincubation sensitizes reoxygenated cells to ionizing radiation, and sensitization can be achieved when the drug is added after irradiation under hypoxic conditions, ruling out a radiochemical mechanism. Tirapazamine is currently in Phase I clinical trials, and muscle cramping has been noted as the most common side effect (451). A series of analogs of tirapazamine were synthesized to determine structure-activity relationships (450). One of these analogs, SR-4482 (**67**),



(67)

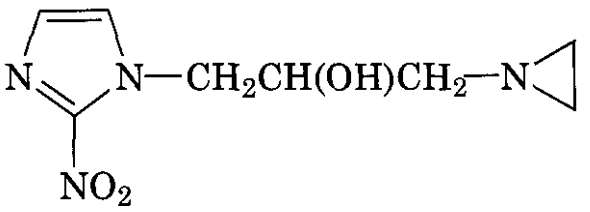
which contains no substituent on the 3-position of the triazine ring, is more toxic to hypoxic cells *in vitro*, but less toxic to mice, than tirapazamine. A relationship was found between the one-electron redox potential of these analogs and toxicity, although SR-4482 did not follow this pattern, and may represent a new class of benzotriazine di-N-oxides (450). RB90740 (68) is the lead compound selected



(68)

from a series of pyrazinemono-N-oxides that are selectively toxic to hypoxic mammalian cells (452). RB90740 has been found to accumulate in murine tumors, compared to normal tissue, providing additional selectivity.

RSU 1069 (69) is the lead compound in a series of 2-nitroimidazoles containing an alkylating moiety on the side chain (453). RSU 1069, which contains an aziridine ring as an alkylating moiety, is more potent than et-



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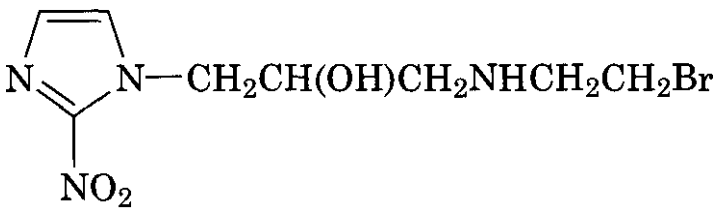
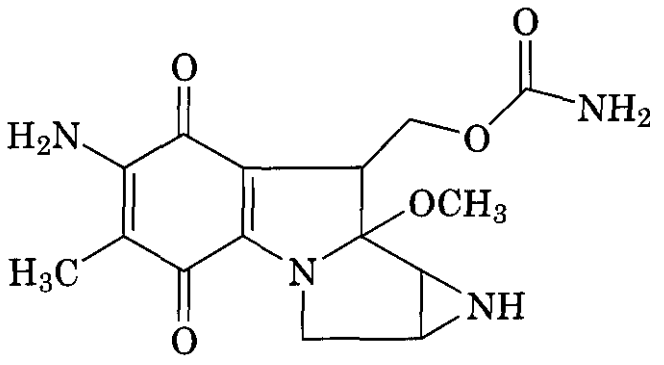
anidazole both as a hypoxic cell radiosensitizer and as a bioreductive drug. However, gastrointestinal toxicity limits the dose than can be administered clinically to levels that would not be expected to provide a benefit in cancer therapy. RB 6145 (70) is a prodrug of



(70)

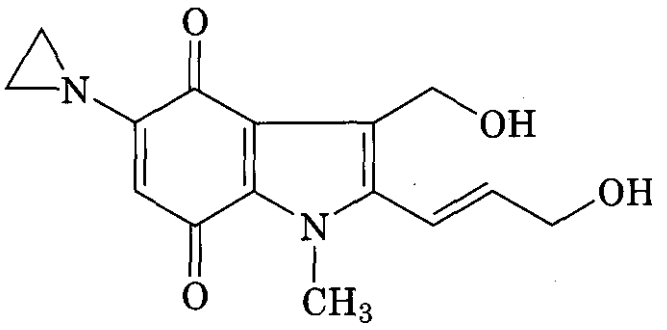
RSU-1069 that has lower toxicity with similar potency (454,455).

Mitomycin C (71) is the prototype for quinone-based bioreductive drugs (456). A clinical trial using mitomycin C and radiation for the treatment of squamous cell carcinoma of the head and neck showed an improvement in local control without enhancement of normal tissue toxicity (457). EO9 (72) is a mitomycin C analog containing an aziridine ring (458). A factor to consider is that the toxicities of bioreductive drugs depend considerably on the relative and absolute activities of cellular reductases; this is particularly a factor for substrates of DT-diaphorase, such as EO9, because DT-diaphorase activity can vary widely (458).



(71)

AQ4N (1,4-bis-{[2-(dimethylamino-*N*-oxide)ethylamino}5,8-dihydroxyanthracene-9,10-dione, 73) is reduced under hypoxia to AQ4 (74), which binds to DNA (459). The terminal half-life for AQ4 is reported to be of the order of 24 h, whereas the terminal half-life for the parent drug AQ4N is 30 min (459). AQ4 is retained in hypoxic cells for days, and is cytostatic (460). Consequently, repopulation of the tumor may be suppressed by exposure of cells to AQ4 while they are hypoxic, even if they subsequently become reoxygenated. The benefit of combined use of AQ4N and radiation in animal tumor studies is obtained, even when the treatments are administered more than 24 h apart in either order, suggesting additivity rather than synergy.



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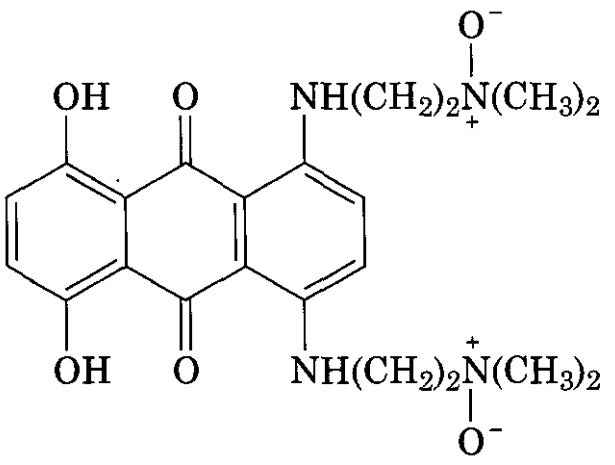

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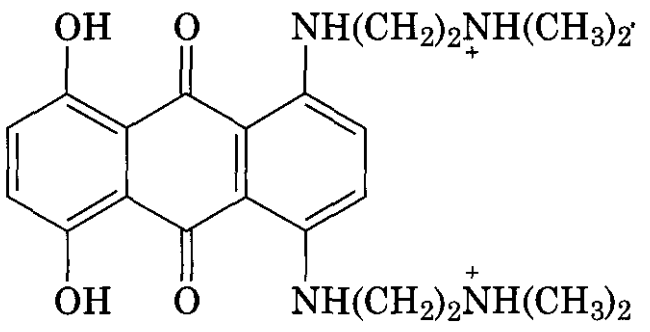

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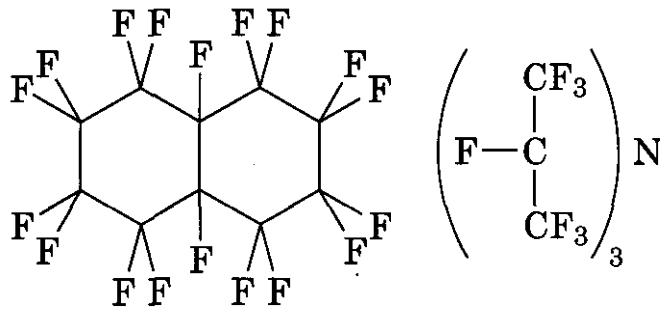
2.6 Radiosensitization by Alteration of Oxygen Delivery

An alternative to using oxygen-mimetic radiosensitizers is to improve oxygen delivery to tumors. Tumor hypoxia can result from one of two mechanisms: growth of the tumor beyond the diffusion distance of oxygen (chronic hypoxia) (461) or intermittent impairment of tumor blood flow (acute or intermittent hypoxia) (462,463).

The response of experimental tumors to radiation can be improved by increasing the oxygen content of the inspired air (464). Clinical trials with hyperbaric oxygen were carried out as early as the 1950s (465–467). Of nine prospective randomized trials, three showed a statistically significant benefit for hyperbaric oxygen (468). However, this strategy is technically difficult and impractical for widespread use in fractionated radiotherapy.

A prospective randomized trial using red blood cell transfusion to increase the hematocrit showed a benefit in the radiation therapy of cervical cancer (469). There is a limit beyond which this approach is counterproductive, in that increasing the hematocrit increases blood viscosity, resulting in poorer tumor perfusion. Furthermore, adaptation to chronically altered oxygenation results in reestablishment of tumor hypoxia (470). Adaptation to oxygenation status has been exploited in animal experiments to improve the tumor radiation response by adapting animals to low oxygen tensions (12% O_2) and then returning them to normal or higher than normal oxygen tensions just before irradiation (471).

Perfluorochemicals have been used to increase the oxygen-dissolving capacity of blood (472). Treatment of tumor-bearing rats with dodecafluoropentane and carbogen was recently reported to completely reverse the hypoxic cell radioresistance in this tumor model (473). Several clinical trials have been carried out with Fluosol (**75**) (Fluosol-DA is an emulsion containing perfluorodecalin and perfluorotripropylamine) (474). Mild liver dysfunction was noted as a side effect. A benefit from the use of Fluosol in combination with radiation was demonstrated in a trial of high grade brain tumors (475). Perflubron (perfluorooctyl bromide) is another perfluorochemical that has shown promise in animal model experiments (76) (476).



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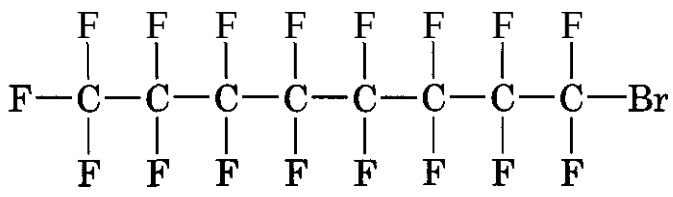

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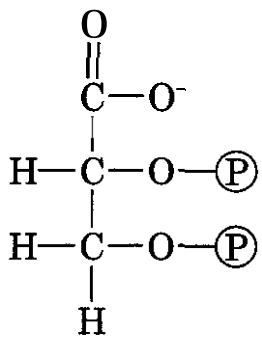
Various forms of hemoglobin have been used to increase the oxygen-carrying capacity of blood in animal experiments. Measurements using the Eppendorf oxygen needle electrode indicate that such treatments can effectively decrease tumor hypoxia (476). Radiobiological experiments in animal models have shown that tumor radiosensitivity is increased by perfluorochemicals or by ultrapurified polymerized bovine hemoglobin, by as much as an enhancement ratio of 3 (476), which is the theoretical maximum expected for total elimination of tumor hypoxia. Carbogen breathing (95% O_2, 5% CO_2) increases the effectiveness of these agents. Hemoglobin derivatized with polyethylene glycol (PEG-hemoglobin) has been demonstrated to improve tumor oxygenation in animal models (477). Derivatization with polyethylene glycol increases the circulating half-life of proteins, increases their solubility, and decreases their immunogenicity (478).

Allosteric modifiers of hemoglobin constitute another class of modifiers of tumor oxygenation (479). 2,3-Diphosphoglycerate (77) is a natural modifier of the oxygen affinity of hemoglobin and has been used to modify the tumor radiation response in experimental animals (480). Other allosteric modifiers of the oxygen affinity of hemoglobin that have been



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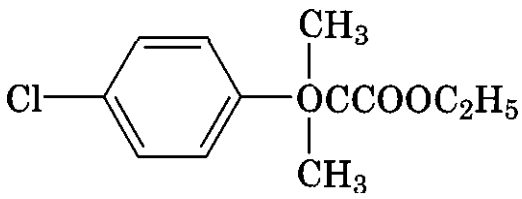
tested as radiation sensitizers include clofibrate (78), bezafibrate, and gemfibnozil (481).

