CHAPTER SIX

Future Strategies in Immunotherapy

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Burger's Medicinal Chemistry and Drug Discovery
Sixth Edition, Volume 5: Chemotherapeutic Agents
Edited by Donald J. Abraham
ISBN 0-471-37031-2 © 2003 John Wiley & Sons, Inc.

1 INTRODUCTION

The immune system is a complex, but remarkably precise cellular and molecular orchestra capable of responding to, eliminating, and providing protection from invading pathogens such as viruses, bacteria, fungi, and even tumors. The primary function of the immune system is to distinguish "self" from non-self. The term "self" encompasses host-derived tissue, whereas non-self includes infectious and non-infectious foreign agents. The immune system consists of many cell types, each specialized to fulfill an important role in the generation of a robust immune response on encounter of an invading agent and to provide life-long protection to the host.

The first section of this chapter will focus on the cellular and molecular components of the immune response and will be discussed in the context of future directions in immunotherapy. The remainder of the chapter will discuss current cutting edge and future immunotherapeutic strategies for cancer.

1.1 Innate Immunity

The two major components of immunity are the innate and adaptive (acquired) immune systems. As part of the innate immune system, physical barriers such as skin and mucosa provide the first line of defense against the outside environment (1) If skin or mucosal barriers (ocular conjunctiva, for example) are broken and an invading agent enters the eye or the blood stream, biochemical barriers such as lysozyme, present in tears, and serum complement provide protection from bacterial invasion and infection. Lysozyme breaks down bacterial cell walls and complement deposits on the surfaces of bacteria and viruses, ultimately resulting in their destruction. The breakdown of complement components also attracts inflammatory cells such as macrophages, neutrophils, and other granulocytes that are important cellular components of the innate immune response (2). These inflammatory cells bridge innate and adaptive immune responses. If these innate components are not successful, or if the antigenic insult is sufficiently large, adaptive immunity is called into play.

1.2 Adaptive Immunity

The adaptive immune system responds specifically to an enormous arsenal of antigens, discriminates between foreign and "self" antigens, and remembers previously encountered antigens so it can respond faster and more effectively to a second antigenic challenge. An adaptive immune response results in production of antigen-specific antibodies and T-cells. Antibodies provide protection against repeated invasion in an infectious disease setting and also coat, or opsonize, agents so that effector cells can destroy them (3). CD4-positive T-helper cells signal other cells in the immune system using a network of cytokines (4), whereas CD8-positive cytotoxic T-lymphocytes (CTL) lyse tumor cells or virally infected cells (5). Multiple gene segment rearrangements of B-cell receptors [immunoglobulins (Ig)] and T-cell receptors generate over a million different specificities in each B-cell and T-cell population to match the antigenic diversity found in nature (6).

Generation of specific immunity requires antigen-presenting cells (APC), including macrophages, dendritic cells (DC), skin-derived DC (Langerhans cells), and B-lymphocytes. These cells enzymatically digest protein antigens and present the derived peptides to the T-cell receptor (TCR) in association with class I and class II major histocompatibility complex (MHC) proteins (7). $CD4^+$ T-helper lymphocytes recognize peptides presented by MHC class II, whereas $CD8^+$ cytotoxic T-lymphocytes (CTL) recognize peptides presented by MHC class I. Class II peptides are derived from proteins outside the cell that have been engulfed by APCs into endosomes. These proteins are enzymatically digested into individual peptides, loaded onto MHC class II and carried to the surface for presentation to $CD4^+$ T-cells. In contrast to class II peptides, class I peptides are derived from within the cell. For example, cells that become infected with a virus present endogenous peptides in

class I molecules to $CD8^+$ T-cells. Tumor cells also present endogenous tumor antigens in context of MHC class I. APC often become infected with viruses and present peptides derived from exogenous and endogenous sources to both CD4 and $CD8^+$ T-cells simultaneously. When an APC and T-cell interact, the TCR is responsible for the specificity of the interaction, and the interaction between a variety of co-stimulatory and adhesion molecules on both APC and the T-cell activate the T-cell, resulting in secretion of appropriate cytokines.

Two subsets of T-helper lymphocytes (Th1 and Th2) have been reported based on the cytokines they secrete. The Th1 subset produces interferon-y (IFN-γ) and interleukin-2 (IL-2) and enhances cell-mediated immunity by activation of macrophages, CTL, and natural killer (NK) cells. The Th2 subset produces IL-4, IL-5, and IL-6, which induce B-cell proliferation followed by differentiation into antibody-secreting plasma cells. These subsets down-regulate each other, such that Th2 cells produce IL-10, which inhibits cytokine production by Th1 lymphocytes. Conversely, IFN-γ produced by Th1 cells inhibits the proliferation of Th2 cells. Although these subsets have been well characterized *in vitro*, it is still unclear whether they exist *in vivo* as distinct populations or as a single T-helper cell population that is capable of exhibiting either phenotype depending on the type of the antigen they encounter (8).

$CD8^+$ CTL are activated only after peptide antigen presentation and co-stimulation by APC. Then they become serial killers and no longer require co-stimulation by APC. Without initial co-stimulation, T-cells may become unresponsive to further antigenic stimulation (anergy). Activated CTL induce target cell death through lytic granule enzymes ("granzymes"), perforin, and the Fas-Fas ligand (Fas-FasL) apoptosis pathways. $CD8^+$ cells are thought to be a major component of antitumor responses and are the activation target of nearly all cellular immunotherapy protocols.

The immune system may eliminate tumors that arise spontaneously. Circumstantial evidence that the immune system is capable of tumor destruction is offered by the fact that individuals with natural or acquired immune deficiencies develop cancer at an increased incidence over the general population. For example, AIDS patients develop tumors at a much high rate than immunocompetent individuals (9). Spontaneous regression of tumors may result from hormonal fluctuations, tumor necrosis caused by abrogation of blood supply, elimination of carcinogens, differentiation, epigenetic mechanisms, apoptosis, and even psychological factors. However, the prevailing view is that immunological mechanisms play a leading role in spontaneous tumor regressions (10).

Although the innate immune system is clearly involved in prevention and early rejection of tumors, two general arms of the adaptive immune system participate in the elimination of established tumors. The humoral arm of the immune system consists of B-lymphocytes that primarily secrete antibodies. The cellular arm of the adaptive immune system is composed of T-cells responsible for tumor cell destruction. Both arms interact with each other intimately. For example, B-cells expressing surface immunoglobulin in peripheral secondary lymphoid organs may bind a soluble tumor protein antigen shed from a tumor (Her-2/neu, for example) (11). That protein is then internalized, processed into peptides and then presented as peptide-class II major histocompatibility complexes to $CD4^+$ T-helper cells that recognize each peptide-class II complex through their TCR. These T-helper cells then secrete cytokines, such as IL-4, that act directly on the B-cell that bound the antigen to help it differentiate into clonal antibody-secreting plasma cells producing high affinity antibody. Circulating soluble antibody may bind to antigen on the surfaces of tumor cells, marking the tumor cells for destruction by effector immune cells such as macrophages, NK cells, or neutrophils by antibody-dependent cellular cytotoxicity (ADCC) or by complement-mediated lysis.

One problem for the cellular arm of the immune system in fighting cancer is that most tumor cells express self-antigens. The immune system is tolerant to self (12), as T-cells strongly reactive to self-peptides are deleted in the thymus early in life (13–15). Therefore, when peptides from a tumor are presented to

T-cells, they do not recognize the peptides as avidly as they would if the cell were infected with a virus and presenting a foreign peptide. However, anti-tumor T-cells exist and can be isolated from peripheral blood and from tumors or tumor-draining lymph nodes (16–19). Tumor cells secrete cytokine that suppress immune cell function such as including transforming growth factor-β and IL-10 (20–29). Several strategies have been employed to rescue and re-activate tumor-specific T-cells, including isolation of tumor-infiltrating lymphocytes (TIL), expansion of TIL, demonstration of autologous tumor lysis, and re-infusion of these TIL into the patient, indicating existence of tumor-specific CTL (30). Recent strategies for activating tumor-specific CTL have focused on the use of dendritic cells, which will be discussed in the latter section of this chapter. Next, we describe antibodies that have been humanized or made chimeric using genetic engineering techniques that are FDA-approved for cancer and anti-inflammatory treatment modalities.

2 ANTIBODY-DIRECTED IMMUNOTHERAPY

2.1 History

The development of "hybridoma" technology by Milstein and Koohler in the mid-1970s revolutionized the generation of specific antibodies for use in research and clinical applications (31). Hybridomas are made by fusing antibody-forming B-cells with an immortal, non-antibody-secreting plasma cell line resulting in a population of hybrid cells that are selected for secretion of an antibody specific for an antigen of interest. The secret to this technology is that the immortal plasma cell line does not secrete antibody and is deficient in a purine enzymatic salvage pathway, hypoxanthine phosphoribosyl transferase (HPRT). When these plasma cells are fused to B-cells and placed in medium containing hypoxanthine-aminopterin-thymidine (HAT), the aminopterin poisons the de *novo* purine synthesis pathway. Unfused cells die, and only the hybridomas survive in HAT, while the B-cell component of the hybridoma provide the purine salvage pathway and the plasma cells contribute unlimited *in vitro* proliferation. Supernatants of bulk cultures of hybridomas are then screened for the presence of antibodies of interest, usually by ELISA. On a "hit" (a well containing hybridomas secreting antigen-reactive antibody), the hybridomas can be subcloned by limiting dilution such that they are monoclonal. Monoclonal hybridomas secreting an antibody of interest can then be mass-cultured for antibody production and re-tested for reactivity against its target and lack of reactivity against other tissues.

2.1.1 Humanized Antibodies. As one might imagine, mouse monoclonal antibodies far outnumber human monoclonal antibodies, because it is relatively easy to immunize mice and obtain splenic B-lymphocytes for hybridoma formation. The drawback of using mouse monoclonal antibodies for passive immunotherapy is that they induce "human anti-mouse antibody" (HAMA) responses when administered to humans (32). Therefore, many mouse antibodies have been "humanized" for therapeutic use in humans (33). Humanization of antibodies consists of exchanging mouse constant domains with their homologous human constant domains, thus decreasing the immunogenicity of the mouse antibody when administered to humans. A diagram of an antibody is shown in Fig. 6.1. To further humanize an antibody, mouse framework variable regions are replaced with human frameworks that provide the scaffolding for the complementarity determining regions (CDR). The problem with replacing mouse framework sequences with their homologous human sequences is that antibody specificity is often lost. There is a delicate balance between specificity and humanization. Computer modeling is most often used to overlay human amino acid sequences on mouse framework structures to determine what gene family of framework regions might least distort the CDRs. Then human framework region DNA is spliced frameworks 1–4, among mouse CDRs 1–3, cloned into an expression vector, and expressed as a recombinant protein from appropriate cells.

2.1.2 Chimeric Antibodies. Chimeric antibodies are less "human" than humanized an-

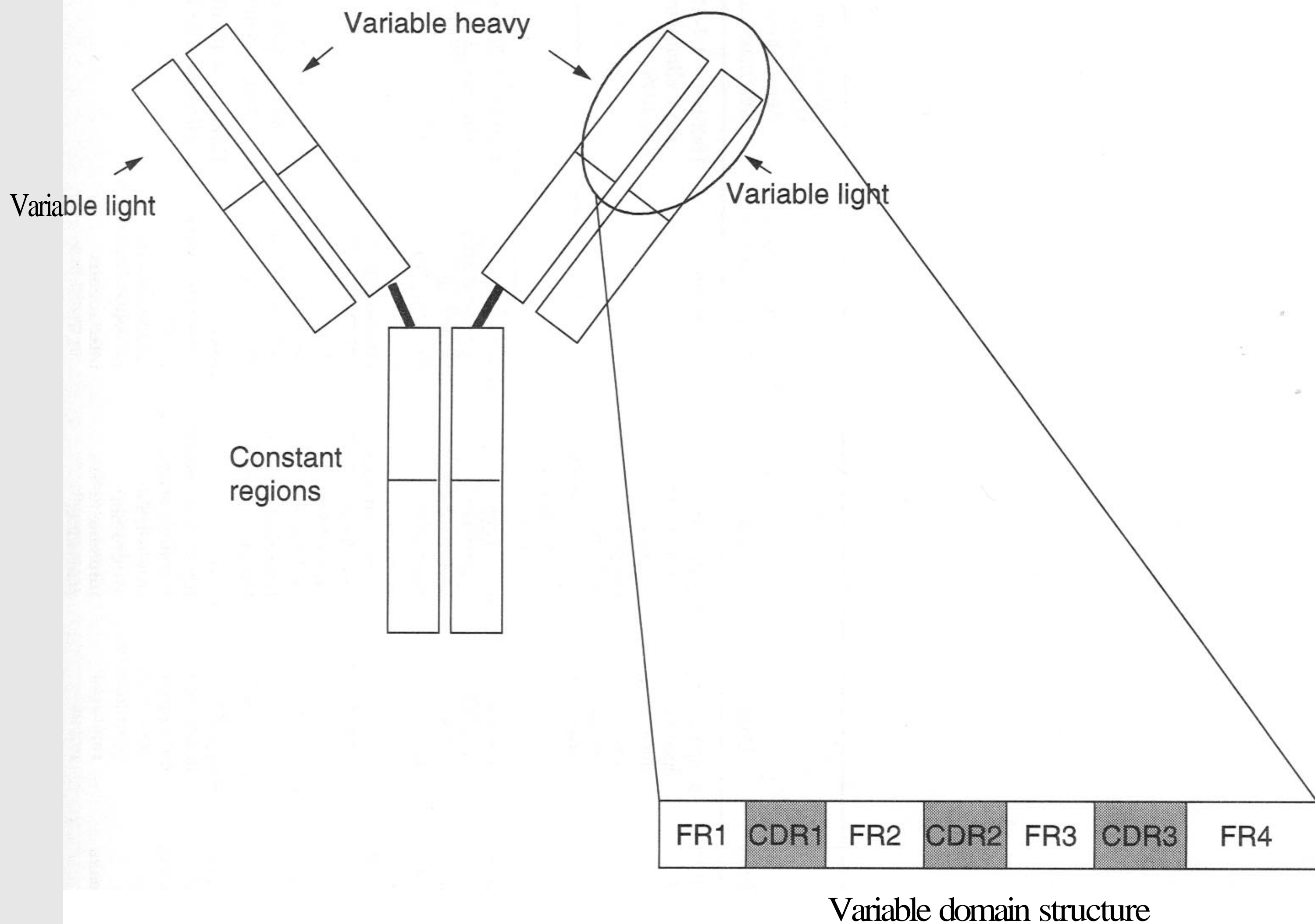

Figure 6.1. General structure of an antibody molecule. IgG1 contains three constant heavy chain domains, CH 1–3, and one variable domain. Each domain is approximately 110 amino acids in length. Variable light chains and constant light chains are noncovalently associated to the heavy chain variable and CH1 domains. Interchain disulfide bonding links the two heavy chains together in the hinge region, whereas intrachain disulfide bonding occurs within domains. The Fc portion of an antibody activates serum complement. The Fc portion binds Fc receptors on effector cells such as macrophages and neutrophils. The Fab contains variable heavy and light chains, and constant heavy and light chains. Fabs may be generated by papain digestion of IgG. Framework (FR) sequences interspersed between complementarity determining regions (CDR) may be exchanged among species, because CDR sequences impart specificity of the antibody.

tibodies, and as such, generally elicit more HAMA responses than a fully humanized antibody. Chimeric antibodies are generated by splicing DNA from murine heavy and light chain variable regions onto human IgG constant region DNA. This means that the entire variable region, including frameworks, is of murine origin, and only the effector, or Fc portion, of the antibody is human. The Fc portion of the antibody imparts effector function and allows antibody-dependent cellular cytotoxicity (ADCC) to occur after the antibody has bound to its target. Specificity is not usually a problem after generating a chimeric antibody, because it retains the natural framework scaffolds as the parent murine antibody.

2.2 Current Antibodies on the Market

2.2.1 Therapeutic Antibodies. Recently, the FDA has approved several humanized monoclonal antibodies for the treatment of various diseases. Because this chapter concentrates on future directions in immunotherapy for cancer, only chimeric or fully humanized antibodies will be described in detail. It should be recognized that antibodies for infectious diseases such as respiratory syncytial virus have been humanized (Palivizumab, Synagis; Medimmune) and are significant products for the treatment of those diseases. It is also important to note that many mouse monoclonal antibodies directed against human tumor an-

Table 6.1 Therapeutic Antibodies

Trade Name (Generic Name)	Target	Manufacturer	Structure1 Chemical Class	Route of Administration	Efficacy/Potency	Dose	Potential Side Effects	Drug Interactions1 Contraindications	Absorption, Distribution, Metabolism, Eliminations
Herceptin (Trastuzumab)	Her-2/neu positive tumors	Genentech	Humanized mouse monoclonal antihody, IgG_1, κ	IV	Mediates ADCC K_d = 5 n*M*	4 mg/kg loading dose; 2 mg/kg weekly dose	Anaphylaxis; cardiotoxicity; infusion reactions; pulmonary events; anemia or leukopenia; diarrhea	None known	Half-life = 1.7–12 days, eliminated by RES
Rituxan (Rituximab)	CD20 positive B-cell neo· plas ms	IDEC and Genentech	Chimeric mouse/human monoclonal antibody, IgG_1, κ	IV	Mediates direct apoptosis, ADCC and complement dependent lysis (CDL) K_d = 8 n*M*	375 mg/m^2 weekly for 4 or *8* doses	Tumor lysis syndrome; severe infusion reactions; severe mucocutaneous reactions; pulmonary events; lymphopenia (B-cell)	Known IgE hypersensitivy to murine proteins, No drug interactions	Half-life = 76.3 h; eliminated by RES
Mylotarg (Gemtuzumab)	CD33 positive leukemias such as acute myelogenous leukemia	Wyeth-Ayerst	Humanized mouse monoclonal antibody, IgG_4, κ 50% conjugated to calicheamicin	N o r central line	Internalization of antibody, Calicheamicin binds DNA and cause strand breaks	9 mg/m^2 2 doses, 14 days apart	Severe myelosuppression; hepatotoxicity; tumor lysis syndrome; infusion reactions; pulmonary events; Mucositis	Known hypersensitivity to calicheamicin, no drug interactions	Half-life = 45–100 h; eliminated by RES
Campath (Alemtuzumab)	CD52 positive leukemia	Millenium and ILEX	Humanized rat monoclonal antibody,	IV	Mediates ADCC and CDL	Dose escalation: 3, 10, 30 m e as	Hematologic toxicity; infusion reactions;	Known hypersensitivities to components of drug; pre-	Half-life = 12 days; eliminated by RES

Campath (Alemtuzumab)	CD52 positive leukemia such as B-chronic lymphocytic leukemia	Millenium and ILEX	Humanized rat monoclonal antibody, IgG_1, κ	IV	Mediates ADCC and CDL	Dose escalation: 3, 10, 30 mg as tolerated. Maintenance dose is 30 mg three times per week.	Hematologic toxicity; infusion reactions; opportunistic infections resulting in grade 3 or 4 sepsis	Known hypersensitivities to components of drug; pre-existing immunodeficiency; no drug interactions	Half-life = 12 days; eliminated by RES
Remicade (Infliximab)	TNF-a	Centocor	Chimeric mouse/human monoclonal antibody, IgG_1, κ	N	Neutralizes the biological activity of TNF-a; K_d = 0.1 nM	3 or 10 mg/kg every 4 or 8 weeks with concurrent methotrexate Tx	Risk of infection; infusion related reactions ANA formation	Known IgE hypersensitivity to murine proteins; no drug interactions	Half-life = 8–9.5 days; eliminated by RES
Enbrel (Etanercept)	TNF-a and TNF-β	Immunex (Amgen)	Dimeric fusion protein between TNF receptor and IgG_1	Subcutaneous	Neutralizes the biological activity of TNF-a	Adults: 25 mg Peds: 0.4 mg/kg	Risk of infection; ANA formation; mild injection site reaction	Known hypersensitivity to components; sepsis	Half-life 102 h; eliminated by RES

tigens have been approved by the FDA as radioconjugates for diagnostic imaging applications. These radiolabeled antibodies search out and bind primary and metastatic tumors, revealing their locations on scanning of cancer patients. Because these radiolabeled antibodies are of mouse origin and have not been humanized using genetic engineering techniques, they will not be discussed here.

Over the past 5–10 years, the promise of antibodies as "magic bullets" in treating disease has been partially realized. The FDA has approved 15 antibodies for use in humans as therapeutic treatments and/or imaging agents at the time of this writing. Therapeutic antibodies for cancer and rheumatoid arthritis are listed in Table 6.1.

2.2.2 Trastuzumab. Herceptin (Trastuzumab) is a prototype for future antibody-based immunotherapeutics. It is a recombinant DNA-produced, humanized monoclonal antibody (IgG_1-κ chain) containing human framework regions and complementarity determining regions (CDRs) of mouse monoclonal antibody, 4D5 (34). It selectively binds to the extracellular domain of the human epidermal growth factor receptor-2 (EGF-2), also known as Her-2 with an affinity of 5 n*M*. The Her-2 protein is overexpressed on approximately 25–30% of primary human breast tumors as well as several other adenocarcinomas. Overexpression of Her-2 is often associated with increased tumor aggressiveness. Because Trastuzumab inhibits the proliferation of tumors overexpressing Her-2, it has been shown to be more effective against aggressive tumors in patients with an otherwise poor prognosis (35, 36). One of the effector mechanisms of Trastuzumab tumor cell killing is ADCC (37). ADCC occurs after multiple antibodies have bound to a tumor cell, exposing the Fc portion of the antibodies so that immune cells with IgG Fc receptors, such as macrophages, neutrophils, eosinophils, or NK cells, bind and either phagocytose the tumor cell or secrete lytic granules that result in tumor cell death. Another potential mechanism of tumor cell killing is induction of p27KIP1 and the Rb-related protein, p130, which results in a significant reduction in the number of cells in S-phase, thereby reducing tumor growth. Trastuzumab also induces phenotypic changes in tumors, which include down-modulation of the Her-2 receptor, increased cytokine susceptibility, restored E-cadherin expression, and reduced vascular endothelial growth factor production (38).

Because Her-2/neu is overexpressed on many adenocarcinomas, including breast tumors, Trastuzumab was tested for efficacy in humans with breast cancer. Phase III clinical trials designed to evaluate Trastuzumab in over 500 patients with metastatic breast cancer, either in combination with paclitaxel or as a single agent, demonstrated a significantly longer time to disease progression, a higher overall response rate, longer duration of response, and higher 1-year survival compared with chemotherapy alone. Trastuzumab is supplied lyophilized and reconstituted to 21 mg/mL with supplied diluent followed by further dilution in 0.95% sodium chloride. In studies using a loading dose of 4 mg/kg followed by weekly infusions of 2 mg/kg, a mean half-life of 5.8 days was observed (range, 1–32 days), with a mean serum concentration between 79 and 123 mg/ml between weeks 16 and 32, respectively. Trastuzumab is approved by the FDA for use in patients with metastatic breast cancer whose tumors overexpress Her-2 and who have previously received one or more chemotherapy regimens for their metastatic disease.

Adverse events from Trastuzumab administration are rare but can result in severe hypersensitivity, including systemic anaphylaxis, urticaria, bronchospasm, angioedema, or hypotension. A recent warning of cardiotoxicity has been issued for Trastuzumab, where its use in patients with cardiac dysfunction has resulted in congestive heart failure. This phenomenon is currently under further evaluation and investigation.

2.2.3 Rituximab. Rituxan (Rituximab) was the first monoclonal antibody to be approved by the FDA for cancer treatment. It is a genetically engineered monoclonal antibody that binds to CD20. CD20 is a B-lymphocyte lineage-restricted differentiation antigen found on normal and malignant B-lymphocytes, but not on other normal hematopoietic cells or antibody-producing plasma cells. CD20 has a mo-

lecular weight of 35 kDa and is a hydrophobic transmembrane protein that regulates cell cycle activation and differentiation during B-lymphocyte development (39). In contrast to Trastuzumab, Rituximab (MW 145 kDa) contains murine heavy and light chain variable regions genetically fused to human IgG1 heavy chain constant regions and human κ chain constant regions; it is a chimeric murine/human antibody (40, 41). The binding affinity for the CD20 antigen is approximately 8 n*M*. Rituximab-mediated killing of CD20-positive tumor cells *in vivo* is largely because of ADCC, and to a minor degree, induction of apoptosis by direct ligation of CD20 and complement-dependent lysis (40, 42).

Rituximab was initially approved for the treatment of low-grade or follicular, relapsed, or refractory CD20-positive B-cell non-Hodgkin's lymphoma. In clinical trials, patients were treated weekly with either four or eight doses of Rituximab at 375 mg/m^2 as an IV infusion at a concentration of 1–4 mg/mL in 0.9% sodium chloride or 5% dextrose in water. The overall response rate for four doses weekly was 48% with six complete responses; for eight doses weekly it was 57% with a 14% complete response rate. The mean serum half-life of Rituxan is dependent on dose, but also depends greatly on tumor burden and circulating CD20-positive tumor cells or B-lymphocytes. Peak and trough serum levels are inversely proportional to the number of circulating CD20-positive cells. After the third or fourth dose, normal B-cells remain depleted for 6–9 months after treatment, after which B-cell numbers returned to normal by 12 months. Retreatment can be attempted, because only 4 of 356 patients developed a human anti-chimeric antibody response. However, patients should be closely monitored on retreatment for serum sickness. Rituximab is currently under study for the treatment of other B-cell malignancies including chronic lymphocytic leukemia, Hodgkins disease, and other lymphoid malignancies which CD20 (43).

Adverse events caused by Rituximab infusion include severe infusion reactions, including hypotension, angioedema, hypoxia, bronchospasm, pulmonary infiltrates, myocardial infarction, ventricular fibrillation, and cardiogenic shock. Tumor lysis syndrome has been observed in patients with high levels of circulation CD20-positive tumor cells, in which rapid reduction of tumor volume is followed by acute renal failure, hyperkalemia, hypocalcemia, hyperuricemia, or hyperphosphatasemia. Patients with known IgE-mediated immediate hypersensitivity reactions to murine proteins should not receive Rituximab.

2.2.4 **Gemtuzumab.** Mylotarg (Gemtuzumab ozogamicin, CMA-676; Wyeth-Ayerst Laboratories) is an FDA-approved monoclonal antibody specific for CD33, a sialoadhesion protein found on leukemic blasts in 80–90% of patients with acute myelogenous leukemia (AML) (44). CD33 is also expressed on normal immature cells of the myelomonocytic lineage. The humanization of Gemtuzumab is similar to Trastuzumab in that it contains mouse CDRs and human framework and constant regions, such that over 98% of the antibody is human. Gemtuzumab is an IgG_4 with a κ light chain chemically linked to the cytotoxic agent, calicheamicin. In the formulation of the drug, 50% of the antibody is linked to *N*-acetyl-γ-calicheamicin through a bifunctional linker at between 4 and 6 moles of calicheamicin per mole of antibody, whereas the remaining 50% is not derivatized (Fig. 6.2) (45). Tumor cell internalization of Gemtuzumab linked to calicheamicin results in release of the cytotoxin from the antibody in the lysosome. Calicheamicin is then free to bind to the minor groove in DNA causing double strand breaks and cell death (46). The exact mechanisms of induction of leukemic cell death by Gemtuzumab without calicheamicin linkage are not yet known, but the binding of the antibody to CD33 on the leukemia cell surface is thought to induce apoptosis (47).

Gentuzumab was approved in May 2000 by the FDA for the treatment of patients 60 years and older in first relapse with CD33$^+$ AML who are not considered candidates for other types of cytotoxic chemotherapy. In combined phase II studies, 142 patients with CD33-positive AML in first relapse demonstrated a 30% overall response rate with Mylotarg therapy alone. Treated patients had relatively high incidences of myelosuppression, hyperbilirubinemia, and elevated hepatic transaminases

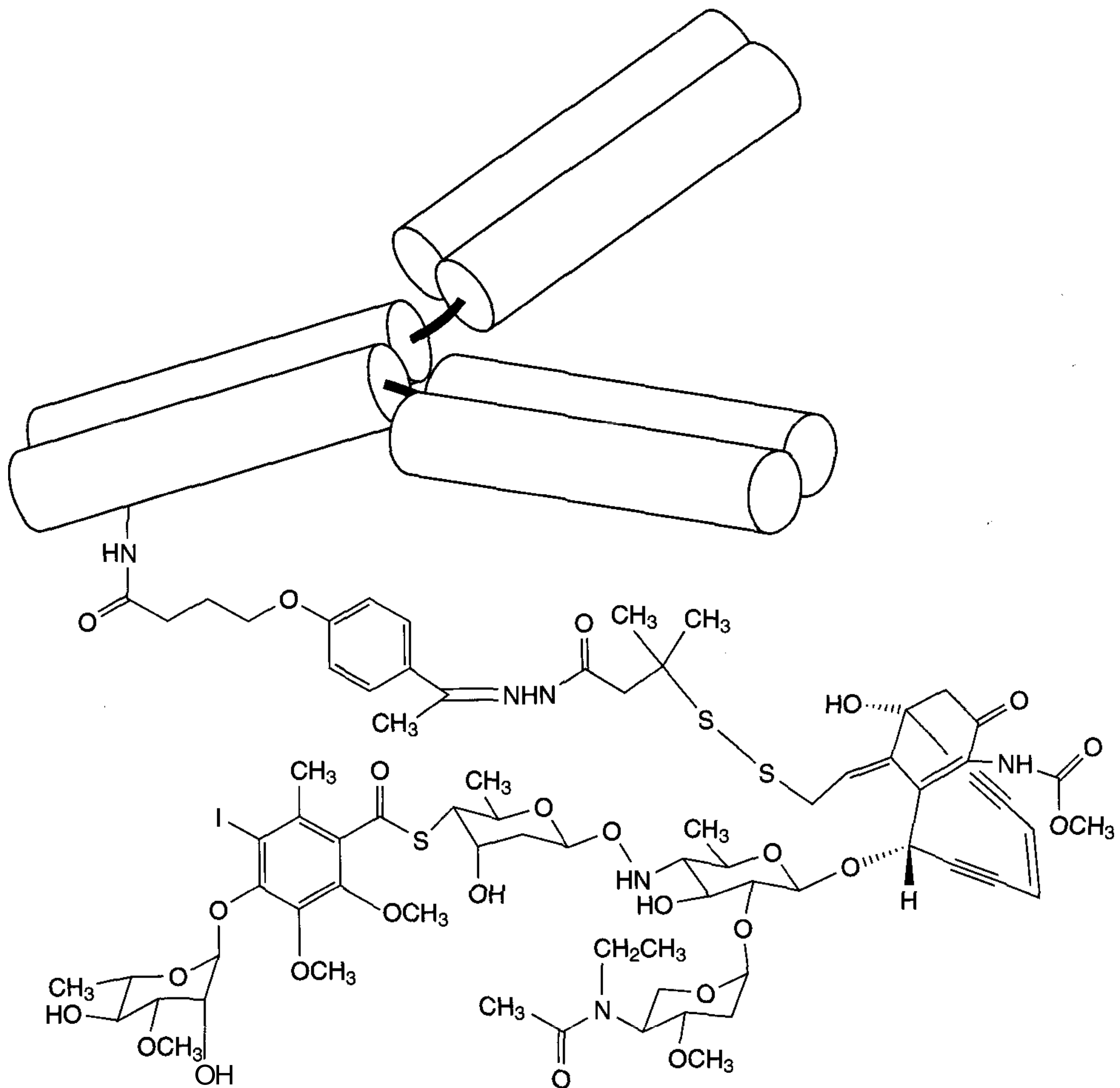

Figure 6.2. Diagram and chemical structure of Gemtuzumab ozogamicin. Calicheamicin (ozogamicin) is coupled to Gemtuzumab at an average loading of 4–6 moles per mole of antibody. The antibody is linked to N-acetyl γ calicheamicin. The molecular weight of the antibody-toxin conjugate is 151–153 kD. The injected formulation consists of 50% conjugated antibody and 50% unconjugated antibody.

(48). The incidences of severe mucositis and infections were low compared with mucositis resulting from conventional chemotherapeutic treatment. Sixteen percent of the patients had a complete response (CR), while 19% had a CR requiring platelet transfusions. The clinical data support the use of Gemtuzumab in AML patients with CD33-positive leukemia.

Adverse events caused by the administration of Gemtuzumab ozogamicin include severe myelosuppression, especially neutropenia, requiring careful hematological monitoring. Patients should be monitored for infection after Gemtuzumab administration and treated appropriately if necessary. Other adverse events associated with Gemtuzumab are hypersensitivity reactions, including anaphylaxis, infusion reactions, and pulmonary events including pulmonary edema, dyspnea, pleural effusions, hypoxia, and acute respiratory distress syndrome. Patients with greater than 30,000 leukemic cells/μL should be considered for leukoreduction to avoid tumor lysis syndrome, similar to that observed with Rituximab therapy.

2.2.5 Alemtuzumab. Campath (Alemtuzumab) is a recombinant DNA-derived humanized monoclonal antibody directed against CD52, a 21- to 28-kDa cell surface glycoprotein. CD52 is expressed on the surfaces of normal and malignant B- and T-lymphocytes, NK cells, monocytes, macrophages, a subset of granulocytes, and tissues of the male reproductive tract, but not on hematopoietic stem cells. Alemtuzumab is an IgG1 with a κ light chain containing human variable framework and constant regions and (CDRs) from a rat monoclonal antibody (Campath-1G). CD52 is a glycosylphosphatidylinositol (GPI)-anchored protein with unknown function (49). Binding of Alemtuzumab to CD52 is thought to result in complement-dependent lysis and ADCC on tumor and normal cells (50).

Alemtuzumab is indicated for the treatment of B-cell chronic lymphocytic leukemia (B-CLL) in patients who have been treated with alkylating agents and who have failed fludarabine therapy. In a study where Alemtuzumab was infused intravenously once weekly for a maximum of 12 weeks over a range of doses, the overall average half-life during treatment was about 12 days. Alemtuzumab administered as a 30-mg intravenous infusion three times per week evaluated in CLL patients demonstrated variable peak and trough levels of the antibody during the first few weeks of treatment, but seemed to level off by week 6. Inter-patient variability was likely caused by tumor burden and circulating numbers of leukemia cells. However, increases in serum levels of Alemtuzumab corresponded to reduction in neoplastic cells. Clinical trials have shown that in previously treated B-CLL patients, partial responses occur at a rate of 40%, with 2–4% CRs. Responses are more likely in blood and bone marrow compared with lymph nodes. The median duration of response is 9–12 months. Because of the strong depleting activity on circulating lymphocytes, it has been used for purging residual disease in B-CLL, followed by autologous stem-cell transplantation (51, 52).

Alemtuzumab is provided as a sterile, clear, colorless, isotonic solution, pH 6.8–7.4, for injection. Each single use vial of antibody contains 30 mg Alemtuzumab, 24.0 mg sodium chloride, 3.5 mg dibasic sodium phosphate, 0.6 mg potassium chloride, 0.6 mg monobasic potassium phosphate, 0.3 mg polysorbate 80, and 0.056 mg disodium edetate. No preservatives are added. Infusions should be initiated at an initial dose of 3 mg with gradual escalation to 30 mg. Careful monitoring of blood pressure and hypotensive symptoms is recommended especially in patients with ischemic heart disease and in patients taking antihypertensive medications. If therapy is interrupted for 7 or more days, Alemtuzumab may be reinstituted with gradual dose escalation.

Adverse events are infusion related and are caused by tumor necrosis factor (TNF)-α and interleukin (IL)-6 release, usually during the first intravenous infusion, and include fever, rigor, nausea, vomiting, and hypotension that responds to steroids. Adverse events are usually less severe with subsequent infusions and can be prevented by with appropriate medication. Antihistamine and acetaminophen are recommended before infusion. Because CD52 is present on many types of leukocytes, immunosuppression resulting from depletion of normal B- and T-lymphocytes usually occurs, resulting in an increased risk for opportunistic infections.

2.3 Anti-Inflammatory Therapeutic Antibodies

Two recombinant antibodies, Infliximab and Etanercept, that bind to and neutralize TNF-a have been approved by the FDA for the treatment of rheumatoid arthritis (RA) and Crohn's disease.

2.3.1 Infliximab. Remicade (Infliximab) is a chimeric monoclonal antibody containing human constant and murine variable regions that inhibits TNF-a from binding to its receptor (53). It binds to TNF-a very strongly, with an association constant of 0.1 nM. TNF-a is secreted as a trimer by macrophages, T-cells, and NK cells. Biological activities of TNF-a include induction of pro-inflammatory cytokines such as IL-1 and IL-6 and increased leukocyte migration through up-regulation of endothelium permeability and adhesion molecules by both endothelial cells and leukocytes. TNF-a also activates eosinophils and neutrophils and induces acute phase proteins as well as enzymes such as matrix metalloproteinases

involved in degradation of synoviocytes and chondrocytes in joint tissue. It is thought to play a major role in mediating tissue damage in RA and other autoimmune diseases. Infliximab has been shown to prevent RA in transgenic mice that develop polyarthritis caused by constitutive expression of TNF-a (54). When administered to mice after joint destruction had been established, damaged joints began to heal.