Pentoxifylline (79), a methylxanthine de-



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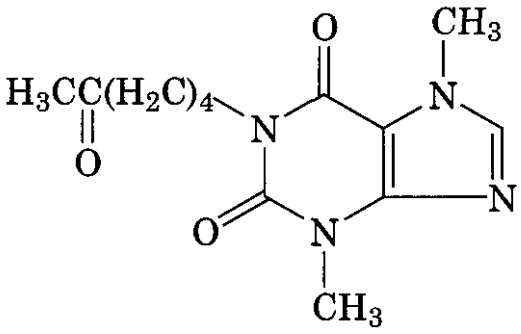
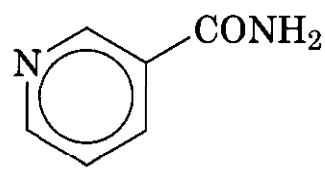


(79)

rivative, increases blood circulation by increasing blood cell deformability and decreasing blood viscosity (482). Pentoxifylline has been reported to increase murine tumor oxygenation and to enhance the radiation response of murine tumors (483). A consideration in the design of strategies for overcoming tumor hypoxia is the type of hypoxia (acute or chronic) that is targeted. Acute hypoxia is caused by intermittent cessation of blood flow. An agent that improves blood flow would be expected to be more effective against acute hypoxia than an agent that increases the oxygen content of blood. Nevertheless, the Eppendorf electrode data indicate that perfluorochemicals and hemoglobin solutions can overcome acute hypoxia, given that the oxygen electron should not have the spatial resolution to detect chronic hypoxia (narrow bands of hypoxia at a distance from a capillary corresponding to the diffusion limit for oxygen).

Nicotinamide (80) has been observed to en-



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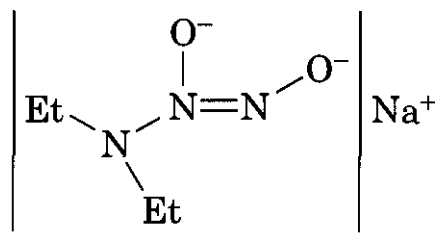
hance the radiation sensitivity of experimental murine tumors to a greater degree than normal tissues (484). This radiosensitization has been attributed to correction of acute hypoxia (485). In clinical studies, the combination of nicotinarnide and carbogen (95% O_2, 5% CO_2) breathing has been shown to improve tumor oxygenation (486). A phase II study combining accelerated radiotherapy with carbogen breathing and nicotinamide ("ARCON") resulted in high local and regional control rates in head and neck cancer (487). Nitric oxide (endothelium-derived relaxing factor) is a potent vasodilator. Drugs that release NO, such as DEA/NO {$(C_2H_5)_2N[N(O)NO]^-$, (81)}



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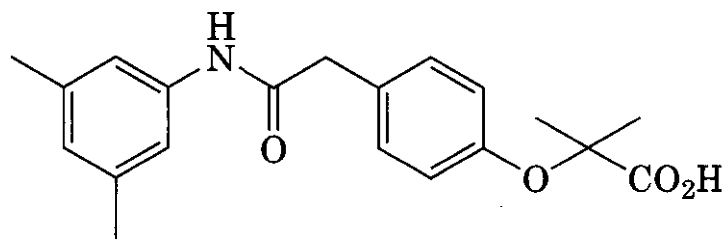
can sensitize tumors to radiation (488), although the mechanism appears to include a direct oxygen-mimetic effect (488). Nitric oxide synthase inhibitors can increase tumor hypoxia (488).

2.6.1 RSR13. RSR13 (efaproxiral sodium, 82) is a synthetic allosteric modifier of hemoglobin, the first of a new class of pharmaceuti-



(82) RSR-13

cal agents. RSR13 is a small molecule that reduces hemoglobin oxygen-binding affinity and enhances the diffusion of oxygen from the blood to hypoxic tissues. RSR13 emulates the function of natural allosteric modifiers of hemoglobin such as hydrogen ions (H^+) and 2,3-diphosphoglycerate (2,3-DPG). This "turbocharging" of oxygen unloading from hemoglobin to tissue emulates and amplifies physiologic tissue oxygenation. This approach has broad clinical applicability in indications characterized by tissue hypoxia, including the use of RSR13 as an adjunct to radiation therapy (RT) and chemotherapy (CT), as well as cardiovascular, surgical, and critical care indications.

By increasing tissue oxygenation, RSR13 reduces tumor hypoxia and enhances the cytotoxic effects of RT and CT in animal models. These effects provide the rationale for RSR13 as an adjunct to RT and CT for the treatment of cancer patients with solid tumors. The goal of adjunctive RSR13 therapy is to achieve maximal concentrations of oxygen in the tumor tissue before administration of RT or during administration of CT, to decrease the hypoxic fraction of cells, and increase the radio- or chemoresponsiveness of malignant tumors. This hypothesis is supported by Phase Ib and II clinical efficacy data from studies using RSR13 combined with RT in the treatment of brain metastases, newly diagnosed glioblastoma multiforme (GBM), and locally advanced, inoperable non-small-cell lung cancer (NSCLC).

RSR13 has been studied for the prevention or treatment of conditions associated with tissue hypoxia in 17 phase I, II, and III clinical studies. RSR13 is being evaluated as a radioenhancement agent in patients receiving RT to treat brain metastases, GBM, or locally advanced, inoperable NSCLC. More than 400 cancer patients have received RSR13 in eight phase I–III radiation oncology studies. In addition, a study is ongoing to evaluate RSR13 as an adjunct to BCNU (carmustine) chemotherapy in patients with recurrent malignant glioma.

2.6.1.1 Mechanism of Action. RSR13 binds to a site on hemoglobin that is separate from the oxygen-binding site; therefore, it is referred to as an ***allosteric modifier*** (489, 490).

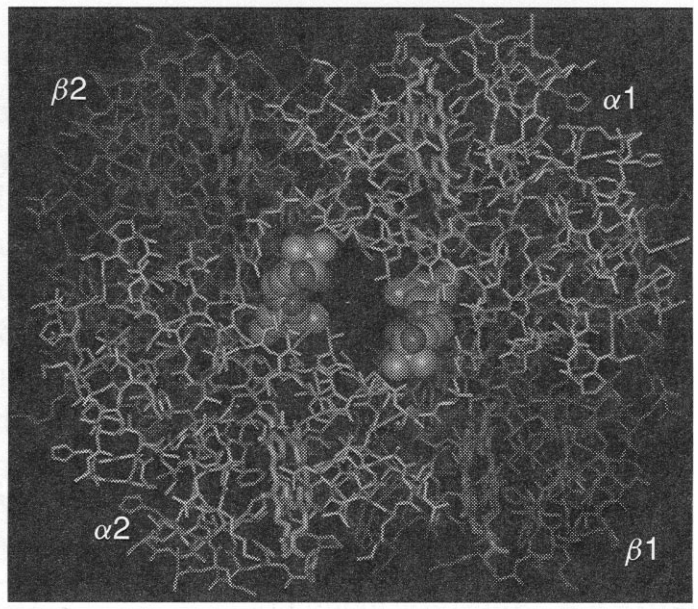


Figure 4.3. X-ray crystal structure of RSR13 bound to hemoglobin.

Natural and synthetic allosteric modifiers regulate oxygen-binding affinity by effecting a change of the hemoglobin tetramer from a high affinity to a low affinity structural conformation. Naturally occurring allosteric modifiers (H^+, CO_2, and 2,3-DPG) modulate hemoglobin oxygen-binding affinity and shift the oxygen equilibrium curve (OEC) to regulate oxygen unloading from hemoglobin to tissues under various physiologic conditions.

RSR13 binds to the central water cavity of the hemoglobin tetramer (Fig. 4.3). This site is different from the binding sites of natural allosteric modifiers, but the effects of RSR13 and the natural allosteric modifiers are similar, as shown in Fig. 4.4. Each molecule induces a rightward shift in the OEC, an effect that is described by an increased p50. The p50 is the oxygen pressure that results in 50% saturation of hemoglobin. Thus, an increase in p50 reflects a reciprocal decrease in oxygen-binding affinity of hemoglobin. Figure 4.4 depicts a p50 shift of 10 mmHg, a typical result in patients receiving a 100 mg/kg dose of RSR13.

The pharmacologic effect of RSR13 has broad clinical applicability in situations characterized by tissue hypoxia attributed to: (1) reduced blood flow (regional or global), (2) reduced oxygen carrying capacity, and/or (3) increased tissue oxygen demand. These therapeutic indications include the use of RSR13 as an adjunct to RT or CT, as well as cardiovascular, surgical, and critical care indications. Preclinical studies have shown that RSR13 can increase normal tis-

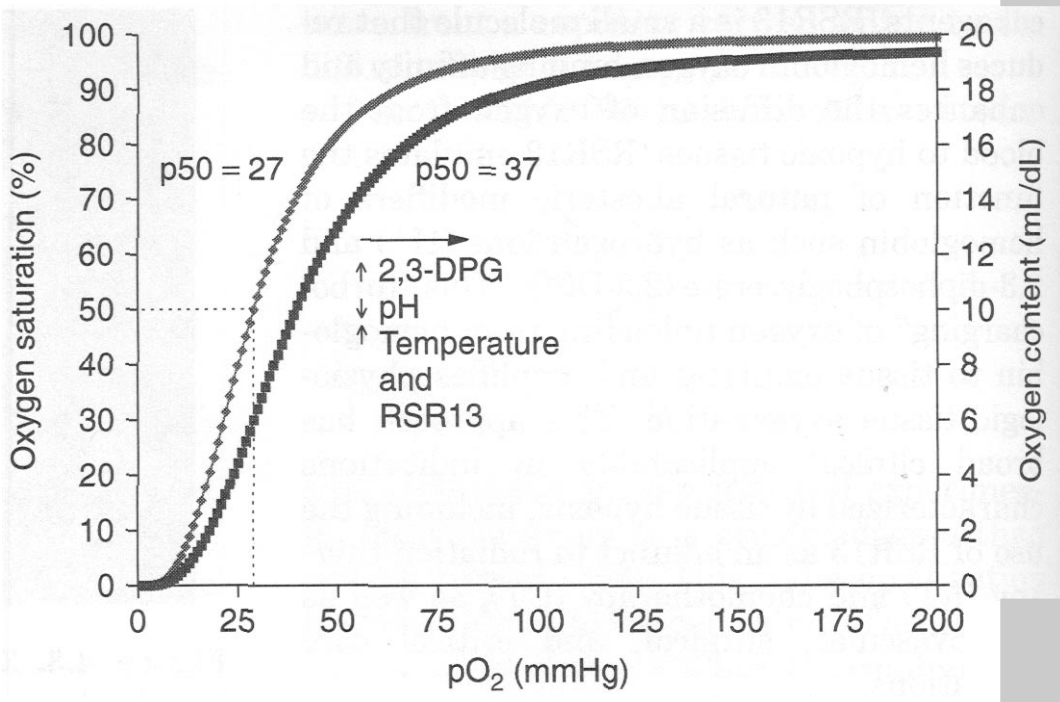


Figure 4.4. Allosteric modifiers of hemoglobin oxygen equilibrium curve for human blood.

sue oxygenation, reduce tumor hypoxia, improve the efficacy of RT and CT, and attenuate the functional and metabolic deficiencies attributed to myocardial and cerebral ischemia.

2.6.1.2 Tumor Hypoxia. Direct oxygen measurements in human tumors have confirmed tumor hypoxia (median partial pressure of oxygen [pO_2] < 20 mmHg) in many types of human tumors (491–495). Mechanisms for chronic or transient ischemic hypoxia may include obstruction of blood flow, inadequate or defective angiogenesis, and unregulated cellular growth that outstrips the capacity of the capillary blood supply (496–500). In general, tumor cells are oxygenated up to a distance of about 150 μm from a functional capillary; beyond that, tumor cells can become oxygen depleted and either die or survive in a hypoxic state. Calculations based on a three-dimensional simulation of oxygen diffusion from a network of vessels, with a geometry derived from observations of tumor microvasculature in the rat, have indicated that an increase in hemoglobin affinity for oxygen should have a beneficial effect on tumor radiotherapy (501). Although this should not be of much benefit in areas where the circulation is completely occluded (i.e., cells are completely anoxic), a recent report that a large proportion of the tumor cells in SiHa xenografts in mice are intermediately hypoxic (oxygen tension between 0.1 and 0.2%)(502) suggests that diffusion-limited hypoxia may be a significant contributor to tumor resistance.

The efficacy of RT can be affected by the extent of tumor oxygenation. Hypoxic tumors are more resistant to cell damage by radiation (503), and tumor hypoxia has been associated with a poor clinical prognosis of patients receiving RT (503–507). Oxygen measurements in human tumors have detected tumor hypoxia in GBM (491), brain metastases (491, 508), squamous cell carcinomas of the uterine cervix (493), and head and neck (492) and breast carcinomas (495). Because hypoxic tumors are substantially more resistant to radiation than oxygenated tumors, even small hypoxic fractions in a tumor may affect the overall response to RT and increase the probability that some tumor cells will survive RT.

Certain alkylating chemotherapeutic agents also require oxygen for maximal cytotoxicity. This may be related to effects of hypoxia on cellular metabolism, decreasing the cytotoxicity of anticancer drugs and enhancing genetic instability, which can lead to more rapid development of drug-resistant tumor cells (498–500, 509).

2.6.1.3 Radiation Therapy Sensitization with RSR13. Unlike most other radiosensitizing agents and strategies, the radiosensitizing effect of RSR13 is not dependent on its entry into the tumor. Instead. RSR13 enhances oxygen release from hemoglobin, thereby increasing the diffusion of oxygen from plasma and the vascular compartment to the hypoxic tumor cells. Enhanced tumor oxygenation is the basis for the radioenhancement and che-

moenhancement effects of RSR13. The fact that RSR13 does not have to enter cells to increase the tumor sensitivity to RT or CT is an important differentiation between RSR13 and other attempts to improve the efficacy of cancer therapy. This is especially important in the setting of primary or metastatic brain tumors, where the blood-brain barrier acts to exclude or impede the entry of chemical agents into the brain parenchyma. Oxygen readily diffuses the blood-brain barrier and the cancer cell membrane to increase tumor oxygenation and, thereby, the effectiveness of therapy.

RSR13 is being developed as adjunctive therapy to standard RT for the treatment of solid tumors. Fractionated RT is administered once per day, 5 days per week. For most palliative indications, such as the treatment of brain metastases, RT is administered daily (Monday through Friday) for 2–3 weeks, for a total of 10–15 treatments. For other treatment regimens, such as the treatment of primary brain cancer and other solid tumors, RT is typically administered daily for 6–7 weeks, for a total of 30–35 treatments. In completed and ongoing radiation oncology studies, RSR13 has been administered as daily doses of 50–100 mg/kg, infused over 30–60 min through a central venous catheter, immediately before RT. This regimen results in the administration of a total of 10, 30, or 32 doses to patients with brain metastases, GBM, or NSCLC, respectively.

Phase II clinical studies suggest that RSR13 improves the efficacy of RT in patients with brain metastases, newly diagnosed GBM, and locally advanced, inoperable NSCLC.

Animal pharmacology studies have shown that RSR13 dose-dependently increases blood p50 (510–514), increases pO_2 in nontumor tissue (512, 515–517), and increases oxygen-diffusive transport in nontumor tissue (518). In rats bearing mammary carcinoma tumors, tumor pO_2 was measured using Eppendorf histograms with the tumor hypoxic fraction expressed as the percentage of readings ≤ 5 mmHg. In this model, RSR13 (150 mg/kg, i.v.) decreased the tumor hypoxic fraction from 36% (controls) to 0% (treated) and increased tumor oxygenation within 30 min after RSR13 dosing (519, 520).

By use of mice with subcutaneous human NSCLC xenographs and applying the noninvasive technique of blood oxygen level dependent magnetic resonance imaging (BOLD-MRI), RSR13 was shown to dose-dependently increase tumor pO_2. The maximum increase in tumor pO_2 was achieved after 200 mg/kg of RSR13 was administered by i.p. injection. In this human tumor xenograft model, RSR13 administered 30 min before 10-Gy radiation enhanced the radiation-induced tumor growth delay by a factor of 2.8. Without radiation, RSR13 had no effect on tumor growth delay (521).

In a mouse model with subcutaneous lung tumors, i.p. RSR13 dose-dependently enhanced the efficacy of fractionated RT (measured as an enhancement of tumor growth delay) by 22, 40, and 69% at 50, 100, and 200 mg/kg, respectively (520). In additional studies RSR13 decreased tumor cell survival when combined with fractionated radiation of mice bearing FSaII fibrosarcomas, squamous cell carcinomas (510), or mammary carcinoma tumors (522). The radioenhancement effect of RSR13 was shown to be oxygen dependent, with no direct cytotoxic effect on the tumor, bone marrow (520–522), or skin (522).

RSR13 was also shown to be an effective chemosensitizer of EMT-6 cells that were exposed to a variety of cytotoxic anticancer drugs in vitro (520). When tested as a single agent in clonogenic assays in vitro, RSR13 was not cytotoxic, and it did not alter the radiation response of bone marrow progenitor cells. These studies indicate that RSR13 has chemosensitizing activity when combined with alkylating/DNA-damaging agents.

The molecular basis of the chemoenhancement activity of RSR13 may involve both hemoglobin-dependent and -independent mechanisms. In *vivo*, RSR13 has demonstrated chemoenhancement in combination with various widely used agents. The effects of chemotherapeutic agents on tumor growth delay and development of lung metastases were potentiated by RSR13 in the Lewis lung carcinoma model (520–523) and the MB-49 bladder carcinoma model (520). In addition, RSR13 demonstrated a marked ability to decrease tumor volumes when given with BCNU in a 9-L gliosarcoma model in rats (524).

2.6.1.4 Clinical Trials with RSR13. A total of 17 Phase I-III clinical studies making use of RSR13 have been completed or are ongoing in patients with cancer (including studies specifically enrolling patients with brain metastases, newly diagnosed and recurrent GBM, and NSCLC), surgical patients, patients with cardiovascular disease, and healthy subjects. Phase II studies in patients with brain metastases, GBM, and NSCLC have been completed. One randomized phase III study in patients with brain metastases is ongoing and, in addition, a phase I/II study is ongoing to evaluate RSR13 as an adjunct to BCNU chemotherapy in patients with recurrent malignant glioma.

The RSR13 dosing in clinical trials, using the drug in combination with radiotherapy, is based on a phase I trial that studied escalation of both drug dosing and frequency of administration (525). According to this open-label study in patients undergoing palliative irradiation to 20–40 Gy in 1015 fractions, RSR13 could be administered daily in doses of up to 100 mg/kg for 10 consecutive treatments through central venous access and supplemental nasal oxygen at 4 L/min. The tolerance of the drug was supported by clinical monitoring of oxygen saturation and associated pharmacokinetic and pharmacodynamic studies. At 100 mg/kg the peak increase in p50 averaged 8.1 mmHg, consistent with the targeted physiological effect (525).

2.6.1.4.1 Brain Metastases. Nearly one-third of patients with systemic cancer develop brain metastases, a complication that profoundly affects the patients' quality of life and survival. In early studies, untreated patients with brain metastases had a median survival time of about 1 month. Without more aggressive treatment, nearly all patients died as a direct result of the brain metastases (526). Even with contemporary treatment, specifically earlier diagnosis, radiation therapy, and systemic chemotherapy, approximately 30–50% of brain metastases patients die as a direct result of the brain metastases (527). Expanding intracranial tumor masses lead to intractable headaches, nausea and vomiting, serious cognitive dysfunction, and one or more focal neurological deficits, including hemiparesis, seizures, visual, speech, and gait disturbances (528). Acute, catastrophic neurological complications, such as intracerebral hemorrhage and brainstem herniation, occur in 5–10% of brain metastases patients (529).

Study RT-008 was a phase II, open-label, multicenter study to assess the effect of RSR13 on enhancing radiation therapy in patients with brain metastases. Patients received a standard 10-day course of whole-brain radiation therapy (WBRT) (3 Gy in 10 fractions = 30 Gy) within 30 min of receiving RSR13 administered through a central venous access device. RSR13 administration began on the first day of WBRT and continued daily for a total of 10 doses.

Patient eligibility was based on histologically or cytologically confirmed breast, NSCLC, melanoma, genitourinary, or gastrointestinal primary carcinoma. Patients were stratified by recursive partitioning analysis (RPA), Class I or Class II, as previously described (529). A more recent analysis by the RTOG (Study 91–04) showed similar median survival times (MST) in each class. The analysis also indicated that no major change in the prognosis of brain metastases patients has been observed in the last 25 years, even with the advent of more aggressive multiagent CT regimens directed at both the primary tumor and extracranial metastases (530). At the time the study was closed there were 57 Class II patients enrolled.

The objective of the study was to compare MST in the study population to that from the RTOG Brain Metastases Database (BMD) through use of both the overall RTOG database and controls case-matched by prognostic factors. Exact case-matched controls were obtained for 38 patients [matching 5 of 5 criteria: age, Karnofsky Performance Status (KPS), extent of metastases, status of primary cancer, and location of primary tumor). RSR13-treated patients had significantly superior overall survival (6.4 months) compared to the historical BMD control group (4.1 months) by Kaplan-Meier estimates of MST ($P = 0.0269$ compared to the overall database) (Fig. 4.5) (531, 532). One-year survival was 23% for RSR13-treated patients compared to 15% for the overall BMD population, and 9% for all case-matched BMD controls.

Further improvements in survival were observed for RSR13-treated patients compared

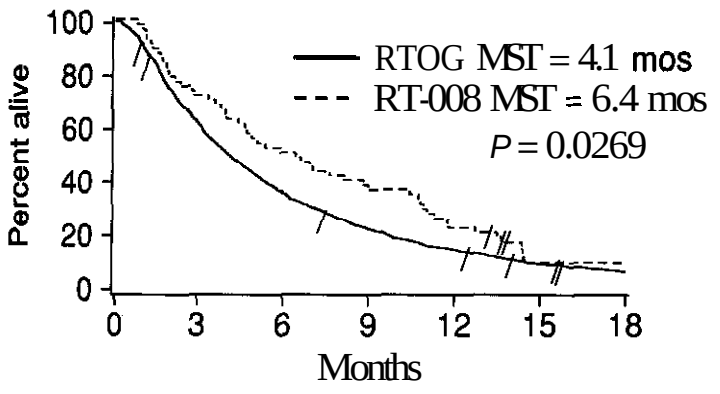


Figure 4.5. Survival results: study RSR13 RT-008 versus RTOG BMD (overall).

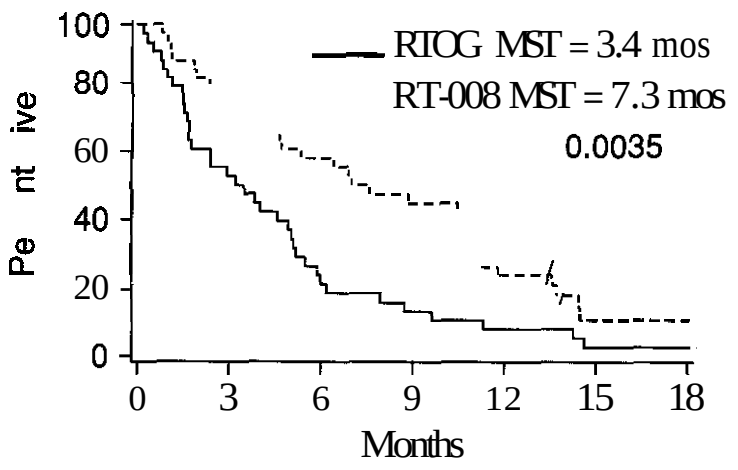


Figure 4.6. Survival results: study RSR13 RT-008 versus RTOG BMD (exact matches).

to exact case-matched controls (7.3 months versus 3.4 months, P = 0.0035, n = 38) (Fig. 4.6). One-year survival was 24% for RSR13-treated patients compared to 8% for exact case-matched BMD controls. All analyses used the log-rank test.

2.6.1.4.2 Glioblastoma Multiforme. Glioblastoma multiforme, or GBM, is a deadly form of primary brain cancer. This condition occurs in about 20% of all brain cancer patients in the United States, or approximately 3400 people per year. The median survival time of patients with GBM is approximately 9 to 10 months. Radiation therapy is the standard of care for GBM and is administered to most patients. The goal of radiation therapy is to prevent or reduce complications and improve survival time.