Infliximab in combination with methotrexate (MTX) is indicated for reducing the symptoms and inhibiting the progression of structural joint damage in patients with moderate-to-severe active rheumatoid arthritis who have had an inadequate response to methotrexate alone. It is also indicated for patients with Crohn's disease who have had inadequate responses to conventional therapy. Clinical studies with Infliximab in combination with MTX in 428 RA patients demonstrated serum half-lives of 8–9.5 days. In clinical trials, approximately 50% of patients receiving either 3 or 10 mg/kg of Infliximab every 4 weeks responded to treatment at a rate of approximately 50%, compared with placebo as measured by the American College of Rheumatology (ACR) response criteria (55). Treatment with Infliximab decreased inflammatory cell infiltration into inflamed areas in the joint, expression of adhesion molecules, E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1), chemoattractants such as IL-8 and monocyte chemotactic protein (MCP-1) and also inhibited expression of matrix metalloproteinases 1 and 3, which are involved in joint destruction (56). The treatment of Crohn's disease with Infliximab alone resulted in better than a 70% response rate compared with placebo within 4 weeks of receiving a single intravenous infusion according to the Crohn's Disease Activity Index (57).

Infliximab is provided as a sterile, lyophilized powder for reconstitution with 10 mL USP sterile water for injection such that the reconstituted material is 10 mg/mL followed by additional dilution into 250 mL of 0.9% sodium chloride. Recommended dose of Infliximab is 3 mg/kg given as an intravenous infusion between 0.4 and 4 mg/mL over a period of 2 h or more.

2.3.2 Etanercept. Enbrel (Etanercept), similar to Infliximab, binds to and neutralizes the biological activity of TNF-a. Etanercept is novel in that it was constructed by fusing cDNA from the extracellular ligand-binding portion of the TNF receptor to cDNA from the Fc portion of IgG1 and has the same approximate molecular weight (150 kDa) as an IgG molecule (58). Because Etanercept contains the TNF receptor, it binds and neutralizes both TNF-a and TNF-β (lymphotoxin) (59), in contrast to Infliximab, which binds only TNF-a. However, Etanercept suppresses the same biologic and pathogenic mechanisms leading to RA as does Infliximab.

Like Infliximab, Etanercept is approved for use in adult patients with moderate-to-severe active RA. However, Etanercept was also approved to reduce the symptoms of moderate-to-severe polyarticular-course juvenile RA (JRA) in patients who have had inadequate responses to disease-modifying anti-rheumatic drugs (60) and for use in patients with psoriatic arthritis in combination with MTX who do not respond to MTX alone (61). Clinical evaluation of subcutaneous administration of Etanercept twice per week for 6 months demonstrated an overall 23% major clinical response, defined as maintenance of an ACR70 (70%) response over a 6-month period. Discontinuation of Etanercept generally resulted in return of symptoms within 1 month. If patients were retreated with Etanercept, they achieved the same response as the initial treatment.

Etanercept is supplied as 25 mg of lyophilized powder for reconstitution with 1 mL of USP bacteriostatic water for injection, resulting in a 25 mg/mL solution that may be self-injected by a patient or physician into the thigh, abdomen, or upper arm.

Risks associated with Infliximab and Etanercept treatment include increased risk of infections such as reactivation of latent tuberculosis, invasive fungal infections, and sepsis. Autoantibodies against DNA and other nuclear components were observed in 10% of patients treated with Infliximab or Etanercept, mimicking a lupus-like syndrome. Patients receiving placebo did not generate lupus-like antibodies. Infusion-related reactions to Infliximab included fever, chills, cardiopulmonary

reactions, urticaria, and pruritus. Because Etanercept is injected subcutaneously, injection site reactions were limited to erythema, itching, pain, or swelling at the site of injection. Thirteen percent or fewer patients receiving Infliximab developed anti-Infliximab specific antibodies, while 11% of patients receiving Etanercept generated anti-TNF receptor-specific antibodies. These antibodies were all non-neutralizing and did not inhibit TNF-a from binding to its cell surface receptor. Administration of Infliximab and Etanercept is associated with increased infections (62), therefore immune responses to vaccines may be affected. Although not studied in-depth, patients may be immunized if necessary, with the exception of live or attenuated vaccines.

3 DENDRITIC CELL IMMUNOTHERAPY

The successful induction of cell-mediated anti-tumor immunity relies on the efficient capture of antigen by antigen-presenting cells, antigen processing, and presentation to T-lymphocytes. Antigen presenting cells (APC) such as B-cells, macrophages, and dendritic cells display antigenic peptides in the context of major histocompatibility complexes (MHC) to T-cells. Although B-cells and macrophages are capable of antigen uptake, processing, and antigen presentation, these APC are ineffective activators of naïve T-cells. In contrast, dendritic cells (DC) are considered the most effective, efficient, and potent APC of the immune system, because they are the only subset of APC that can present antigen to naive T-cells resulting in T-cell activation (63). Cell-mediated tumor cytotoxicity is the desired outcome of tumor immunotherapy. Modalities aimed at the induction of specific anti-tumor T-cell responses by DC are extremely promising in the fight against cancer. The following sections emphasize pre-clinical and clinical studies employing DC to induce active, specific immunity against tumors.

3.1 Dendritic Cell Physiology

The first studies involving DC generation were performed by isolating $CD34^+$ progenitor cells from the bone marrow and culturing these cells in the presence of GM-CSF and TNF-α (64, 65). While this was an effective means of DC generation, a relatively small number of DC were obtained, because of the limited number of progenitor cells present in bone marrow. Subsequent studies demonstrated that DC could be generated from peripheral blood mononuclear cells (PBMC) directly isolated from blood. Monocyte-derived DC can be generated *ex* vivo by culturing monocytes with a cocktail of cytokines such as GM-CSF and IL-4 or IL-13, yielding an immature population of DC (66, 67). Immature DC can be driven to a mature phenotype with the addition of inflammatory cytokines such as IL-1β and TNF-a, bacterial derived products [lipopolysaccharide (LPS)], recombinant CD40L (costimulatory molecule found on T-cells), or double-stranded RNA (68–72). The ability to effectively generate DC in vitro, coupled with the fact that DC efficiently present antigens, makes this subset of immune cells attractive candidates for the treatment of cancer and other diseases.

3.2 T-Cell Activation by Dendritic Cells

Immature DC are efficient at antigen uptake through micropinocytosis, receptor-mediated endocytosis, or phagocytosis; however, they have low T-cell stimulatory capacity (73). Conversely, while mature DC are less efficient at antigen uptake and processing, they are very efficient presenters of antigenic peptide to T-cells, resulting in the initiation of immune responses (73, 74). Mature DC express high levels of MHC molecules, high levels of co-stimulatory molecules (such as CD80, CD86, and OX40L), and adhesion molecules (i.e., CD54 and CD11c) that bind to counter-receptors on T-cells (73, 75, 76). These multiple interactions between DC and T-cells play a major role in the activation of T-cells specific for antigenic peptide/MHC complexes displayed by DC (Fig. 6.3). DC also secrete a milieu of cytokines and chemokines involved in the recruitment and activation of T-cells. Chemokines are small molecules that serve as chemical attractants for lymphocytes. DC-CK and RANTES are two examples of such chemokines that attract naïve and memory T-cells toward DC displaying antigenic peptide (77). The production of cytokines such as interleu-

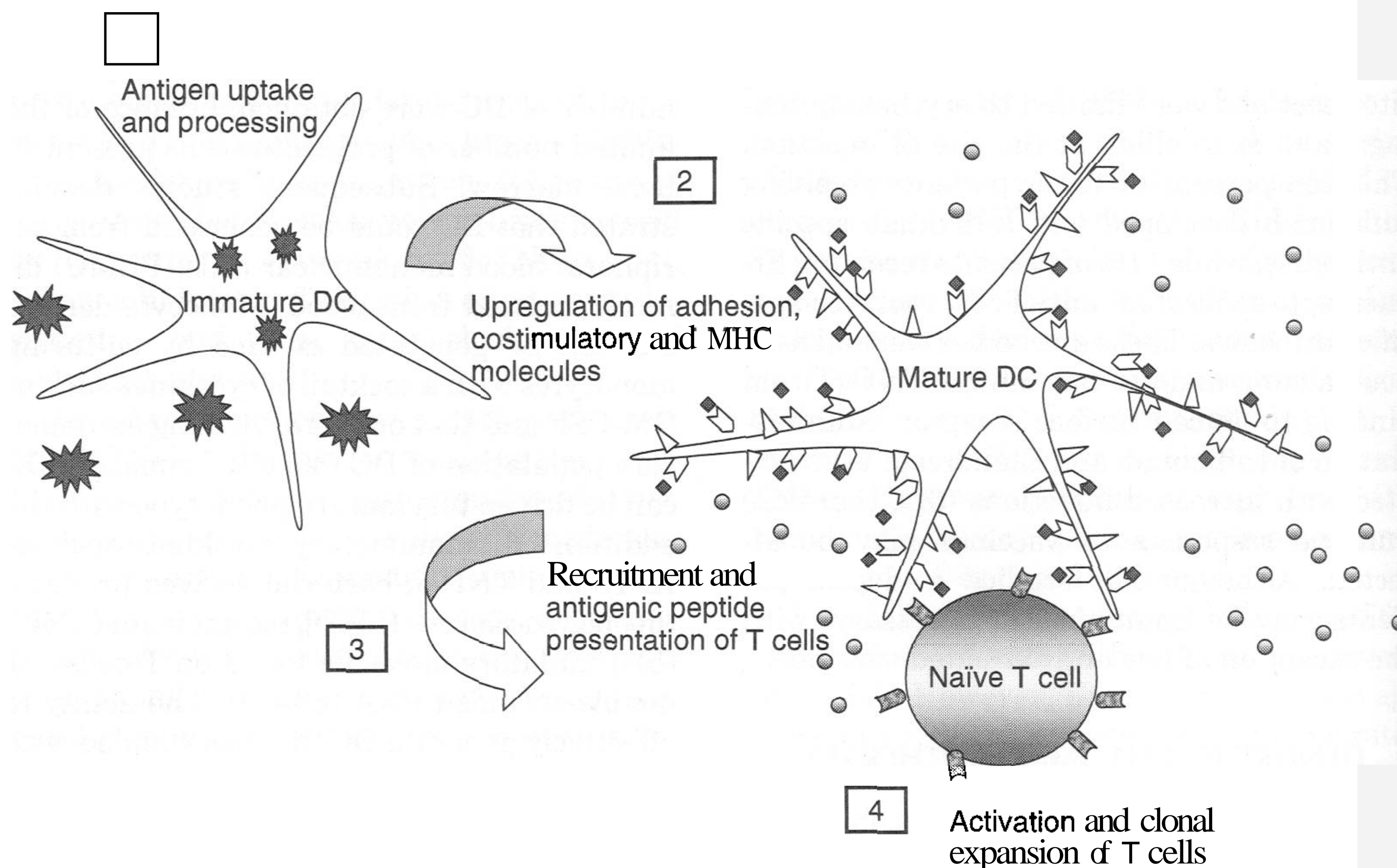

Figure 6.3. Antigen presentation and activation of naïve T-cells by mature DC.

kin-12 (IL-12) and IL-15 by DC are important cytokines for T-cell activation (78).

3.3 Immunotherapy Using Dendritic Cells

Recently, DC have been used in numerous clinical trials for the treatment of cancer. Immunization with DC is not toxic in either healthy subjects or cancer patients; no dose-limiting toxicity has been observed (79). The induction of tumor-specific T-cell responses has been detected in patients that have received DC immunotherapy. Several clinical trials are currently in progress investigating the safety and efficacy of immunotherapy of cancer with DC. Ex viuo incubation of DC with a source of tumor antigens is necessary to load tumor-derived antigenic epitopes on DC. Large numbers of DC generated ex *vivo* can be manipulated to enhance tumor antigen presentation and then re-administered to the patient to study the efficacy of DC immunotherapy. DC have been shown to induce strong anti-tumor immune responses both in uitro and in viuo. Early vaccination protocols involved DC pulsed with synthetic HLA-binding peptides. Since then, many other strategies involving DC have been investigated, such as DC-tumor cell fusions, DC transfected with tumor RNA or viral vectors, and DC exposure to tumor apoptotic bodies or exosomes. We will discuss current DC immunotherapy strategies in further detail in the following, sections. Table 6.2 provides a brief listing of the advantages and disadvantages of DC immunotherapy modalities.

Antigenic peptides are presented to T-cells by APC in the context of human leukocyte antigen (HLA) molecules. Numerous peptide epitopes have been identified and studied for both $CD8^+$ and $CD4^+$ tumor-specific T-cells (reviewed in ref. 80). Table 6.3 provides examples of HLA class I-binding peptide epitopes recognized by CD8+ CTL. Many tumor-associated antigens have been identified and may serve as targets for immunotherapy. Melanoma-specific cells can be propagated in *vitro*, therefore facilitating the identification of immunogenic melanoma antigens. However, there has been less success in the identification of tumor-associated antigens derived from other cancers because of the difficulty in generating tumor-specific CTL lines for *in* uitro studies. The use of peptides in immunotherapy is an attractive approach because

Table 6.2 Advantages and Disadvantages of DC Immunotherapy Modalities

	Advantages	Disadvantages
RNA transfection of DC	Simple technique; no requirement of tumor antigen identification	Difficult to measure transduction efficiency; potential oncogenic transformation of DC
DC-tumor cell fusion	Presentation full repertoire of tumor antigens possible with DC-derived costimulation; no requirement of tumor antigen identification	Low fusion efficiency; availability of autologous tumor cells; DC may not home to secondary lymphoid organs
Tumor cell lysate loading of DC	No requirement of tumor antigen identification; multiple epitopes presented by DC	Availability of autologous tumor cells
Apoptotic tumor cell loading of DC	May carry proteins that aid in loading peptides onto MHC; no requirement of tumor antigen identification; multiple epitopes presented by DC	Availability of autologous tumor cells
Peptide-pulsed DC	Ease of peptide synthesis; GMP manufacture straightforward known immunogen; multiple single peptides may be used to broaden T-cell response	Single antigenic epitope restricted by HLA haplotype
Viral vector transduction of DC	Long-lived, gene expression; may incorporate multiple defined antigens and/or costimulatory molecules	DC often resist transduction; safety: infectious agents

peptides are easily synthesized to good manufacturing process (GMP) specifications. However, vaccination strategies of DC pulsed with peptide require more study to determine optimal dosing and route of administration.

Immunodominant peptides derived from tumor-associated antigens such as gp100 (melanoma),Her-2/neu (overexpressed on adenocarcinomas such as breast cancer), MAGE peptides, and carcinoembryonic antigens (CEA) have been used to vaccinate patients in several clinical trials (81–84).

A well-studied tumor antigen is gp100, a melanocyte lineage-restricted glycoprotein that is expressed by most melanoma cells (17). Bakker et al. (85) demonstrated that an HLA-A*0201-restricted gp100 immunodominant epitope (residues 209–217) was recognized by

Table 6.3 Examples of HLA Class I Binding Tumor Peptides*

Classification	Gene	HLA-Allele	Peptide Epitope	Reference
Melanocyte differentiation antigens	$gp100_{(209-217)}$	A2	ITDQVPFSV	85
	$tyrosinase_{(1-9)}$	A2	MLLAVLYLL	86
	$MART\text{-}1_{(27-35)}$	A2	AAGIGILTV	17
Shared tumorltestis antigens	$MAGE\text{-}1_{(161-169)}$	A1	EADPTGHSY	87
	$NY\text{-}ESO\text{-}1_{(53-62)}$	A31	ASGPGGGAPR	88
Non-mutated, overexpressed tumor antigens also expressed on normal tissue at low levels	$Her\text{-}2/neu_{(369-377)}$	A2	KIFGSLAFL	89
	$CEA_{(61-70)}$	A3	HLFGYSWYK	90
	$MUC\text{-}1_{(950-958)}$	A2	STAPPVHNV	91
	$PSA_{(146-154)}$	A2	FLTPKKLQCV	92
Tumor-specific antigen caused by point mutation in normal gene	$P53_{(264-272)}$	A2	LLGRNSFEV	93
	$\beta\text{-catenin}_{(29-37)}$	A24	SYLDSGIHF	19
	$HSP70\text{-}2M_{(286-294)}$	A2	SLFEGIDIY	94
Viral gene products	$HPV\ E6_{(11-20)}$	A2	YMLDLQPETT	95

Table is not a complete listing of tumor antigens and peptides.

tumor infiltrating lymphocytes derived from melanoma patients. This gp100 epitope was modified to enhance peptide binding to HLA-A*0201 as an attempt to increase peptide immunogenicity (86). The modified peptide, g209 2M, enhanced CTL responses from patients with melanoma compared with the wild-type gp100 peptide in uitro (86). Early peptide immunotherapy trials involved g209 2M peptide administration in combination with IL-2 therapy (87). Vaccinations were well tolerated, and objective clinical responses were noted in 38% of patients (87). However, it was unclear whether these clinical responses were truly because of peptide therapy or a result of IL-2 administration. Future studies confirmed that peptide-specific CTL were induced in patients immunized with the g209 2M peptide post-vaccination (88), demonstrating the feasibility of therapeutic peptide vaccines.

Recently, clinical trials involving DC-peptide therapy have begun. Patients with ovarian or breast cancer were vaccinated with DC pulsed with either Her-2/neu or MUC-1 derived peptides (89). Peptide-specific CTL responses were detected as measured by cytokine secretion and lysis of tumor cells. Another recent clinical trial involved pulsing autologous DC with the following melanoma peptides: g209 2M, MART-1 (AAGIGILTV), or tyrosinase (YMDGTMSQV) before administration in 16 patients with melanoma (90). Minimal toxicity was observed. Peptide-specific T-cell responses were generated in *vitro* against gp100 or tyrosinase from five patients; four of five patients demonstrated either tumor regression or stable disease. Immune responsiveness in this study correlated with clinical benefit. Transient increases in T-cell stimulation to MAGE peptides were also observed in six of eight patients enrolled in a separate DC clinical trial (91). These studies suggest that peptide-pulsed DC provide clinical benefit to melanoma patients.

Autologous DC were pulsed with MAGE-3A1 tumor peptide and administered to advanced stage IV melanoma patients who received five DC vaccinations (three intraderrnal and two intravenous injections). CTL specific for MAGE-3A1 were generated in 8 of 11 patients (92). Tumor regression of metastases was evident in 6 of 11 patients. Interestingly, $CD8^+$ T-cells were absent from non-regressing lesions as well as the expression of MAGE-3 mRNA.

Several factors may play a role in the limited successes with DC-peptide immunotherapy. One reason may be that the optimal dose and route of administration are not yet known. Second, if tumor cells down-regulate the tumor antigen from which the peptide is derived, DC-peptide presentation to T-cells may be futile. Therefore, strategies using multiple peptides from multiple antigens are most likely to elicit effective responses with minimal tumor escape variants.

A current limitation of peptide immunotherapy is the applicability to a large patient population. Peptides are specific for HLA haplotypes, such that only cancer patients whose haplotype matches that of the tumor-derived peptide may be candidates for a given single peptide therapy. However, recent studies investigating peptide cross-reactivity for HLA molecules determined that a peptide with high affinity for one particular HLA class I allele can also bind to other alleles (93). Additionally, medical science is increasingly tailoring therapies to the individual.

Another problem facing immunotherapeutic approaches designed to stimulate T-cells involves self-tolerance. Thymic education of T-cells is a selection process in which T-cells that bind with high affinity to self-peptides are deleted through apoptosis (94). If these deleted T-cells were specific for a tumor antigen, then only low affinity T-cells would remain (13, 95, 96). These low affinity T-cells are incapable of responding strongly to tumor antigen without strong stimulation provided by DC. However self-specific T-cells can be found in the periphery (97–99). The existence of these self-specific T-cells, albeit with a low affinity, may initiate an anti-tumor immune response. These cells alone are insufficient to completely resolve tumor burden. Therefore, attempts have been made to increase both the HLA affinity and peptide immunogenicity of tumor-derived peptide similar to the modified gp100 peptide, g9 209 2M.

Several studies have shown that increased affinity of a peptide for MHC molecules is an important parameter in determining peptide immunogenicity (100–102). By modifying self-

peptides, there is potential to activate T-cells that are either tolerant or have a weak affinity for self to initiate an immune response. Altered peptide ligands for p53, Her-2/neu, MAGE, carcinoembryonic antigen (CEA), and MART-1 tumor peptides have been successfully synthesized that have higher affinities for HLA molecules compared with wild-type, and elicit CTL responses *in vitro* (103,104).

With the knowledge that tumor-derived peptides can be modified to increase HLA binding coupled with the potential that these peptides may cross-react with several HLA alleles, these findings may greatly impact the design of HLA-binding peptides for cancer immunotherapy applicable to a broad range of patients.

3.4 Dendritic Cell Immunotherapy Approaches for Undefined Tumor Antigens

An important issue in optimizing DC vaccines is choosing an ideal tumor antigen for DC loading. Tumor cell lysates, apoptotic tumor bodies, and tumor cells can be used as immunogens in DC cancer therapy for the development of anti-tumor strategies. For several tumor types, antigenic epitopes are unknown. In contrast to peptide immunotherapy, using tumor-derived products bypasses the need to consider HLA haplotypes and the identification of specific tumor-derived antigens. Several of these treatment modalities will now be reviewed, which demonstrate specific anti-tumor responses against tumors with undefined tumor antigens.

3.4.1 Apoptotic Tumor Cells. In an effort to overcome the limitations of HLA restriction and identification of specific tumor-associated antigens, whole tumor cells containing unidentified antigenic components as an antigen source have been investigated. Apoptotic tumor cells (ATC) have been studied to determine the effectiveness of these cells to serve as sources of antigen. Hoffmann et al. (105) generated a $CD8^+$ T-cell line specific for a squamous cell carcinoma of the head and neck cell line (SSCHN) to determine if immunogenic peptides could be presented by DC cultured with ATC. DC generated from healthy donors were tested for the ability to uptake ATC. Immature DC ingested ATC and were able to effectively present tumor antigens and induce a T-cell–mediated anti-tumor immune response more effectively than DC incubated with tumor-derived lysate preparations. An increase in CTL number and lytic function was observed when DC were loaded with pancreatic apoptotic tumor cells compared with cell lysate (106). The mechanism of enhanced DC priming using ATC is still unknown. Studies using ATC are ongoing in many laboratories and promise to yield data supporting their use in DC immunotherapy.

3.4.2 Tumor Lysates. Compared with vaccination approaches directed against a single tumor antigen, tumor cell lysates contain an array of tumor-derived antigens that have the potential of inducing broad T-cell responses against multiple antigens expressed by tumor cells, either previously identified antigens or unknown tumor-derived antigens. Although many vaccines employing tumor lysates have been prepared in the past, the addition of DC to the vaccine should lead to more robust immune responses than previously observed.

Tumor lysate-pulsed DC were tested in a phase I trial of pediatric patients with solid tumors (107). Patients were vaccinated with immature DC pulsed separately with either tumor cell lysates or keyhole limpet hemocyanin (KLH; an immunological tracer molecule). DC were administered intradermally every 2 weeks for a total of three vaccinations. Fifteen patients (10 of which completed all DC vaccinations) diagnosed with neuroblastoma, sarcoma, and renal malignancies were immunized without observable toxicity. IFN-y secreting T-cells were detected *in vitro* in 3 of 7 patients against tumor lysates and 6 of 10 patients for KLH. Five patients showed stable disease, including three who had minimal disease at time of vaccine administration and remained free of tumor 16–30 months post-vaccination. This trial demonstrated that tumor lysate or KLH-pulsed DC generated specific T-cell responses, thereby inducing regression of metastatic disease.

In another study, autologous tumor lysate-pulsed DC were administered intradermally to adult patients with stage IV solid malignancies every 2 weeks for three cycles with varying amounts of DC (108). The vaccine for this

phase I clinical trial was composed of a mixture of lysate-pulsed DC and DC pulsed with KLH. No severe toxicity was reported in 14 patients that received three DC vaccinations. $CD4^+$ and $CD8^+$ T-cells were detected at the vaccination sites. Two patients with melanoma experienced a partial and a minor response, respectively. In other tumor types, phase I trials of patients with advanced gynecological malignancies and a malignant endocrine carcinoma demonstrated that vaccination with DC-autologous tumor lysates was safe, well-tolerated, and immunologically active with no significant adverse effects (109, 110). Tumor lysates were also derived from ovarian cancer cells and used to evaluate the potential of lysate-pulsed DC to induce tumor-specific T-cell responses against autologous tumors (111). DC-lysate stimulated proliferation of autologous T-cells and CTL-mediated lysis of autologous tumor cells. These effects were abrogated using anti-MHC class I and anti-CD8 antibodies. Furthermore, T-cells cultured with autologous tumor lysate-pulsed DC secreted cytokines that play an important role in immune cell recruitment, such as GM-CSF, TNF-a, and IFN-y. T-cells cultured with tumor lysates in the absence of DC resulted in no lytic activity, further confirming that DC play a major role in the initiation of anti-tumor immune responses.

In another study, DC generated from melanoma patients were loaded with either a melanoma peptide, gp100 (280–288), or melanoma tumor cell lysate to determine the ability to induce cytotoxic autologous T-cell activation (112). Weekly stimulations of PBMC were performed with the lysate and peptide DC preparations. $CD8^+$ CTL displayed strong lytic activity against melanoma cells irrelevant of the DC stimulations (peptide versus lysate). These findings indicate that a variety of DC immunotherapy strategies are likely to be effective at inducing CTL responses. Collectively, these studies demonstrate that the administration of tumor lysate-pulsed DC is non-toxic and effective at inducing immunological responses against autologous tumor.

3.4.3 Tumor RNA. Studies involving exposure of DC to purified tumor-derived RNA have shown that RNA is efficiently taken up by the DC, resulting in presentation of tumor antigens to T-cells. RNA extracted from metastatic colon cancer or lung cancer were loaded onto autologous DC (113). In vitro lysis of autologous tumor cells was observed when RNA-transfected DC were incubated with autologous T-cells. Loading of DC with RNA followed by potent stimulation of T-cells is an interesting contrast to the resistance of DC to transfection with plasmids.

A phase I trial involving patients with metastatic prostate cancer was conducted to determine the safety and efficacy of DC transfected with mRNA encoding for prostate-specific antigen (PSA) to induce specific anti-PSA T-cell responses (114). At all doses of mRNA-transfected DC administered, no evidence of dose-limiting toxicity or adverse effects were observed. Furthermore, PSA-specific T-cell responses were detected in vitro in all patients who received the vaccine.

This approach has also been applied to cervical cancer using autologous DC transfected with RNA encoding for E6 and E7 (115), oncoproteins constitutively expressed by many cervical carcinomas. Antigen-specific CTL responses were observed both in vitro and in *vivo.* Human cervical carcinoma cells expressing the E6 and E7 products were lysed by CTL.

These studies provide a rationale for the development of immunotherapy using DC transfected with RNA encoding for tumor antigens. DC transfected with tumor RNA may emerge as a method for inducing immune responses against tumor antigens. Clinical trials demonstrating vaccine safety are promising for the development of anti-tumor cellular vaccines.

3.4.4 DC-Tumor Cell Fusion. Hybrid cells composed of tumor cells fused to DC have been generated using either polyethylene glycol (PEG) or electrofusion techniques, in which these hybrid cells possess antigen presentation characteristics of DC as well as tumor antigens derived from tumor cells (Fig. 6.4). The distinct advantage of a fusion product between tumor cell and a DC is that the hybrid contains the co-stimulatory molecules that tumors rarely express. The hybrid also contains

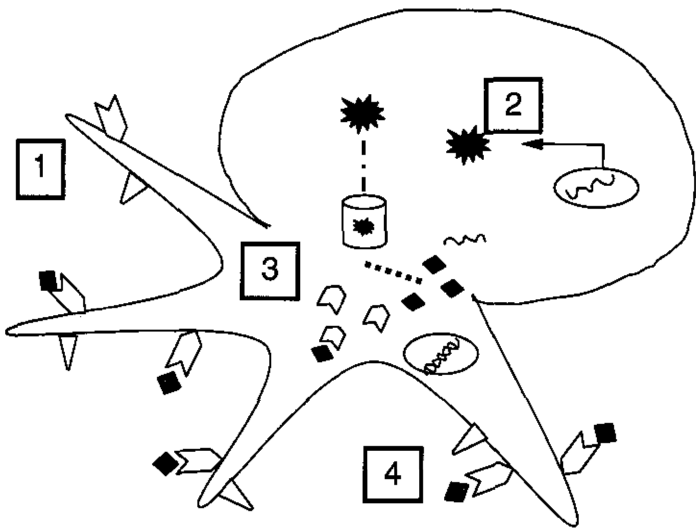

Figure 6.4. Tumor cell-dendritic cell hybrid. (1) On fusion, DC express costimulatory molecules, adhesion molecules, and MHC molecules necessary for T-cell activation. (2) Tumor cells transcribe genes that encode for tumor-derived antigens. (3) Tumor antigens can be processed by DC machinery and assembled onto MHC molecules. (4) Tumor-derived peptides can be presented in context of MHC molecules on the dendritic cell to tumor-specific T-cells.

multiple tumor antigens, including unidentified antigens that are endogenously processed and presented by MHC class I molecules.

Gong et al. (116) demonstrated that fusion between DC and autologous tumor cells is a feasible approach for immunotherayy. DC were fused with either autologous human breast or ovarian carcinoma cells (116, 117). These DC-tumor hybrids expressed both tumor-associated antigens and co-stirnulatory molecules naturally expressed by DC. *In vitro*, hybrids induced both autologous T-cell proliferation and lysis of autologous tumor cells by responding CTL.

Fusion products of autologous renal cell carcinoma cells and allogeneic DC were tested in a phase I clinical trial (118). Post-vaccination with these fusion hybrids, four patients rejeded all metastatic tumor lesions and two patients demonstrated tumor regression greater than 50% in strong partial responses.

Taken together, these studies indicate that the development of a "super" DC that can both stimulate and activate T-cells through DC-associated co-stirnulatory molecules and presentation of tumor antigens derived from tumor cells may be a feasible approach for cancer immunotherapy. Currently, fusion efficiency is relatively low, ranging from 5–50% DC-tumor cell hybrids. This obstacle is currently being further addressed in several pre-clinical and clinical studies to maximize the clinical effectiveness of DC-tumor cell of hybrid cells.

3.4.5 Exosomes. Exosomes are membrane-bound vesicles released from either tumor cells or DC that have processed tumor cells. They are of endocytic origin ranging from 60 to 90 nm. Exosomes are thought to be secreted upon the fusion of multivesicular endosomes with the plasma membrane. Red blood cells, platelets, B- and T-cells, and DC have been found to secrete exosomes. Recent proteomic studies demonstrated that exosomes display MHC molecules (both class I and class II), co-stirnulatory molecules, and several adhesion molecules (119). The mechanism of action of exosomes *in vivo* is not well defined. Exosomes may stimulate T-cells directly through surface MHC molecule expression or they may be ingested by other APC that could potentially further present peptide contained within the exosome. Wolfers et al. (120) demonstrated that upon DC uptake of tumor cell exosomes, presentation to $CD8^+$ T-cells resulted in potent anti-tumor effects in *in vivo* murine models, indicating that tumor-derived exosomes transfer antigenic tumor material to DC. Injection of DC-derived exosomes sensitized with tumor peptides induced anti-tumor immune responses in mice and eradicated previously established tumors (121). This anti-tumor effect of DC exosomes was dependent on $CD8^+$ T-cells as determined by T-cell depletion experiments *in vivo.* Recently, the first protein map of DC-derived exosomes was established (119), which may provide substantial insights into the exploitation of exosomes for immunotherapeutic use.

3.5 Dendritic Cells Transduced with Viral Vectors

One promising strategy for the future of immunotherapy is transduction of DC with recombinant, replication-incompetent viruses expressing multiple or single tumor antigens, cytokines, or co-stirnulatory molecules. The goal of DC-based immunotherapy using viral vectors is to express one or more transgenes in DC so that endogenous and exogenous presentation of antigenic tumor peptides occurs in a favorable immunostimulatory environment

using the complete repertoire of co-stimulatory molecules present on DC. The advantages of viral transduction of DC are as follows. (1) Presentation of multiple epitopes derived from the same or multiple antigens may reduce the probability that mutant tumor cells will escape immunological surveillance. (2) Antigen presentation by multiple MHC alleles provides even greater diversity of peptide epitopes. (3) Cytokines and co-stimulatory molecules can be co-expressed along with the tumor protein, eliciting stronger activation of T-cells. (4) Finally, the viral transduction procedure itself may mature DC, enhancing antigen presentation capacity and activation of T-cells (122). Next, promising viral vector systems for active specific immunotherapy will be discussed.

3.5.1 Adenoviral Transduction of DC. DC have been shown to be uniformly resistant to nearly all transfection techniques *in* vitro (123). Additionally, monocyte-derived DC do not undergo cell division and are therefore resistant to transduction by retroviral vectors (124). The fact that adenoviruses do not require active cell division for infection is a distinct advantage of the adenoviral system (125). Thus, it is possible to transduce DC with recombinant adenovirus. The problem is that DC express very low levels of the receptor for adenovirus, "coxsackie adenovirus receptor" (CAR) (126). One solution is to transduce DC with recombinant adenovirus encoding CAR as the transgene (127). In lieu of transducing DC with CAR so that they can be transduced again with the gene of interest, multiplicities of infection (MOI) as high as 1000 must be used to achieve high transduction efficiency (124, 128).

In a pre-clinical study, replication-deficient recombinant adenoviruses encoding human gp100 or MART-1 melanoma antigens were used to transduce human DC. The study demonstrated that human monocyte-derived DC could be infected at an MOI between 100 and 500 independent of the CAR. DC transduced with this replication-deficient adenovirus also elicited tumor-specific CTL *in* vitro from patients with $gp100^+$ metastatic melanoma (129). These findings led to a clinical trial evaluating the safety, dose-limiting toxicity, and maximum tolerated dose of autologous DC transduced with adenoviruses encoding the MART-1 and gp100 melanoma antigens with or without interleukin-2 therapy in patients with stage III or IV melanoma. Although the preclinical results are encouraging, no results have been published from this trial as of this writing.

3.5.2 Pox Virus Transduction of DC. Human DC have also been successfully transduced with various pox viruses including fowl pox and modified vaccinia ankara (MVA), a highly attenuated vaccinia virus. Pre-clinical work characterized the transduction efficiency of W A encoding human MUCl (tumor antigen that is aberrantly glycosylated on malignant cells) and IL-2 with monocyte-derived DC. MUCl expression in DC was from 27–54% of the cells, which also secreted biologically active IL-2. Over 72 h post-transduction, cytopathic effects of MVA resulted in decrease of the transgene expression. Transduction of both immature and mature DC did not alter expression of the MUCl and IL-2 transgenes (130).

Other pre-clinical work demonstrated that human DC can be transduced with W A expressing tyrosinase (melanoma-associated antigen). These transduced DC stimulated anti-tyrosinase CTL responses from melanoma patients who had received smallpox vaccination earlier in life (131). This data demonstrate the use of MVA transduced DC and negate the argument that immunodominance by the pox vector itself will overwhelm any tumor antigen-specific immune response.

Another pre-clinical study that has led to a clinical trial in patients with carcinoembryonic antigen-positive (CEA) malignancies is evaluation of fowlpox transduced DC expressing three co-stimulatory molecules: B7–1, intercellular adhesion molecule-1, and leukocyte function-associated antigen-3 (TRICOM). The goal of the study was to determine if DC infected with TRICOM would have an enhanced capacity to stimulate T-cell responses (132). Although the study was performed in a murine system, it showed that poxvirus vectors overexpressing of a triad of co-stimulatory molecules can significantly improve the efficacy of dendritic cells in priming specific immune responses.

These studies led to an NCI-sponsored clinical trial to determine the safety and feasibility of active immunotherapy comprising autologous DC infected with recombinant fowlpox-CEA-TRICOM vaccine in patients with locally recurrent or metastatic malignancies expressing CEA (133). In this ongoing study, autologous DC are harvested and infected with fowlpox-CEA-TRICOM vaccine. Patients receive their infected DC intradermally and subcutaneously followed by autologous DC mixed with CMV pp65 peptide and autologous DC mixed with tetanus toxoid SC and intradermally on day 1. Treatment was repeated every 3 weeks for a total of 4, 8, or 12 immunizations in the absence of unacceptable toxicity. CMV pp65 peptide and tetanus toxoid were administered so that immune responses to naïve and recall antigens can be monitored. We look forward to the results of this promising trial (134).

4 CONCLUSIONS

Many exciting developments have occurred in the field of immunotherapy in the past several years. This chapter has highlighted humanized antibody therapeutics, many of which have already been approved by the FDA, and the newest techniques in DC therapies for cancer. Clinical acceptance of humanized, chimeric, and diagnostic monoclonal antibodies has fostered growth in sales in 2000 of 1.8 billion dollars to nearly 3 billion dollars in 2001. We can expect to see many more humanized and chimeric antibodies in the immediate future.