Patients with GBM received a standard 6-week course of cranial RT (2 Gy in 30 fractions = 60 Gy) after RSR13, at a dose of 100 mg/kg, was administered over 30 min through a central venous access device. RSR13 administration began on the first day of RT and continued daily for a total of 30 doses. RT was administered within 30 min after completion of each RSR13 infusion.

Patient eligibility was based on histologically confirmed supratentorial grade IV astrocytoma (GBM).

RT-007 was a study sponsored by the National Cancer Institute (NCI) and conducted by the New Approaches to Brain Tumor Therapy (NABTT) CNS Consortium. Survival results of the intent-to-treat analysis for this study have been published (533, 534). An intent-to-treat analysis was performed after all 50 patients had completed a minimum follow-up of 14 months. Survival data were compared to those from an NABTT historical database. The NABTT historical database consisted of 152 patients pooled from multiple studies. NABTT and RSR13 studies were performed in the same general time period (1994–2000).

Patients in the RSR13 and NABTT studies were predominantly male and Caucasian. Mean values for baseline parameters by study ranged from 53 to 62 years for age, from 83 to 88 for KPS, and from 62% to 83% for history of surgical resection. The distribution of patients by RPA class included 6 patients in Class III, 32 patients in Class IV, and 12 patients in Class V (535).

The MST was greater for RSR13-treated patients compared to the NABTT historical database: 12.3 months vs. 9.7 months and was statistically significant as determined by the Wilcoxon test ($P = 0.02$) and the log-rank test ($P = 0.04$) (Fig. 4.7).

2.6.1.4.3 Non-Small-Cell Lung Cancer. Non-small-cell lung cancer, or NSCLC, is a type of cancer that occurs in approximately 130,000 patients per year in the United States. RSR13 is currently being evaluating as a radiation enhancer for the treatment of patients with locally advanced, inoperable Stage IIIA and IIIB NSCLC. Radiation therapy for treatment of Stage III NSCLC is intended to prevent or reduce complications and control local tumor growth in the chest. The overall median survival time of patients with Stage III NSCLC is approximately 9 to 12 months.

Study RT-010 was a phase II, nonrandomized, open-label, multicenter efficacy and safety study. Patients with locally advanced

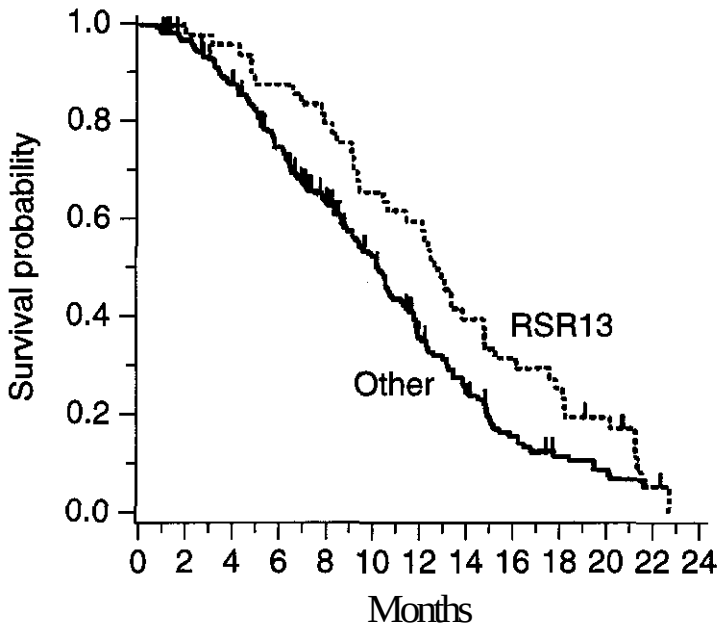


Figure 4.7. Kaplan-Meier survival distribution: study RSR13 RT-007 vs. NABTT historical database.

inoperable (Stage IIIA or IIIB) NSCLC received induction chemotherapy [paclitaxel (225 mg/m^2 i.v.) and carboplatin (area under the curve = 6, i.v.) every 3 weeks × two cycles] followed by standard thoracic RT with RSR13 over 6–7 weeks (up to 32 fractions/doses). RSR13 was administered at an initial dose of 75 mg/kg through a central venous access device just before daily RT. Dose reduction to 50 mgkg or increase to 100 mg/kg was allowed per protocol depending on patient tolerance.

Objectives of this study were to evaluate complete and partial response rates in the chest (radiation portal), overall survival, progression-free interval in the chest, time to disease progression outside the radiation portal, and toxicities and adverse events associated with RSR13 and RT after induction chemotherapy.

The study was conducted in 47 evaluable patients with locally advanced, inoperable, Stage IIIA/IIIB non-small-cell lung cancer. The objectives of this study were to evaluate overall survival, progression-free interval in the chest, complete and partial response rates in the chest (radiation portal), and time to disease progression outside of the radiation portal. The patients received two courses of induction paclitaxel and carboplatin chemotherapy followed by daily RSR13 combined with chest radiation therapy for 32 doses.

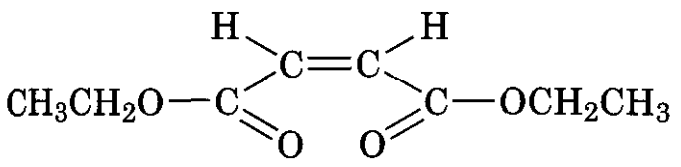
The overall response rate was 89%, with complete and partial response rates of 9% and 80%, respectively (536). Median time to first progression was 9.9 months. Median tumor progression-free survival time in the radiation portal was 24.8 months, whereas median progression-free survival time outside the portal was 11.3 months. The median survival was 20.6 months, 1-year survival rate of 68%, and an estimated 2-year survival rate of 43% (537).

2.6.1.5 ***Summary of RSR13 Results.*** The preclinical and clinical studies of RSR13 indicate that RSR13 increases oxygen release from red blood cells and increases the pO_2 in hypoxic tissues. Tumor hypoxia can limit the efficacy of radiation therapy; pretreatment of patients receiving radiation therapy for brain metastases, primary glioblastoma multiforme, and non-small-cell lung cancer with RSR13 appears to increase their chances for survival.

2.7 Radiosensitization by Depletion of Endogenous Protectors

Radiosensitization of hypoxic cells should be achievable by either increasing the concentration of oxygen-mimetic sensitizers or by decreasing the concentration of endogenous protectors. as discussed in Section 2.4. The concentration of endogenous protectors is too low to effectively compete with oxygen for reaction with DNA radicals, at oxygen concentrations found in well-oxygenated tissues. However, endogenous protectors become effective at low oxygen tensions and can undermine the effectiveness of electron-affinic sensitizers by competing with them for reaction with DNA radicals. Several human tumor cell lines have been reported to have high glutathione levels and to be correspondingly resistant to hypoxic radiosensitization with misonidazole (538). A strategy for selective radiosensitization of hypoxic cells is therefore to deplete endogenous radioprotectors.

Because glutathione (GSH) is the principal intracellular thiol, it has been the principal target for depletion. Selective depletion of GSH can be accomplished with weak electrophiles that are substrates for GSH *S*-transferases (539). Diethylmaleate (DEM, 83) is one agent that meets these criteria (540). The enzyme-catalyzed reaction is substantially faster than chemical reactions with other endogenous nucleophiles at low DEM concentrations (540, 541). GSH depletion with low con-



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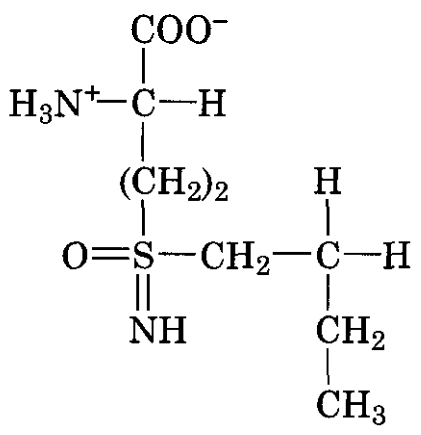
centrations of DEM sensitizes hypoxic cells to radiation and enhances the radiosensitizing effect of nitroimidazole radiosensitizers, without affecting aerobic cell radiosensitivity (541). Tumor sensitization can be achieved *in vivo* with DEM without enhancing normal tissue radiosensitivity, except for a slight enhancement of skin reactions (540). Enhancement of misonidazole tumor radiosensitization by DEM has also been demonstrated *in vivo* (36). The relative activities and substrate specificities of the various isozymes of GSH S-transferase (542,543) in human tumors will be a consideration for optimization of this approach for human use. For example, it has been reported that GSH S-transferase π is overexpressed in many human tumors (544).

Another strategy for depletion of GSH is to inhibit its synthesis. *L,S*-Buthionine sulfoximine (*L,S*-BSO, 84) is one of a series of substrate analogs that inhibit y-glutamyl cysteine synthetase, which is rate limiting for GSH synthesis (544, 545). GSH becomes depleted over a period of 4–24 h, depending on cell type, as GSH is lost to catabolism. *L(S,R)*-BSO is in clinical trials as a chemosensitizer (546). Early clinical results with bolus administration of *L(S,R)*-BSO indicated that tumor GSH depletion was not consistently achieved; however, continuous infusion trials appear to have been more successful in depleting tumor GSH (547–549). There have been no reports of clinical toxicity with *L(S,R)*-BSO, although it does enhance the myelotoxicity of melphalan (547), and it has not yet been determined whether it produces a therapeutic gain as a chemosensitizer in the clinic. A possible explanation for the difficulty in achieving tumor GSH depletion in the clinic is that *L(S,R)*-BSO treatment can lead to increased expression of y-glutamyl-cysteine synthetase messenger RNA (550). Tumor GSH depletion with *L,S*-BSO in a murine model system is less than expected from *in vitro* studies (551). Nevertheless, enhancement of etanidazole radiosensitization has been observed when GSH is depleted with *L(S,R)*-BSO in animal model systems (36). Enhancement of etanidazole-induced neurofilament degradation in a spinal cord organotypic model by *L,S*-BSO was not greater than the expected enhancement of hypoxic tumor cell radiosensitivity, suggesting a therapeutic gain may be achievable by the combination of *L,S*-BSO and etanidazole (552).



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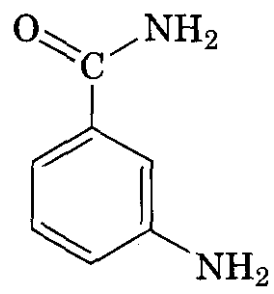
2.8 Radiosensitization by inhibition of DNA Repair

The sensitivity of mammalian cells to ionizing radiation is very dependent on DNA double-strand break repair capacity (553), particularly at the radiation doses that are used in cancer therapy. Cell lines that are deficient in DNA double-strand break repair are radiosensitive (554,555). Inhibition of DNA repair has great potential for tumor radiosensitization (556, 557), but strategies for selective sensitization of tumors remain elusive (558).

2.8.1 PLD Repair Inhibitors. One strategy is to inhibit a type of cell recovery from radiation damage that is called ***potentially lethal damage repair*** (PLDR). PLDR is not yet understood at the molecular level. PLDR is functionally defined as an increase in survival when cells are held under nutrient-deprived conditions, in comparison to the survival of cells that are immediately plated in fresh medium after irradiation (559).

Poly(ADP-ribose) polymerase (PARP) binds to single-strand breaks (SSB) in DNA (560), and is activated by DNA single-strand breaks. Inhibitors of this enzyme, such as 3-aminobenzamide (3AB, 85) inhibit SSB repair and inhibit PLDR (561). Cleaver et al.



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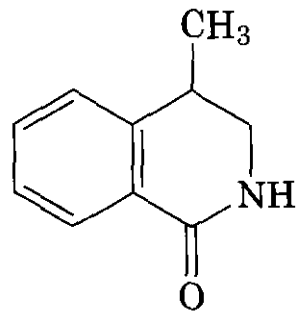
(562) cautioned that 3AB has other effects on the cell and may not be acting by inhibition of PARP, particularly considering that the concentrations required for inhibition of PLDR are much higher than the K_i value for isolated PARP. Utsumi and Elkind (561) reported that two types of PLDR are inhibited by 3AB, at different concentrations of 3AB. A variety of compounds inhibit PARP (563) and there is currently intensive activity in the development of more effective PARP inhibitors. PD 128763 [3,4-dihydro-5-methyl-1(2 *H*)-isoquinolinone; (**86**)] inhibits PARP at a concen-



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tration about 50 times lower than 3AB, and sensitizes both exponentially growing and stationary phase mammalian cells to ionizing radiation (564).

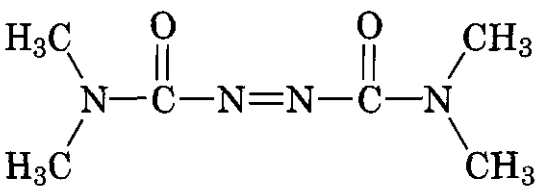
Other PLD repair inhibitors include actinomycin D (565), 9-β-D-arabinofuranosyladenine (ma-A) (566), 1-β-D-arabinofuranosylcytidine (ma-C) (567), cordycepin (3'-deoxyadenosine) (568), 3'-deoxyguanosine (569), and aniosotonic media (570). Irradiation of cells under aerobic conditions in the presence of high concentrations of misonidazole results in inhibition of PLD repair, even though misonidazole does not radiosensitize exponentially growing aerobic cells (571). Some DNA-repair inhibitors can also alter the survival curve of exponentially growing mammalian cells (572). Much remains to be understood about the mechanisms of radiosensitization by these agents and about the differences in repair enzymology, if any, between tumors and normal tissue, before this approach can be used effectively in the clinic.

2.8.2 Radiosensitization by Reaction with Protein Sulfhydryls. Most exposed intracellular cysteine thiol groups normally occur in the reduced state (573), given that glutathione is kept highly reduced and enzymes catalyze redox reactions between intracellular glutathione and protein thiols/disulfides (574). In many cases oxidation or alkylation of these protein sulfhydryl groups results in loss of enzyme activity (575). Consequently, treatment of cells with thiol oxidants or thiol-binding reagents affects many metabolic processes (576).

It has long been recognized that thiol oxidants or thiol-binding reagents sensitize cells to ionizing radiation (577–580). Part of this effect is attributed to depletion of endogenous radioprotectors. However, this does not explain radiosensitization that is observed when thiol reagents are added after irradiation because endogenous chemical radioprotectors will have acted within 10 ms of irradiation (399). Recent studies have indicated that protein thiol loss results in inhibition of DNA double-strand break repair (581, 582), and that this can account for the postirradiation sensitization.

There are two approaches that can be taken to deplete protein thiols. One is to oxidize intracellular glutathione, which in turn will oxidize protein thiols. The other is to use thiol-binding reagents.

Diazenes have been used as reagents for selective oxidation of intracellular glutathione (582–584). Diamide [diazenedicarboxylic acid bis(*N*-*N*'-dimethylamide), (**87**)] sensitizes mammalian cells to ionizing radiation (576, 585, 586). The predominant finding is that it decreases the shoulder of the radiation survival curve. Similar results were obtained with the diamide analogs DIP (**88**) and DIP + 1 (89) that penetrate cells more slowly, resulting in less drug toxicity (587) and with the diamide analog SR-4077 [diazenedicarboxylic acid bis(*N*-*N*'-piperidide), (**90**)] that is similar in reactivity to diamide, but less cytotoxic (582). This is in contrast to the effect of glutathione



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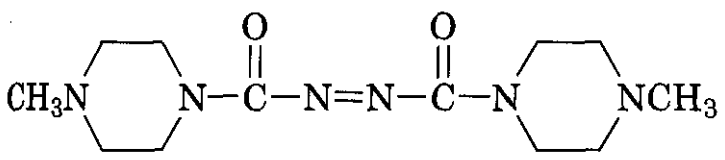

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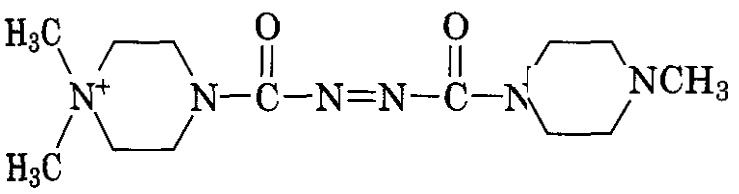

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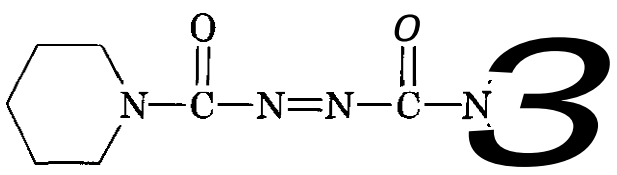
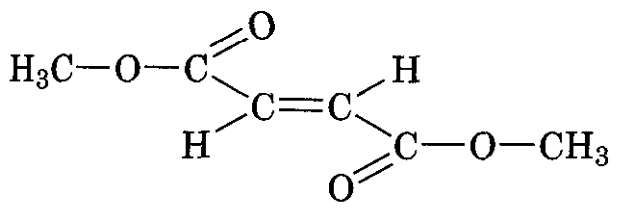


(90)

depletion, which is a change of the exponential dope of the survival curve **(36).** Radiosensitization by thiol oxidation can be achieved, even if the oxidant is added after irradiation, eliminating rapid radiation chemistry as a factor. Radiosensitization by glutathione oxidants is associated with inhibition of DNA double-strand break repair (581,588).

Dimethyl fumarate (DMF, 91) is a thiol-binding agent that depletes glutathione and protein thiols (589). DMF sensitizes hypoxic and oxygenated cells when added after irradiation and this effect is associated with inhibition of DNA repair. Additional sensitization of hypoxic cells is observed if DMF is added before irradiation, and this has been attributed to depletion of glutathione (589).



(91)

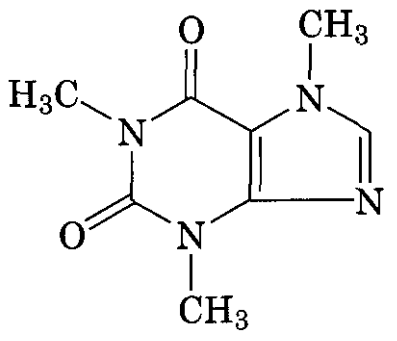
2.9 Radiosensitization by Perturbation of Cellular Metabolism

2.9.1 Perturbation of Energy Metabolism. Several studies have indicated that radiation sensitivity can be altered by treatment of mammalian cells with uncouplers or inhibitors of oxidative phosphorylation (590). A potential selective effect on hypoxic tumor cells is suggested by the observation that 5-thio-D-glucose can sensitize hypoxic cells to radiation (591), given that inhibition of glycolysis would be expected to have a greater impact on hypoxic cells than on oxygenated cells. Beyond selectivity toward hypoxic cells, 2-deoxy-D-glucose may selectively sensitize cancer cells that depend on aerobic glycolysis, while protecting bone marrow (592,593).

The mechanism of radiosensitization by modulators of energy metabolism is not clear. No radiosensitization was observed when low concentrations of 2-deoxyglucose and rotenone were used in combination to achieve a steady-state decrease in the adenylate energy charge of Chinese hamster ovary cells, even though very low energy levels were maintained for up to 4 h and DNA repair was inhibited (594). This result suggests that a complete collapse of energy metabolism may be required to achieve radiosensitization, and that the mechanism may involve secondary events, such as failure to maintain ionic homeostasis, rather than simply a lack of ATP to carry out repair. It could be that the processes that result in damage fixation are equally inhibited by a low energy state, and that both damage fixation and damage repair recover when the ATP is restored. It is possible that under certain conditions, the balance could be shifted the other way, inhibiting damage fixation while permitting repair. This possibility is suggested by observations of radioprotection with uncouplers of oxidative phosphorylation (595).

An additional consideration arises in *vivo* because inhibition of oxidative phosphorylation will decrease oxygen consumption, providing a means of sensitization of chronically hypoxic cells by increasing the diffusion distance of oxygen.

2.9.2 Abrogation of G_2 Delay. Ionizing radiation induces cell-cycle arrest at both the G_1 and the G_2/M checkpoints (596). The purpose of cell-cycle arrest is presumed to be to allow time for DNA repair before progressing through the cell cycle (597), although the precise mechanism of "repair" involved is not clear. In the case of a G_1 arrest, abrogation of the arrest, for example, by transfection of a mutated form of the *p53* gene, does not result in increased cell killing (598), but has been associated with increased mutagenicity and carcinogenicity (599). Abrogation of the G_2/M arrest, on the other hand, can enhance radiation cell killing. The classic example of a radiosensitizer that acts by this mechanism is caffeine (92) (600–602).



(92)

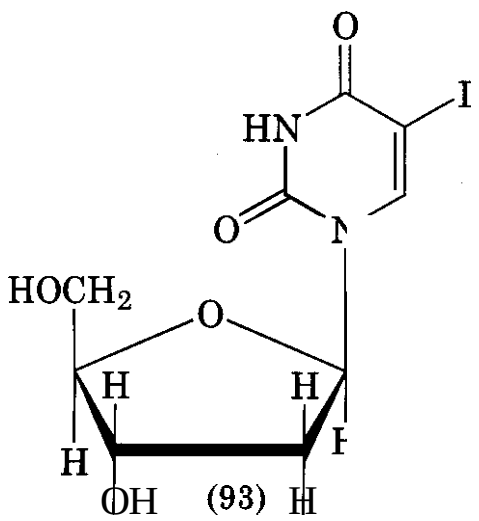
G_2/M arrest appears to be an effect on cellular regulation, rather than simply a physical consequence of damage. Cyclin B1 mRNA and protein levels have been observed to decrease in correlation with radiation-induced G_2/M arrest (603, 604). Cyclin B1 is required for the G_2/M transition. Cyclin Bl forms a complex with $p34^{cdc2}$ kinase called mitosis promoting factor (MPF). MPF is kept inactive by phosphorylation. Caffeine can inhibit the protein kinase that phosphorylates $p34^{cdc2}$ (605). MPF is thought to trigger entry into mitosis upon its dephosphorylation by cdc25 (606, 607), by phosphorylating key proteins such as histones and lamins. Metting and Little (608) reported that failure to dephosphorylate the $p34^{cdc2}$-cydin B1 complex accompanies radiation-induced G, arrest in HeLa cells. Caffeine treatment results in increased $p34^{cdc2}$ kinase activity and increased histone mRNA in irradiated cells (609).

An interesting possibility is suggested by a study by Powell et al. (610), in which they found that $p53^-$ cells were sensitized more than $p53^+$ cells by caffeine. They suggested that this might be a basis for a therapeutic gain because many tumors have mutated forms of p53.

Other agents that abrogate the radiation-induced G_2/M arrest include staurosporine, 2-aminopunine, cordycepin, 6-dimethylaminopurine, theobromine, and theophilline (611–613). Staurosporine, a protein kinase inhibitor, can override both the G_1 and G_2/M blocks induced by irradiation (611). Hallahan et al. (614) reported radiosensitization of human squamous cell carcinoma cell lines with the protein kinase inhibitors sangivamycin and staurosporine.

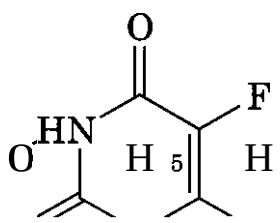
2.9.3 Radiosensitization by Growth Factors and Cytokines. Radiation sensitivity can be altered with growth factors and cytokines, although it is likely that the effect of a particular agent of this type will be substantially dependent on the context of its use. Hallahan et al. (615) reported that TNF-α inhibits PLD repair in several human tumor cell lines when added 4–12 h before irradiation. Epidermal growth factor has been reported to radiosensitize human tumor cells that overexpress the EGF receptor (616, 617). Insulin and insulin-like growth factor 1 have been reported to inhibit PLD repair in human tumor cells (618).

2.9.4 Halogenated Pyrimidines. Substitution of a halogen atom for the hydrogen at the 5-carbon position of uracil or cytosine has produced a series of compounds that interfere with nucleotide metabolism. Radiosensitization by halogenated pyrimidines was noted in the late 1950s (619, 620). Radiosensitization by 5-iododeoxyuridine (IdUrd, 93) requires incorporation into DNA and correlates with the percentage of thymidine replacement. The mechanism may involve enhancement of initial damage to DNA, inhibition of DNA repair, or alteration of cell-cycle kinetics, but has not been clearly established (621). A rationale for selective tumor radiosensitization is that the cells in some tumors proliferate more rapidly than the stem cells of the limiting normal tissue. Early clinical trials with Bromodeoxyuridine (BrdUrd) in the treatment of head and neck cancer resulted in considerable normal



tissue toxicity, indicating that this rationale does not apply to all tumors (622). More recent trials have focused on brain tumors (623), where there appears to be a larger differential in proliferation rates between the tumor and the critical normal tissue cells (621). The limitation of this approach is that noncycling tumor cells will not be sensitized; the effectiveness of the treatment will depend on the percentage of cells that incorporate the analog. Combinations with biochemical modulators are being devised to enhance incorporation in tumor cells (621).