Cellular immunotherapies using DC are now logistically and technologically feasible because closed system processing laboratories become more common. However, several parameters must be optimized to realize the full potential of DC in cancer immunotherapy. Vaccination schedules, route of DC administration, and the number of DC for effective immune responses must be resolved. The life span of DC *in vivo* post-vaccination and whether these cells effectively home to lymphoid tissues to encounter and activate T-cells also must be further investigated. Lastly, clinical trials involving DC immunotherapy need to combined with other treatment modalities that are non-immunosuppressive to fully develop efficient anti-tumor therapies. Before poisoning the immune system with current cytotoxic chemotherapies, hopefully a paradigm shift will occur to activate and manipulate patients' own immune systems to therapeutic advantage, employing active specific immunotherapy. Therapeutic antibodies have now been accepted as viable treatments for cancer and other diseases. We are optimistic that some of the immunotherapeutic strategies outlined in this chapter will become front-line therapies in the future.

5 ABBREVIATIONS

ADCC	antibody dependent cell mediated cytotoxicity
APC	antigen presenting cell
CDR	complementarity determing region
CTL	cytotoxic T lymphocyte
DC	dendritic cell
HAMA	human anti-mouse antibody
HLA	human leukocyte antigen
IFN	interferon
Ig	immunoglobulin
IL	interleukin
MHC	major histocompatibility complex
NK	natural killer cell
PBMC	peripheral blood mononuclear cell
TCR	T cell receptor
TIL	tumor infiltrating lymophocyte
TNF	tumor necrosis factor

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CHAPTER SEVEN

Selective Toxicity

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Edited by Donald J. Abraham
ISBN 0-471-37031-2 © 2003 John Wiley & Sons, Inc.

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1 INTRODUCTION

Selective toxicity was popularized by Professor Adrian Albert beginning with his lectures at University College London in 1948 and the first edition of his book in 1951. Subsequent editions appeared until the seventh edition in 1985 (1)The latter was reprinted in Japanese as two volumes in 1993 and 1994 (2, 3).

Today, the concept of selective toxicity largely is limited to chemotherapy and antibiotic therapy. How does one design a cancer chemotherapeutic agent that will kill a malignant cell and not interfere with mitosis in a benign cell? How does the biochemistry of a bacterial cell differ from that of a mammalian cell so that the antibiotic is toxic to the bacterium and "ignored" by the patient's cells?

It is important to realize that nearly all types of drug therapy can be thought of as selectively toxic. With the possible exception of some forms of hormonal replacement therapy, a drug's desired pharmacological response is actually an intervention in normal biochemical processes. Consider the nonsteroidal anti-inflammatory drugs (NSAIDs) that inhibit cyclooxygenase (COX). Although reducing inflammation is the desired response for these agents, the drugs are toxic to the enzyme. β-Adrenergic agonists are exogenous ligands for the same receptors as epinephrine but are not subject to the same regulatory controls (biosynthesis, release, reuptake, metabolic disposal) as for the endogenous hormone. Herbicide and pesticide use in agriculture increasingly is based on the agent being selective for a specific plant or taking advantage of some specific property of the insect that differs from those of farm animals and humans.

1.1 Categorization of Therapeutic Agents

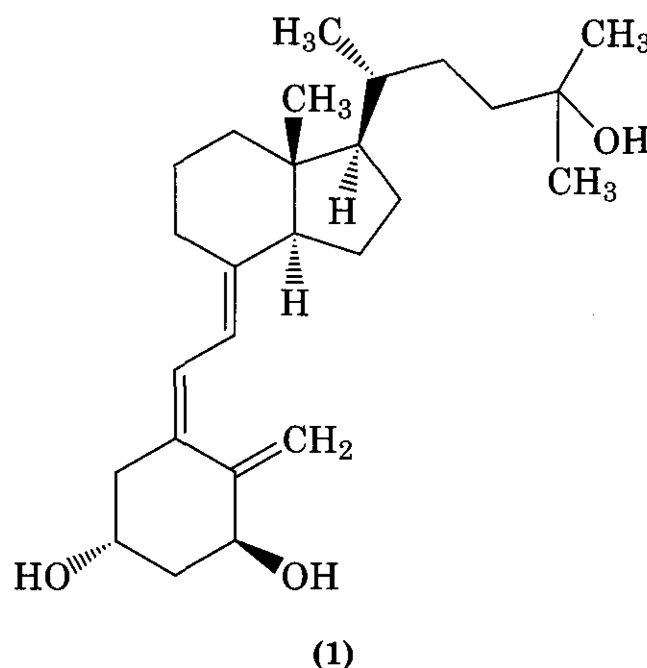
Focusing on humans, most drugs are agonists, antagonists, or replacement agents. Hormone replacement therapy is the basis for the use of insulin by a person with type 1 diabetes (formerly called insulin-dependent diabetes mellitus or IDDM) and levothyroxine for a patient with a thyroid deficiency. Insulin and thyroxine act only on specific receptors. As long as the dosing is correct, patients do not experi-

ence unpleasant adverse reactions. Excessive insulin causes severe, life-threatening hypoglycemia, leading to insulin shock. Because of the general distribution of thyroxine receptors throughout the body, excessive thyroxine accelerates intermediate metabolism in several organs.

This concept of replacement therapy can be extended to the administration of vitamins and minerals to a patient whose body stores have been depleted, or has a medical diagnosis for which increased administration of nutritional supplements is indicated (e.g., digestive disorders, malignancies, clinical depression, weight control with low calorie diets). The nutritional agents combine with specific receptors or, usually after a metabolic transformation, act as coenzymes (e.g., niacin-NAD/NADP, thiamine/thiamine pyrophosphate, folic acid/tetrahydrofolates, and cobalamin/adenosyl cobalamin). Again, when the dosing is correct, the patient does not experience adverse effects. Excessive doses of some vitamins can cause neuritis (pyridoxinelvitamin B_6), hypercalcemia (cholecalciferol/vitamin D_3 and ergocalciferol/vitamin D_2), and liver damage (retinol/vitamin A).

Agonists mimic the endogenous ligand when combining with the ligand's receptor. In theory, there should be excellent selectivity, but many times the receptors for the ligand are scattered widely throughout the body and located in many organs. Thus, an adrenergic agonist such as phenylephrine will constrict blood vessels while simultaneously increasing the heart rate. For some patients, the latter could lead to life-threatening tachycardia. Depending on the receptor, the active form of cholecalciferol (vitamin D_3), 1,25-dihydroxycholecalciferol (1) causes the synthesis of a calcium transport protein in the intestinal mucosa and regulates cell division. An analog of 1,25-dihydroxycholecalciferol, calcipotriene (Dovonex, 2) is used topically for psoriasis. If administered internally, the patient could experience severe hypercalcemia, leading to calcification of the soft tissues and blood vessel walls. In the context of selective toxicity, calcipotriene has poor selectivity.

Most drugs dispensed today are antagonists. (See other chapters for discussions of partial agonists and antagonists.) The desired goal is to "block" responses in the cholinergic, adrenergic, rennin/angiotensin, and other integrated systems. Rarely are these biochemical systems localized in a specific organ. Inhibiting one enzyme can have unforeseen metabolic consequences downstream. A classic example is the angiotensin-converting enzyme (ACE) inhibitors that produce an irritating cough (to the patient and patient's family). Angiotensin-converting enzyme has more than one substrate: angiotensin I and bradykinin (Fig. 7.1). The desired target was inhibiting the formation of angiotensin II from angiotensin I and was the focus for the development of the ACE inhibitors (e.g., captopril, enalapril, and lisinopril). Angiotensin-converting enzyme also degrades bradykinin, one of the many peptides involved with pain production and inflammation. ACE inhibitors



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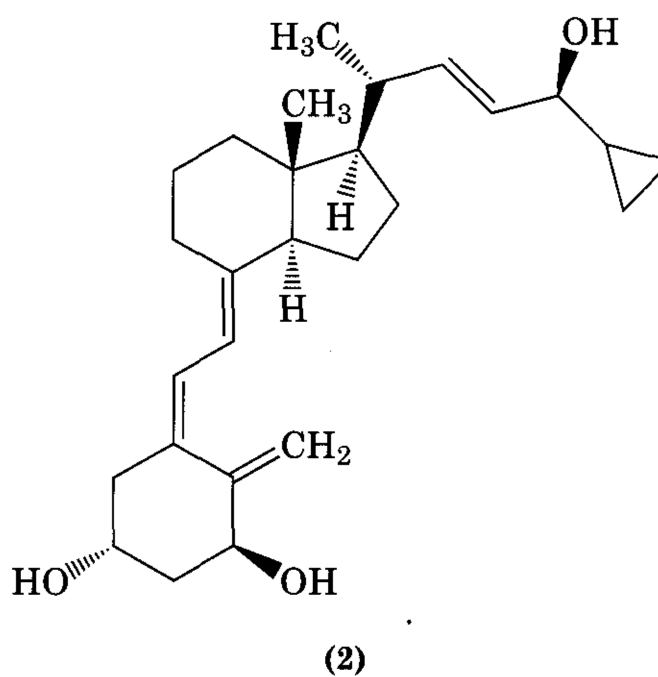

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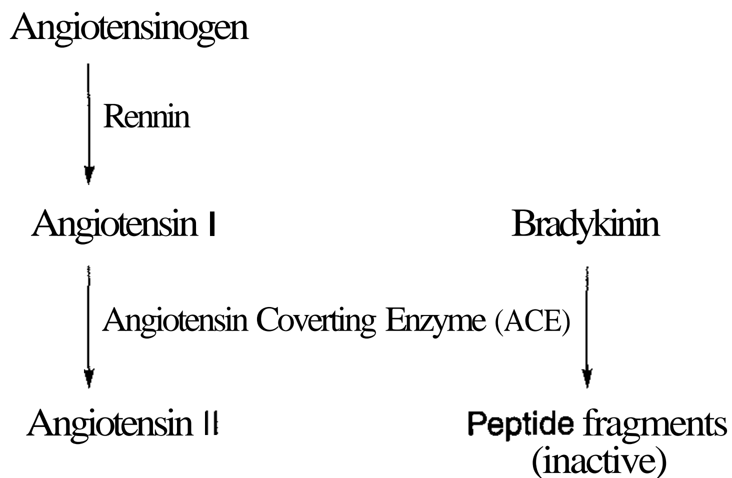


Figure 7.1. Rennin-angiotensin converting enzyme biochemistry.

cause production of excess bradykinin and may be the cause of the annoying dry cough characteristic of these drugs.

An important approach to increasing the selectivity of a pharmacological agent is to thoroughly understand the biochemistry of the system where drug therapy is indicated. By going downstream from the angiotensin-converting enzyme and employing an angiotensin II antagonist, there are fewer adverse reactions.

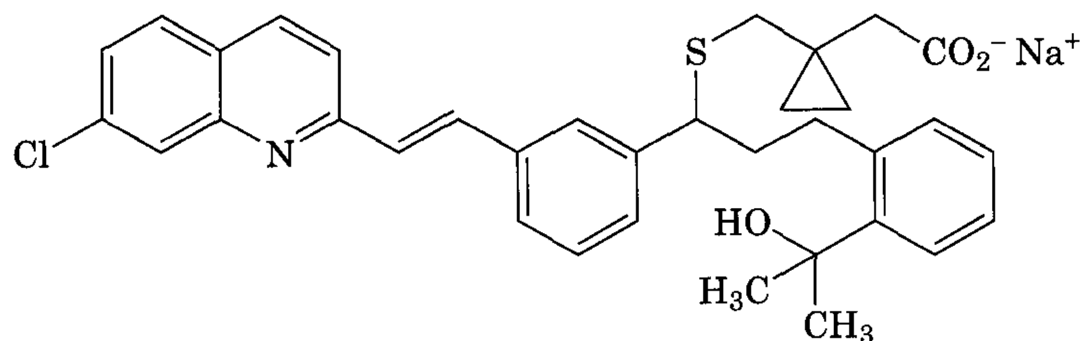
Asthma is a complex inflammatory disease of the respiratory system. One of the family of chemical mediators are the leukotrienes synthesized from arachindonic acid by the lipoxygenase enzyme complex (Fig. 7.2). The leukotrienes are one of the mediators of bronchoconstriction. In the treatments of asthma, the leukotriene D_4 (LTD_4) receptor antagonists, montelukast sodium (3)and zafirlukast (4), exhibit less adverse responses than the 5-lipoxygenase inhibitor, zileuton (5), which interferes with the formation of LTA_4, LTC_4, LTD„ and the LTE_4 family of leukotrienes, all of which are chemical mediators of the immune response at several sites.

Distribution of enzyme types is another way to increase the selectivity. The cyclooxygenase (COX) isozymes are distributed unevenly among the various organs. What is now known as COX-1 is a constitutive enzyme found in many organs. COX-2 is inducible by cytokines and appears at sites when there is inflammation. Although not free of adverse reactions, celecoxib and rofecoxib do show fewer side effects in patients who must take these drugs on a chronic basis compared to the older COX-1 inhibitory nonsteroidal anti-inflammatory drugs (NSAIDs).

1.2 Principles of Selectivity

Professor Albert, after examining the various ways that selectivity of biological response is obtained from both natural products or synthetic agents, concluded that there are three possible ways that a pharmacologically active agent exerts selectivity: (1)comparative distribution, (2) comparative biochemistry, and (3)comparative cytology (4).

1.2.1 Comparative Distribution. Comparative distribution can be caused by differences in physical area where absorption occurs or differences in the drug's biodistribution. An insecticide accumulates in the insect because the insect has more exposed surface area relative to that of animals in the same environment. Radioactive iodine is used both to diagnose diseases of the thyroid and to destroy the thyroid gland because it accumulates in the thyroid as a result of specific iodine transport proteins. Parasites that remain in the intestinal tract are easier to treat because many of the newer drugs also remain in the intestinal tract. A rapidly dividing cell line will preferen-



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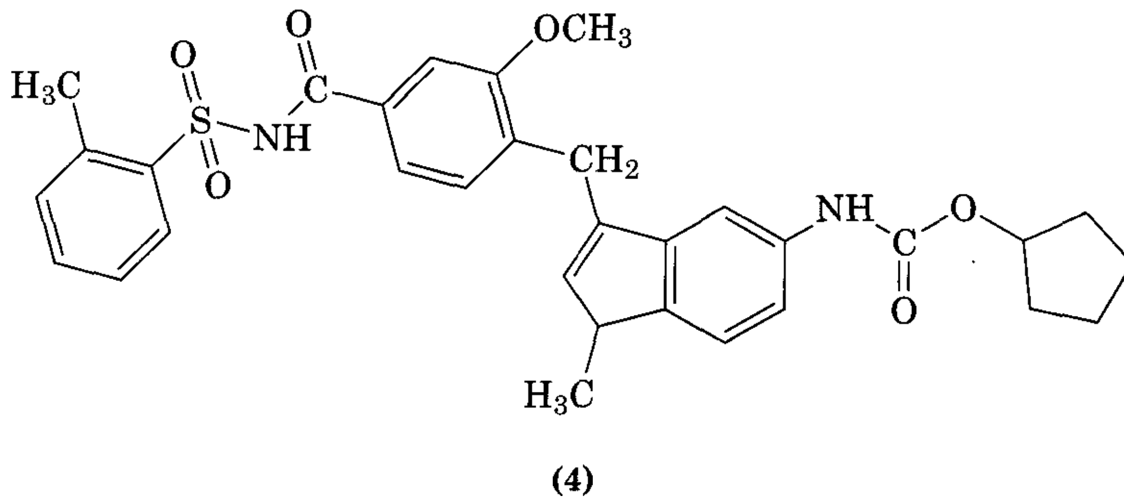

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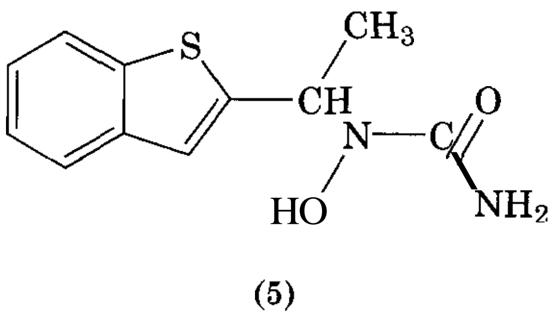


(5)

tially incorporate a number of drugs, including those used in the treatment of cancer.

There are several examples where drugs cause adverse responses in patients because of poor comparative distribution. Most of the drugs used in the treatment of cancers do not differentiate between benign tissues, which are constantly dividing (bone marrow, intestinal mucosa, hair follicles), and malignant cells
(5). The result is immunosuppression and/or
anemia, diarrhea or constipation, and loss of
hair. **A** problem with the current antiviral drugs (see below) is that they cannot intercept free viruses but are effective only when the virus is inside a cell and dividing. These drugs cannot differentiate between virus-infected cells and cells free of virus.

1.2.2 Comparative Biochemistry. Comparative biochemistry is the basis for successful antibiotic therapy. The antibiotic class is one of the main reasons that human life expectancy has increased, producing a significant older population. The β-lactam antibiotics, penicillins and cephalosporins, inhibit

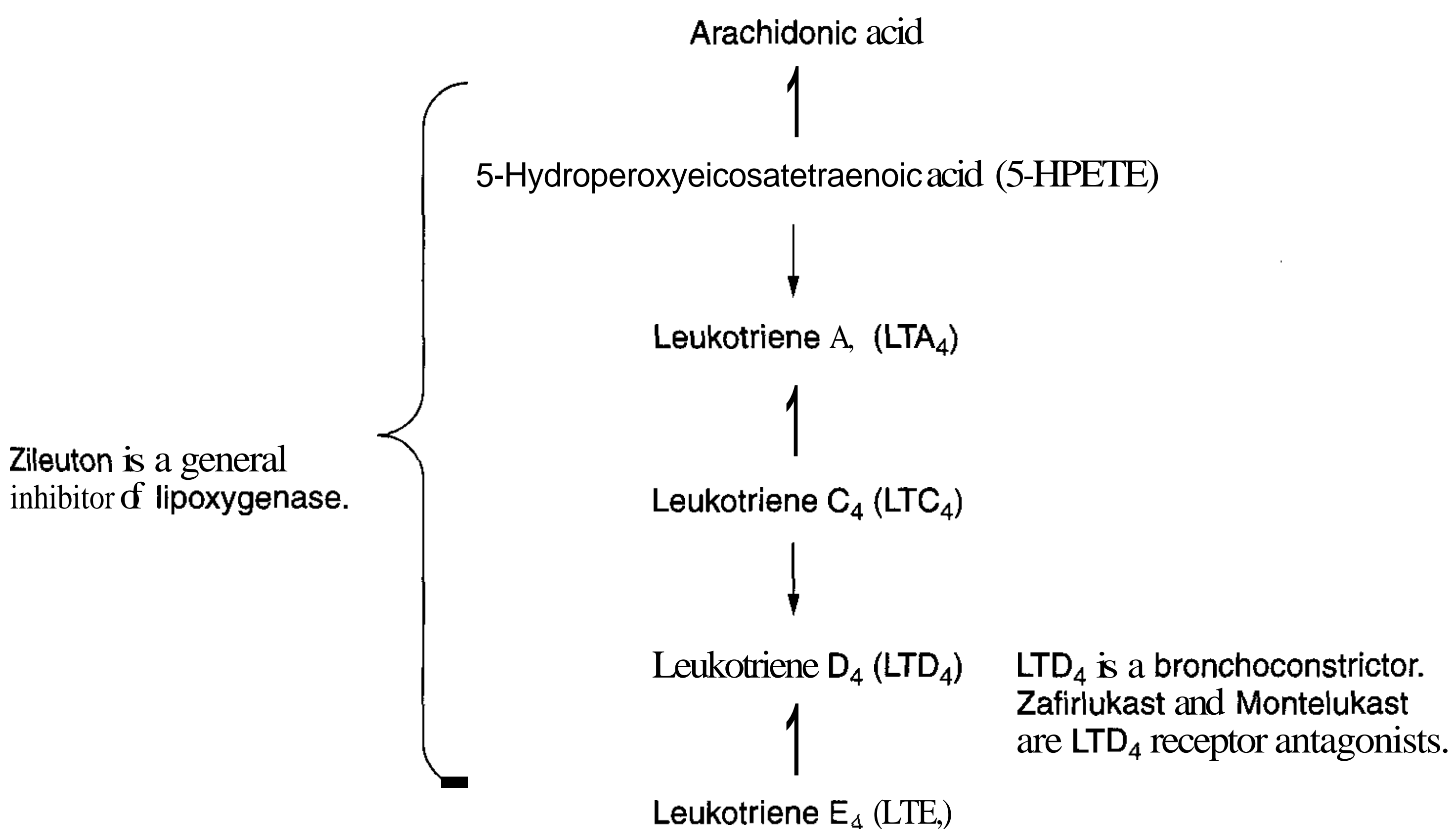


Figure 7.2. Outline of leukotriene biosynthesis.

bacterial transpeptidase, thus preventing the microorganism from completing the synthesis of its cell wall. Mammals do not have cell walls and, thus, are not affected by β-lactam antibiotics. Our lipid bilayers have completely different biochemistry from that of a bacterium. The various drugs that inhibit the biosynthesis of folic acid or its reduction to tetrahydrofolic acid (trimethoprim, sulfonamides) are bacteriocidal or bacteriostatic and do not affect mammals that cannot synthesize folic acid. (That is why folic acid is a vitamin.) Pyrimethamine shows a preference for plasmodia dihydrofolate reductase relative to the human enzyme and, therefore, has found use in the treatment of malaria (6).

In contrast, it has been difficult to develop antiviral and antifungal drugs that are selective for the infectious agent. Viruses are complete parasites in that they can reproduce only inside the host cell. Many viruses are dependent on the host cell's polymerases, ribosomes, and transfer RNAs. Most of today's antiviral drugs not only poorly differentiate between viral and human polymerases, but they also cannot distinguish between cells infected with the virus versus noninfected cells. Even inhibitors preferential for RNA-dependent DNA polymerase (reverse transcriptase), an enzyme not found in mammalian cells, can inhibit mammalian DNA-dependent DNA polymerase, leading to suppression of the patient's bone marrow.

Acyclovir and its analogs probably are the most successful antiviral drugs in use today. They are indicated for herpes simplex I and II and varicella zoster (chicken pox). The herpes virus has one of the more complicated viral genomes, coding for over 160 genes. One of these structural genes codes for thyrnidylate kinase, which is different from the mammalian kinase enzyme. The viral enzyme will phosphorylate inactive acyclovir (actually a prodrug), producing active acyclovir monophosphate (Fig. 7.3). The latter is phosphorylated next to the diphosphate and finally the triphosphate, which is the active antiviral drug. None of the mammalian kinases can significantly phosphorylate inactive acyclovir to the active form.

Fungal metabolism is more similar to mammalian metabolism than to bacterial metabolism (Fig. 7.4). Whereas fungi and plants produce ergosterol rather than cholesterol, fungi and mammals follow the same pathway to lanosterol from mevalonate and squalene (7). In contrast, plants produce cycloartenol from mevalonate and squalene.

Many of today's antifungal drugs take advantage of the fact that fungi produce ergosterol rather than cholesterol; however, ergosterol biosynthesis is very similar to that of cholesterol. Several of the antifungal drugs inhibit the C-14 demethylase that removes the 14-methyl group from lanosterol, thereby preventing the subsequent synthesis of ergosterol. For this reason, antifungal drugs that inhibit ergosterol's biosynthesis show poorer selectivity, with the result that many antifungal drugs can only be used topically, far away from the patient's organs that synthesize cholesterol.

1.2.3 Comparative Cytology. The third principle, comparative cytology, refers to the comparative taxonomic structure of cells. Examples include plant vs. animal cells; undifferentiated malignant cells vs. fully differentiated mature benign cells; the patient's immune system recognizing nonself cells, which produces the basis for monoclonal antibody therapy; cell wall vs. cell membrane; mitochondria vs. chloroplasts; and the presence of mitochondria in aerobes vs. their absence in anaerobes (8). There is overlap between comparative cytology and the first two principles. It is not uncommon to see selectivity caused by two and possibly all three principles, although usually one predominates. Penicillins and cephalosporins are selective for bacteria because of comparative cytology (cell wall vs. cell membrane) and comparative biochemistry (transpeptidase in bacteria).

1.2.4 Comparative Stereochemistry. The fourth principle of selectivity that is being used today in drug design is comparative stereochemistry. Most receptors are chiral and respond differently to drugs of different chirality. Increasingly, the Food and Drug Administration requires new drug applications to resolve racemic mixtures to determine which stereoisomer is biologically active. Drugs with chiral centers may exhibit more

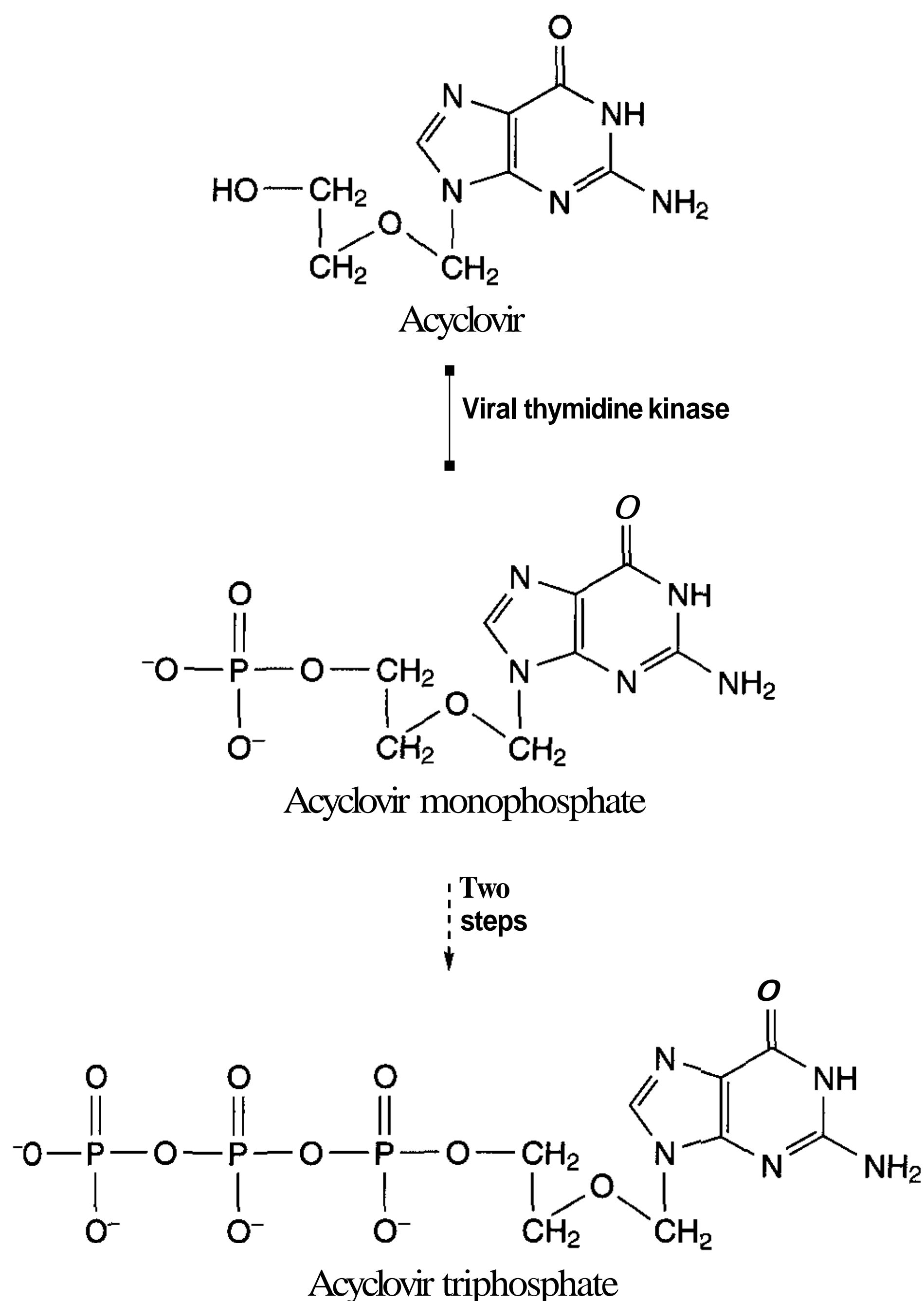
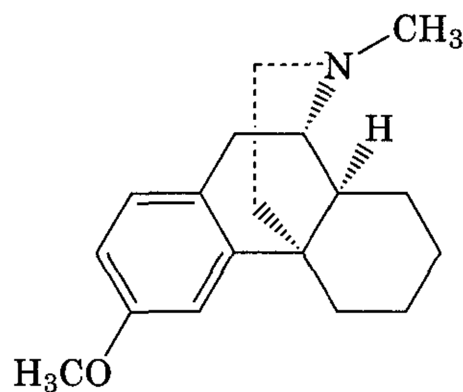


Figure 7.3. Acyclovir (acycloquanosine) activation.

complex **selectivity**. This can be caused by flexibility at the receptor, variations in transport across cell membranes to the site of action, and differences in metabolism for each **stereoisomer**. **A** classic example is the antitussive **dextromethorphan** (**_d_-3-methoxy-_N_-methylmorphinan**) (**6**), which is devoid of opiate **activity** compared to that of the **levo** isomer (*l*-3-methoxy-N-methylmorphinan) (7).

The remainder of this chapter consists of a brief review of strategies to enhance selectivity in biological response. Most examples consist of commercially available pharmacologically active agents, but there are descriptions



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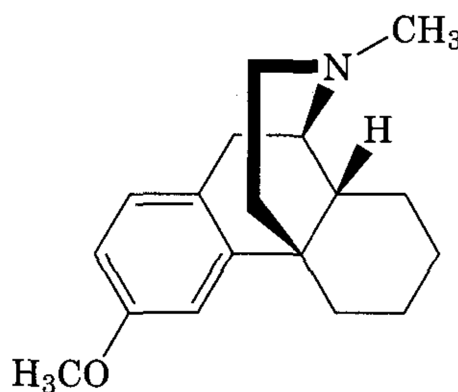

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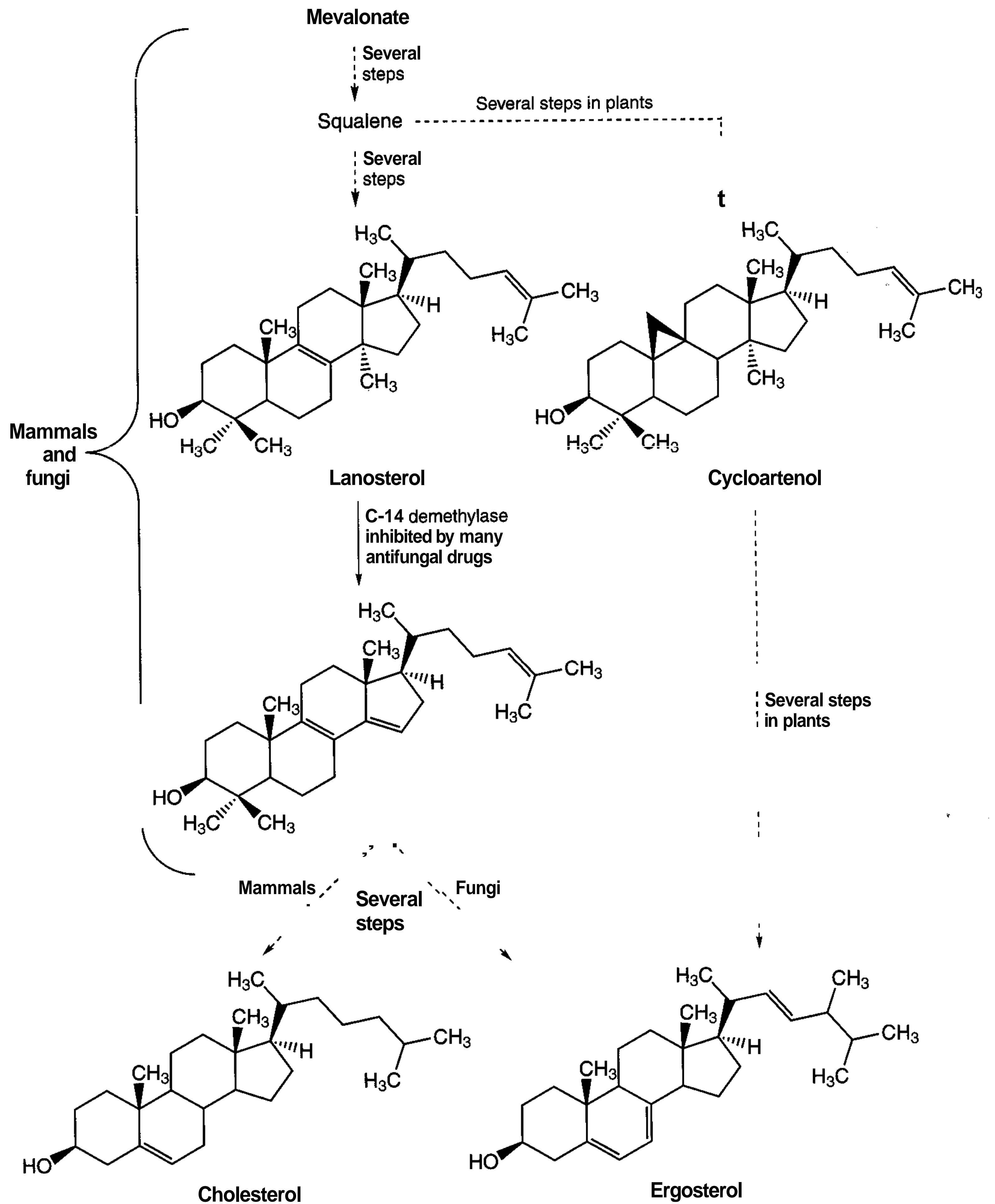


Figure 7.4. Cholesterol-ergosterol biosynthesis.

of recent research involving strategies that may or may not result in commercial products. Although Professor Albert's three principles are presented in each example, a standard pharmacological or toxicological classification is used.

2 EXAMPLES OF SELECTIVE TOXICITY (9)

2.1 Cancer Chemotherapy

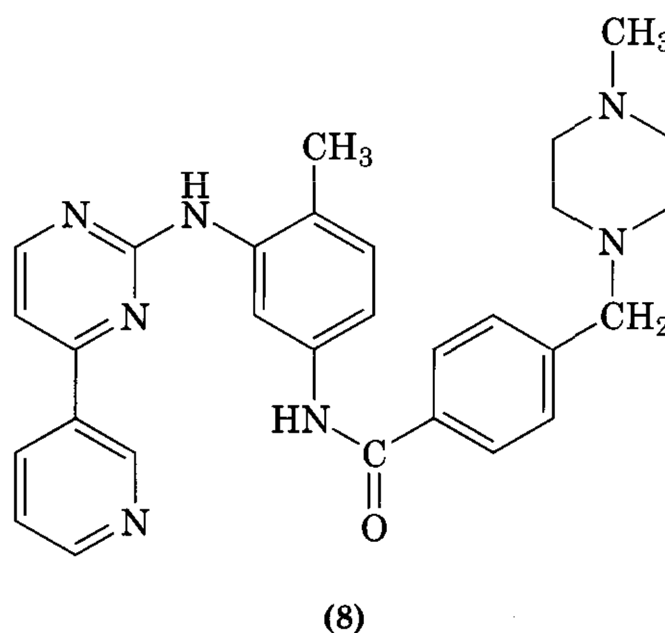
2.1.1 Monoclonal Antibodies *(Comparative Cytology).* Monoclonal antibodies were going to be the fulfillment of Ehrlich's "magic bullets," particularly in the treatment of cancer where selective toxicity has been poor. They would carry the cytotoxic drug to the malignant cell where it would be released. The antibody would ignore benign cells. Alternatively, the antibody itself would attach to the malignant cell, providing the initial step for further response by the patient's immune system involving complement, macrophages, and T killer cells. This goal still has to be reached, but significant progress is being made.

The first impediment is the main source of monoclonal antibodies. They come from diverse nonhuman sources such as rodents and sheep. Repeated injections sensitize the patient to the animal protein. A partial solution as been "humanizing" the monoclonal antibody by using human genes to code for the constant region of the heavy and light chains and the animal genes for the variable regions. The result is that now approximately 70–75% of the immunoglobulin is human.

In addition, the malignant cell is not recognized by the patient's immune system as nonself because its surface contains essentially the same self-antigens as the patient's benign cells. Otherwise, the patient's immune system likely would have killed the malignant cells before they grew into a detectable mass. Therefore, the monoclonal antibodies developed in an animal where human cell surface antigens are foreign, also will respond to benign cells.

The most successful product to date that addresses this problem is trastuzumab (Herceptin) indicated for metastatic breast cancer containing cells that overexpress the HER2 proto-oncogene. This particular gene codes for a transmembrane receptor structurally related to the epidermal growth factor receptor. Trastuzumab binds to this receptor. This overexpression is seen in only 25–30% of primary breast cancers. Also, the gene is normally expressed in other cells, which means trastuzumab is not as selective as would be desired.

2.1.2 Imatinib *(Comparative Biochemistry).* The search for selective metabolism in malignant cells has focused on unique proteins synthesized by the cell that are involved in cell division. In the case of chronic myeloid leukemia, imatinab [Gleevec, (**8**)] inhibits a protein-tyrosine kinase formed by the Ber-Abl gene. The latter, and its protein product, are not found in normal cells. Although this drug does show selectivity, tyrosine kinases are common enzymes and imatinib also inhibits this tyrosine kinase receptor in platelet-derived growth factor and stem cell factor. Therefore, the patient can experience thrombocytopenia and neutropenia.



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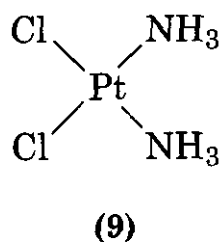
2.1.3 Cisplatin/Carboplatin *(Comparative Distribution).* It has been known almost from the time of its serendipitous discovery that the *cis* analogs of the platinum-containing cytotoxic agents, cisplatin (9) and carboplatin (**10**), are much more cytotoxic than the *trans* isomers. Both the *cis* and *trans* compounds enter the growing cell and form inter- and intrastrand crosslinks within the DNA double helix. The kinetics of this binding is very different

between the two isomers. Further, DNA repair is more efficient with the *trans* isomers. The net result is that the cis isomer remains in the DNA long enough to be cytotoxic (10).



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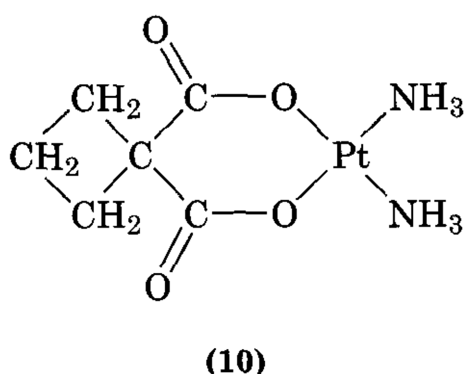

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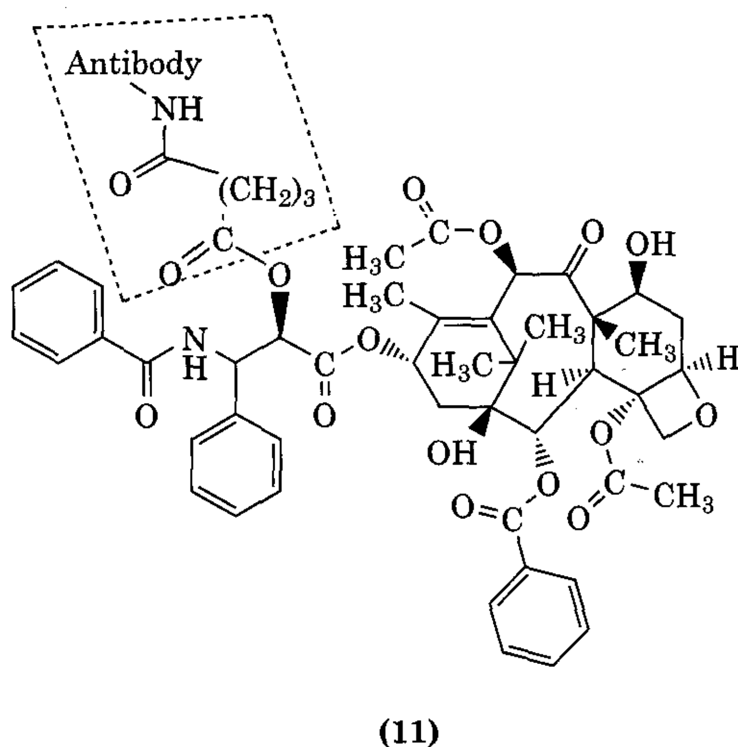
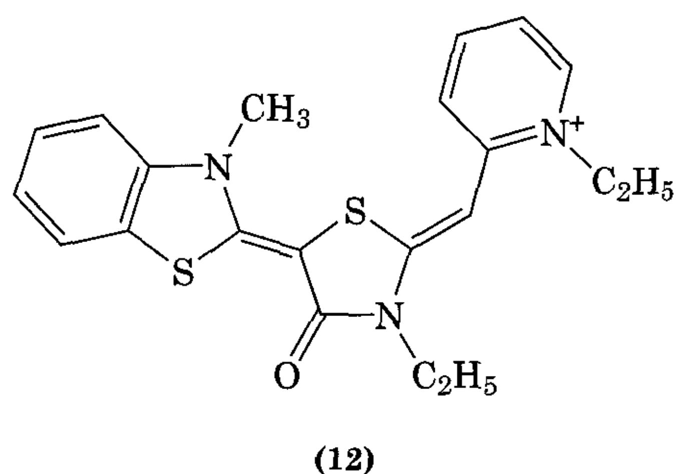
2.1.4 Newer (Not Commercially Available) Selective Approaches to Cancer Chemotherapy

2.1.4.1 Paclitaxel Antibody Conjugate (Comparative Cytology). The rationale for this project was to combine the best properties of two drug types and overcome each of their undesirable characteristic~Paclitaxel is a very effective anticancer drug but has poor solubility and lacks tumor specificity. Monoclonal immunoglobulins have good solubility properties and better tumor specificity, but lack good therapeutic efficacy. The goal was to develop a conjugate that would be inactive until attached to the malignant cell. The conjugate was produced by synthesizing 2'-glutaryl paclitaxel from glutaric anhydride. The latter's free carboxyl group was derivatized with *N,N'*-carbonyldiimidazole followed by addition of the monoclonal antibody (**11**). The binding character of the antibody was not altered by adding it to paclitaxel, and paclitaxel's cytotoxicity also was not affected when tested in rat neuroblastoma cell lines (11).



(11)

2.1.4.2 Cationic Rhodacyanine Dye Analog (Comparative Distribution). One cytotoxic target in a malignant cell is the cell's mitochondria, the membranes of which are negatively charged. Therefore, a positively charged molecule lipophilic enough to pass through the cell's hydrophobic membrane's lipid bilayer might be one way to obtain comparative distribution within the cell. It has been found that the positively charged rhodyacyanine dyes pass through the hydrophobic cell membrane lipid bilayer and concentrate in the negatively charged mitochondria membranes. Of course, both malignant and benign nucleated cells have mitochondria. The selectivity claimed for the rhodacyanine dyes (**12**) is based on the observation that carcinoma cells have a higher mitochondrial membrane potential relative to that of benign cells (12).



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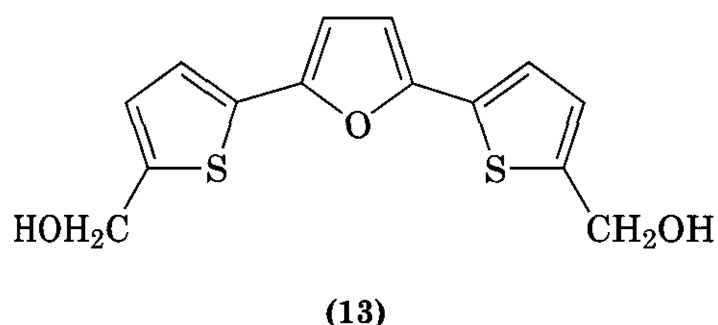
2.1.4.3 Tricyclic Thiophene (Comparative Distribution and Comparative Metabolism). The National Cancer Institute uses a panel of 60 human tumor cell lines to develop a "fingerprint" that, when analyzed using appropriate software, provides leads to mechanisms of cytotoxicity. An example is a tricyclic thiophene (13) that showed selective accumulation and metabolism in renal tumor cell lines.

Preliminary work in cell cultures and extracts indicate that a CYP 450 enzyme in the malignant cells may be responsible for forming an oxidized product that binds to proteins inside the cell. The latter leads to the cytotoxic response (13).



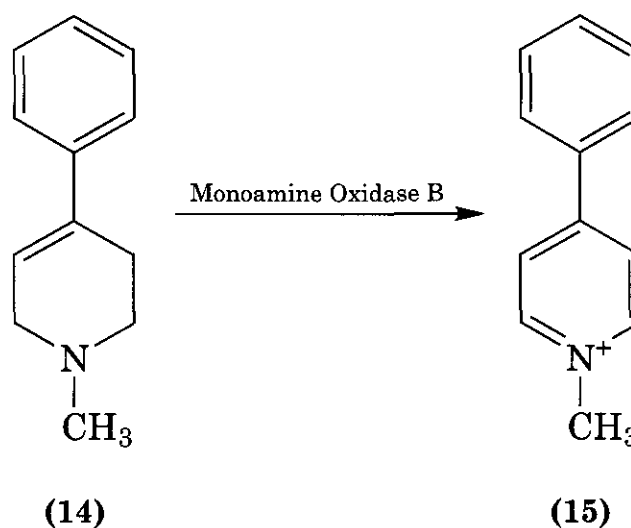
(13)

2.1.4.4 *Diphtheria Toxin (Comparafive Biochemisfry).* Rhabdomyosarcomas are characterized by a fusion protein formed from two abnormal genes that may contribute to the tumor cell's immortality, possibly by inhibiting apoptosis. By a process called protein swapping, the gene for diphtheria toxin A-chain is inserted into the malignant cell by a plasmid vector, thereby producing a fusion protein containing the toxin's A-chain and causing these tumor cells to undergo programmed cell death. Cells lacking the defective genes do not experience protein swapping and the gene for the toxin A-chain is not expressed (14).

2.1.5 Summary. The examples used in the treatment of cancers are attempts at trying to increase the selectivity in cancer chemotherapy using Professor Albert's three comparative principles. Nearly all of the drugs used today in cancer chemotherapy interfere with cell replication. Unfortunately, it has been difficult to specifically target malignant cells while sparing normal cells (i.e., gastric lining, skin, bone marrow). The end result is the use of very toxic drugs in the treatment of disease. If it were not for the lethality of most cancers, cancer chemotherapeutic drugs would not be approved by the regulatory authorities.

2.2 Dopaminergic Receptors

2.2.1 Receptor Destruction *(Comparafive Cytology).* Although selective toxicity in drug design focuses on mechanisms that avoid adverse reactions, the concept can be applied to models that help explain how receptors are destroyed during the disease process. Parkinson's disease involves destruction of dopaminergic receptors. Molecular probes that are very specific to dopaminergic receptors targeted in Parkinson's disease and do not affect other receptors in the brain are useful in determining how the disease progresses. One such probe is 1-methyl-4-phenylpyridinium [MPP^+, (**15**)] obtained by monoamine oxidase B oxidation of 1-methyl-1,2,3,6-tetrahydropyridine [MPTP, (**14**)]. MPP^+ inhibits mitochondrial complex I. It has been suggested that this inhibition results in less ATP for the neuron, depolarization of the mitochondria, and generation of reactive oxygen species, which leads to neuronal death. This model does not explain why dopaminergic neurons are more susceptible than other aerobic cells to MPP^+. In a series of experiments using another mitochondrial complex I inhibitor, rotenone, it appears that MPP^+ is specific to the structure of the dopaminergic receptor (15).



(14) **(15)**

2.3 Anti-Infectives

2.3.1 introduction. The approach to anti-infective therapy is based on the drug's being selectively toxic to the pathogen and, ideally, being "ignored" by the patient's cells. This group of widely prescribed drugs is covered in more detail in a separate chapter. Thus, this discussion is limited to a representative set of examples. Table 7.1 provides a summary of sites where antibiotics exert their antibacterial activity. The reader should refer to the anti-infectives chapters for discussions of structure-activity relationships (SARs), including how to reduce the

Table 7.1 Summary of Sites Where Antibiotics Exert Their Antibacterial Effect

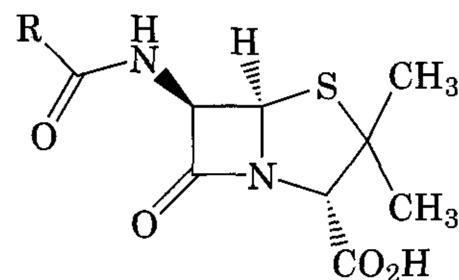
Site of Action	Antibiotic Class
Bacterial cell wall (peptidoglycan) synthesis	β-Lactams, Bacitracins, Vancomycin
Bacterial ribosomal units	Aminoglycosides, Tetracyclines, Macrolides
Organization of bacterial membrane	Polymixins, Colistins, Gramicidins
Bacterial DNA gyrase/topoisomerase	Quinolones
Folic acid biosynthesis	Sulfonamides
Dihydrofolate reductase	Trimethoprim

risk of antibiotic resistance and improve the drug's pharmaceutical properties.

Most of the examples in this section are based on Professor Albert's principle of comparative biochemistry. Comparative cytology also plays a role, although most of the time this principle frustrates the drug designer and clinician. Many times antibiotics will be effective against Gram-positive but not Gram-negative bacteria. This difference often is attributed to the additional proteoglycan layer on the outside of the cell wall of Gram-negative bacteria. At the macro level, the additional barrier that keeps the gram stain from entering the bacterium also hinders the antibiotic from entering the cell.

2.3.2 Examples Based on Chemical Class

2.3.2.1 β-Lactams (Comparative Biochemistry). The β-lactam antibiotics consist of two major groups, penicillins (16) and cephalosporins (17), both of which are still the most widely prescribed drugs indicated for bacterial infections. They exhibit classic selective toxicity because they inhibit a key enzyme, transpeptidase, in the biosynthesis of the bacterial cell wall, a structure not found in the lipid bilayer of mammalian cell membranes. The inhibition is complex and involves the antibiotic covalently binding to "protein-binding proteins" (Fig. 7.5). The carbonyl carbon of the sterically strained β-lactam ring is attacked by the serine hydroxyl at the protein's active site, forming a stable covalent protein-antibiotic conjugate. In general, the β-lactam antibiotics are very safe. Adverse reactions are usually found in a small subset of patients who are allergic to this group of drugs.



(16)

2.3.2.2 Aminoglycosides (Comparative Biochemistry and Comparative Distribution). In contrast with the β-lactam antibiotics, the aminoglycoside antibiotics have more structural diversity (Fig. 7.6). Examples include streptomycin, the gentamycin family, the kanamycin family, and the neomycin family. The one structural characteristic they have in common is one or two amino hexoses connected to a six-mem-

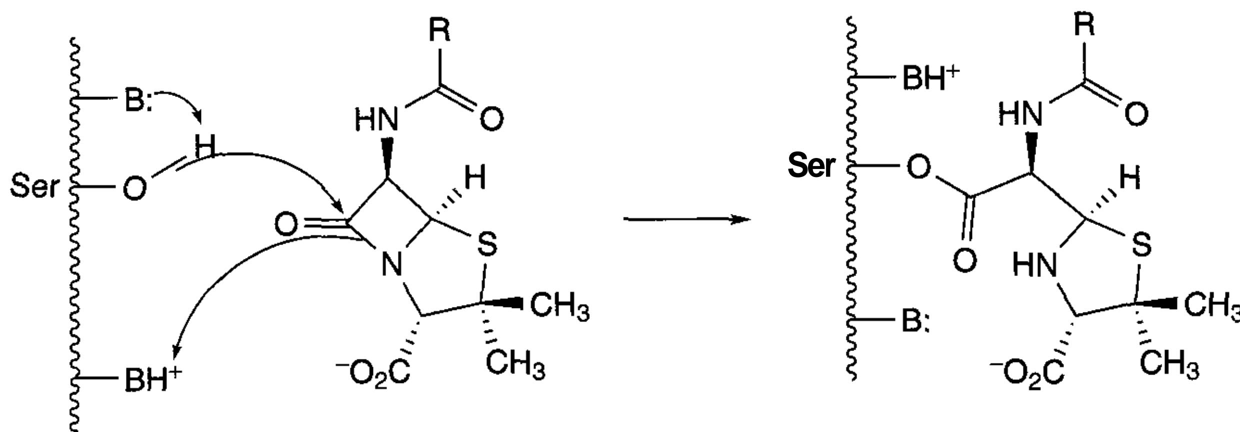
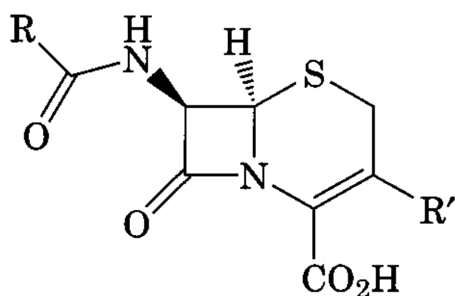


Figure 7.5. Penicillin mechanism.



(17)

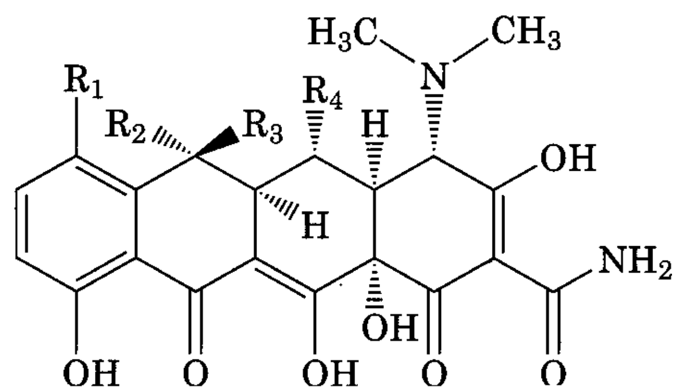
bered ring substituted with alcohols and amino moieties. Sometimes these are called **aminocyclitols.** Because the latter do not have an **anomeric** carbon, they are not sugars.

The aminoglycosides bind to the bacterial 30S ribosomal unit, causing inhibition of initiation of protein synthesis and sometimes misreading of the genetic code. Fortunately, the ribosomal units in mammalian cells are sufficiently different that the aminoglycosides do not readily bind to mammalian ribosomes.

Unfortunately, the arninoglycoside antibiotics do have severe toxicities. The neomycin **family** has such severe nephrotoxicity that these drugs usually are only administered topically. One of the most distressing and common adverse responses is ototoxicity, which can lead to permanent deafness. Although the biochemical mechanism of this toxicity is poorly understood, these antibiotics concentrate in the lymphatic tissue of the inner ear. The half-lives of the aminoglycosides are five to six times longer in the otic fluid compared to that of the plasma (16). This is an example in which comparative distribution increases the toxicity of the drug.

2.3.2.3 Tetracyclines (Comparative Biochemistry and Comparative Distribution). Like the **aminoglycoside** antibiotics, the tetracyclines (18) preferentially bind to the 30S ribosomal subunit, thus preventing elongation of **peptide** chains. In addition, the tetracyclines are actively transported into the bacterium, causing the drug to concentrate in the susceptible cell. Mammalian cells lack this active transport system for tetracyclines. Interestingly, some resistant bacteria have an active efflux system that **can** pump the tetracycline back out of the cell.

The tetracyclines are considered relatively nontoxic. Their ability to chelate di- and trivalent cations, particularly **divalent** calcium, can be a problem during certain periods of a patient's development. Tetracyclines are **de-**

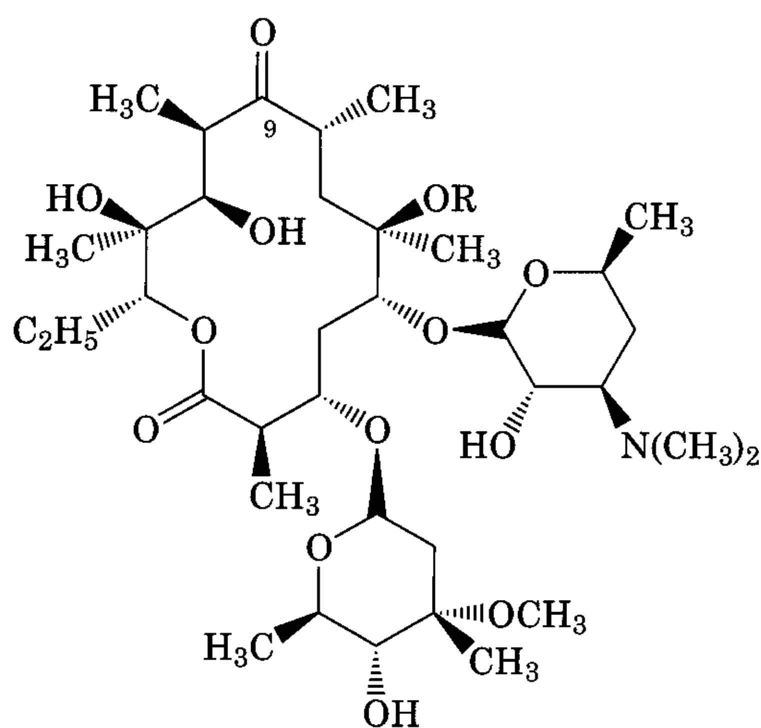


(18)

posited in the bones of fetuses and growing children. They also are deposited in the developing teeth, producing discoloration. Therefore, tetracyclines normally are **contraindicated** for infants and pregnant women, although this problem is easily handled by prescribing an alternate antibiotic.

2.3.2.4 Macrolides (Comparative Biochemistry). Erythromycin ($R = H$) **(19), clarithromycin** ($R = CH_3$) **(19),** and azithromycin (20), produced by ring expansion of erythromycin, reversibly bind to the bacterial **50S** ribosomal subunit. The result is inhibition of the growing protein chain. (Carbon 9 is labeled on erythromycin for reference.) Selective toxicity is achieved because these agents do not bind to mammalian ribosomes.

The macrolide antibiotics are not as selective as other anti-infective **drugs.** They **can** cause severe epigastric distress, possibly functioning as a **motilin** receptor **agonist,** which stimulates **gas-**



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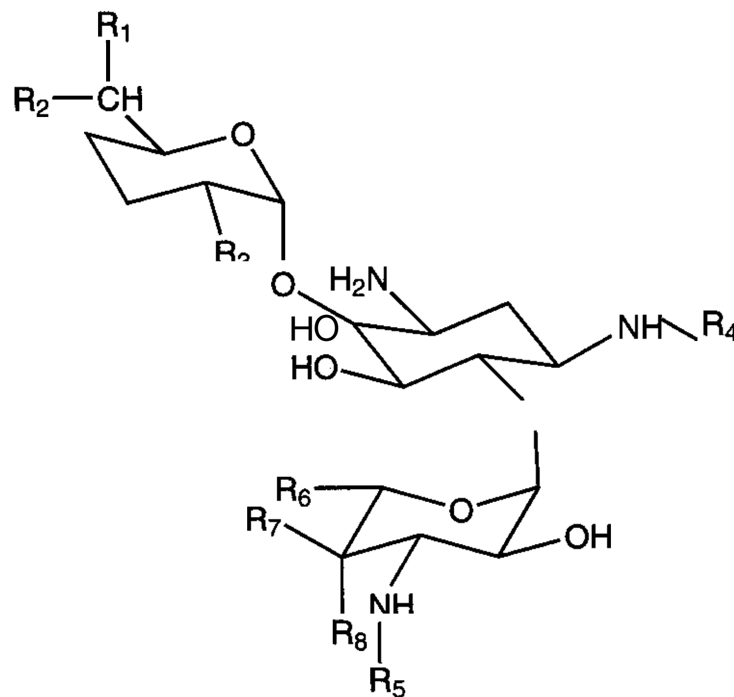
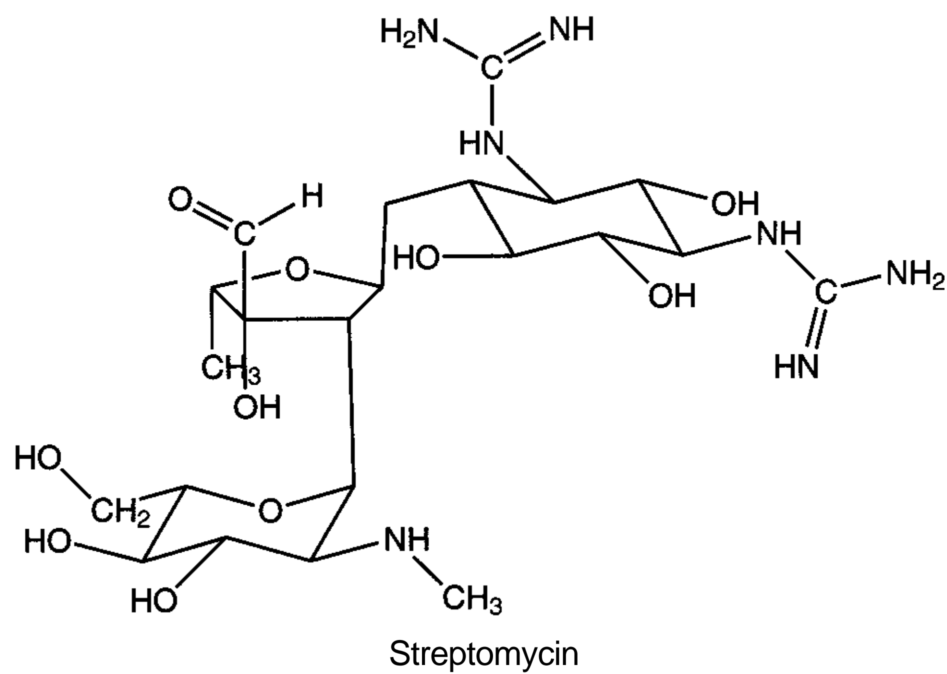
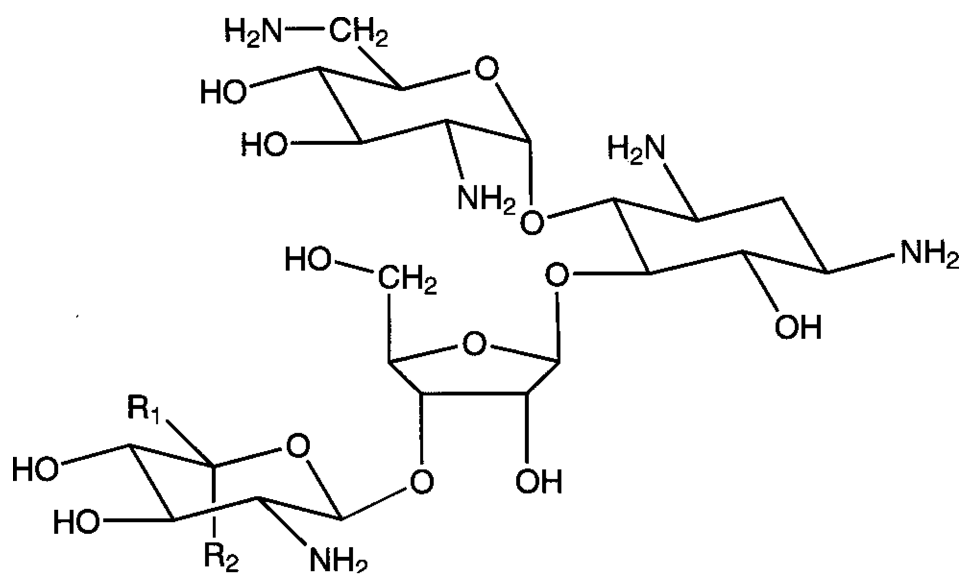


Gentamycin C_1: $R_1 = R_2 = R_5 = R_7 = CH_3$; $R_3 = NH_2$; $R_4 = H$; $R_8 = OH$
Kanamycin A: $R_1 = NH_2$; $R_2 = R_7 = H$; $R_3 = OH$; $R_4 = R_5 = R_8 = H$; $R_6 = CH_2OH$

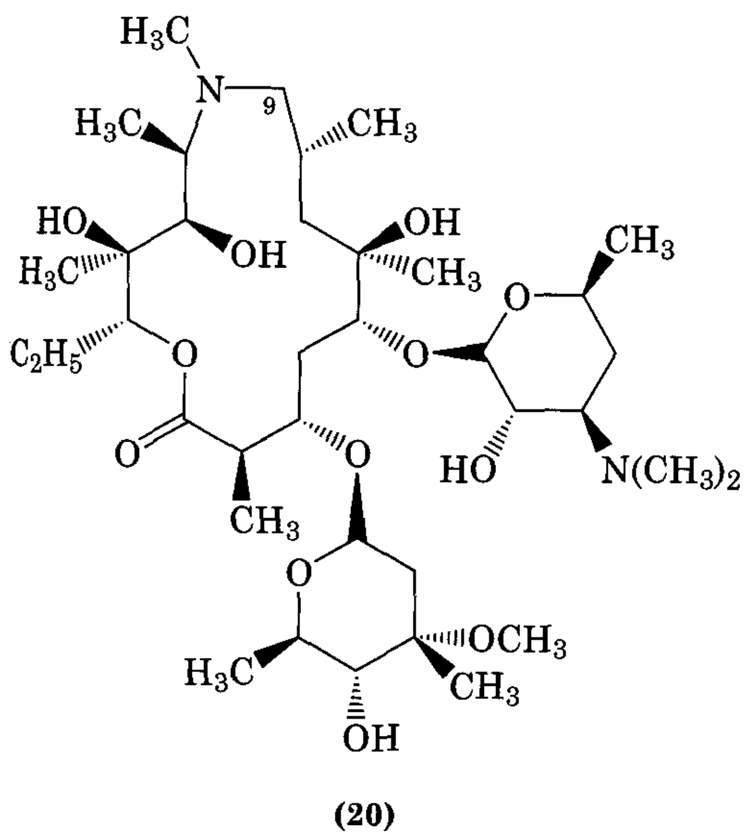


Streptomycin



Neomycin B: $R_1 = H$; $R_2 = CH_2NH_2$
Neomycin C: $R_1 = CH_2NH_2$; $R_2 = H$

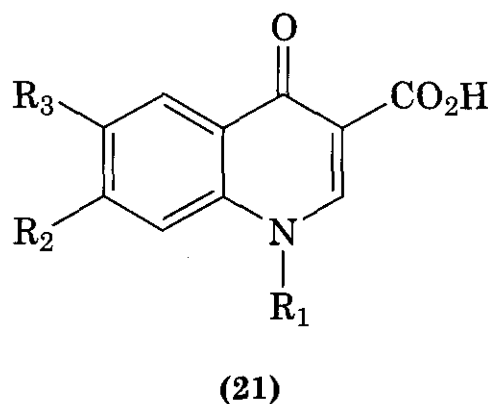
Figure 7.6. Representative aminoglycosides.



(20)

tric motility. This group also inhibits cytochrome P450 isozymes, CYP1A2 and CYP3A4. Table 7.2 lists the clinically relevant interactions. In each case, erythromycin causes an increase in serum concentrations of the drugs whose metabolism is inhibited by the antibiotic.

2.3.2.5 Quinolones/Fluroquinolones (Comparative Biochemistry). The quinolones/fluroquinolones (**21**) are one of the newer chemical classes of antibiotics and are completely synthetic. Their mechanism of action is unique among the antibiotics. The quinolone group targets two bacterial DNA topoisomerase II enzymes, also known as DNA gyrases. DNA gyrase relaxes and reforms the DNA supercoil that is necessary for DNA to first be read and then reformed during replication. Selective toxicity arises because mammalian cells do not have DNA gyrases, although they do have a topoisomerase II. The latter requires a much higher dose of quinolones for inhibition to occur. Although no group of drugs administered internally is completely nontoxic, the quinolones show good selectivity.



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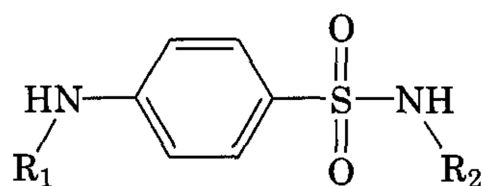
2.3.2.6 Sulfonamides/Sulfanilamides and Diaminopyrimidines (Comparative Biochemistry and Comparative Distribution). The discovery of sulfonamides (**22**) was the start of modern antibacterial chemotherapy and gave credence to the "magic bullet" ideal in drug design. The sulfonamide pharmacophore has several important applications in drug design. Although there are very few antibacterial sulfonamides still be-

Table 7.2 Erythromycin-Drug Interactions[a]

↑ Serum Levels	Remarks
CYP1A2 Inhibition	
Theophylline	Rifampin inhibits CYP1A2 metabolism of theophylline.
CYP3A4 Inhibition	
Warfarin	Recommend monitoring when beginning or stopping erythromycin.
Cisapride	Cisapride is contraindicated in patients taking erythromycin.
Alprazolam	Patients on these drugs should be monitored for increased sedation.
Diazepam	
Midazolam	
Triazolam	
Atorvastatin	Patients should be monitored for statin adverse reactions including liver and muscle damage.
Cerivastatin	
Lovastatin	
Simvastatin	
Sildenafil	Lower doses should be considered.
Astemizole	Astemizole is contraindicated in patients taking erythromycin.
Cyclosporine	Serum levels should be monitored.

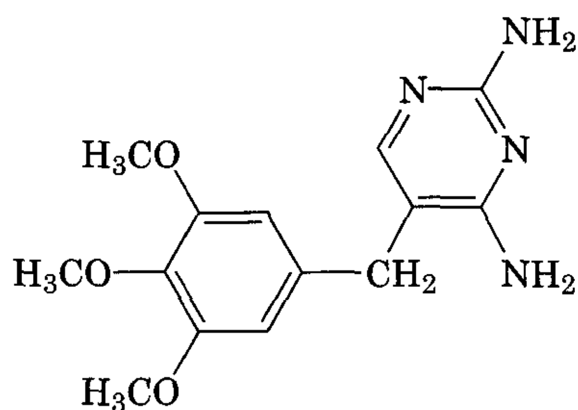
[a]*Pharmacists Letter, Document* 150401 (2001).



(22)

ing used in medicine, the sulfonamide moiety is found in diuretics (furosemide, thiazide, and thiazide-like, carbonic anhydrase inhibitors) and oral hypoglycemics (chlorpropamide, glyburide, glipizide, glimepiride, and other sulfonylureas).

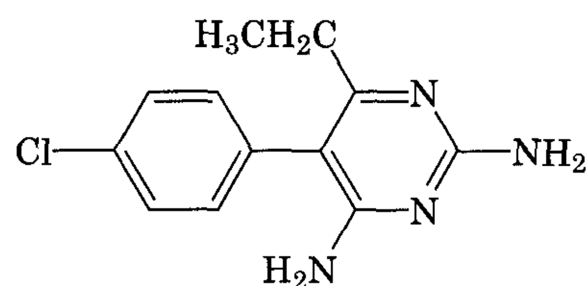
The sulfonamides are selective for a key reaction found only in bacteria. Most bacteria synthesize their own folic acid. In contrast, humans obtain their folic acid from food and vitamin supplements. Therefore, in bacteria sulfonamides block folic acid biosynthesis by competitive inhibition of dihydropteroate synthase, which is the enzyme used by bacteria to incorporatep-aminobenzoic acid to form dihydropteroic acid (Fig. 7.7). Upon the addition of glutamic acid to the latter, the bacteria synthesize dihydrofolic acid (FAH_2, FH_2, DHF). A second antibiotic, trimethoprim (**23**), selectively inhibits bacterial dihydrofolate reductase. As long as the patient's folic acid status is adequate, there is minimal metabolic toxicity from the sulfonamides or trimethoprim.



(23)

In contrast with these two antibacterial antibiotics, methotrexate (24) is one of the most used cytotoxic drugs for malignancies and, in lower doses, an immunosuppresive in autoimmune diseases (i.e., psoriasis and rheumatoid arthritis). Methotrexate inhibits mammalian dihydrofolate reductase (DHFR), which is found in every cell that uses one of the coenzyme forms of folic acid. Because of its poor comparative biochemical selectivity, it is common to administer one of the tetrahydrofolates as an antidote for methotrexate toxicity.

Dihydrofolate reductase also is a potential site for antifungal antibiotics. The problem was to find a drug that is selective for the fungal version of this enzyme. The result was pyrimethamine (**25**), which shows a preference for plasmodia dihydrofolate reductase relative to the mammalian enzyme.