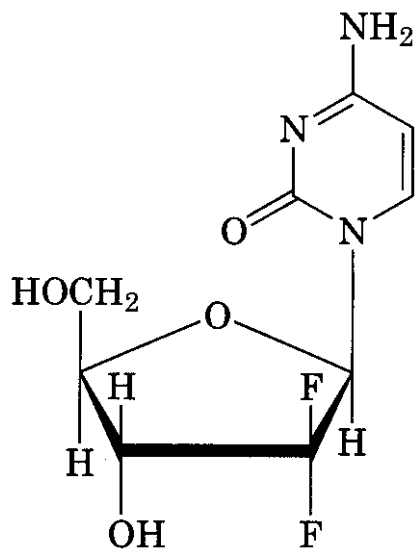
5-Fluorouracil (5-FU, 94) is a cytotoxic



(94)

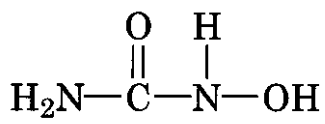
agent that is used in cancer chemotherapy. A number of clinical trials have shown a benefit for the combined use of 5-FU and ionizing radiation (621, 624), but it is not clear whether the effect is additive or synergistic, nor is it clear whether this represents a true therapeutic gain, in that increased normal tissue toxicity is also observed. 5-FU is thought to act principally by inhibition of thymidylate synthetase (TS), but it is also incorporated into RNA and can alter cell-cycle kinetics. Because inhibition of TS blocks de *novo* synthesis of thymidine, 5-FU can also be used to enhance IdUrd incorporation into DNA (625). More specific TS inhibitors appear to retain radiosensitizing activity, suggesting that this is the dominant mechanism of radiosensitization (626).

Gemcitabine [2′,2′-difluorodeoxycytidine (dFdC), (95)] radiosensitizes human tumor



(95)

cells to radiation (627). This effect has been attributed to inhibition of ribonucleotide reductase, resulting in decreased deoxyribonucleotide pools (628). Selectivity could be achieved by differences in deoxycytidine kinase activity, given that this enzyme is required to activate this antimetabolite (629). Hydroxyurea (96), another inhibitor of ribo-



(96)

nucleotide reductase, has been tested as a radiosensitizer in clinical trials with some encouraging results (630).

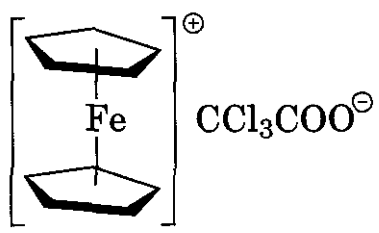
Another strategy for inhibition of ribonucleotide reductase is to use 5-chloro-2'-de-oxycytidine or analogs thereof (631), which need to be metabolized by cytidine deaminase or deoxycytidylate deaminase to become active inhibitors, because it has been observed that a number of tumor cell lines express high levels of these enzymes (632). The most effective strategy appears to be to use this drug in combination with tetrahydrouridine (a cytidine

deaminase inhibitor) to inhibit activation by cytidine deaminase because the greater differential between tumor and normal tissue appears to be in deoxycytidylate deaminase.

2.10 Radiosensitizers for which the Mechanism of Sensitization has not been Established

2.10.1 Metal Ion Complexes. A property of metal ions that could be useful in radiosensitizer design is the ability to readily exchange single electrons. Single-electron exchange could serve as a mechanism either for radioprotection or for radiosensitization. Some metal complexes that have been found to be hypoxic cell radiosensitizers are electron affinic. However, the mechanism of radiosensitization has generally not been established for this type of compound because metal ions also have the potential of interaction with thiol groups, disturbing DNA structure, and upsetting cellular metabolism (633).

Ferricenium salts (97) are very efficient radiosensitizers of hypoxic mammalian cells, achieving an enhancement ratio of 2 at 10 μM, with little effect on oxygenated cells (634). Unlike other electron-affinic sensitizers, these compounds predominantly decrease the shoulder of the radiation survival curve. In that sense they resemble agents that inhibit DNA repair. Cu(I) salts have been reported to radiosensitize hypoxic bacterial or mammalian cells, but the mechanism has not been explored (635). Metalloporphyrins, and particularly Co(III) complexes, have been reported to preferentially radiosensitize hypoxic mammalian cells (636). Teicher et al. found various Co(III) and Fe(III) complexes to be hypoxic cell radiosensitizers (637). SN 24771 (98) is a cobalt (111)-nitrogen mustard complex that is selectively toxic to hypoxic cells (638).



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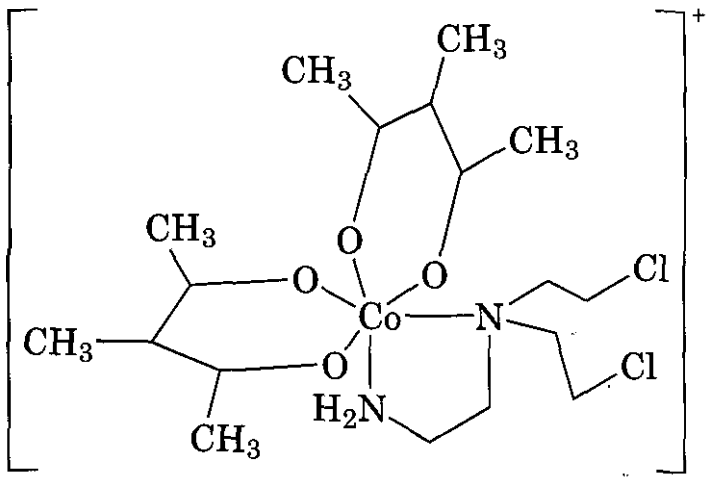

(98)

Cytotoxic platinum-containing drugs have been reported to have a more than additive effect on cell killing in combination with ionizing radiation (346–399, 639). Clinical trials with cisplatin and radiation in inoperable non-small-cell lung cancer have shown better tumor control (640). Carboplatin appears to be as effective as cisplatin in sensitizing human lung cell lines to radiation in *vitro*, and is less toxic clinically (639). Because Pt is not redox active, the mechanism of radiosensitization is most likely related to Pt binding to cellular macromolecules. Cisplatin cytotoxicity is related to its ability to produce DNA-DNA crosslinks. However, the mechanism of interaction with radiation has not yet been established.

Metal complexes of electron-affinic nitroheterocyclics have been synthesized, with the rationale that the ability of metals to bind to DNA will localize the electron-affinic agents to DNA and therefore improve their efficacy as hypoxic cell radiosensitizers (641,642). Rh(II) complexes appear to be particularly effective.

2.10.2 Thiols and Miscellaneous Compounds. Although the best radioprotectors are thiols, several thiols, including isocysteine, 13-homocysteine, and D-penicillamine, have been reported to be radiosensitizers (643). Thioglycol and thioglycolic acid also cause sensitization (644). Thiamine diphosphate (645), riboflavin (646), and menadiol sodium phosphate (Synkovit) (647) act as sensitizers in animals. Demecolcine sensitizes mice when administered 12 h before irradiation, but is radioprotective when given 48 h before irradiation (648). Sensitization of mice or rats

has also been reported for pentobarbital (6491, nalorphrine (6501, butanone peroxide (651), methylhydrazine (6521, and cupric salts (653).

2.10.3 Bacterial Sensitizers. Several compounds were found to sensitize bacterial cells to radiation in a series of studies in the 1960s in which the mechanism of sensitization is not clear. These include hadacidin (654), chloral hydrate and other halides (655), quaternary heterocycic salts, including phthalanilides, phenaziniums, and isoindoliniums (6561, methylhydrazine (652), methylglyoxal (6571, tetracyclines (658), and irradiated cupric salts (659).

3 SUMMARY AND PROSPECTS FOR FUTURE DEVELOPMENT OF CLINICAL RADIATION MODIFIERS

A variety of approaches have been developed for diminishing the effects of radiation on normal tissues or enhancing tumor cell killing by ionizing radiation. Nevertheless, it is not yet clear that these strategies will provide a therapeutic gain in radiation therapy. Results obtained with model systems do not always apply to more complicated biological systems. Many of these compounds have multiple pharmacological actions, which are sometimes antagonistic. Most often, agents have been tested clinically at suboptimal doses because of limiting toxicities, and the results have been inconclusive. The possibility that radioprotectors could protect the tumor makes it difficult to test radioprotectors in the clinic. Unless the rationale for selective radioprotection of normal tissues or radiosensitization of the tumor is foolproof, there is a risk in clinical testing because the clinical impact is often not known for several years. Nevertheless, several clinical trials are in progress, mostly with sensitizers, and substantial new information is expected from these trials within the next few years.

Several problems make it particularly difficult to develop clinical strategies for modification of radiation therapy. One problem is selective and effective drug delivery. Many of these compounds are metabolized or chemically altered before they reach the target cells. Electron-affinic hypoxic cell radiosensitizers or bioreductive drugs that are excellent in vitro can be metabolically inactivated before they reach the hypoxic tumor cells. Reducing agents that might be excellent protectors in vitro can be inactivated by oxidation in *vivo*. Tumor vasculature is chaotic, creating problems in drug delivery (660). The intermittent vascular occlusion that creates problems in drug delivery is the same phenomenon that creates the hypoxic areas that need to be sensitized.

Selective drug delivery to the tumor is a difficult problem. Boron neutron-capture therapy, for example, depends entirely on selective uptake of the sensitizer in the tumor, and animal studies have suggested that this can be achieved, although the clinical results have not been as impressive. It is not enough to deliver more sensitizer to the tumor; it needs to be delivered to all the target cells in the tumor. The exponential relationship between dose and cell killing means that an equal increment in radiation dose is required for each log of cell kill. It is therefore of little use to sensitize 90% or 99% of the cells; this only decreases the dose necessary to kill the first log or two of cells, but has no impact on the remaining 10^7 or 10^8 cells. The situation in normal tissues is the reverse. It is not enough, to protect some of the cells in the normal tissue because tissue damage can occur even if a small subset of these cells are killed, if their function is vital. In the case of early boron neutron-capture trials, it appears that accumulation of the sensitizer in the normal tissue vasculature negated the potential therapeutic gain that was expected from lower overall sensitizer concentrations in the normal tissue.

Despite the problems, the outlook for radiation modifiers is good. The radiation dose-response relationships for both tumor cure and normal tissue damage are steep, so that the challenge for modifiers is not too great; even a 20% shift in either response curve should have a large impact on clinical results. The classical view of the mechanism of action of ionizing radiation is that all of the biological effects can be accounted for by cell killing that results from clustered DNA lesions. That particular mechanism does not lend itself easily to differential modification of tumors and nor-

mal tissues, given the same radiation chemistry in both cases, with the exception that hypoxic tumor cells can be targeted with specific strategies. However, it is now recognized that ionizing radiation produces subtle changes in cell function, in addition to classical reproductive cell death, and that these other effects of radiation may be modifiable with agents that do not affect classical reproductive cell death (661).

Cytokine cascades persist for months after radiation, possibly contributing to tissue fibrosis, as a pathogenic mechanism (662,663). Dittman et al. (664) suggested that radiation-induced differentiation of progenitor fibroblasts could be related to the development of tissue fibrosis, and they found that the Bowman-Birk proteinase inhibitor can inhibit radiation-induced premature differentiation of these cells. Delayed mutagenesis and cell death that occurs many cell divisions postirradiation could be attributed to an induced hypermutability (665). There are indications that mutagenesis may be inhibitable with strategies that would not affect the formation of clustered DNA lesions that are thought to be responsible for classical reproductive cell death (666). Apoptosis is a regulated mechanism of cell death that occurs in certain cell types more than others, and can be modified with agents that do not affect classical reproductive cell death (324,667,668). Vascular effects of radiation may be mediated in part by bioactive products that can be specifically antagonized (669–671).