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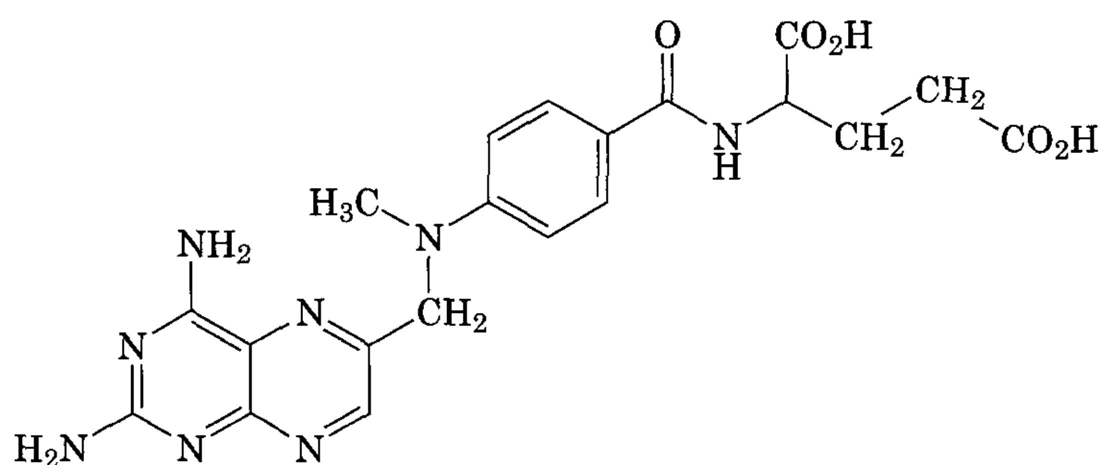
2.3.3 Examples Based on the Target Organism. When the pathogen's biochemistry and cell structure becomes more mammalian-like, it becomes more difficult to target a specific site that is significantly different from that found in the host. In some cases, the pathogen



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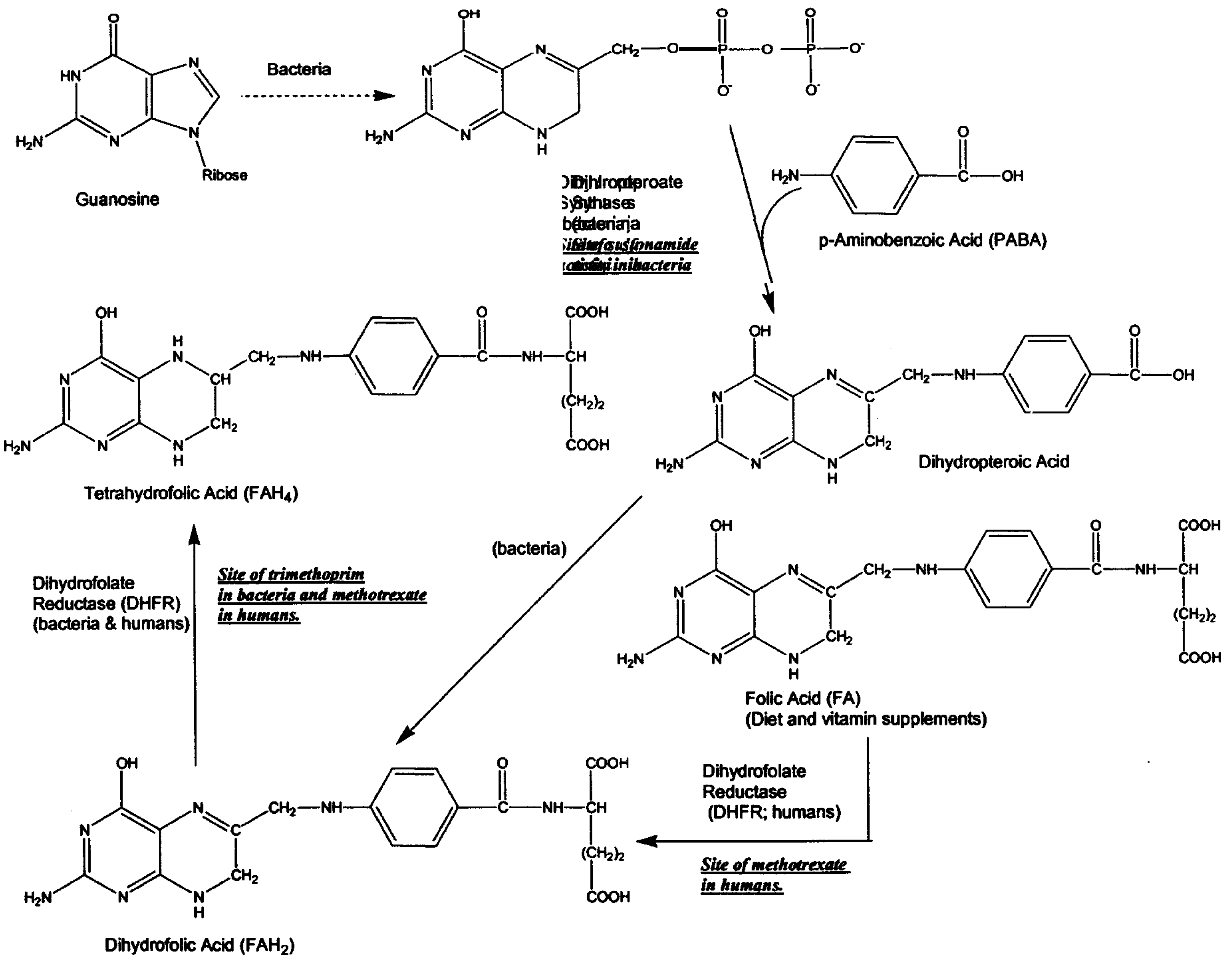
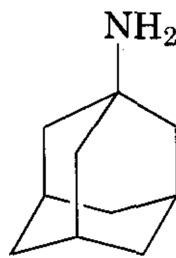


Figure 7.7. Sites of inhibitors of folate biosynthesis.

is able to hide from the host's immune system. This creates a problem getting the drug to the microorganism, which leads to reduced selectivity.

2.3.3.1 Antiviral Drugs (Comparative Biochemistry). Viral biochemistry duplicates much of the host's biochemistry because it is a complete parasite. A virus cannot reproduce unless it is inside the host's cells where it uses many, if not all, of the cell's DNA and RNA polymerases, ribosomes, and t-RNAs. In contrast with bacteria where most antibiotics are effective against a wide variety of microorganisms, antiviral drugs are specific for narrow virus types. This is because the virus biochemistry tends to be specific for the virus type. Amantadine (26) and rimantadine (27) only block the uncoating of Influenza A, whereas oseltamivir (28) and zanamivir (29) inhibit viral neuraminidase found in influenzas A and B. Both sets of antiviral drugs show good selectivity for the virus.



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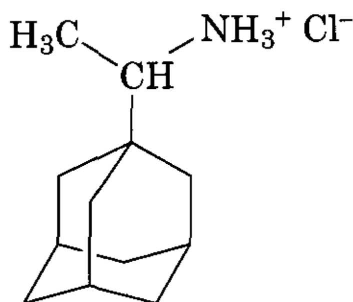
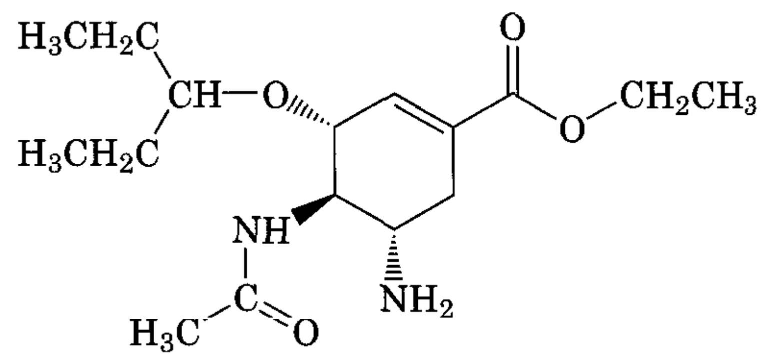


(27)

In contrast, the large number of drugs used to treat human immunodeficiency virus infections target enzymes (RNA-dependent DNA polymerase and HIV protease) specific to this virus and, in general, show poor selectivity for a variety of reasons. See Table 7.3 for examples and the antiviral chapter for more information.

2.3.3.2 Anfimycobacferial Drugs (Comparative Biochemistry and Comparative Cytology).



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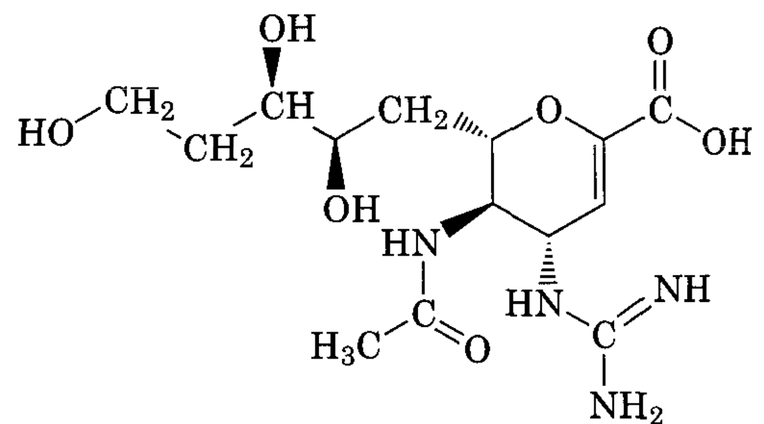


(29)

Mycobacteria-caused diseases are difficult to treat because the bacterium's complex outer membrane makes it difficult to get the drug inside the microorganism. It also is very slow growing, making it less susceptible to drugs that will stop the mycobacterial's reproduction: One of the standard drug treatments is isoniazid (30), which, after oxidative activation, inhibits mycolic acid biosynthesis. Mycolic acid is an essential part of the mycobacterium's cell wall. In contrast with the β-lactam antibiotics that show excellent selectivity, isoniazid has a boxed warning regarding adverse responses involving the liver, particularly in older patients whose lifestyle may have damaged this organ. Rifampin (31) shows a significant preference for DNA-dependent RNA polymerase. Even though the selectivity for the bacterial enzyme is very good, rifampin also induces the CYP1A2 and CYP3A4 isozymes and can be the cause of clinically significant drug-drug interactions (Table 7.4) that result in decreased serum concentrations of drugs metabolized by these isozymes.

2.3.3.3 Antifungal Drugs (Comparative Biochemistry). Selective toxicity is more difficult with these targets. Most are structurally based on ergosterol, the central fungal sterol, and its

Table 7.3 Representative Antiviral Drugs Classified by Virus

Virus	Drug	Site of Activity	Remarks
Influenza Type A	Amantadine Rimantadine	Blocks uncoating or entrance of the virus into the cell	Good selectivity. Restricted to Influenza Type A virus.
Influenza Types A and B	Oseltamivir Zanamivir	Viral neuraminidase	Good selectivity. Effective against both Influenza Types A and B virus.
Herpes Simplex I and II (HSV I and II), Varicella zoster (VZV)	Acyclovir Famciclovir	Substrate for herpes thymidine kinase	Good selectivity. This group of drugs are very poorly phosphorylated by the host cell's kinases.
Human Immunodeficiency Virus (HIV)	Zidovudine Didanosine Zalcitabine Lamivudine Stavudine Abacavir Delavirdine Nevirapine Efavirenz	HIV-RNA-dependent DNA polymerase (reverse transcriptase)	Although mammals do not have reverse transcriptase, the inhibitors also inhibit the host cell's DNA-dependent DNA polymerase.
	Indinavir Ritonavir Saquinavir Nelfinavir Amprenavir Lopinavir	HIV protease	This group of drugs are very effective, but they have a large number of adverse reactions. The mechanisms of these complications are poorly understood.
Cytomegalovirus (CMV)	Foscarnet Cidofovir	Viral DNA-dependent DNA polymerase	There is a preference for the viral polymerase, but these drugs also inhibit the host cell's DNA polymerase.

biosynthesis. Because ergosterol and its biosynthesis are so similar to that of cholesterol, antifungal drugs show poor selectivity when used internally. Thus, several antifungal drugs are limited to topical use. Several of the groupings are summarized in Table 7.5. More detailed information is to be found in the chapter on antifungals.

Table 7.4 Rifampin-Drug Interactions[a]

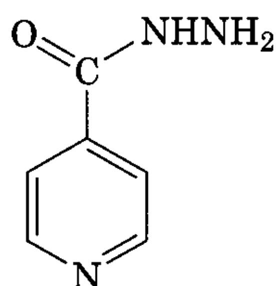
↓ Serum Levels	Remarks
CYP1A2 Induction	
Theophylline	Rifampin induces CYP1A2 metabolism of theophylline.
CYP3A4 Induction	
Warfarin	Recommend monitoring when beginning or stopping rifampin.
Phenytoin	Recommend monitoring when beginning or stopping rifampin.
Delavirdine Indinavir Nelfinavir Ritonavir Saquinavir	Depending on the clinician and the patient, rifampin can be contraindicated for these anti-HIV drugs.
Oral contraceptives	Nonhormonal methods of birth control or warn the patient to refrain from intercourse.

[a]Pharmacist's *Letter*, Document 150401 (2001).

Table 7.5 Antifungal Drugs Classified by Site of Action

Site of Drug's Action	Drug	Remarks
Ergosterol in fungal membrane	Amphotericins	Poor selectivity: Complexes with cholesterol (whose structure is very similar to that of cholesterol) in mammalian kidneys. Selectivity might be increased by forcing amphoteracin into an extended conformation.[a]
Fungal C-14α-demethylase	Ketoconazole, Fluconazole, Itraconazole, Clotrimazole, Miconazole, Terconazole	Most are limited to topical use because of poor selectivity. The same enzyme is found in the biosynthesis of cholesterol in mammals.
Fungal squalene-2,3-epoxidase	Tolnaftate, Naftifine, Terbinafine	Most are limited to topical use because of poor selectivity. The same enzyme is found in the biosynthesis of cholesterol in mammals.

[a]H. Resat, F. A. Sungur, M. Baginski, E. Borowslu, and V. Aviyente, J. *Comput.-Aided Mol. Des.*, 14,689–703(2000)



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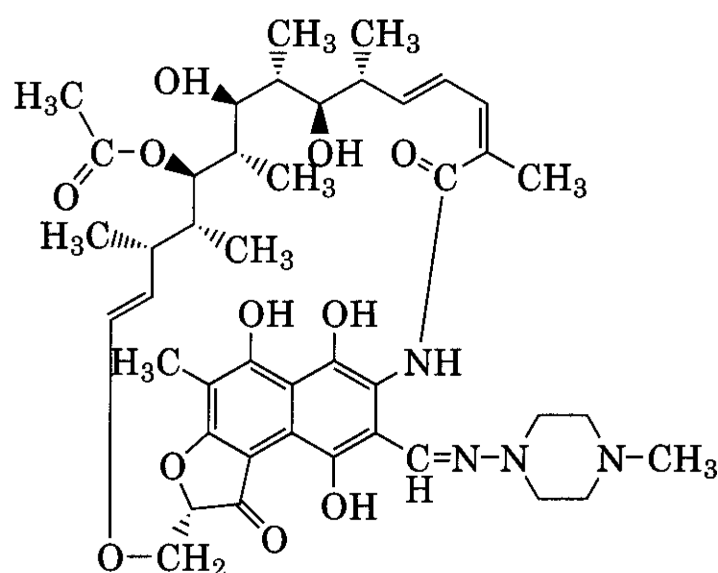


(31)

2.3.3.4 Drugs Used to Treat Parasitic Infections (Comparative Biochemistry). The selectivity for receptor sites specific to parasites generally is not good. There are problems with patient compliance because of significant adverse reactions. Drugs must be taken over long periods of time and are costly for a patient population generally living in economically deprived areas. The result is that diseases caused by parasites are difficult to treat. Malaria, the most common disease in this group, involves hundreds of millions of people. The quinine-based drugs, which are widely used to treat this disease, interfere with the last steps in the patient's heme biosynthesis that cause an increase in porphyrins, which may be toxic to the parasite. With the site of action being an essential component of the patient's metabolism, these drugs can be very toxic to the patient. See the chapter on drugs used to treat malaria and other parasitic infections for more information.

3 DRUG CHIRALITY

Many drugs are asymmetric. At least 25% are marketed as racemic mixtures (17). The United States Pharmacopeia recognizes this in defining the chemical standards for many drugs (18). Examples include:

Clomiphene Citrate, USP (Fig. 7.8)

Clomiphene citrate contains not less than 98.0% and not more than 102.0% of a mixture of the (*E*)- and (2)-geometric isomers of $C_{20}H_{28}ClNOAC_6H_8O_7$, calculated on an anhydrous basis. It contains not less than 30.0% and not more than 50.0% of the 2-isomer.

Doxepin Hydrochloride, USP (Fig. 7.9)

Doxepin hydrochloride, an (*E*)- and (*Z*)-geometric isomer mixture, contains the equivalent of not less than 98.0% and not more than

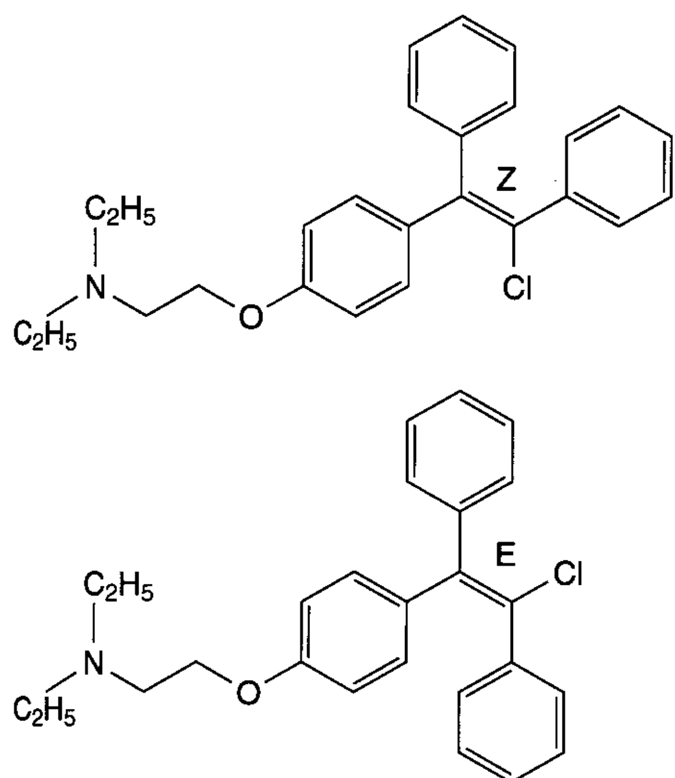
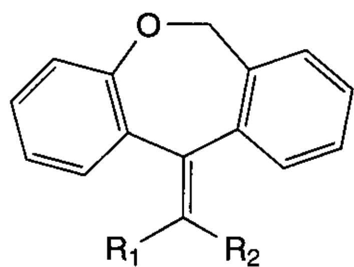


Figure 7.8. Clomiphene isomers.

102.0% of doxepin ($C_{19}H_{21}NOAHCl$), calculated on a dried basis. It contains not less than 13.6% and not more than 18.1% of the (*Z*)-isomer and not less than 81.4% and not more than 88.2% of the (*E*)-isomer.

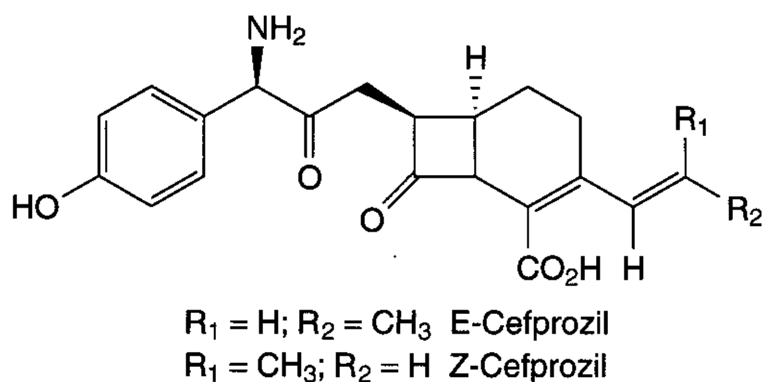
Cefprozil, USP (Fig. 7.10)

Although not specifically defined as a mixture, it is clear from the assay procedure that it is a mixture. The chromatographic procedure requires preparation of a resolution solution consisting of a mixture of equal volumes of the Cefprozil (2)-isomer standard preparation and of the stock solution used to prepare the Cefprozil (*E*)-isomer standard preparation. The final line in the assay reads, "Calculate the quantity, in micrograms of cefprozil ($C_{18}H_{19}N_3O_3S$) in each mg of the Cefprozil taken by adding the values, in micrograms per mg, obtained for the cefprozil (2)-isomer and for cefprozil (*E*)-isomer."



$R_1 = CH_2CH_2N(CH_3)_2$; R_2 = H Z-Doxepin
R_2 = H; $R_2 = CH_2CH_2N(CH_3)_2$ E-Doxepin

Figure 7.9. Doxepin isomers.



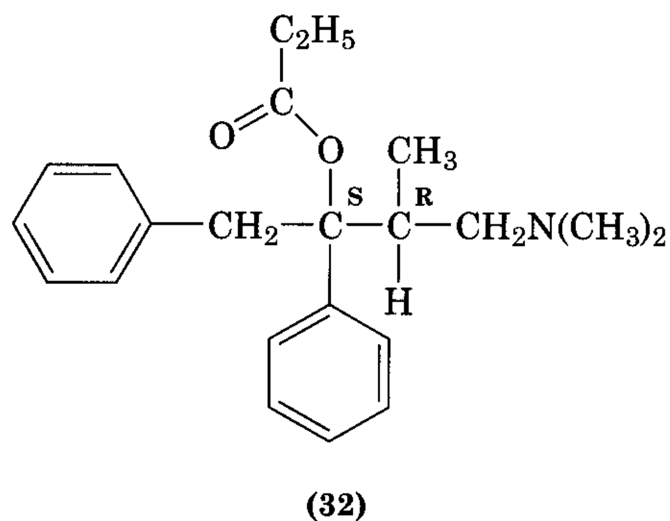
R_1 = H; $R_2 = CH_3$ E-Cefprozil
$R_1 = CH_3$; R_2 = H Z-Cefprozil

Figure 7.10. Cefprozil isomers.

It now is realized that many times only one isomer contains the pharmacological activity. The routes of a drug's metabolic degradation also may vary with the isomer. In a few cases, the adverse responses may be stereoselective. Here are a few representative examples in which stereoselectivity can be significant (19).

3.1 Propoxyphene

Dextropropoxyphene [Darvon, (**32**)] is marketed as an analgesic, and levopropoxyphene (**Novrad**) as an antitussive (33). (Note that the



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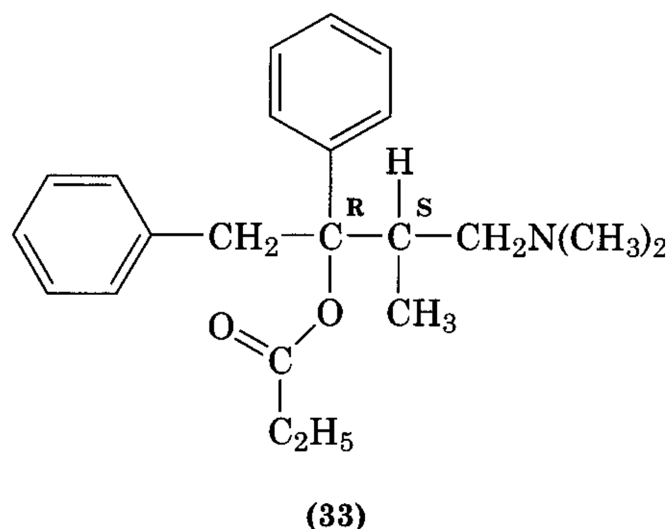
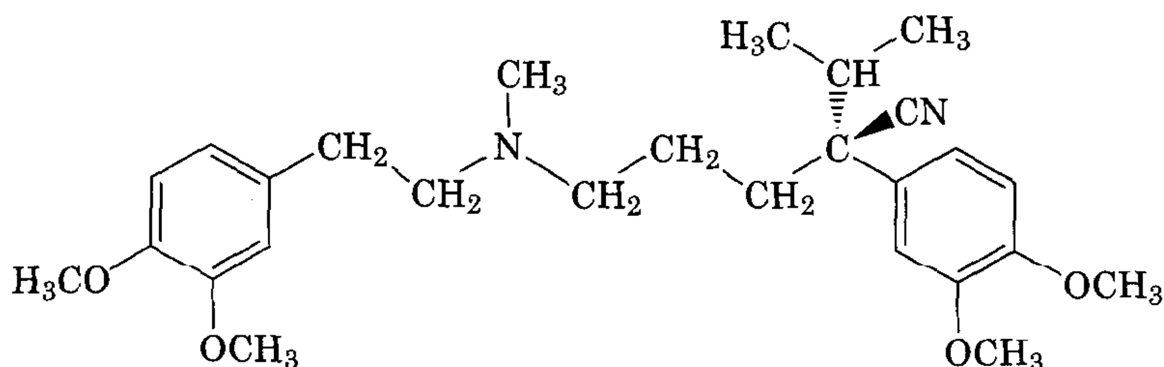


(33)

brand name *Novrad* is Darvon spelled backward and, therefore, the mirror image of Darvon.)

3.2 Verapamil

This calcium channel antagonist illustrates why it is difficult to conclude that one isomer is superior to the other. S-Verapamil **(34)** is the more active pharmacological stereoisomer than the less active R-verapamil (**35**), although the former is more rapidly metabolized by the first-pass effect. (First-pass refers to orally administered drugs that are extensively metabolized as they pass through the



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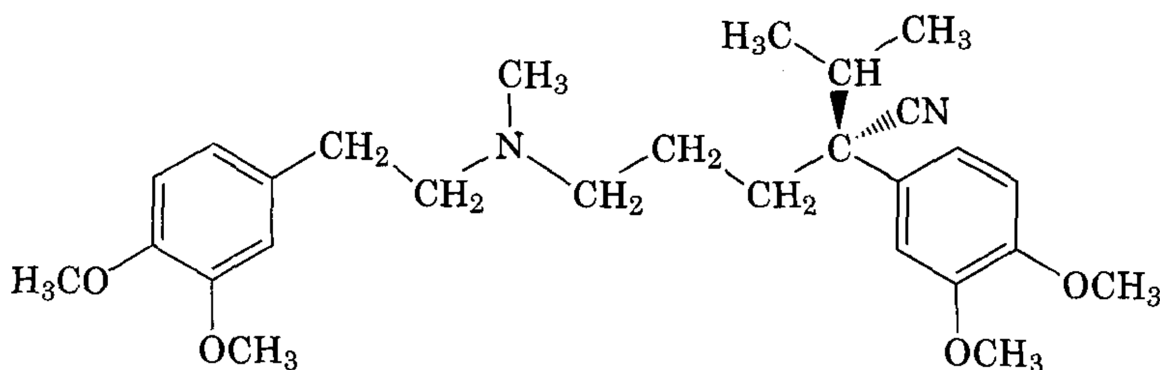

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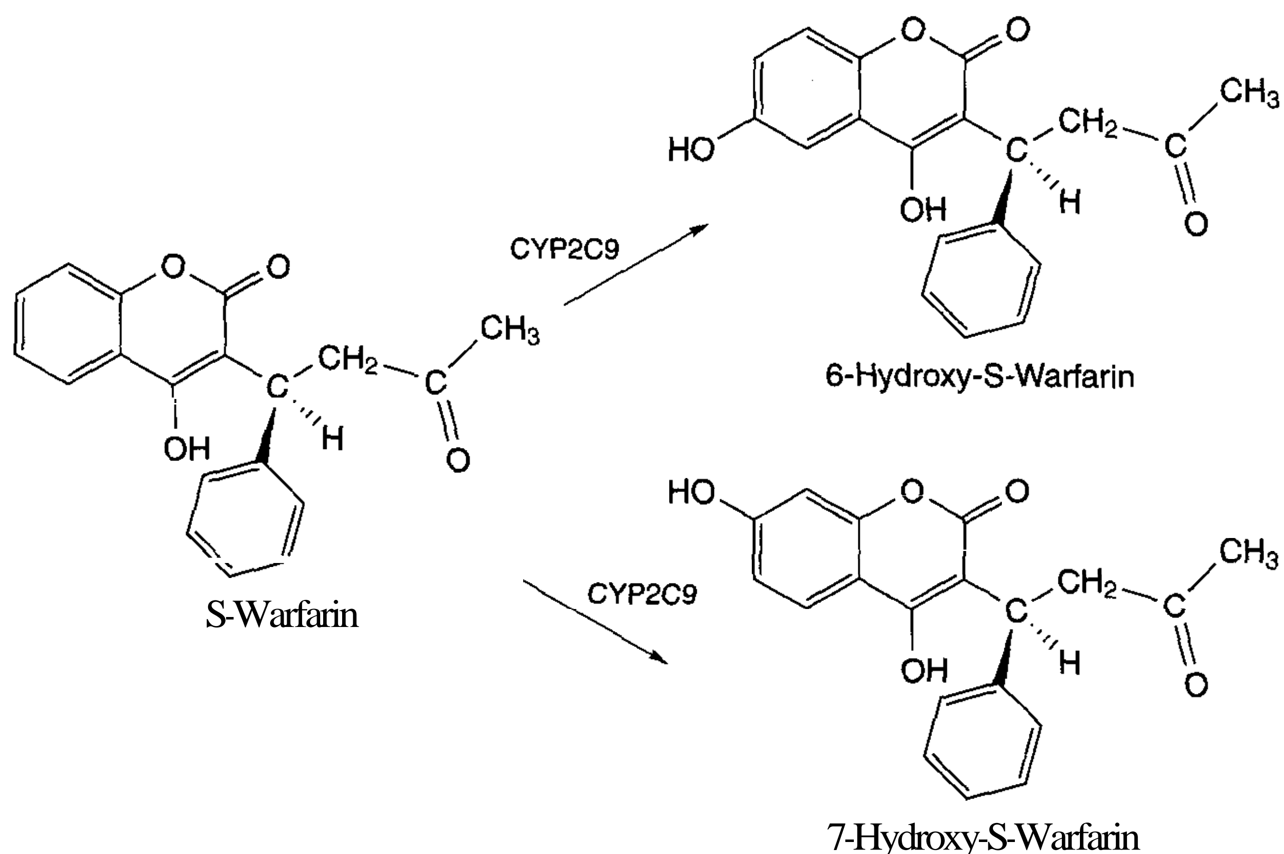


Figure 7.11. CYO450 2C9 metabolism of **S-warfarin.**

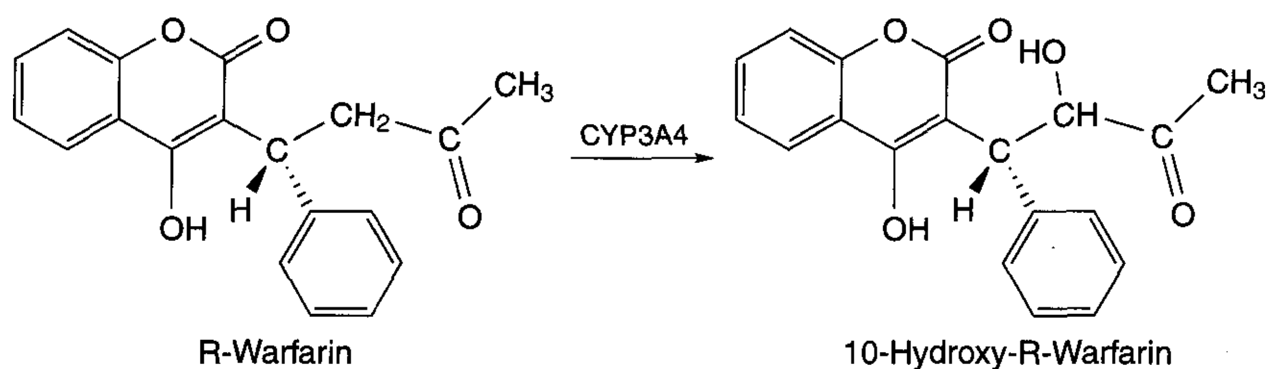


Figure 7.12. CYP450 3A4 metabolism of R-warfarin.

liver. It is not as significant when the drug is administered parenterally because the drug is dispersed before reaching the liver.) Therefore, intravenous administration of the racemic mixture of verapamil produces a longer duration of action than when administered orally because the more potent S-isomer will be metabolized more slowly.

3.3 Warfarin

The S-isomer of warfarin is more active and is metabolized by the CYP450 2C9 (CYP2C9) isozyme (Fig. 7.11), whereas the R-isomer is metabolized at a different position by CYP450 3A4 (CYP3A4) (Fig. 7.12) (20–22). The fact that two different CYP450 isozymes are required for warfarin metabolism and polymorphism is seen with CYP2C9 increases the chances of drug-drug interactions with this potent anticoagulant. Indeed, the package insert lists 22 different pharmacological classes of drugs that can alter warfarin's pharmacological response, as measured by prothrombin timelinternational normalized ratio (PT/INR). Specific examples are found in Table 7.6.

3.4 Other Examples

There are many synthetic products where the drug is marketed as a specific stereoisomer. Early SAR studies on the phenethyl amines showed that the substituent stereochemistry on both the a-carbon (amine carbon) and β-carbon (benzylic carbon) is crucial. Dextrorotatory substituents on the β-carbon increase central activity relative to that of the enantiomeric levorotatory analog. In contrast, levorotatory substituents on the β-carbon increase peripheral activity relative to that of the dextrorotatory analog. As already mentioned, the dextrorotatory opiates have antitussive activity and low abuse potential whereas the levorotatory opiates are analgesics with such high abuse potential that most are classified as Schedule II drugs by the Drug Enforcement Agency. Sometimes the generic name indicates the specific isomer: dextroamphetamine (Fig. 7.13), dexamethasone (**36**), dextromethorphan (**6**), levamisole (**37**), levobupivacaine (**38**), levothyroxine (Fig. 7.13), and levodopa (Fig. 7.13).

Table 7.6 Potential Inhibitors and Inducers of Warfarin Metabolism[a]

Warfarin Isomer	CYP3A4 Inducers	CYP3A4 Inhibitors
R-warfarin	Carbamazepine, phenobarbital, dexamethasone, nevirapine, phenytoin, rifabutin, rifampin	Amiodarone, diltiazem, erythromycin, azole antifungals, norfloxacin, zafirlukast, zileuton
	CYP2C9 Inducers	CYP2C9 Inhibitors
S-warfarin	Carbamazepine, phenobarbital, phenytoin, primadone, rifampin	Amiodarone, cimetidine, azole antifungals, fluoxetine, isoniazid, sertraline, zafirlukast

[a]*Pharmacists Letter, Document* 150401 (2001).

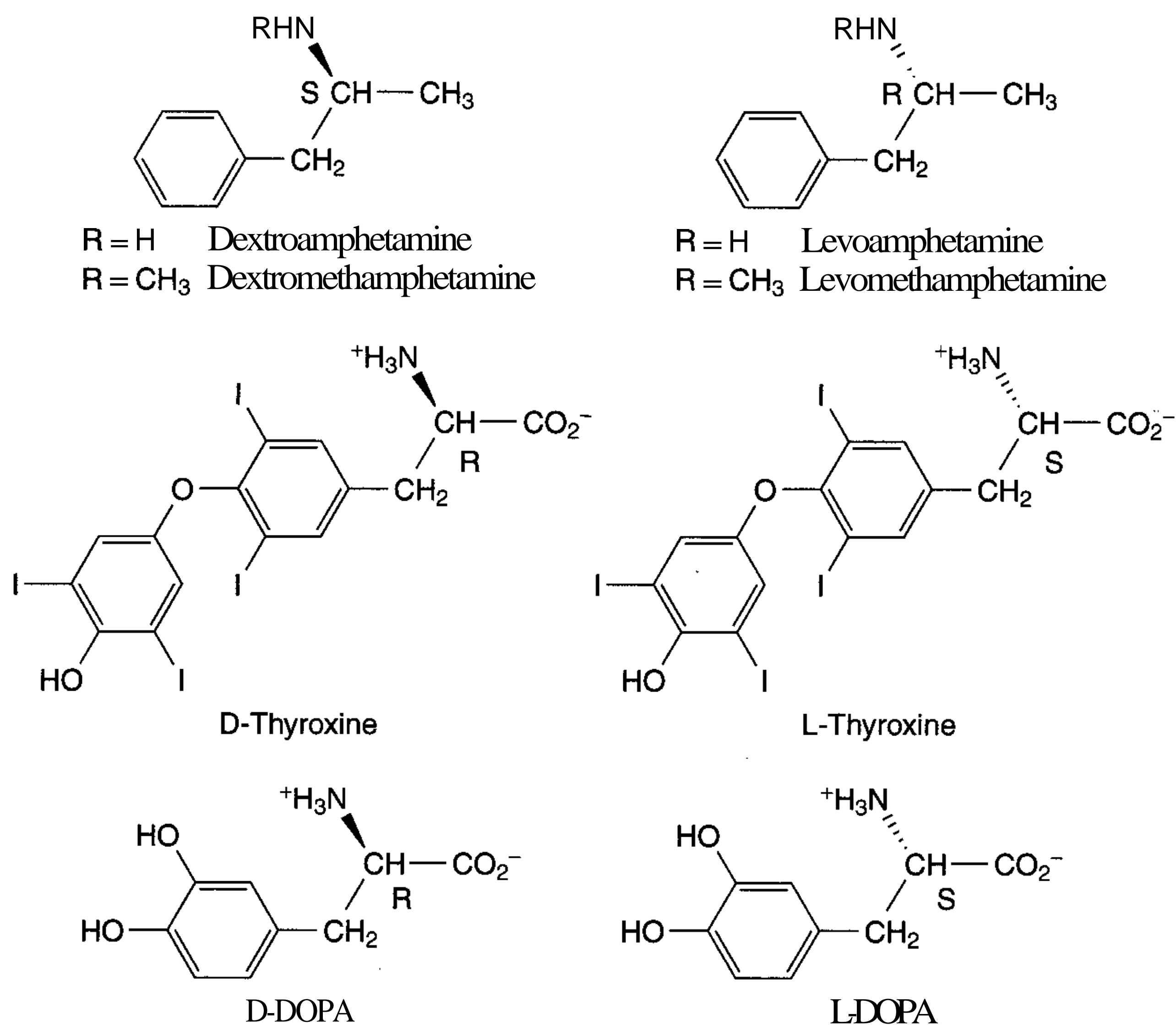
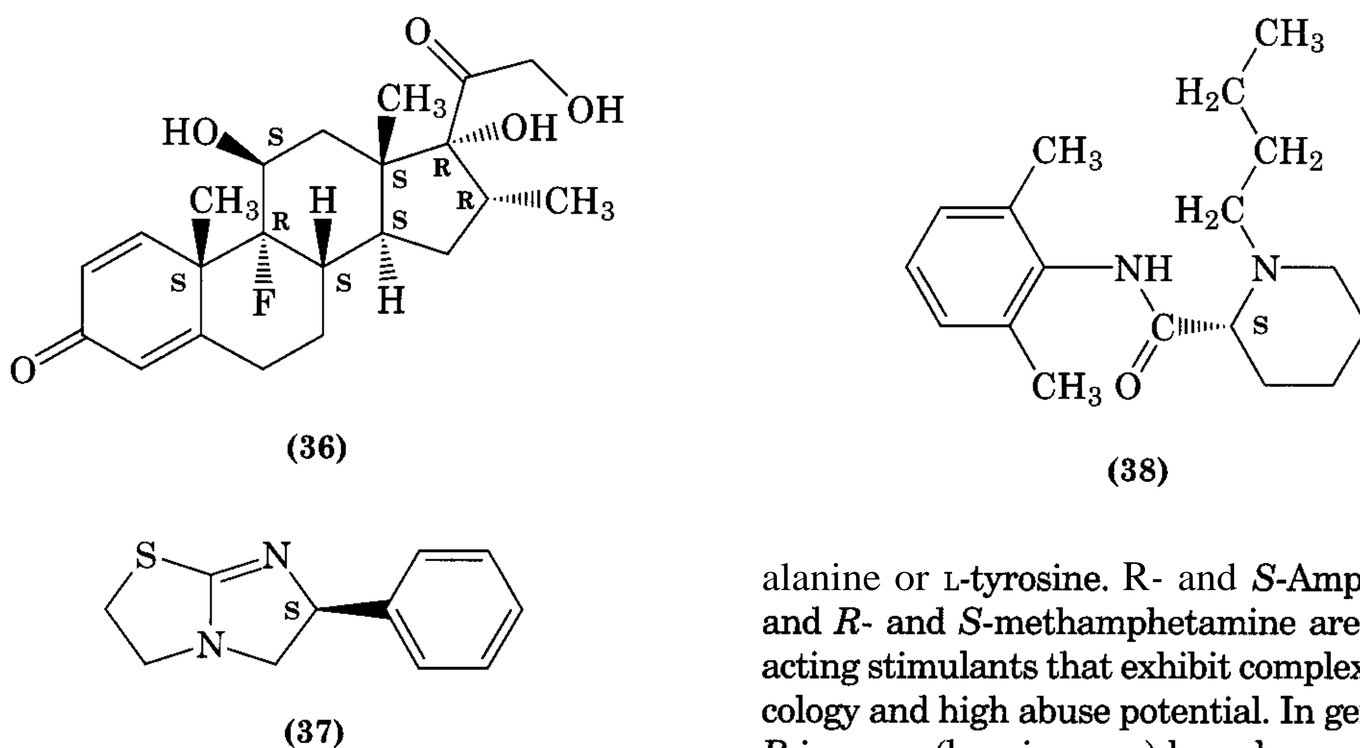


Figure 7.13. Stereoisomers based on L-phenylalanine.



Other than its use as an analytical tool, the optical rotation does not provide information as to the molecule's shape, which, of course, determines fit at the receptor. Figure 7.13 contains drawings for a group of drugs based on L-phenylalanine or L-tyrosine. R- and *S*-Amphetamine and *R*- and *S*-methamphetamine are centrally acting stimulants that exhibit complex pharmacology and high abuse potential. In general, the *R*-isomers (levo isomers) have less central nervous system (CNS) activity but stronger cardiovascular effects compared to that of their *S*-isomers (dextro isomers).

Steric differences in the two thyroxine isomers also cause differences in biochemical response. Both *S*- and *R*-thyroxine bind to thy-

roglobulin. The S-isomer (L-thyroxine) has about six times the antigoiter activity relative to that of R-thyroxine (D-thyroxine). The difference is more pronounced with the triiodothyronine isomers. S-Triiodothyronine has 13 times the antigoiter activity relative to that of R-triiodothyronine (23, 24). The fact that *R*-thyroxine is not devoid of hormonal activity is evidence for its being contraindicated in euthyroid patients who were prescribed the drug for hypercholesterolemia. (R-Thyroxine is no longer sold in the United States.)

Finally, there is L- or D-DOPA. This drug is the natural metabolite from hydroxylation of L-tyrosine. Keeping in mind that some peptide antibiotics contain D-amino acids, one would not expect D-DOPA to be a good substrate for DOPA decarboxylase. Further, repeated studies show that D-DOPA (R-isomer) does not have significant neurotrophic effects (25).

Levamisole [S-isomer; (**37**)] is an interesting drug. Its original indication was the treatment of worms in animals, possibly by blocking the parasite's cholinergic receptors. Later it was found that levamisole restores the immune response in humans when given with the anticancer drug 5-fluorouracil for the treatment of certain cancers of the colon. In contrast the R-isomer (dexamisole) shows little anthelmintic activity (26, 27).

A recent drug is the local anesthetic, levobupivacaine, the S-isomer (**38**) of bupivacaine. Many of the local anesthetics affect the neurons servicing the cardiac muscle. Sometimes this can be useful for the treatment of cardiac arrhythmias. On the other hand, particularly when administered in large amounts such that plasma levels become significant, some local anesthetics may cause depression of the myocardium, decreased cardiac output, heart block hypotension, bradycardia, and ventricular arrhythmias. Bupivacaine has been resolved with the S-isomer showing less cardiotoxic responses but still good local anesthetic activity (28).

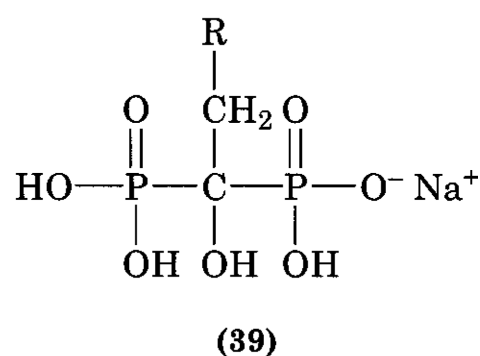
4 ADDITIONAL PHARMACOLOGICALLY ACTIVE AGENTS

See the specific chapters for more complete discussions for each of the following pharmacological groups.

4.1 Bisphosphonates *(Comparative Distribution)*

Osteoporosis is an increasing problem in an aging population. Its mechanism is very complex because bone metabolism is intricate. Besides its structural or support role, bone can be considered as the body's calcium reservoir used to maintain calcium homeostasis. That means that there has to be a means for calcium to be stored as hydroxyapatite when not needed and removed from bone as blood levels begin to decrease. For purposes of this discussion, consider that osteoporosis results when the osteoblast (bone-forming) and osteoclast (bone-resorption) cells are not in balance.

Two widely used drugs (**39**), alendronate (R = 2-n-propylamine; **Fosamax**) and risedronate (R = 3-pyridyl; Actonel), used for reducing the rate of calcium loss from bone tissue, belong to the bisphosphonates class. Both are administered orally and are widely distributed in the tissues and then concentrate onto the hydroxyapatite, where there are osteoclast cells. Although the mechanism of their toxicity is not understood, both drugs can cause local irritation and, for some patients, actual damage to the gastric linings. Even without damage, patients can experience severe gastric pain such that adherence is poor. Patients are advised to take either drug in an upright position with a full glass of water in the morning, 30 min before eating breakfast. It must be remembered that the bisphosphonates will be taken by the patients for the rest of their lives or until a better drug is discovered. Of the two drugs, risedronate appears to exhibit less adverse reactions, but it contains the same warning regarding taking it in an upright position with a glass of water. The selectivity of the current bisphosphonates is poor.



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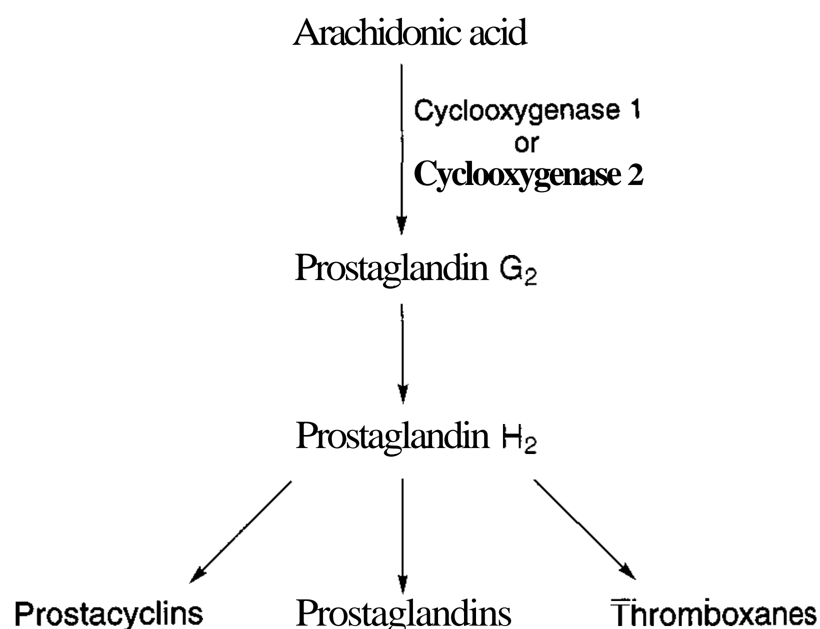
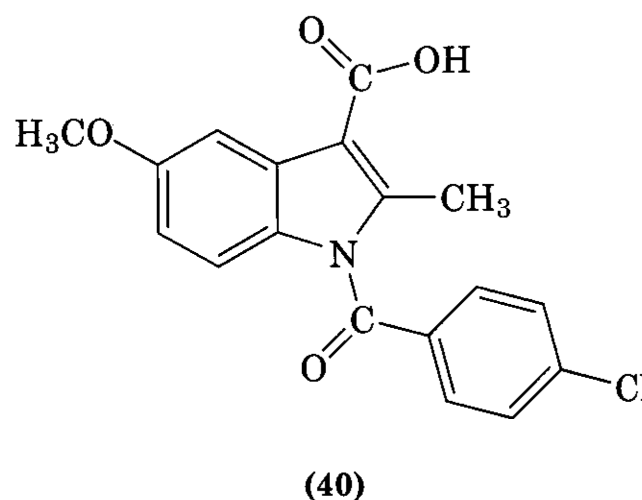


Figure 7.14. End products from the cyclooxygenase cascade.

4.2 Inhibitors of Cyclooxygenase I (COX-1) and II (COX-2) (Comparative Biochemistry and Comparative Distribution)

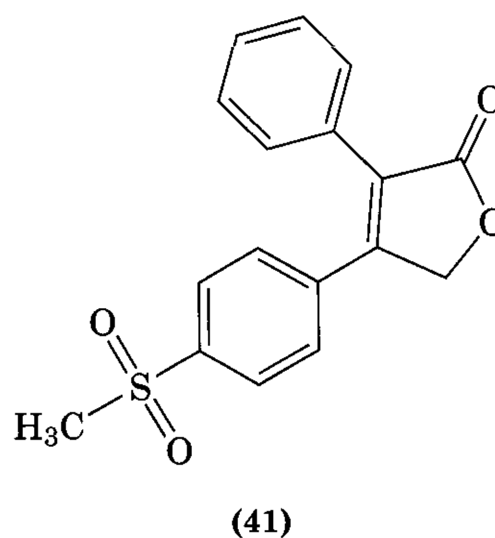
Beginning with aspirin through today's nonsteroidal anti-inflammatory drugs (NSAIDs), the cyclooxygenase inhibitors have been very beneficial for the control of inflammation and fever. It now is realized that COX exists in at least two isozyme forms. COX-1 is constitutive and is found in many tissues. Its inhibition can reduce the integrity of the tissues where inhibition of COX-1 occurs. Note the outline shown in Fig. 7.14. Inhibition of the COX enzymes will reduce levels of **prostacyclins**, prostaglandins, and **thromboxanes**. Examples include the gastric lining and the **kidney** tubules. Although the NSAIDs are reasonably specific for cyclooxygenase, this enzyme is located at the beginning of an essential set of reactions, producing a diverse group of active compounds. There is an important difference between COX-1 and COX-2. Whereas COX-1 is constitutive, COX-2 is induced in tissues where inflammation is occurring. Therefore, selectivity is increased by designing NSAIDs that preferentially inhibit COX-2 in tissues experiencing inflammation without affecting COX-1 in healthy tissue (29, 30).

Fortunately, there are sufficient differences in the receptor sites of these two isozymes that compounds with reported good **selectivity** can be designed. The NSAIDs such **as indomethacin** (Indocin), (40) that inhibit COX-1 have a carboxylate group that binds to the **guanidium** residue of an **arginine.** COX-2 has a valine where



(40)

COX-1 has a **bulkier isoleucine.** The latter's larger hydrophobic side **chain** blocks entrance to a pocket that both enzymes contain. The net result is that the older **acidic** NSAIDs **preferentially** bind to COX-1 and the newer COX-2 **inhibitors, rofecoxib** [**Vioxx,** (**41**)] and **celecoxib** [**Celebrex,** (**42**)], do not bind well to the COX-1 site because the **bulky** aromatic **ring** on the COX-2 inhibitors cannot enter the cleft "guarded" by COX-1's isoleucine.



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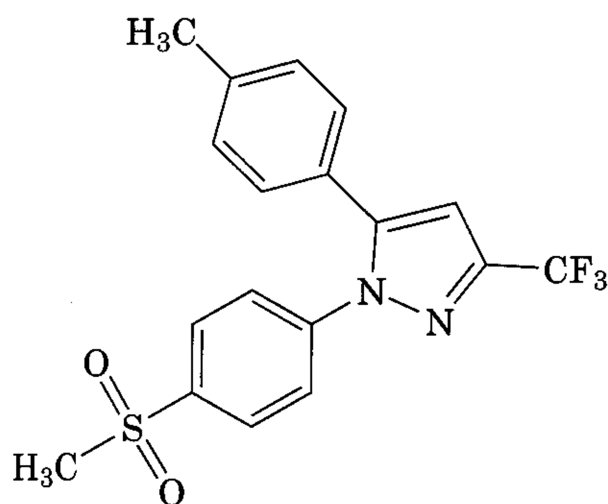

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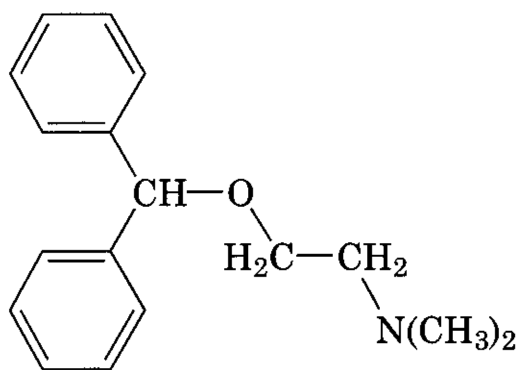
4.3 Antihistamines *(Comparative Biochemistry* and *Comparative Distribution)*

With two distinct classes of receptors and significant differences in their locations, antihistamines have good to excellent selectivity. First, there are the **H1** and H2 receptor classes. Histamine is produced by a variety of cells. Patients taking the **H1** antihistamines do so in response to the release of histamine from mast cells. These are located in the respiratory passages, skin, and gastrointestinal tract and are the cause of what patients refer to as an allergic response. The "first generation of H1 antihistamines" represented by **di**phenhydramine [Benadryl, (**43**)] and **chlor**-



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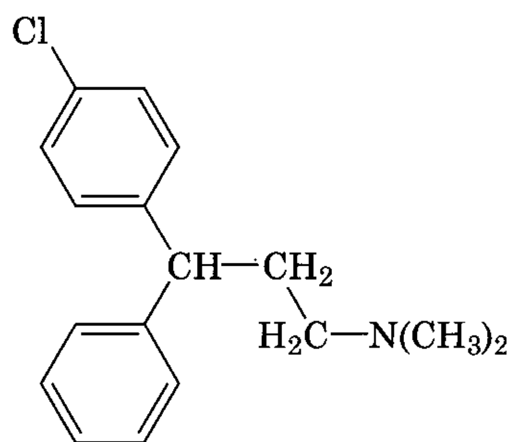
pheniramine [Chlor-Trimeton, (**44**)] are effective, but they cross the blood-brain barrier, causing mild to significant sedation. Indeed, some of the early antihistamines are used as nonprescription sleep aids. They also could show anticholinergic effects. Sometimes the combination of crossing the blood-brain barrier and anticholinergic activity was put to good use in antinausea drugs, particularly when caused by motion sickness. The familiar



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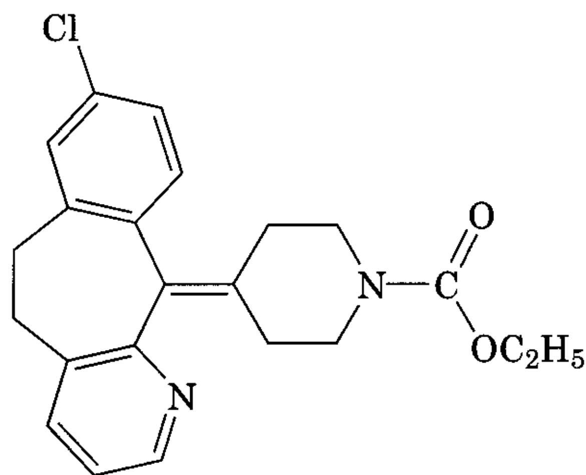
Dramamine is the chlorotheophylline salt of diphenhydramine (43). Thus, useful as these products are, the first generation of antihistamines show poor selectivity.

In contrast, the second generation of H1 antihistamines, loratidine [**Claritin,** (**45**)] and fexofenadine [**Allegra,** (**46**)] show better **selec**-



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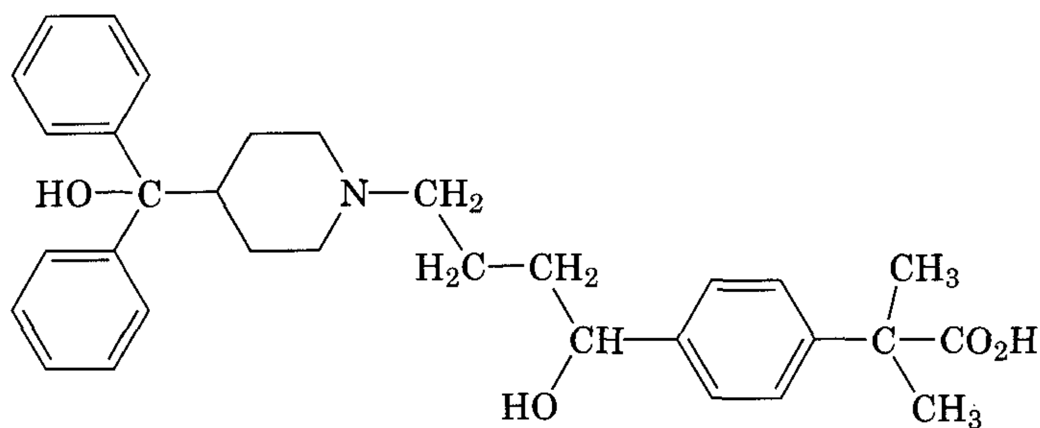
tivity because they are less likely to cross the blood-brain barrier, and, therefore, preferentially inhibit peripheral **H1** receptors. **As** can be seen from their structures, they tend to be larger molecules. Nevertheless, their distribution is not an either-or situation. The **second**-generation **H1** antihistamines still have central effects.

The H2 antihistamines, or blockers, are mainly used to reduce the secretion of gastric HCl. Their structure-activity relationships are significantly different from those of the **H1** antagonists. Using cimetidine [Tagamet, (**47**)] as the prototypical molecule, H2 antagonist structure-activity relationships are based on the histamine structure. The selectivity of this group of drugs for the H2 receptor in the **gas**-



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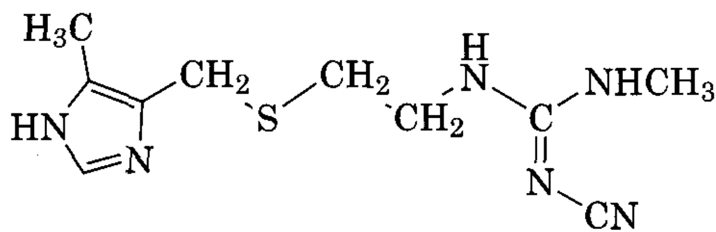
tric lining is remarkable when one considers how widely distributed are the H2 receptors. Although no drug is without adverse reactions, the H2 antagonists are approved for nonprescription use.



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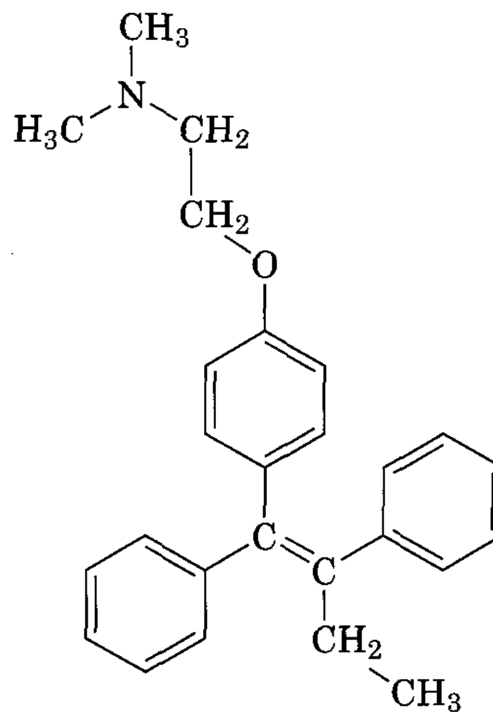

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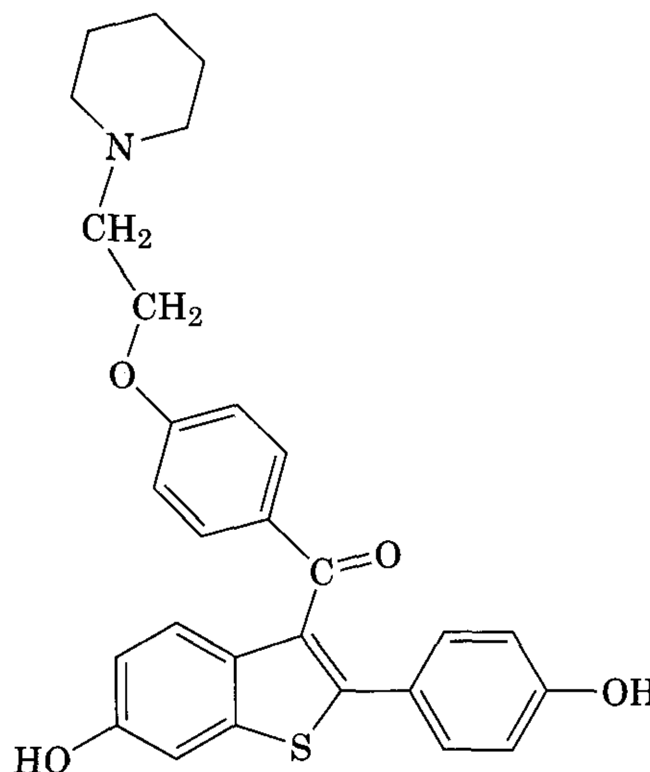
4.4 Selective Estrogen Receptor Modulators (Cornparative Biochemistry)

The pharmacology of the selective estrogen receptor modulators (**SERMs**) is complex (see the chapter covering this group). They are not simple agonists or antagonists. Rather they can be considered variable agonists and antagonists. Their selectivity is very complex because it is dependent on the organ where the receptor is located.

This complexity can be illustrated with **tamoxifen** [Nolvadex, (**48**)], which is used for estrogen-sensitive breast cancer and reducing bone loss from osteoporosis (**31**). Prolonged treatment, however, increases the risk of endometrial cancer. Thus, tamoxifen is an estrogen antagonist in the mammary gland and an agonist in the uterus and bone. In contrast, raloxifene [**Evista**, (**49**)] does not appear to have many agonist properties in the uterus, but like tamoxifen is an antagonist in the breast and agonist in the bone.



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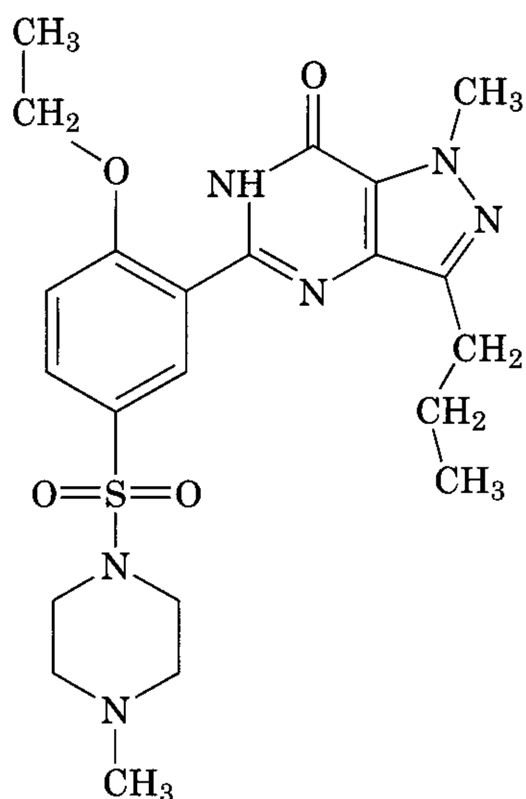
4.5 Phosphodiesterase Inhibitors

Inhibitors of phosphodiesterase type 5 have an important role in maintaining a desired lifestyle: treatment of erectile dysfunction caused by a variety of conditions. Originally developed for the treatment of angina (and not effective for this purpose), male test subjects reported the ease of having an erection, and the rest is history (32). **A** complex mechanism is involved. Nitric oxide (NO) activates **guanylate** cyclase, forming cyclic GMP (**cGMP**), which is hydrolyzed by a phosphodiesterase. Sildenafil [Viagra, (**50**)] and the newer compounds, cialis (51)and **vardenafil** (**52**), inhibit the phosphodiesterase. The selectivity is good, but it must be remembered that cyclic GMP, like cyclic AMP, is ubiquitous and, therefore, the phosphodiesterases required to hydrolyze these chemical transmitters are also ubiquitous. There are a wide variety of **phosphodiesterase** isoforms and their distribution tends to be uneven. In other words, phosphodiesterase type 5 tends to be found in the corpus **cavernosum** and type 6 in the retina. Depending on the organ and enzyme isoform, sildenafil shows a 10 to 8500 times preference for the type 5 isoform (33, 34). More information on this group of drugs is found in another chapter of this series.



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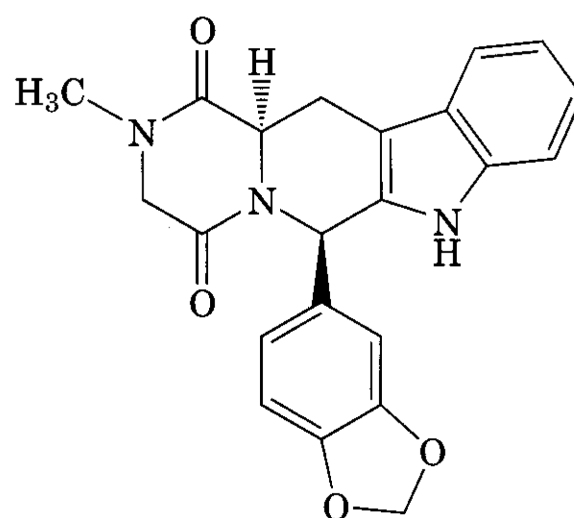

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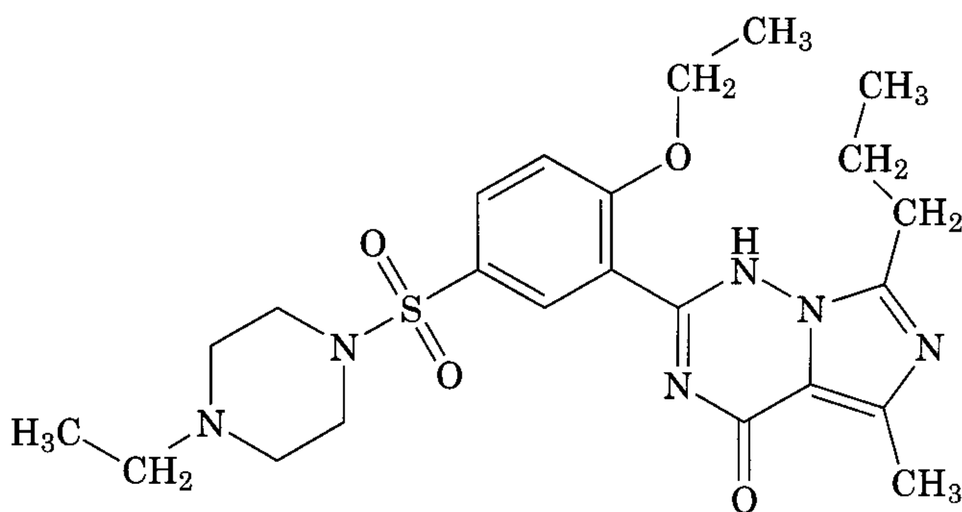
5 INSECTICIDES

This chapter closes with a brief description of the type of selectivity seen with insecticides. Today an insecticide must show good selectivity by targeting the insect and not affect the plant or mammal consuming the plant. Ideally, exploiting the principle of comparative biochemistry would be the goal, but many

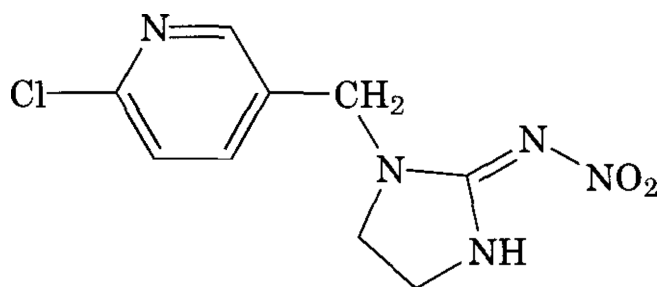


(51)

times the principle of comparative distribution is used by designing an insecticide to be nonsystemic and remain on the surface of the plant. Usually it can be washed off. Alternatively, a systemic insecticide is desired to kill sucking insects. Another approach is to design an insecticide that will degrade before the plant is harvested. Although the thrust is to protect humans and animals that consume the plants, safety of farm workers and food processors also is important (35, 36). The following classification is based on the target.

5.1 Neurotoxic Insecticides *(Comparative Biochemistry* and *Comparative Distribution)*

5.1.1 Neonicotinoids. The prototype compound that has wide use is imidacloprid (53). This group preferentially binds to the nicotinic acetylcholine receptors of insects relative to those found in animals (37). Their lipophilicity also tends to increase their safety for aquatic species.



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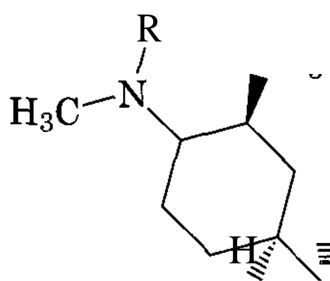

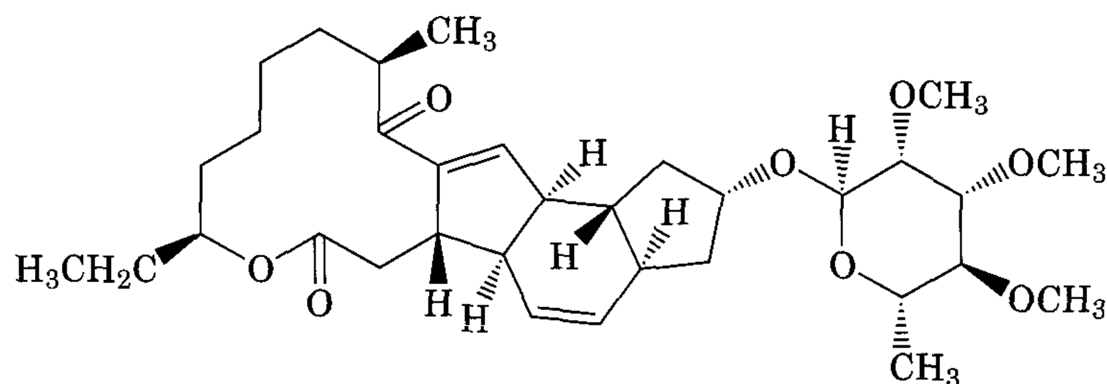

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5.1.2 Spinosyns. A commercial mixture of the natural products spinosyns A and B (54)is reported to selectively activate insect acetylcholine receptors. They appear to have good selectivity for the insect receptor.

5.2 Insect Growth Regulators *(Comparative Biochemistry)*

Many of the insecticides in this class mimic insect juvenile hormone, including the insect-molting hormone, 20-hydroxyecdysone. This group of compounds are considered to have excellent selectivity because hormonal control of development in insects differs significantly from those of vertebrates. The growth regulators prevent the insect from undergoing normal maturation.

5.3 Inhibitors of Oxidative Phosphorylation *(Comparative Biochemistry* and *Comparative Distribution)*

This group tends to show specificity for either mitochondrial complex I or III. The basic selectivity is not as good as the neurotoxins and insect growth regulators. Mammals have similar mitochondrial complexes. Selectivity can be increased by administering the insecticide as a proinsecticide that is converted to the active chemical by an enzyme found only in the

insect. Pharmacokinetic properties also may help increase selectivity.

6 CONCLUSION

Selective toxicity is an important goal and concept that must be used in designing a successful biologically active molecule. Today's drugs increasingly are being used to treat complex disease processes whose target receptors are found at several locations throughout the patient's body. There is selectivity within these receptors. The challenge continues to be to discover these differences and then use the principles of comparative biochemistry, distribution, cytology, and stereochemistry in designing new and better drugs.

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CHAPTER EIGHT

Drug Resistance in Cancer Chemotherapy

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ISBN 0-471-37031-2 © 2003 John Wiley & Sons, Inc.

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1 INTRODUCTION

Chemotherapy is a mainstay of cancer therapies given to patients today. From the introduction of systemic chemotherapy in the late 1940s, an individual's response has been recognized to be dependent on many factors unique to the host, as well as the specific histologic and genetic subtype of malignancy. The design of rational, effective chemotherapeutic protocols involves using agents to which the tumor is susceptible that have different mechanisms of action, nonoverlapping dose-limiting toxicities, and that attack the malignant cells during different phases of the cell cycle. However, because of baseline genetic alterations in tumor cells and the fact that malignant cells can acquire pleiotropic changes in the presence of chemotherapy, tumors may still become refractory to both drugs they have been exposed to as well as to drugs with which they have never been treated. The latter case, termed multidrug resistance, can involve compounds with completely unrelated structures and mechanisms of action (1, 2). Clinical resistance occurs as the resistant clones are positively selected for during a course of chemotherapy (3). Despite the emergence of new classes of chemotherapeutic agents and the widespread use of rational protocol design, the study of the panoply of mechanisms of drug resistance continues to be essential for using this tool effectively for the most resistant subtypes of malignancies.

This chapter will attempt to describe some examples of acquired resistance in cancer cells, and discuss strategies employed therapeutically to overcome this resistance. Additionally, some somatic mutations also alter the susceptibility of a tumor to chemotherapy, and these will be discussed in brief. It is important to note, that although beyond the scope of this chapter, somatic polymorphisms that affect the amount of drug or dose intensity of a drug regimen given to a patient can ultimately impact the induction of resistance in tumor cells exposed to subclinical doses of drug and are also important clinical considerations concerning drug resistance.

2 MECHANISMS OF RESISTANCE

In 1984, Goldie and Coldman published a landmark paper attempting to use a mathematical model to follow the emergence of clinical drug resistance (4). This model attempted to describe clonal heterogeneity in tumors that provided the means for positive selection of resistant clones in the presence of a constant fraction of cell-kill during chemotherapy (Fig. 8.1). The Goldie-Coldman hypothesis of acquired mutations during therapy directly impacting resistance provides a basic framework for understanding drug resistance in the clinical setting.

The cancer cell exhibits unique molecular properties, which render the cell unable to halt replication in the presence of DNA damage. Thus, cancer cells can continue to survive despite DNA damage, can rapidly incorporate new molecular configurations that confer a survival benefit, and can replicate rapidly and efficiently, increasing the speed at which molecular mechanisms of resistance can be incorporated into the population of cells. These changes include increased gene copy, mutations, altered transcription, and epigenetic changes. The overall phenotype of a cancer cell that has incorporated these changes in its genome is a cell that either no longer accumulates drug, no longer makes the drug's target protein, or alters the target protein in such a way that the protein no longer binds or is affected by the drug (see Table 8.1).

2.1 Alterations in Drug Targets

One of the first mechanisms of acquired resistance to chemotherapy by the tumor cell was determined to be an alteration of the protein targeted by the drug, either by loss or gain of function. Additionally, some tumor cells can amplify copies of the genes encoding the drug's target and transcribe and translate more of the target molecules, overwhelming the drug's cytotoxic ability. Finally, some preexisting somatic mutations that are present in the tumor cell confer resistance to therapy and must be considered when choosing active agents for individuals.