The implication of these new findings is that new types of modifiers can be developed that target a specific aspect of the mechanism of action of ionizing radiation. If the mechanism is more important to normal tissue than to the tumor, or vice versa, the modifiers would not have to be delivered differentially and selectivity would be achieved by mechanistic differences.

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CHAPTER FIVE

Synthetic Antiangiogenic Agents

OREST W. BLASCHUK
McGill University, Royal Victoria Hospital
Montreal, Quebec, Canada

J. MATTHEW SYMONDS
Adherex Technologies Inc.
Ottawa, Ontario, Canada

Contents

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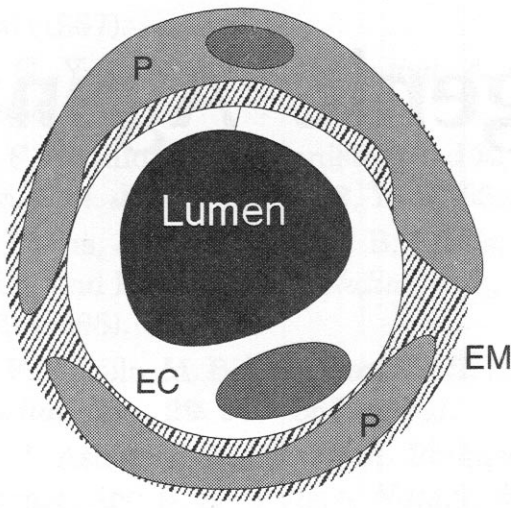


Figure 5.1. Structure of microvasculature. Endothelial cells (EC) form the wall of the microvessel and are surrounded by an extracellular matrix (EM) and pericytes (P).

1 INTRODUCTION

This chapter focuses on well-defined chemical compounds that antagonize specific targets believed to be critical regulators of the angiogenic process. These compounds have mainly been tested in the clinic for their ability to disrupt tumor-driven angiogenesis. It is noteworthy that very few antiangiogenic agents have thus far been successful in clinical trials.

2 FORMATION OF BLOOD VESSELS

2.1 Structure of Blood Vessels

Endothelial cells line the lumens of blood vessels (1,2). In capillaries (i.e., small blood vessels, or microvessels),the endothelial cells are surrounded by a single layer of pericytes embedded in a basement membrane (also known as the basal lamina), whereas in large blood vessels (i.e., arteries and veins), endothelial cells rest on a lamina, which is encircled by multiple layers of smooth muscle cells (Fig. 5.1). Pericytes (which express a-smooth muscle actin) and the smooth muscle cells are thought to regulate blood flow, as well as to stabilize the vasculature (1–3).

2.2 General Description of Angiogenesis

The process by which new vasculature arises from preexisting blood vessels is known as angiogenesis (4, 5) (Fig. 5.2). This process remains imperfectly understood. Endothelial cells are nonmigratory and quiescent (6). These cells become "activated" when the cumulative effects of proangiogenic stimuli are sufficient to overcome the influence of local antiangiogenic regulators (5). Endothelial cells of normal blood vessels rest on a basement membrane (1,2) (Fig. 5.1). For angiogenesis to proceed in response to a stimulus, the endothelial cells must first detach from one another and degrade this basement membrane (4, 5, 7). Once released from these physical constraints, the endothelial cells begin migrating in a column toward the source of the proangiogenic stimulus. Cell division occurs at the front of the advancing column, whereas cell differentiation occurs in the rear. The differentiated endothelial cells adhere to one another and begin the process of forming a new capillary. Maturation of the capillaries is believed to be regulated by newly recruited pericytes, which are thought to suppress endothelial cell proliferation, as well as to collaborate with the endothelial cells in the synthesis of a basement membrane (2, 3, 8, 9).

3 COMPOUNDS THAT INHIBIT ANGIOGENESIS

3.1 Inhibitors of Proteolysis

The major structural components of blood vessel basement membranes are collagen IV, laminin, and heparan sulfate proteoglycans (HSPGs) (10–13). Endothelial cells use a group of zinc-binding endopeptidases, known as matrix metalloproteinases (MMPs), to degrade these components and breach the basement membrane (13–19). They also use these enzymes to migrate through the extracellular matrix of the perivascular stroma (which is predominantly composed of collagens I, III, and VII, fibronectin, and HSPGs) toward the proangiogenic stimulus (13, 14).

A number of synthetic substrate analog inhibitors of MMPs with antiangiogenic activity have been developed (20, 21) (Table 5.1). Two of the most potent broad-spectrum inhibitors

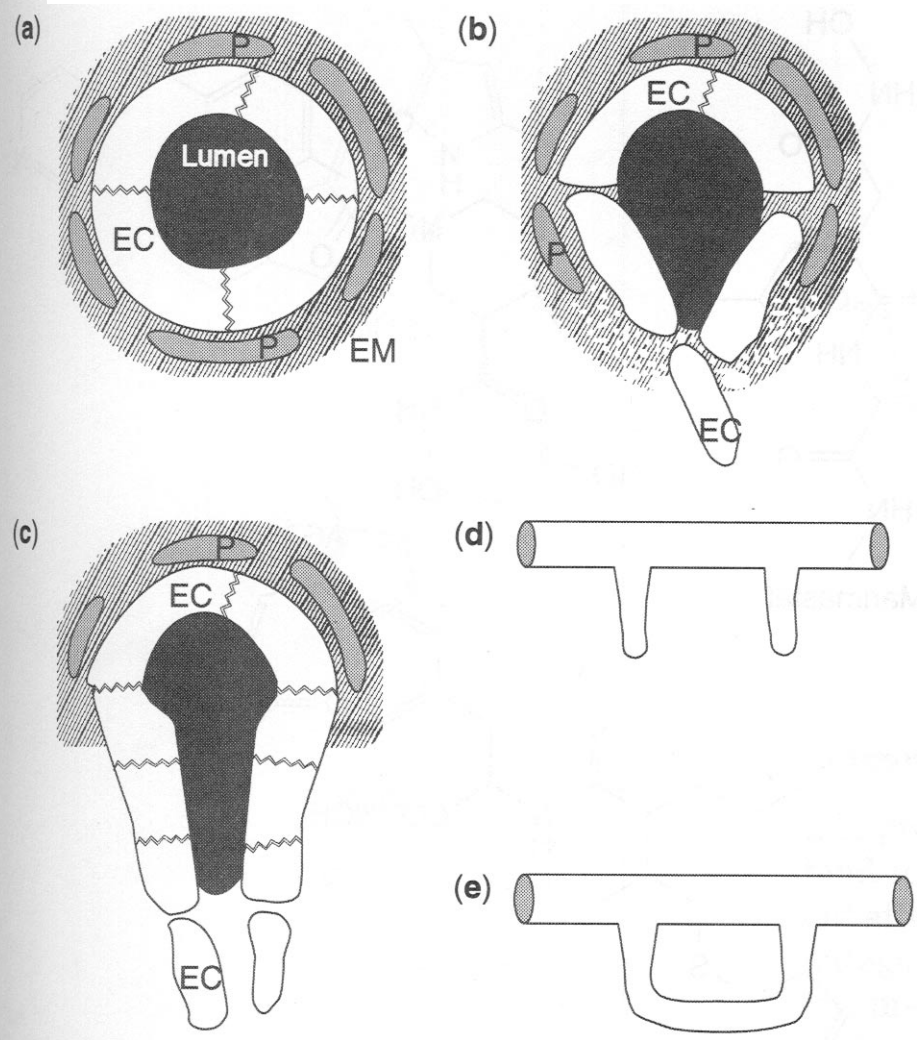


Figure 5.2. The angiogenic process. (a) The lumen of a mature microvessel is formed by endothelial cells (EC), which are supported by pericytes (P) and an extracellular matrix (EM). (b) After receiving an angiogenic signal, endothelial cells secrete enzymes (matrix metalloproteinases) that degrade the extracellular matrix, thus allowing them to migrate into the surrounding stroma. (c, d) The migrating endothelial cells proceed to form a microvascular column, or sprout. (e) Finally, the sprouts coalesce to from intact vessels. Further details concerning the angiogenic process are given in the text.

of MMP activity, marimastat and batimastat, are collagen peptidomimetics coupled to the zinc-binding group, hydroxamic acid (Fig. 5.3) (14, 15, 22, 23). These inhibitors bind to the active site of MMPs, which contains a zinc atom. They have been shown to be capable of inhibiting the formation of microvessels *in vitro* (24) and angiogenesis in animal models (25). Unfortunately, clinical trials using these and other MMP inhibitors to treat angiogenic-based diseases (such as cancer) have not been overly successful (20, 21). The reasons for the lack of success of MMP inhibitors in the clinic remain obscure, although it has become obvious that the roles played by MMPs during angiogenesis *in vivo* require further clarification (21).

3.2 Inhibitors of Growth Factor Receptor Function

The ability of receptor tyrosine kinases (RTKs) and their growth factor ligands to regulate angiogenesis has been extensively documented (5, 8, 9, 17, 26, 27). RTKs are integral membrane glycoproteins that exist as monomers on the cell surface (26, 27). They form dimers upon binding to their ligands. This triggers their intracellular kinase domain to catalyze the transfer of phosphate from ATP to protein substrates, thus initiating signaling cascades that ultimately cause changes in gene expression (26–30).

Vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and platelet-

Table 5.1 MMP Inhibitors with Demonstrated Antiangiogenic Activity[a]

Inhibitor	Class	Company
Marimastat	Peptidomimetic	British Biotech
Batimastat	Peptidomimetic	British Biotech
AG3340	Nonpeptidomimetic	Aguoron Pharmaceuticals
Bay 12-9566	Nonpeptidomimetic	Bayer Corporation
BMS-275291	Nonpeptidomimetic	Bristol-Myers Squibb
CGS 27023A	Nonpeptidomimetic	Novartis Pharmaceuticals

[a]Modified from Ref. 20.

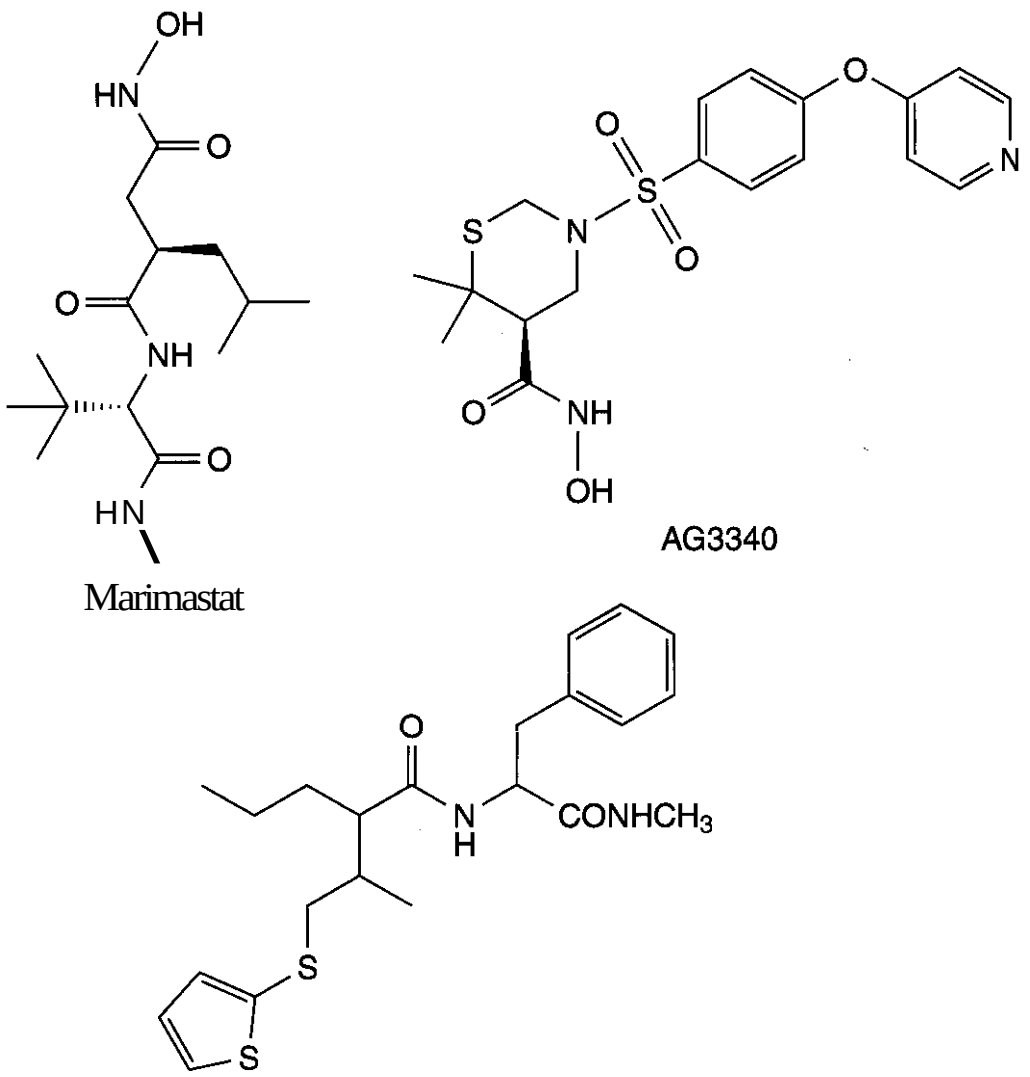


Figure 5.3. Structures of matrix metalloproteinase inhibitors.