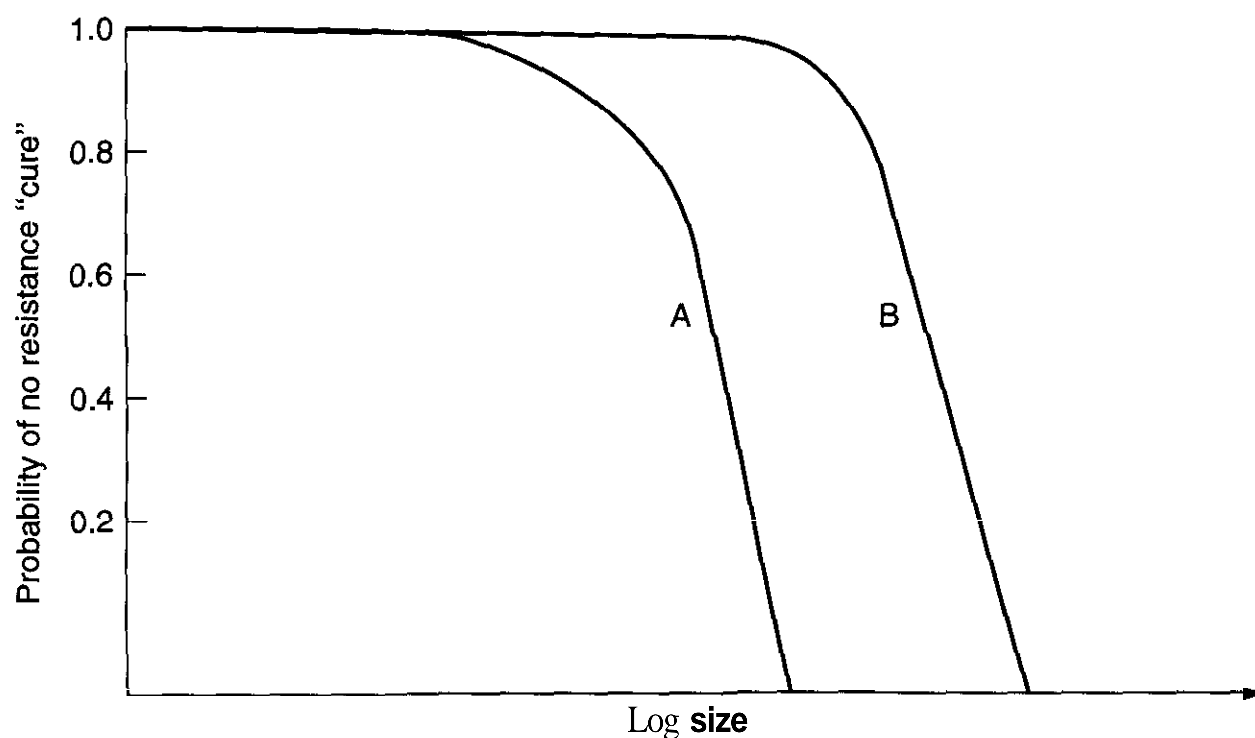

Figure 8.1. Goldie-Coldman model of tumor growth versus acquisition of drug-resistant clones measured by mutation rate. As acquired mutations increase over time, the probability of cure with chemotherapy decreases secondary to the emergence of drug resistance. Plot of two mutation rates, A and B, where A > B (function $p = e^{-\alpha N}$, where "the function defines the probability of there being zero resistant cells present for any given value of the tumor size and the mutation rate to resistance"). As tumors approach the steep portion of the curves, any increase in number of resistant cells significantly impacts the probability of success with single agent therapy, and combination chemotherapy should be considered. Adapted with permission from J. H. Goldie and A. J. Coldman AJ, The genetic origin of drug resistance in neoplasms: Implications for systemic therapy. *Cancer Res.,* 44, 3643–3653 (1984).

A classic example of a tumor cell altering the target of drug therapy was the isolation of tumor cells that had increased levels of intracellular dihydrofolate reductase (DHFR) after exposure to the antifolate, methotrexate (5–7). DHFR is required to maintain intracellular pools of reduced folates, which serve as one-carbon donors for the pathways responsible for *de novo* purine synthesis, pyrimidine synthesis, and ultimately for the synthesis of amino acids. Because of its critical role in the folate pathway, this enzyme is the target of

Table 8.1 Mechanisms for Tumor Cell Resistance to Chemotherapy

Mechanism for Resistance	Example
Alterations in drug targets	
Increased expression of drug's target	DHFR, methotrexate
Increased expression of biologic target	Bcr/abl, imatinib mesylate (Gleevac, STI571)
Somatic genetic variation	Thymidylate synthase, 5-fluorouracil
Tumor mutations in drug target	Topoisomerase II, epipodophyllotoxins
Alterations in intracellular retention of drug	
Tumor decreases influx of drug	RFC, methotrexate
Tumor increases efflux of drug	MDR, MRPs, BCRP and substrates
Tumor affects pharmacologic path responsible for retention of drug	FPGS, methotrexate
Alterations in drug detoxification pathways	Cellular glutathione pathway, alkylators
Increased DNA repair	OGAT, nitrosureas
Defective apoptosis	*p53, bcl2*
Epigenetic changes	Methylation of genes involved in drug's cytotoxicity pathway

the 4-amino folate analogs, or antifolate drugs (e.g., methotrexate). Some tumors that developed resistance to methotrexate over time were found to have amplified the *DHFR* gene, and subsequently increased levels of expressed DHFR enzyme (5, 6). These cells had increased DHFR activity, and an increased concentration of methotrexate was required to kill these cells. Mutation of DHFR has also been documented in some cell lines with acquired methotrexate resistance, resulting in either a DHFR molecule that is catalytically less active than wild-type DHFR or is less able to bind antifolates, but this has not yet been documented in patient samples (8, 9).

Another mechanism of resistance is to amplify genes encoding oncogenic fusion proteins. A new area of active investigation is the production of antitumor agents that target tumor tissue, avoiding damage to normal host tissue. One strategy is to target oncogenic fusion proteins, expressed in tumor cells with chromosomal translocations that result in an expressed protein. For example, BCR/ABL is a chimeric protein, whose expression is the result of the unbalanced translocation of chromosomes 9 and 22. BCR/ABL has been identified in both chronic and acute lymphocytic leukemias. The expressed protein is a tyrosine kinase. STI571 (Gleevac) was developed for its ability to inhibit the tyrosine kinase of BCR/ABL. In *vivo*, STI571 is able to successfully eradicate malignant lymphoblasts on initial exposure to the drug. However, patients uniformly relapse after therapy. Studies done with cells in culture show that these cells develop resistance due to either amplification of *bcr/abl* after prolonged exposure to STI571 or due to a single amino acid substitution in the threonine residue of the Abl portion of the fusion protein that is required for hydrogen bond formation with the drug molecule (10). Therefore, the early clinical results need to be viewed with caution, and additional investigation of combination therapies is currently underway.

Additional mechanisms of resistance occur when somatic variations of gene sequence affect a drug target. 5-Fluorouracil (5FU) is a chemical analog of uracil, and in *vivo,* is converted to its active metabolite 5-fluoro-2-deoxyuridine monophosphate (5-Fd-UMP). 5-Fd-UMP inhibits thymidylate synthase (TS), an enzyme required in the de *novo* pyrimidine synthesis pathway. Somatic polymorphism in the enhancerlpromoter region of (TS) have been documented, which involve a variable number of tandem repeats (VNTRs), and have been shown to influence cellular response to 5FU exposure (and potentially antifolate exposure) (11, 12). Cells whose promoters contain three repeats have higher TS activity than do cells with two repeats because of enhanced TS expression. Patients with a homozygous genotype for two tandem repeats have lower TS activity; therefore, tumor cells derived from this somatic background will respond well to 5FU therapy when compared with patients with a homozygous genotype for three repeats (13). Therefore, prospective pharmacogenetic screening of the host's somatic TS promoter region may help predict both tumor response to conventional doses of 5FU, as well as consider either alternative drug regimens or increasing 5FU dosing for patients with higher TS activity.

Finally, cancer cells can acquire structural changes in drug targets over time. Topoisomerase II is an enzyme targeted by several anticancer drugs because of its normal cellular role in affecting nuclear structure and function. Topoisomerases induce transient breaks in the phosphodiester backbone of DNA. Class II topoisomerases are necessary for the inducement of double-strand breaks during DNA synthesis, transcription, and chromosomal segregation, resolving torsional strain induced during the unwinding of DNA. Topoisomerase II inhibitors (epipodophyllotoxins and anthracyclines) stabilize the topoisomerase 11-DNA covalent bond intermediate after scission has occurred, creating DNA damage that ultimately results in cell death. Multiple mutations in topoisomerase II have been identified in tumor cells. These mutations have been shown in *vitro* to induce resistance either through reduction in enzyme activity or alteration in protein structure that prevents binding of drug to the topoisomerase molecule (reviewed in Ref. 14).

2.2 Alterations in Intracellular Retention of Drug

By reducing the drug's ability to enter the cell or by rapid efflux of the drug from the cell, the cancer cell effectively removes the ability of the drug to exert its effect. This can be accomplished by either reducing copies of the genes for transporters required for the drug's influx into the cell or by increasing copy number of genes encoding transporters that rapidly efflux either the parent drug or the activated drug metabolite from the cell. Additionally, the cell can decrease metabolism of the parent drug, preventing the formation of toxic activated metabolites.

Altered influx of a drug as a mechanism of tumor resistance has been documented for therapy with methotrexate. The reduced folate carrier (RFC) is an ATP-dependent transporter that actively imports reduced folates into the cell against a concentration gradient for their use in DNA, RNA, and amino acid synthesis. Classic antifolates (e.g., methotrexate) are also transported via RFC into the cell. Mutations in RFC have been isolated in both human and rodent cells with acquired resistance to methotrexate (15). These mutations in RFC resulted in decreased methotrexate accumulation in the tumor cells, and therefore less cytotoxicity when these cells were exposed to increasing doses of methotrexate (15). Additionally, some human leukemia cells have been found to decrease expression of RFC, conferring marked resistance to antifolate therapy (16).

By increasing the efflux of drug molecules from the cell, resistance can be achieved. A subset of ATP binding cassette (ABC) transporters are localized to the plasma membrane of cells, and when expressed, confer resistance to multiple structurally unrelated drug compounds (this family of transporters is summarized with relevant references at http://www.nutrigene.4t.com/humanabc.htm) (17). These include the multidrug resistance protein (MDR), the multidrug resistance-related protein (MRP) and related family members, and the breast cancer resistance protein (BCRP). Expression of these gene products is often turned on in tumor cells after they acquire alterations or deletions in the regulator genes normally controlling their expression (e.g., P53 or N-MYC) (18–21). Therefore, tumor cells can either increase expression of several ABC transporters after the transformation event, thus conferring *de* novo resistance to a broad spectrum of drugs or increase expression of these transporters after exposure to a drug in vivo. Because these transporters do transport a spectrum of compounds, up-regulation of ABC transporters in tumor cells not only yields cells that are resistant to drugs they have had previous exposure to, but also to drugs to which they have not yet been exposed. This limits the number of agents that can then be effectively used for eradication of these tumors in *vivo*.

MDR1, otherwise known as the P-glycoprotein (Pgp), was first identified in cells selected for multidrug resistance (22). It was subsequently discovered that cells that overexpress MDR1 acquire resistance to several compounds (primarily cationic and hydrophobic in structure), including etoposide, anthracyclines, taxanes, vinca alkaloids, steroids, and actinomycin D (23). A somatic mutation in *MDR1* has been identified (SNP in exon 26, C3435T) that confers a twofold decrease in duodenal expression of MDR1 and a fourfold reduction of activity in homozygous mutant individuals (24). The allele frequency for this mutation is ethnically distributed, with high frequency of the C allele found in African populations (25). The presence of somatic mutations in *MDR1* may have prognostic value for patients treated with chemotherapeutic agents that are substrates for this transporter.

MRP was first isolated in 1991 by Cole and colleagues, who were studying a small cell lung cancer cell line with acquired resistance unrelated to the expression of MDR1 (26–28). Since the discovery of MRP, many related family members have also been isolated (e.g., MRP2–6), each of which confer resistance to a signature spectrum of anionic compounds. (Table 8.2). Up-regulation of expression of MRPs in tumors, both at diagnosis and at relapse, have been documented. These tumors have been shown both in vitro and in vivo to be less responsive to chemotherapy (reviewed in Ref. 29).

Somatic mutations in *MRP2* that result in expression of an inactive transporter have

Table 8.2 Multidrug Resistance Transporters and Chemotherapy Substrates (as Validated *In Vitro*)

MDR1	Vinca alkaloids, anthracyclines, taxanes, actinomycin D; epipodophyllotoxins, steroids
MRP1	Doxorubicin, daunomycin, vincristine, etoposide, methotrexate
MRP2	Vinca alkaloids, cisplatin, CPT-11, methotrexate
MRP3	Etoposide, teniposide, methotrexate, vincristine
MRP4	Methotrexate, purine antimetabolites
MRP5	Purine antimetabolites
BCRP	Mitoxantrone, camptothecins, anthracyclines

been identified as the causative mutations in Dubin-Johnson Syndrome, an inherited disorder characterized by chronic mild hyperbilirubinemia (30–32). Patients who are heterozygotes for these mutations also excrete excess bilirubin byproducts in urine. It is suspected that mutations in this transporter may also contribute to hepatic toxicity with some anticancer drugs, and this is under current investigation.

BCRP was first identified in breast cancer cell lines with acquired resistance to mitoxantrone. Unlike MDR and the MRPs, BCRP is a "half molecule" transporter and is thought to function as either a homo- or heterodimer. This transporter also transports several anticancer agents (Table 8.2) (33, 34). Interestingly, a polymorphism has been identified in the third transmembrane domain of this protein that alters its substrate profile (35). At amino acid 482, three variants were isolated with nonredundant amino acid substitutions (arginine, glycine, and threonine). The frequency of the SNPs responsible for these substitutions is being investigated and may prove to be an additional somatic mutation that can alter individual response to chemotherapy. Additionally, the cell type in which BCRP is expressed may affect its substrate profile, especially if it is determined that this protein can function as a heterodimer. Conceivably, different cell types might express different "partners" for BCRP function, which would alter its substrate specificity profile.

Alterations in the pharmacologic retention of a drug molecule within the cell are also documented as a mechanism for resistance. Methotrexate is an anionic compound with a monoglutamate moiety, to which intracellular fclylpolyglutamate synthase (FPGS) adds additional glutamate molecules. Polyglutamylated methotrexate is highly anionic and is no longer a substrate for either passive or active efflux from the cell. These polyglutamylated metabolites are active inhibitors of all proteins in the folate metabolism pathway normally inhibited by the parent molecule and provide prolonged toxicity when retained within the cell. In childhood acute lymphoblastic leukemia (ALL), patients with T lymphoblastic ALL have lower overall formation of polyglutamylated MTX than do patients with B lineage ALL. Because T lineage ALL is known to have a worse prognosis than B lineage ALL, the mechanism by which this occurs was investigated in primary ALL blasts. T lineage blasts were found to have lower FPGS activity than B lineage blasts, providing the mechanism by which less active metabolite was formed, and the tumor cells were more resistant to therapy (35–37).

2.3 Alterations in Drug Detoxification

Cells use secondary metabolism pathways (e.g., phase II metabolism) for the primary purpose of making drug molecules more water soluble in order to efflux them from the cell. By enhancing phase II metabolism, cancer cells can rapidly excrete drugs, thereby reducing their overall exposure time to the cytotoxic agents. For example, alterations in the glutathione conjugation pathway can alter a tumor cell's sensitivity to drugs that are significantly excreted via conjugation with glutathione.

γ-Glutamlycysteinylglycine, or reduced glutathione, is nucleophilic and conjugates with electrophilic atoms in three primary reactions: (1) nucleophilic addition of glutathione to electrophiles (e.g., detoxifies epoxides), (2) reduces lipid and DNA hyperoxides (e.g., thymine hydroperoxide), and (3) readily directly reduces some free radicals (e.g., hydroxy and

carbon). Glutathione is present at high concentrations in tissues (0.1–10 m*M*) but is present in highest concentration in the liver (5–10 m*M*). At these high concentrations, glutathione can undergo nonenzymatic conjugation with nucleophiles. Cancer cells are noted for decreased glutathione concentrations when compared with the somatic tissues from which they are derived. Decreased intracellular glutathione pools prevent detoxification of nucleophiles. However, this decreased intracellular concentration of glutathione in some cell types is caused by overexpression of MRPs 1 a n d , which use glutathione to cotransport anticancer agents from the cell, even when these compounds are not conjugated to GSH (e.g., vincristine) (38). Conversely, some cancer cells have been shown to have elevated intracellular glutathione concentrations (two- to threefold), providing resistance to vinblastine, adriamycin, and VP-16 (39).

In tissues with low glutathione concentrations, glutathione-S-transferases are important to mediate glutathione conjugation to nucleophiles. There are multiple GST isozymes that have extensive substrate overlap. In humans, four cytosolic subfamilies of GSTs are important for drug detoxification: π, μ, θ, and a. Although these enzymes are polymorphic, with μ and θ mutations comprising the majority of polymorphisms within the population, tumor cells usually up-regulate only the α and π isoforms, thereby making these the most important predictors of tumor resistance. GSTs are active as either homo- or heterodimers. In *vitro* assays of GST activity in tumor cells has shown that in *vivo* tumor cells have increased GST activity, which renders them resistant to alkylating agents (38). Drugs with convincing data for the impcrtance of glutathione conjugation in *vivo* include chlorambucil, melphalan, nitrogen mustard, acrolein, BCNU, hydroxyalkenals, ethacrynic acid, and steroids (40).

2.4 Increased DNA Repair

By increasing either the rapidity of DNA repair or decreasing the efficiency of repair, cancer cells can overcome some of the DNA damage exerted by certain chemotherapeutic agents. One example is 06-alkyl guanine alkyl transferase (OGAT), a constitutively expressed DNA repair protein, which removes alkyl groups from the 06-position of guanine in DNA. Tumor cells that exhibit high OGAT activity are resistant to agents that form *O*6-alkyl adducts, such as the nitrosureas (e.g., BCNU) and triazene compounds (e.g., procarbazine and temozolomide). Agents are currently in development that inactivate OGAT, rendering tumors sensitive to nitrosourea and triazene therapy (41).

2.5 Defective Apoptosis

Apoptosis, or programmed cell death, is an energy-dependent process by which cells undergo an orderly series of intracellular events leading to cell death. This process is initiated by a cell in response to specific stimuli, such as DNA damage. Apoptosis is required for maintaining appropriate function and structure of normal proliferating for renewable tissues. Disruption of the normal programmed cell death response prevents cells from self-destructing when irreversible damage takes place, and they survive with this damage. Because they do not apoptose, these cells can continue to replicate unchecked. Two examples of genes that are commonly mutated in cancer cells are *p53* and *bcl2*.

p53 is a transcription factor, which in its wild-type form, both represses and initiates specific promoter transcription in addition to several other cellular functions. In normal cells, *p53* is activated in response to DNA damage, triggering either growth arrest or apoptosis depending on the current stage of the cell cycle (Fig. 8.2). Greater than 50% of tumors have been documented to contain functional inactivation of p53. The cancer cell genome is rendered unstable secondary to these alterations in *p53* or other changes that inactivate the apoptotic pathway (e.g., *bcl-2*). This allows for multiple secondary and tertiary molecular changes within the cancer cell genome (both in coding and noncoding regions), some of which are exploited in the presence of drug to allow for survival of the cell despite therapy. Cells that have mutant *p53* show a propensity to amplify DNA and show a distinct growth advantage to cells with wild-type *p53*. Therefore, these tumor cells can continue to divide, even under the selective pressure of chemotherapeutic agents whose mechanism of ac-

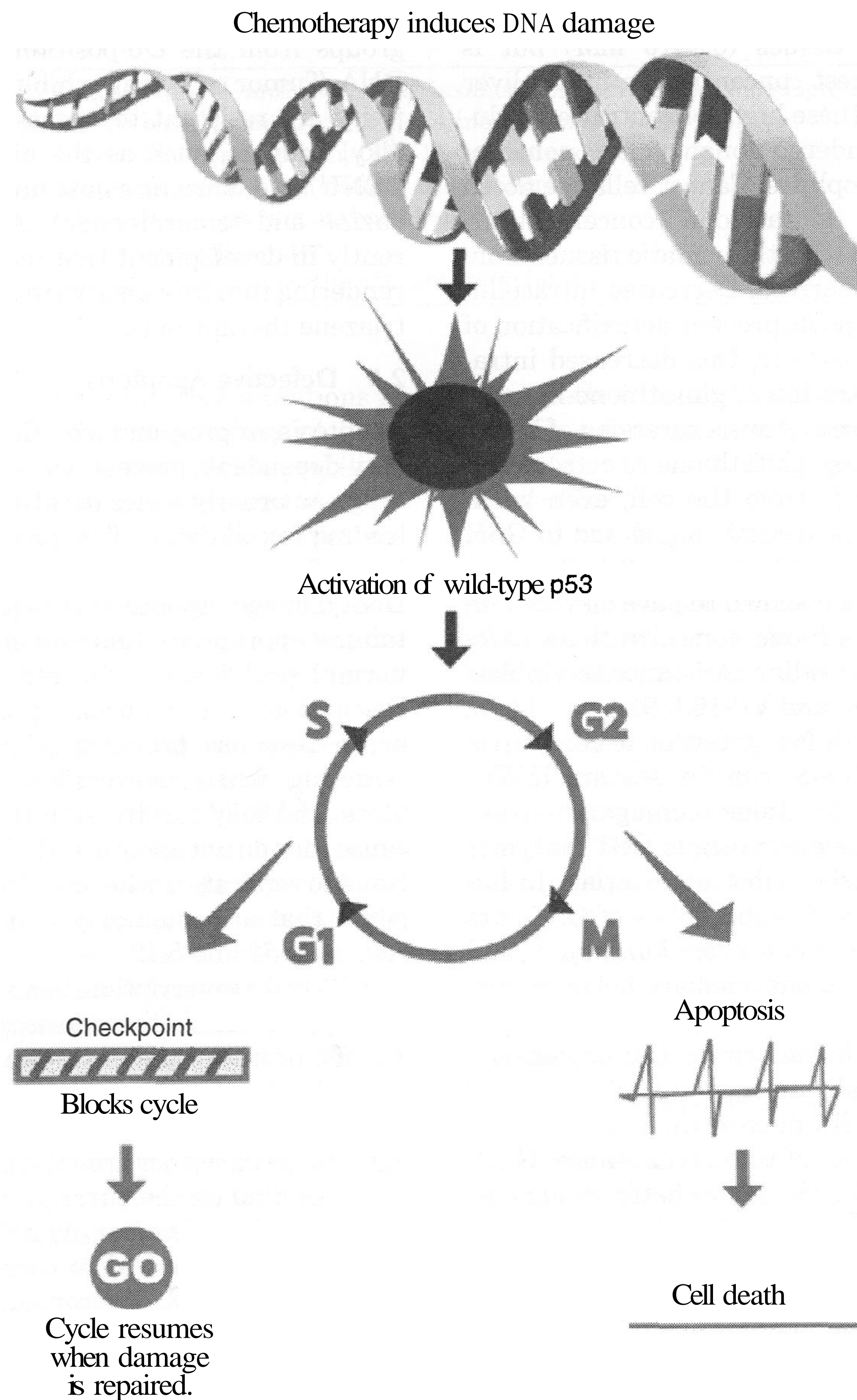

Figure 8.2. Chemotherapy induces DNA damage, stimulating activation of p53. Based on the stage of the cell cycle the cell is in when the damage takes place, different pathways are activated. When damage occurs early in the cell cycle, progression is arrested until DNA damage is repaired. Late in the cell cycle, apoptosis is stimulated.

tion is to damage DNA. Lowe et al. showed that normal mouse embryo fibroblasts lacking the *p53* gene were resistant to doxorubicin, 5-FU, and etoposide (42, 43). When the *p53* gene was transfected into these cells, the cells became sensitive to these drugs and died via the apoptotic pathway (44).

bcl2 is an oncogene, the expression of which inhibits apoptosis. In tumor cells, *bcl2* is activated, allowing cell to survive in the face of

cellular damage that would normally signal for programmed cell death, thus making these cells more resistant to chemotherapy-induced cytotoxicity. In *vitro* models of cell lines with *bcl-2* activation show that these cells continue to divide and grow despite high concentrations of drug in the media (45).

2.6 Epigenetic Changes

Changes in the cancer cell phenotype that do not alter the genotype (i.e., epigenetic changes) are an additional source of drug resistance. One example is the process of methylation/demethylation of DNA. Epigenetic silencing of tumor suppressor genes has been documented in human cancers. Methylation of DNA occurs in eukaryotic cells. DNA methyltransferase catalyzes the reaction, whereby a methyl group is transferred to the carbon 5 position of the cytosine ring within a 5'-CG-3' sequence. Methylation of genes silences expression by preventing binding of transcription factors and recruiting binding of other proteins that limit the accessibility of the gene to the cell's transcription machinery. Demethylation of a gene activates its expression. Normal methylation patterns are disrupted in many tumor cells, with tumors exhibiting decreased methylation in areas of repetitive DNA and an increase in methylation of promoter CpG islands. Hypermethylation of tumor suppressor genes has been documented in many tumor cells, and tumor-specific patterns of gene methylation has identified a subset of patients whose tumors display widespread methylation abnormalities. These tumors are referred to as displaying the CpG island methylate phenotype (CIMP), and these patients tend to respond less well to chemotherapy. The exact mechanism by which this occurs is not known, but investigators are currently determining whether this phenomenon extends to drug targets or enzymes that catalyze drug activation/detoxification pathways.

3 STRATEGIES TO OVERCOME RESISTANCE

The future strategy to overcome resistance will be to individualize a patient's therapy prospectively, employing both knowledge of the patient's somatic mutations in drug metabolizing enzymes, drug receptors, and drug targets with knowledge of tumor-specific changes that affect cytotoxicity of agents normally given systemically to patients. Some strategies used clinically are pharmacokinetic-based strategies, pharmacogenetic-based strategies, and basic tumor biology-directed strategies for dosing. The future of individualization for patients lies in developing algorithms for dosing, based on a synthesis of all of these methods, to determine an overall phenotype for each patient prospectively.

3.1 Pharmacokinetic Monitoring

One strategy to overcome tumor cell resistance to chemotherapy is to employ pharmacokinetic strategies to further intensify doses of drugs by giving the maximally tolerated dose of drug as close together as possible. For example, by using 24-h methotrexate infusions clinically, one can presumably overcome the resistance phenotype seen in tumors overexpressing certain ABC transporters *in vitro,* whereby long exposure to methotrexate was able to overcome the high levels of resistance observed after short (4-h) exposure (e.g., MRP1-3) (46-48). Additionally, higher serum concentrations of methotrexate can overcome the resistance achieved by increased quantities of DHFR intracellularly or decreased expression of the reduced folate carrier. Unfortunately, not all interpatient variability can be determined prospectively for pharmacokinetic strategies to be completely effective prior to an individual's initial exposure to drug. Additionally, pharmacokinetic-guided dosing can be labor intensive for both the patient and medical staff.

3.2 Pharmacogenetic Monitoring

Pharmacogenetic-based strategies are currently used to identify monogenic traits in patients that would alter either a predisposition to toxicity or the efficacy of response. Currently, these strategies are not used prospectively in patients to alter dosing *a priori,* but rather once a patient experiences toxicity or doesn't respond to therapy, genotyping is performed. The future of pharmacogenetic monitoring, however, will be to develop dosing

based on multiple genotypes in an individual (49–51), creating rational selection of drugs and doses for individual patients.

One example of a well-characterized polymorphism in drug metabolism that is currently used to screen patients treated with thiopurines is the SNPs present in thiopurine methyltransferase. 6-Mercaptopurine is a thiol-substituted analog of hypoxanthine, used to treat patients with acute lymphoblastic leukemia. TPMT methylates both the parent compound (6-MP) and the active phosphorylated metabolites of 6-MP, resulting in detoxification of the compound. Patients with homozygous mutations in (G238C) (TPMT*2) or (G460A and A719G) (TPMT*3A) experience profound neutropenia when exposed to thiopurines (52–54). Heterozygotes for these autosomal codominant mutations experience intermediate toxicity, with as many as one-third of these patients requiring a dose-reduction of thiopurines during therapy for ALL (55). Therefore, these somatic mutations alter the ability of patients to receive maximal doses potentially altering outcome in some studies.

3.3 Biologic Inhibition of Tumor Cell Proaerties (ABC Transporters)

Cyclosporine A, (CSA) and PSC833 are agents that block MDR1-mediated efflux of substrates in vitro and have been used systemically to inhibit MDR1 during chemotherapy, with mixed results (56). CSA has had some improvement in efficacy for systemic chemotherapy, probably because of the fact that it is a nonselective inhibitor and also inhibits BCRP-mediated efflux.

4 CONCLUSIONS

Drug resistance continues to provide an obstacle for administering chemotherapy to patients. Although some patients respond very well to chemotherapy, a significant number of treatment-refractory tumors are still under study. Ultimately, the combined knowledge of patient-specificfactors that alter our ability to administer optimal doses of chemotherapy and tumor-specific factors that result in decreased efficacy of chemotherapy will be used to more efficiently dose patients. As new drugs emerge for bioselective treatment of tumors, understanding induction of resistance continues to be essential for providing the best therapy to patients. With the powerful genomics tools that are becoming more available, it will soon be feasible to monitor both tumor and somatic variations in DNA sequence, expression of proteins, and in vitro response to therapy before initiating therapy in patients, thereby providing information required to reach the ultimate goals of reducing the incidence of side effects, improving response to therapy, and preventing the induction of resistant malignant clones in *vivo*.

5 ACKNOWLEDGMENTS

I would like to acknowledge Peter Adamson, M.D. (Children's Hospital of Philadelphia) for his editorial comments and Evan Katz (Children's Hospital of Philadelphia) for designing Fig. 8.2.

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CHAPTER NINE

Antiviral Agents, DNA

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Burger's Medicinal Chemistry and Drug Discovery
Sixth Edition, Volume 5: Chemotherapeutic Agents
Edited by Donald J. Abraham
ISBN 0-471-37031-2 © 2003 John Wiley & Sons, Inc.

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1 INTRODUCTION

Antiviral chemotherapy is conceptually no different than chemotherapy of other infectious agents, but the therapeutic targets are more limited. Viruses are obligate intracellular parasites that rely heavily on their host cell to produce progeny virions. The virus may supply as few as two nonstructural proteins to the production line, but may borrow dozens of cellular proteins to gain access to the cell, produce the components of new virions, assemble them, and export them from the cell. The genome sizes of viruses range from 25% to <1% the size of typical pathogenic bacteria. Furthermore, because they borrow heavily from cellular metabolic pathways, they tend to encode proteins that are similar to cellular proteins. Viruses have relatively few proteins with unique activities not found in the host cell. Thus, the difference between inhibiting a viral protein and its cellular equivalent may be small. With most viruses, one is forced to concentrate on a small number of targets and find compounds that can exploit the sometimes subtle differences between viral proteins and cellular proteins with similar functions. Occasionally, a more effective approach has been to not attack the virus directly, but rather to stimulate the body's own defenses to fight it more effectively. Some of the more effective antiviral agents have been compounds that stimulate the immune response against the virus.

2 THE VIRUSES

There are seven families of DNA viruses that are pathogenic for humans. These pathogens come from the Adenoviridae, Hepadnaviridae, Herpesviridae, Polyomaviridae, Papillomaviridae, Parvoviridae, and Poxviridae families. Herpesviruses, hepadnaviruses, and papillomaviruses are well established as human health problems and as targets for antiviral chemotherapy. The biology of adenoviruses, parvoviruses, polyomaviruses, and poxviruses has been intensively studied, but these viruses have not been pursued as drug targets to the extent of the other viral families.

DNA viruses have assumed more importance as pathogens as the prevalence of patients with suppressed immune function has increased. This increased prevalence comes in turn from two sources: greater use of organ transplantation and the AIDS epidemic. Most of the DNA viruses are ubiquitous in the human population, but several become a serious health threat in the absence of a fully functional immune system. The lack of immune surveillance allows them to propagate essentially unchecked. In particular, the overall health threats posed by herpesviruses, hepatitis B virus (HBV), papillomaviruses, and polyomaviruses are worsened by immunodeficiency.

DNA viruses are defined as having their genome in the form of DNA for the infectious phase of their life cycle, although HBV goes through an RNA intermediate during the replication cycle. Their genomes vary in size from approximately 5000–300,000 base pairs (by comparison, human cells contain about 3×10^9 base pairs). The biology and clinical details of these viruses has been reviewed in detail elsewhere (1); only the features most likely to be important to drug development will be discussed here. We start with a general discussion of traits that apply to most viruses, followed by specific discussions of each family.

Most of the DNA viruses are chronic viruses—they infect their host and establish a persistent infection in specific cell types that may last for the lifetime of the host. Two requirements of such a long-lasting relationship are a host cell in which it can remain indefinitely and a means of avoiding detection by the immune system. For instance, α-herpesviruses tend to infect nondividing cells in which they can "lie low" by turning off the expression of most viral genes. On the other hand, y-herpesviruses infect dividing cells, but they have evolved special mechanisms for persisting in these cells and evading the immune system. Their genome is designed to be recognized similarly to host cell DNA, such that cellular machinery replicates it when the cell divides. They have also tailored the proteins needed to maintain their presence in dividing cells to evade the immune system. Some relationships are not as sophisticated; for in-

stance, most HBV and papillomavirus infections are destroyed by the immune system, but rates of production of virions are high enough to ensure long-term propagation of the viruses in a portion of the infected population. In this portion, a chronic infection will be established that will last for years to decades, thus providing a reservoir of virus in humans.

The following are the general steps needed by all viruses to produce progeny.

1. Adsorption and entry. Adsorption to the cell is mediated through interactions between ligands on the viral surface and specific cellular receptors, often involving multiple cellular proteins. Entry is usually by one of two routes, either fusion of a viral membrane with the cellular membrane or internalization by endocytosis and release of the virus from internal vesicles. These steps create the potential for good drug targets, and drugs to block retroviral binding have been developed. However, our understanding of the virus-receptor interactions for DNA viruses is not yet sufficient to be used for drug development.
2. Uncoating and processing. The extent of uncoating varies between species. In some cases, the DNA genome, with a minimal set of associated proteins, is transferred from the capsid to the nucleus. At the other extreme, some viruses, such as poxviruses, do a minimal amount of uncoating, and the entire replication process takes place in a specialized viral structure constructed by the virus in the cellular cytoplasm. Other steps may be required to prepare the genome for use, such as circularization of the linear herpesvirus genome after removal from the capsid or conversion of the partially double-stranded HBV genome to a completely double-stranded, covalently closed circular form. Again, uncoating and processing steps have not yet been exploited for drug development.
3. Transcription of early proteins. These include both regulatory proteins and proteins needed for replicating the viral genome. While critical to the production of progeny virions, achieving selectivity in inhibiting transcription of specific genes has posed a severe limitation, independent of whether the genes are cellular or viral. Herpesviruses provide an additional potential target, in that the productive cycle may be delayed indefinitely after infection (i.e., the virus becomes latent), in which case a reactivation step is needed to move the virus into the lytic cycle. Reactivation is a potential drug target, and in some cases, the reactivation pathways have been partially mapped. However, it is largely another form of transcriptional regulation and has not been profitably pursued as a drug target.
4. Viral genome replication. This is the primary focus of attention for all DNA viruses except papillomaviruses, polyomaviruses, and parvoviruses, which lack a virally encoded polymerase. Polymerase is the obvious target, but accessory proteins provide additional possibilities. Inhibitors of the helicase activity of papillomaviruses have been identified, but drugs have not yet been developed against this enzyme. Nucleoside kinases of herpesviruses represent another piece of the replication machinery that has played a central role in drug development, because they permit the use of drugs that are inactive in uninfected cells. These kinases perform the first addition of a phosphate to nucleoside analogs. Analogs that are poorly recognized by cellular kinases only get converted to the active form in infected cells. To the extent that the analog is selective for the viral kinase, toxicity is limited by the fact that the addition of the negative charge blocks the activated nucleoside analog from crossing the cellular membrane; therefore, uninfected cells are protected from exposure to the activated drug. This is a critical factor in providing specificity to several herpesvirus drugs and will be discussed in the context of those drugs.

Unlike RNA-dependent polymerases, DNA-dependent polymerases have relatively high fidelity, i.e., they are not as error-prone. This decreases the likelihood of

resistance mutations accumulating in the viral population and has contributed to the emphasis on polymerase as a drug target. There are two types of exceptions to this generalization, however. The first is HBV, which has a polymerase with a high mutation rate. This is reflected in the relatively rapid appearance of strains of virus resistant to drugs. The second is loss of immune surveillance, which allows increased viremia with the accumulation of more mutations, even from viruses with a low overall error rate.