derived growth factor (PDGF) are considered to be the most important positive regulators of angiogenesis (1, 5, 8, 9). VEGF and FGF stimulate endothelial cell migration, proliferation, and survival (1, 9, 17, 28, 31, 32), whereas PDGF regulates pericyte recruitment to immature microvessels (3, 8). In addition, VEGF is a potent inducer of vascular permeability, causing the detachment of endothelial cells from one another within microvessels (33, 34). This is likely to be the first step in the angiogenic process. Endothelial cells express the RTKs for VEGF, PDGF, and FGF (VEGF-R, PDGF-R, and FGF-R, respectively), whereas pericytes express FGF-R and PDGF-R (2, 35).

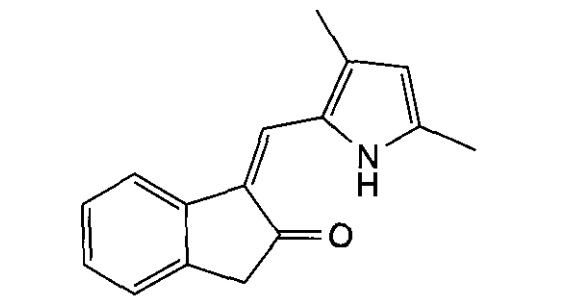
Two inhibitors of VEGF-R (also known as Flk-1/KDR) kinase activity are SU5416 and ZD4190 (Table 5.2) (26, 36). SU5416 and ZD4190 have indolinone and quinazoline core structures, respectively (Fig. 5.4). A third broad-spectrum RTK inhibitor with an indolinone structure is SU6668 (37). Crystallographic studies have shown that SU6668 interacts with amino acids at the entrance to the ATP-binding site in the RTKs.

Because VEGF, FGF, and PDGF are all positive regulators of angiogenesis, it is not surprising that SU6668 is a more potent inhibitor of angiogenesis than agents (such as SU5416) that target only a single angiogenic factor (35, 37). SU6668 has been shown to inhibit VEGF- and FGF-induced endothelial cell proliferation *in vitro* and cause endothelial cell apoptosis within tumors *in vivo* (35, 37).

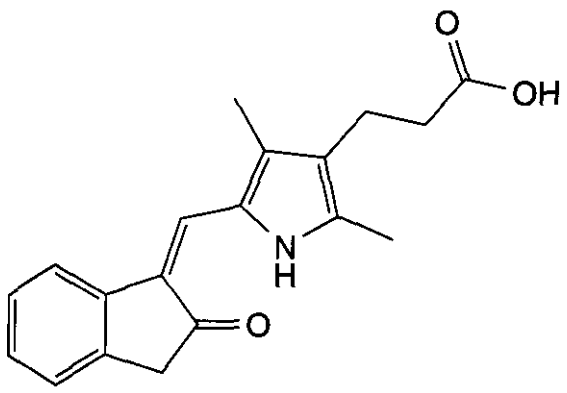
Table 5.2 RTK Inhibitors with Demonstrated Antiangiogenic Activity"

Inhibitor	Target	Company	Clinical Trial
SU5416	VEGF-R	Sugen	Phase III (colorectal cancer)
SU6668	VEGF-R, FGF-R, PDGF-R	Sugen	Phase I
ZD4190	VEGF-R	AstraZeneca	Discontinued

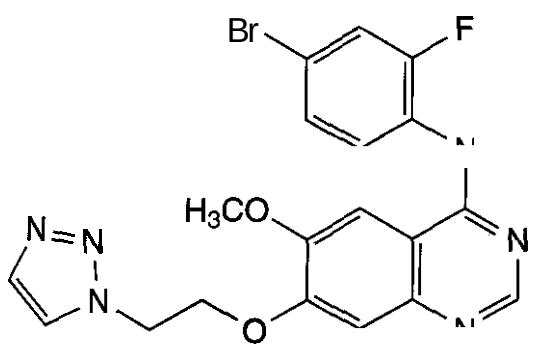
"Modified from Ref. 26.



SU5416



SU6668



ZU4190

Figure 5.4. Structures of receptor tyrosine kinase inhibitors.

Furthermore, this agent also caused a decrease in pericyte vessel coverage within the newly formed vasculature of tumors in *vivo* (35). Collectively, these observations suggest that agents capable of simultaneously antagonizing the activity of multiple RTKs should be effective inhibitors of angiogenesis in the clinic.

3.3 Inhibitors of Endothelial Cell Migration

Integrins mediate the migration of endothelial cells through the extracellular matrix (38–41). These cell surface receptors are heterodimers composed of α- and β-subunits (42, 43). The integrins $\alpha_v\beta_5$, $\alpha_v\beta_3$, and $\alpha_5\beta_1$ have been shown to play pivotal roles during angiogenesis (38–41). These integrins all recognize the amino acid sequence Arg-Gly-Asp (RGD), which is found within many extracellular matrix proteins (such as fibronectin) (44). They bind to RGD-containing proteins with different affinities, thus making it possible to design antagonists that are specific for individual integrins (45–47).

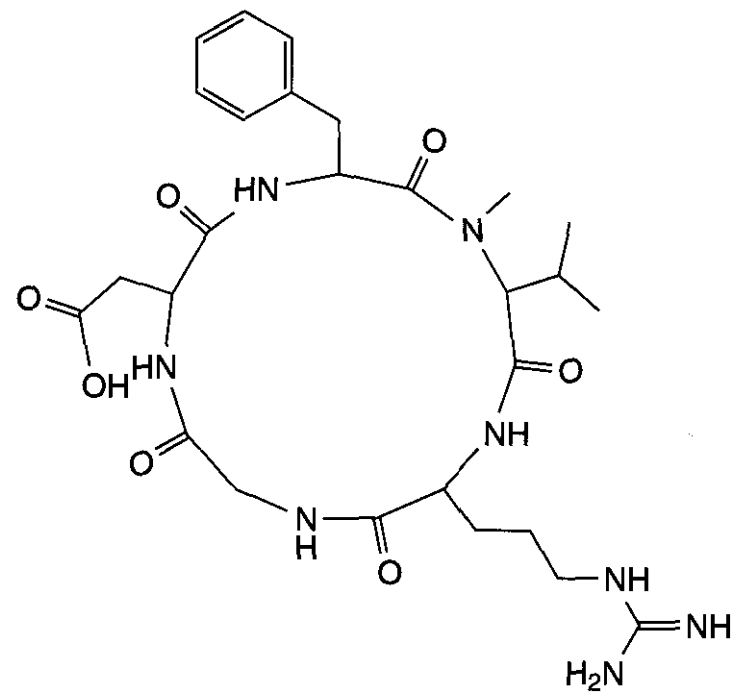


Figure 5.5. Structure of cyclo(RGDf-N(Me)V-).

Linear (44) and cyclic (41, 45, 46) peptides containing the RGD sequence have been developed that inhibit the interaction between integrins and various RGD-containing extracellular matrix proteins. Cyclic RGD-containing peptide antagonists of integrin $\alpha_v\beta_3$ have been shown to block angiogenesis (41, 45). Currently, the integrin $\alpha_v\beta_3$ antagonist cyclo (RGDf-N(Me)V-) (code EMD121974; Merck, Darmstadt, Germany) (Fig. 5.5) is in phase 1/11 clinical trials for Kaposi's sarcoma, brain tumors, and solid tumors (48).

3.4 Disruptors of the Endothelial Cell Cytoskeleton

Microtubules are an integral component of the endothelial cell cytoskeleton (49). They are in-

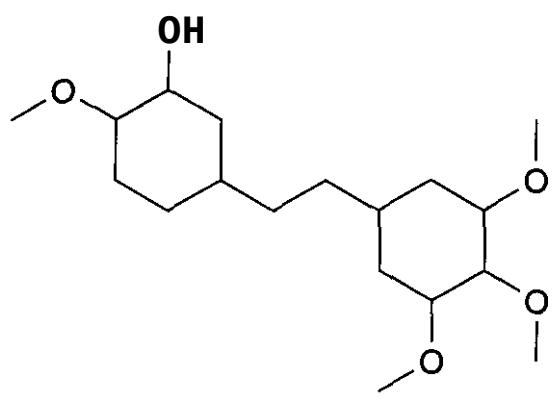


Figure 5.6. Structure of combretastatin **A4.**

Table 5.3 Examples of Angiogenesis-Related Conditions[a]

Malignant tumors	Synovitis
Trachoma	Hemophilic joints
Meningioma	Psoriasis
Hemangioma	Pyogenic granuloma
Angiofibroma	Atherosclerotic plaques
Diabetic retinopathy	Hypertrophic scars
Neovascular glaucoma	Retrolental fibroplasias
Macular degeneration	Scleroderma
Vascular restenosis	Vascular adhesions
Corneal graft neovascularization	Dermatitis
Arteriovenous malformations	Endometriosis
Hemorrhagic telangiectasia	Rheumatoid arthritis

[a]From Ref. 63.

volved in regulating endothelial cell shape and division (49–52). Microtubules are composed of tubulin polymers (53). Drugs capable of interfering with tubulin polymerization would therefore be expected to be effective antiangiogenic agents (54).

Combretastatin A4 (Fig. 5.6) is a tubulin-binding compound that was initially isolated from the South African tree ***Combretan caffrum*** (55, 56). This lipophilic ***cis*** stilbene destabilizes microtubules, thus affecting the cytoskeleton of dividing endothelial cells, causing apoptosis and inhibiting angiogenesis (53, 54, 56–58).

The solubility of combretastatin A4 was increased by attaching a phosphate moiety (59). The resulting compound, combretastatin A4 3-o-phosphate is a water-soluble prodrug whose phosphate moiety is cleaved ***in vivo*** by serum phosphatases to generate the active drug. This prodrug (developed by Oxigene Inc.) is currently in phase I clinical trials to evaluate its effects on tumor vasculature (54, 60).

4 SUMMARY AND FUTURE DIRECTIONS

Synthetic antiangiogenic compounds directed against four main groups of targets are currently being tested in the clinic: MMP, RTK, integrin, and microtubule antagonists. The preliminary clinical results obtained using most of these antagonists have been encouraging.

In view of the success of RTK antagonists in the clinic, a potentially promising area of investigation would be the development of compounds that antagonize the interaction between growth factors and their RTKs. Furthermore, in light of the clinical results obtained using antagonists of cell surface adhesion molecules (integrins), it would seem reasonable to target other adhesion molecules such as VE-cadherin and N-cadherin (1).

Only a few synthetic drugs have thus far been developed that are directed against specific proteins involved in angiogenesis. Although angiogenesis has been well described from a morphological perspective, there is a need to more precisely define the molecular mechanisms regulating this process (61, 62). Only then can new drugs be developed that are capable of modulating angiogenesis.

Most of the clinical trials to date have focused on determining the ability of antiangiogenic agents to prevent malignant tumor neovascularization. Angiogenesis has also been implicated in a wide variety of nonneoplastic diseases (Table 5.3) (63). Further clinical research is necessary to determine whether antiangiogenic agents will be useful in the treatment of these diseases.

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