5. Synthesis of late proteins. Late in the viral life cycle, structural proteins are synthesized, including nucleocapsid proteins needed to bind to DNA, capsid/coat proteins that provide the shell of the virus, and in the case of lipid membrane–coated viruses, glycoproteins that serve as the ligands for cellular receptors. Whereas genes expressing these proteins are easy to identify in the viral genome and the proteins are usually synthesized in large quantities, there is no precedent for directly blocking their synthesis.
6. Processing and assembly. Two forms of processing have been exploited as drug targets. First, processing of the viral genome is needed in some cases. A set of herpesvirus proteins needed to cleave the genome to monomeric length and package it into capsids has been identified (2–4). Inhibitors of these proteins have recently been developed. Second, processing of the structural proteins is generally needed. Many of them are glycosylated by cellular glycosylation enzymes. Of more immediate interest, proteolytic cleavage is generally needed, either to cleave polyproteins to their individual proteins or to perform a maturation step, in which the morphology of the virus changes either after packaging is complete or after the virus has been released from the cell. Whereas the nature of this maturation step is not functionally well defined, protease inhibitors constitute one of the two major classes of currently licensed drugs for treatment of HIV-1, establishing a precedent for this approach. Recently, inhibitors of cytomegalovirus protease have also been developed.
7. Egress of the virion from the cell. Whereas the mechanisms by which viruses leave cells are understood to varying extents, this step has not been pursued for drug development.

The pathology of DNA viruses comes in three guises:

1. The destruction of the cell as an obligatory part of the production of progeny virus. All of the herpesviruses are capable of this, and it is the primary cause of damage by the α- and β-herpesviruses. This is also the primary cause of the pathology of adenoviruses, parvoviruses, and poxviruses.
2. Damage to the cell or organ resulting from the host immune response to the virus. This accounts for much of the pathology of the HBV; the loss of infected hepatocytes by T-cell–mediated destruction, coupled with high rates of viral infection, cause liver function to decrease to inadequate levels.
3. Stimulation of proliferation of the infected cell with the eventual generation of tumors. Tumorigenicity is an unintended consequence of the evolution of some viruses to regulate cellular proliferation, and it is a consequence from which the virus gains no benefit. This form of pathology is, in a sense, the most insidious of the three, because the tumors generally arise from the latent state of the virus or from a virus that has been unintentionally integrated into a cellular chromosome. In both cases, the viruses present very few protein targets for exploitation, either by the immune system or by drugs. The most serious clinical consequences of papillomaviruses and y-herpesviruses are a result of this mechanism.

The classic examples of tumor viruses among DNA viruses are papillomaviruses, HBV, and y-herpesviruses, particularly Epstein Barr virus (EBV) and human herpesvirus-8 (HHV-8, KHSV). EBV presents two

types of proliferative disorders. The first is an acute, usually benign and self-limiting, proliferation of B-cells when infection occurs as an adult, which is associated with mononucleosis. The second generally occurs years after the initial infection and causes tumors to form in a variety of cell types, usually of lymphoid or epithelial origin. Papillomaviruses cause proliferation of epithelial cells, which becomes more aggressive over time, with some cells eventually becoming neoplastic. The role of hepatitis B in forming hepatocellular carcinoma is less well understood, but it may require the integration of the usually episomal genome into a host chromosome. This provides the potential for disruption of regulation of both viral and cellular gene synthesis. For the herpesviruses, and to a lesser extent the papillomaviruses, the quantitative significance of virally induced tumors has increased with the increase in depression of immune system function, either because of infection by HIV or because of the need prevent organ rejection after transplantation.

2.1 Herpesviruses

Herpesviruses are ubiquitous viruses, both in terms of the number of species that they infect and the proportion of human population infected. They are widely distributed throughout the animal kingdom, with about 100 species identified thus far, of which eight infect humans. An estimated 50–90% of adult humans are infected with at least one species of herpesvirus. This percentage points out the importance of latency in the life cycle of herpesviruses—they spend most of their time in what is functionally a state of dormancy, and they have effectively evolved to evade immune surveillance. The importance of evading the host immune system is shown by the fact that herpesviruses are among the more common pathogens when immune surveillance is removed or impaired.

Herpesviruses have large double-stranded linear genomes that range in size from approximately 120,000–230,000 kb. After infection, the genomes become circular, and they remain in the nucleus as an episome. The protein composition of herpesviruses is complex; typically at least 30 proteins are found in the virion and at least twice that many are encoded in the genome. They are enveloped viruses, with several glycoproteins on the membrane surface. They encode an unusually large number of enzymes involved in replication of the viral DNA. There are seven proteins that are required to reconstruct herpes simplex virus-1 (HSV-1) DNA replication in vitro (5). In addition to these enzymes, additional enzymes involved in nucleotide metabolism and other steps are encoded. This wealth of viral machinery has made herpesviruses one of the most popular viral targets for drug development, with DNA replication receiving the majority of the attention. However, there are other targets that are now coming into their own, including a protease and enzymes required for processing the DNA and packaging it into capsids.

Understanding the entry of herpesviruses into cells has been complicated by the fact that there are multiple viral proteins involved in entry, and eliminating expression of them individually may not block infectivity (6–11). As pointed out by Roizman and Sears (12), this multiplicity of potential entry mechanisms may stem from the fact that herpesviruses infect multiple cell types, and different entry mechanisms may be needed for different cell types. If there is redundancy in entry pathways within the most commonly infected cell types, developing entry inhibitors of herpesviruses may be technically prohibitive.

DNA synthesis is carried out by viral enzymatic machinery in a manner that resembles that of host DNA replication. The exception is that the origin of replication is better defined for herpesviruses than for cellular DNA. The polymerases, helicases, primases, processivity factors, and single-stranded DNA binding proteins are nearly identical in function between virus and host (herpesviruses borrow their topoisomerase activity from the host cell). Fortunately, the structural differences in the proteins are sufficient to allow drugs to discriminate between the two. The need for herpesviruses to encode genes for enzymes involved in nucleotide metabolism may be a function of the amount of viral DNA that is synthesized per cell. Replication of viral DNA can result in an amount nearly equivalent to

the amount of host cell DNA, thus placing a large burden on the cellular nucleotide pools.

Herpesviruses are categorized into three classes, α, β, and y. There is overall conservation of the machinery for nucleotide metabolism, DNA replication, and virion assembly and packaging between the classes (13–15). They differ in the genes that are the determinants of their tissue tropism.

The human a viruses are HSV-1, HSV-2, and varicella zoster virus (VZV). These viruses are characterized by a rapid reproductive cycle and rapid spread in cell culture. They are latent primarily in sensory ganglia. HSV-1 is classically associated with cold sores, although it can infect any organ. Cold sores are the most common form of recurrent HSV-1 infection, with 17–30% of humans having at least one recurrence per year (16, 17). The most common route of infection is through the mouth, with primary infection ranging from asymptomatic to mild mononucleosis (18, 19). HSV-2 is mostly associated with genital infections. Its common transmission route is sexual contact (20–22), and infection is usually delayed until the onset of sexual activity. Otherwise, the infections by the two viruses share many similarities. Approximately 20% of the adult population is predicted to be infected with HSV-2 (23), although this rate can vary widely depending on the level of sexual activity (24, 25).

VZV results in chicken pox (varicella) on initial infection and shingles (herpes zoster) if it is reactivated later in life. Approximately 95% of the population is predicted to be infected (26, 27). This proportion should decrease with the recent introduction of a vaccine against VZV (28, 29). The site of entry of VZV is likely to be respiratory mucosal epithelium. From there, it is thought to replicate in lymph nodes and infect reticuloendothelial cells, moving from these cells to cutaneous epithelium (30). It is at this site that the skin lesions occur. VZV infects T lymphocytes, epithelial cells, and dorsal root ganglia (31–33). The source of latent virus is cells of dorsal root ganglia (34–37). The cause of the reactivation from these ganglia, leading to herpes zoster, is not understood.

The mechanisms used for entry, transfer to the nucleus, and for DNA and protein synthesis and assembly seem to be similar to HSV. Egress from the cell differs between the two, at least in cell culture. VZV is largely cell-associated, with little free virus found in cell culture (38–40). Whether this reflects a difference in viral spread *in vivo* is unclear.

The β-herpesviruses are more slowly replicating viruses. They include cytomegalovirus (HCMV), HHV-6, and HHV-7. β-Herpesviruses represent important opportunistic infections in cases of immunodeficiency. In a prospective study of 60 liver transplant patients, one-half were found to have active HCMV and HHV-7 infections, and a third had active HHV-6 infections (41).

HCMV is estimated to be responsible for about 8% of mononucleosis cases (42) and is a leading cause of birth defects caused by infection of the fetus (43). It is also the most frequent opportunistic viral infection found in HIV patients, with loss of vision because of retinitis the most common outcome (44). In a study conducted during the 1980s, 81% of AIDS patients were found to have signs of actively replicating HCMV at autopsy (45). Thirty to forty percent of AIDS patients with $CD4^+$ lymphocyte counts $<50/mm^3$ developed retinitis before the use of multidrug combinations for HIV infection (46).

The primary site of infection is mucosal epithelium. HCMV spreads throughout the body through infection of leukocytes, although the specific cell type is not well defined (47–52). HCMV infects cells of fibroblastic, endothelial, epithelial, macrophage, and muscle lineages (53). Myeloid cells are the most likely reservoir for latent infection (54–57).

The cellular receptors for β-herpesviruses have not been identified. Entry and transfer of the DNA to the nucleus seems to occur through pathways analogous to other herpesviruses (58). The synthesis of early proteins, DNA, and late proteins follows the pattern of a-herpesviruses, but the timescale is longer. HCMV requires approximately three times longer in cell culture to complete the infectious cycle than HSV-1 (59).

HHV-6 and HHV-7 are genetically similar to HCMV and resemble other herpesviruses in their mode of entry, lytic production of progeny virions, and exit from the cell. HHV-6 infects several cell types, including T-cells, epithelial and endothelial cells, and neurons (60–

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66). HHV-7 is found almost exclusively in T-cells (67–69). Infection rates for these two viruses have been estimated at 20–100% of the population (70–73).

The human y-herpesviruses include EBV and HHV-8. The y-herpesviruses have evolved two mechanisms for DNA replication, one used for production of progeny viruses (the lytic cycle) and the other simply for maintaining the presence of the viral DNA in the cell (latency). The latter is needed because these viruses primarily infect cells that either are programmed to divide or are reprogrammed by the virus to divide. The latent replication pathway maintains the copy number of viral genomes in the daughter cells. Note that this pathway is not entirely unique to y-viruses. Other herpesviruses are replicated by cellular polymerases after infection to increase the viral genome copy number. However, latency is more complex in the y-herpesviruses, and this is the only class that is associated with tumors.

The pathology of the y-herpesviruses is counterintuitive, because whereas the other two classes do their damage by lysis of the cell when viruses are released, almost all of the diseases associated with y-herpesviruses occur during the latent phase of their life cycle. With a- and β-herpesviruses, it is possible in principle to essentially eliminate clinical damage by preventing production of new viruses. With y-viruses, clinical damage can be lessened by this approach in that blocking virogenesis decreases the number of infected cells carrying latent genomes. However, it is the latent cells themselves in which disease occurs, so treating the disease state directly requires eliminating the latent cells. This is a tall order, and thus far, drug development has focused on the productive phase of the life cycle rather than the latent phase.

EBV was first identified because of its capacity to induce tumors in B-cells. It is a ubiquitous virus, with nearly 100% of children being infected in developing countries (74). In more developed countries, infection is delayed, and about one-half of the children are infected. However, by adulthood, greater than 90% of the population is infected. EBV is one of the etiological agents associated with infectious mononucleosis, and this is the usual outcome of acute infection in adults. Latent infection is associated with a variety of malignancies, including B-cell lymphomas, Hodgkin's disease, leiomyosarcomas (muscle tumors), T-cell lymphomas, and nasopharyngeal carcinoma (75–84).

B-cells are the primary host cells for latent infection by EBV (85–87), although it is not clear whether the initial site of infection is B-cells or mucosal epithelium (88, 89). Binding of EBV to B- cells is accomplished through the viral glycoprotein gp350 (90, 91) and the cellular protein CD21 (92, 93). The virus is internalized through endocytosis, and the DNA is transferred to the nucleus (94, 95). The initial response is establishment of a latent infection, with only rare cells undergoing a lytic infection. During its latent phase, EBV synthesizes up to eight proteins and several small RNAs. These proteins activate resting B-cells to divide, and one of them, EBNA-1, participates in the latent replication of the viral DNA (96–101). The cascade of events that leads to the switch from latent infection to lytic infection is initiated by the activation of the viral protein BZLF1, and forcing the expression of this protein is sufficient to induce lytic infection (102, 103). While the details vary, the basic outline of the lytic production of progeny virus is the same as for the other classes of herpesviruses.

HHV-8 is the etiological agent of Kaposi's sarcoma (KS) (104, 105), the most frequently found neoplasm in AIDS patients (45). It is also associated with body cavity–based lymphoma and multicentric Castleman's disease. Retroviral therapy often results in resolution of KS and clearance of HHV-8. This does not seem to be a result of a direct effect of the HIV inhibitors on HHV-8, but rather a result of restoration of immune function after effective treatment of the HIV infection (106). The entry and exit of the latent phase are similar to EBV, and lytic production of daughter virions resembles other herpesviruses.

2.2 HBV

An estimated 350 million people worldwide are infected with HBV (107). The disease caused by HBV results not so much from the action of virus per se, but rather the damage that occurs to liver tissue as a result of the immune response to the virus. This liver dam-

age can lead to hepatic fibrosis, cirrhosis, liver failure, and hepatocellular carcinoma (HCC), although HCC may have a more direct viral component (108–110). Chronic HBV infection is associated with an approximately 150-fold increase in the likelihood of developing HCC (111,112). A vaccine is available for HBV, but difficulties of distribution and the large population of currently infected individuals have maintained a relatively high incidence of infection thus far (113). Thus, there is likely to remain a need for effective HBV treatment regimens in the foreseeable future.

Infection of adults is usually from sexual contact or exposure to contaminated blood (114, 115). The primary infection can be asymptomatic, or it may result in acute hepatitis. Most patients will resolve the primary infection (116,117); about 95% of patients infected as adults clear the virus after an acute infection, although only about 10% of children infected before 4 years of age do so (118,119). The remaining cases develop a persistent infection. This persistent infection can range from asymptomatic to high levels of viremia. Even for asymptomatic cases, there is a high incidence of HCC over the course of 30 years.

HBV is a DNA virus 3.2 kb in length with a primary target of the liver (120). In persistently infected individuals, about 10^{11} virions are released into the blood per day, and these virions have a half-life of about 1 day (121). The virus enters the cell in a partially double-stranded form and is converted to completely double-stranded, covalently closed, supercoiled DNA (cccDNA) in the nucleus (122–125). RNA is synthesized from this form, both for translation into viral proteins and for synthesis of genomic DNA. The combination of an RNA genomic intermediate and the cccDNA form presents two barriers to HBV antiviral development. First, the reverse transcriptase of HBV polymerase shares the feature of lack of fidelity with other reverse transcriptases. This lack of fidelity allows drug-resistant mutants to develop relatively easily. Second, the cccDNA form of the genome is quite stable. Drugs that block HBV polymerase activity have no effect on the cccDNA, and the infected cells remain and resume production of virus if treatment is stopped. At this time, the only way to remove the cccDNA is to destroy the infected cell by T-cell–mediated recognition of HBV epitopes. This is a slow process; HBV treatment generally requires time periods in excess of a year. The time required for removal of nuclear genomes is often longer than the time needed for resistance mutations to HBV inhibitors to develop, necessitating changes in treatment or additional treatments.

The proteins encoded by HBV are an important diagnostic tool in monitoring the state of the disease. HBV synthesizes four groups of proteins. The polymerase is both an RNA- and a DNA-dependent polymerase. The core protein (HBcAg) is the structural protein of the nucleocapsid. The core open reading frame also encodes a longer variant, referred to as HBeAg (126–131), whose function is unclear, but it is the best barometer of the outcome of an infection. A high level of HBeAg indicates a high level of viral replication. As an effective immune response is mounted against an HBV infection, HBeAg protein disappears from the serum, and antibodies to HBeAg appear; a process referred to as seroconversion. The strongest predictor of survival is seroconversion, with clearance of HBeAg and development of antibodies to HBeAg (132). The loss of HBeAg is not a perfect barometer, however, because mutations that result in the loss of expression of HBeAg can occur. Another indicator of the state of an infection is an increased presence of hepatocyte enzymes in serum. Serum alanine transferase (ALT) levels are used as an indication of the extent of liver cell necrosis.

HBV treatment presents a conundrum. On the one hand, the presence of elevated ALT and antibodies against HBeAg is an indication of liver damage resulting from an immune response to cells carrying surface HBV antigens. On the other hand, some amount of this response is necessary to clear the liver of cells carrying nuclear DNA copies of the genome. When effective treatments for HBV replication (e.g., lamivudine) are stopped, there is often a transient increase in ALT and viral DNA levels in serum. These symptoms usually clear without treatment and are probably a result of an immune response to cells where virion production resumed when the selection pressure of the drug was removed (133).

Stimulation of the immune response to HBV by interferon-a (IFN-a) is an approved treatment method for HBV. However, only a small proportion of patients have responded well to modulating the immune response alone. Thus, small molecule inhibitors of HBV replication are critical to treating HBV. Furthermore, the development of strains resistant to monotherapy has dictated the need for additional anti-HBV drugs that can be used in combination therapy.

Several species play host to closely related viruses, and two of these have seen extensive use as models for human HBV infection. The first model is infection of duck hatchlings with duck hepatitis B virus (DHBV) (134). The second is chronic infection of woodchuck with woodchuck hepatitis virus (WHV). With the exception of interferon, comparison of compounds that have been studied both in clinical trials and in the woodchuck model shows good agreement between the two with regard to relative potencies of compounds (135).

2.3 Papillomaviruses

The papillomaviruses were initially grouped with the polyomaviruses in the Papovaviridae family. However, the genetic and phenotypic differences between them are sufficiently great that papillomaviruses and polyomaviruses are now placed in separate families. Papillomaviruses are small double-stranded DNA viruses which can exert dramatic effects on the proliferation of the infected cell. Over 70 distinct genotypes have been identified as infecting humans (136), and this variety is reflected in a variety of phenotypes observed. Phenotypic differences between strains are manifested in two ways: first, the cell type and anatomical location that is infected, and second, the severity of the infection. Papillomaviruses are highly restricted in the cell types that they infect. They infect epithelial cells (and in some cases, fibroblasts), but their tropism is more narrowly defined in that they infect only a specific type of epithelium, e.g. genitalia, bottoms of feet or hands, etc. About 40 of these strains infect mucosal, primarily anogenital, epithelium (137). The cellular proliferation that they support takes a number of morphological forms — warts, cysts, intraepithelial neoplasias, and papillomas, etc. The final outcome of the proliferation is a function of the infecting strain. For the so-called low risk strains, these growths are usually benign, although they can be quite debilitating. For a subset of high risk strains (strains 16, 18, 31, 33, 45, others), these proliferating cells have a high propensity to develop into tumors (138, 139). About 95% of cervical cancers are caused by the high risk strains of HPV (140–142).

Papillomaviruses gain entrance to the body through breaks in the epithelium, which expose the basal epithelial cells (143). These cells are the sites of the initial infection, and they remain latently infected. They are constantly dividing, with one of the daughter cells migrating upwards toward the epithelial surface. As they move upward, the cells begin a complex differentiation program. Only when they are well along this differentiation pathway does virion production occurs. The progeny viruses are then shed at the skin surface. The primary route of transmission of anogenital viruses is sexual contact (144–146).

As is the case for other latent viruses, the balance between viral activity and immune surveillance is an important factor in the clinical outcome. Whereas the effect of immune suppression is not as dramatic for papillomaviruses as for herpesviruses, there is nevertheless an increase in pathological severity in immunosuppressed patients (147–149).

Papillomaviruses are non-enveloped viruses approximately 55 nm in diameter (150), with a genome of 7000–8000 base pairs. The capsid is composed predominantly of the L1 coat protein, with L2 as a minor substituent. Virus-like particles (VLPs) can be assembled from recombinant L1 and L2 in the absence of DNA. These VLPs are the primary focus of vaccine development. The genome consists of three parts: a regulatory region (long control region, LCR), an early region (E) encoding the nonstructural proteins, and a late region (L) encoding L1 and L2. The LCR contains binding sites for both E2 and a number of cellular transcription regulators. These factors are both positive and negative regulators, something that is very important with regard to progression to cancer.

Papillomaviruses encode three types of potential drug targets, two of which are currently of interest for medicinal chemistry.

1. E1 and E2. E2 is a regulatory protein for both transcription of viral genes and for viral DNA replication. It recognizes a 12-nucleotide binding site and activates transcription of viral genes (151,152). A subset of the E2 binding sites is included in the origin of replication, and E2 binding is obligatory for efficient assembly of a replication complex at the origin (153, 154). E2 also has a role in ensuring that the viral genome gets distributed properly during mitosis and maintained in the nucleus during interphase (155). In addition to positively regulating transcription, E2 negatively regulates the synthesis of E6 and E7 RNAs. In tumor cells, this control is usually (though not always) abrogated by ligation of the viral DNA into a host chromosome at a point in the viral genome, such that the E2 binding sites are displaced from the E6 and E7 genes (156). Transcription of the E6 and E7 genes is consequently up-regulated, resulting in a cascade of events that lead to cellular proliferation. This mode of action of E2 complicates its use as a drug target, because inhibiting the negative regulation could actually stimulate E6- and E7-mediated proliferation. E1 is the only contribution of the virus to the DNA replication machinery. It is a site-specific DNA binding protein that has ATPase and DNA helicase activity (157, 158), and is responsible for recognition of the viral origin of DNA replication and helping recruit cellular replication proteins to this origin (153, 159). E1 then provides the essential helicase activity for the replication complex.
2. E6 and E7. The primary effect of these proteins is to inhibit cellular proteins controlling cell division. E7 binds the pRB protein to inactivate it (160, 161), and E6 binds p53 and activates a pathway that leads to its degradation (162, 163). E6 and E7 determine the carcinogenic potential of papillomaviruses. In high risk strains (e.g., 16, 18), they form strong associations with their cellular targets. In low risk strains (e.g. 6, 11), this association is weaker (164). The low risk strains efficiently cause cell proliferation, but cells become malignant less frequently than with high risk strains. The regulation of p53 activity alone by E6 is not sufficient to account for the activity of E6. An additional effect of E6 is to form a complex with the focal adhesion protein paxillin, disrupting the cytoskeletal structure (165).
3. The structural proteins L1 and L2. While these proteins are currently of interest for vaccine development, our understanding of papillomaviral entry has not lent itself to use of these proteins as targets for small molecule inhibitors.

These targets have proven remarkably elusive because papillomaviruses have developed such an intricate relationship with the cell. The pathogenesis of these viruses occurs during their latent phase, when viral DNA is being replicated but no infective virus is produced. Furthermore, the virologist's favorite target has been taken away—there is no viral polymerase. E1 helicase is an essential part of the replication machinery, but attempts to identify effective inhibitors of the helicase have not yet been successful. The end result has been that infection has been treated with blunt instruments, either by removing most of the infected cells surgically or with cytotoxins, or by stimulating an immune response against the virus. Success by these approaches has been limited, leaving room for small molecule inhibitors of viral activities.

2.4 Polyomaviruses

Of the 11 viruses currently in the Polyomaviridae family, two of them, BK and JC, infect humans. They are small double-stranded DNA viruses, with a diameter of about 40 nm and genome sizes of about 5000 base pairs. The most common route of infection is likely to be respiratory (166,167). They seem to disseminate through the body through B-cells, and viral DNA can be detected in 5–40% of healthy subjects (168,169). The tissue tropism is different for JC and BK viruses, with BK propagating in epithelial or fibroblastic cells and JC infecting neuronal cells.

JC virus causes progressive multifocal leukoencephalopathy (PML) in immunodeficient patients (170). This disease results from the destruction of the cells that produce the my-

elin sheaths that surround nerves. BK virus is associated with a wider variety of disease states, although it is quantitatively less important than the JC virus. These diseases include retinitis, nephritis, pneumonia, encephalitis, and kidney diseases associated with renal transplantation (171–177).

Polyomaviruses encode three capsid proteins and two nonstructural proteins. The nonstructural proteins provide both an enzymatic activity and regulatory functions. The smaller of the two (t antigen) is dispensable for lytic infection but interacts with cellular proteins involved in cell cycle regulation (178–181). The larger protein (T antigen) is a cornucopia of activities. It is a replication protein that recognizes the origin of replication, partially unwinds it on binding to it, and attracts cellular replication proteins to the site to initiate replication (182–188). It then becomes a replicative helicase, which remains associated with the cellular replication machinery to complete replication of the viral genome (189, 190). In addition, it combines two of the activities carried out by E7 and E6 of papillomaviruses: (1) it binds to Rb protein and the related p107 and p130 proteins, causing the release of the E2F transcription regulator, thus promoting progression of the cell into S phase (191, 192); and *(2)* it binds to p53, marking it for degradation (193, 194).

T antigen is the only protein that polyomaviruses contribute to the replication of their genome, thus, once again a viral polymerase is unavailable. However, experience with papillomaviruses has indicated that these viruses activate poorly understood pathways that increase the sensitivity of the cellular polymerases to certain polymerase inhibitors, the current example being cidofovir. This activity thus provides some selectivity of the drug for infected cells, even though it is targeting a cellular polymerase. The possibility exists that similar mechanisms will exist for polyomaviruses.

Binding and entry into the cell are poorly understood for polyomaviruses. JC virus seems to bind to a sialic acid–containing surface protein (195), and there is a report that BK virus may bind to phospholipids (196). If these observations are related to viral entry, it is unlikely that they will be the entire story; there are likely to be multiple receptor factors. The bound virus enters the cell by endocytosis and is transferred to the nuclear membrane, where the DNA genome becomes uncoated and transferred into the nucleus. The nonstructural proteins are synthesized as early proteins, and DNA synthesis and late protein synthesis follow. Capsids are assembled in the nucleus (197, 198). Packaging of DNA and egress from the cell are not well understood for polyomaviruses.

2.5 Adenoviruses

Adenoviruses infect multiple tissue types and are the causative agent of an estimated 3% of respiratory disease in humans (199). Antibodies to adenoviruses can be found in about 50% of children (200), and adenoviruses have been detected in 5–50% of pediatric patients presenting with lower respiratory tract infections (201, 202). The virus enters its host through the mouth, nose, or eyes, and the primary sites of replication are respiratory epithelium, the eyes, and the gastrointestinal tract. Low levels of viral replication can persist for months to years, but the mechanisms supporting this persistency are not well understood (203–205).

Whereas adenoviruses are a less significant problem during immunosuppression than some other classes of viruses, immune dysfunction can exacerbate adenovirus infections. A study of 572 transplant patients indicated that 17% had an active adenovirus infection in the 150 days following transplantation; six of these infections were lethal (206). Adenovirus infection is more likely to lead to pneumonia in immunocompromised patients than in immunocompetent individuals (207–211).

Adenoviruses were discovered about 50 years ago and they grow well in cell culture, factors that led to their development as model viruses for studying both cellular and viral processes, including gene expression and regulation, RNA splicing, and cell cycle control. Adenovirus and papovavirus SV40 were key models for determining the enzymology by which semiconservative DNA replication occurs in eukaryotes (212). The discovery that human adenoviruses could cause malignant tumors in rodents led to intensive searches for

adenovirus DNA in human tumors (213), but there is no evidence that adenovirus causes malignancy in the species from which it was derived. In recent years, much effort has gone into developing adenoviruses as gene therapy vectors (214–217). However, they have not received much attention with regard to development of chemotherapeutic agents.

Adenoviruses are non-enveloped viruses measuring 70–100 nm in diameter. They have an outer shell composed of seven peptides and an inner core with four peptides (218, 219). The linear double-stranded DNA genome consists of approximately 36,000 base pairs. They are rapidly replicating viruses, with a life cycle of about 24 h in cell culture. Binding and entry of adenoviruses is relatively well understood. Two cellular proteins, the CAR protein (a member of the immunoglobulin superfamily) and a member of the integrin family, form the cellular receptor (220,221). The virus is internalized through endocytosis (222–224), released into the cytoplasm, and transported to nuclear pores, where the viral DNA is released into the nucleus.

Entry of the DNA into the nucleus is followed by early mRNA synthesis, the products of which both cause the cell to enter S phase and are needed for viral DNA synthesis. Adenoviruses encode their own DNA polymerase (225, 226). Replication requires minimally three viral proteins and two cellular proteins and differs significantly from the host cell DNA replication. Late protein synthesis is timed to begin at the time of DNA replication. The late gene products are assembled into capsids in the nucleus, after which a DNA molecule is packaged in the capsid (227). Enzymes for packaging the DNA into capsids and a virally encoded protease are both needed for assembly and maturation of the virions (228, 229). These are both steps that have been successfully employed as drug targets in herpesviruses.

2.6 Parvoviruses

The Parvoviridae family contains several significant animal pathogens, including one human pathogen, B19. Antibodies to B19 have been found in 30–50% of humans (230–233), indicating that exposure to the virus is much broader than the pathogenesis associated with it. However, it is associated with several disease states, including red cell aplasia, fifth disease (often confused with measles in children), and fetal wastage (232, 234–238). Acute infection of adults results in nonspecific influenza-like symptoms, followed about a week later by symptoms of fifth disease (239–241). The primary cell types infected are of the erythroid lineage (242, 243). In immunocompromised patients, the most significant complication is red cell hypoplasia (244, 245).

Parvoviruses are the smallest of the human DNA viruses, with virions approximately 20–25 nm in diameter. The potential use of parvoviruses as gene therapy vectors (246, 247), as well as the existence of B19, has spurred interest in this family of viruses. Parvoviruses are non-enveloped viruses, with a capsid composed of either two or three proteins. This capsid contains one copy of the approximately 5000 base single-stranded genome (248). Both ends of the DNA contain palindromic sequences that are important for DNA replication (249–251). The DNA contains two large open reading frames, each of which codes for two to four proteins by a combination of differently spliced products and different translational start sites (248, 252–254).

As might be expected based on their size, parvoviruses are among the most dependent of viruses on host cell functions. They require that the host cell enter S phase to replicate, but unlike herpesviruses, papillomaviruses, or polyomaviruses, they do not encode a mechanism for activating the cell to do so (255–257). Two mechanisms are used by parvoviruses to bypass this block to productive viral infection, and the viruses fall into two genuses based on which approach they use. The first is to simply wait for the cell to enter S phase, the approach taken by the autonomous parvoviruses, including B19. The second is to depend on a helper virus, such as adenovirus or a herpesvirus, to activate the cell; this is the approach adopted by the helper virus dependent parvoviruses. Adeno-associated virus (AAV) is the prototype for this genus of parvoviruses. AAV integrates into a site on chromosome 19 after infection, where it remains until it is rescued by an adenovirus or a herpesvirus (258,259). Thus, it has a mechanism for maintaining la-

tency. Whereas autonomous parvoviruses can develop a persistent infection, there is currently no evidence for a latent state.

Parvoviruses are relatively, though not entirely, species specific (260). Determinants on the capsid proteins needed for entry have been mapped, and for B19, the cellular receptor is globoside, the blood group P antigen (261). Entry is mediated through an endocytotic pathway, and the viral DNA is delivered to the nucleus. Replication of the viral DNA is through a mechanism that relies on the palindromic sequences for initiation of synthesis. The salient features of parvovirus replication from a medicinal chemistry perspective are as follows: (1) DNA synthesis is by the cellular DNA polymerases; (2) replication generates concatamers of the genome, which must be cleaved; and (3) the virus supplies the helicase activity. Both of the latter activities come from the NS1 protein in B19, or the Rep 78/68 protein in AAV (262–265). After translation of the coat proteins, they self-assemble in the nucleus, where the genomic DNA is packaged.

2.7 Poxviruses

Poxviruses are unique among human DNA viruses because they are complex DNA viruses that replicate entirely in the cytoplasm of host cells, in structures constructed by the virus (266). Their dimensions are approximately 350 by 270 nm, with genome sizes ranging from 130,000 to 300,000 base pairs (267). These genomes encode up to about 200 open reading frames (268–275). They contain virally encoded systems for carrying out both transcription and replication of viral genes and complex schemes for regulating these processes. In short, there are more potential drug targets encoded by poxviruses than for any other virus discussed in this chapter. However the extent of development of antipoxviral therapies has been limited, and their biology will therefore not be outlined in more detail here. It is reviewed by Moss (276).

Before the 19th century, poxviruses were a serious human health threat in the form of smallpox. With the introduction of vaccines and a campaign against smallpox on the part of the World Health Organization, smallpox was declared eradicated in 1980. Whereas laboratory stocks still exist, the last documented infection was reported almost 25 years ago. Nevertheless, there are scenarios whereby poxviruses could become a health threat (277).

1. It would be a formidable biological weapon. A generation has grown up unvaccinated, and therefore susceptible, and current vaccine stocks are likely insufficient to stop its spread.
2. Related poxviruses may have the potential to become more virulent. As an example, human monkeypox outbreaks have occurred in central Africa within the last decade. Increased virulence or host range of this or a related poxvirus could have a significant health impact.
3. Recombinant vaccinia viruses have clinical potential as vehicles to deliver antigens from pathogens. However, in immunocompromised patients, vaccinia virus itself could become pathogenic.

While poxviruses may not generate much activity as drug targets in the near future, it is worth remembering that they are not extinct. They have generated significant human pathogens in relatively recent history. It is possible that others could again emerge from this family, a scenario played out from the retrovirus family with devastating consequences by HIV-1 in the 20th century.

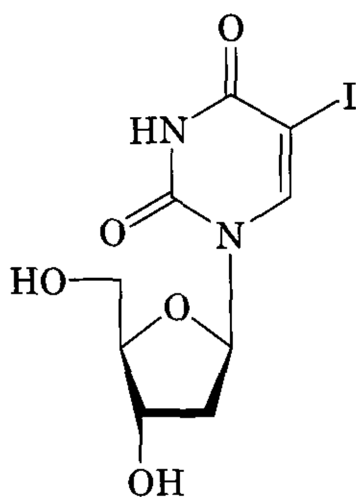
3 ANTIVIRAL COMPOUNDS FOR DNA VIRUSES

The majority of approved drugs and compounds under development for DNA viruses are nucleoside analogs, although there are exciting developments in other target areas that will be discussed. This discussion is organized around the virus families. The currently available drugs are discussed first, followed by compounds under development. A factor of increasing concern with regard to treating DNA viruses is the development of resistance to drugs, and this topic will be discussed for currently available drugs.

3.1 Herpesviruses

3.1.1 Currently Approved Drugs. This section starts with acyclovir and its progeny, and

follows with other nucleoside analogs. Foscarnet is not a nucleoside analog, but functionally it shares many of their features and will be discussed with them. Whereas a wide variety of antiherpesvirus nucleosides have been synthesized, much anti-herpesvirus drug development has focused on guanosine and thymidine analogs. The root of thymidine analog development was idoxuridine (5-iodo-2′-deoxyuridine, IDU) (1)(278), which was licensed for topical treatment of herpesviruses. Acyclovir (ACV) (2) is both the ancestor of and the paradigm for development of many of the purine nucleoside inhibitors. Discovered in the 1970s, it demonstrated a combination of metabolic stability and low toxicity that provided the springboard for development of a series of antiherpesviruses drugs (see Ref. 279 for a review). The relatively low toxicity derives from three sources. First, ACV is an analog of 2-deoxyguanosine, which must be activated by conversion to the triphosphate. Phosphorylation to ACV monophosphate is carried out efficiently by the thymidine kinases of α- and γ-herpesviruses but very poorly by cellular enzymes. These thymidine kinases, unlike their cellular counterparts, bind a wide range of nucleosides in addition to thymidine. Second, the first phosphorylation results in localization of the monophosphorylated form to infected cells because the charged phosphate prevents translocation across the plasma membrane. Thus, ACV is concentrated in the infected cells. Third, ACV is recognized more efficiently by viral than cellular polymerases, providing another source of selectivity.



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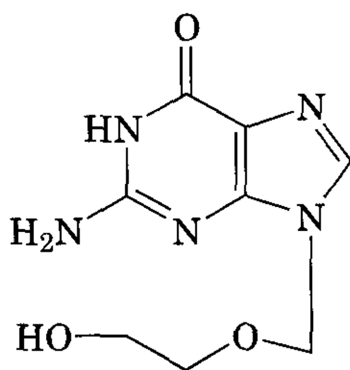


(2)

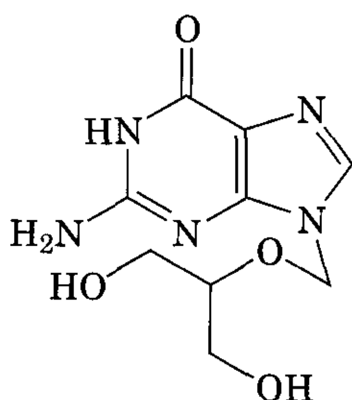
Further phosphorylation of ACV by cellular kinases leads to the triphosphate, which is incorporated into DNA by the viral DNA polymerase. Because it lacks an acceptor for the incoming phosphate of the next nucleotide, ACV acts as a chain terminator. Specifically, work of Reardon and Spector demonstrated that ACV is a competitive inhibitor with respect to deoxyguanosine triphosphate (dGTP), and that incorporation of ACV results in a large increase in K_m of the next incoming base (280).

Acyclovir has been a very successful drug, with good potency against HSV and VZV and with few side effects. The attractive features of the ACV paradigm are delivery of the drug in an inactive form, which is activated primarily in infected cells and selectivity for the viral DNA polymerase over cellular enzymes. However, the specificity provided by the requirement for a viral kinase, along with the relatively low bioavailability of ACV, limit the diseases that it can be used to treat. ACV is a standard treatment for HSV-1, HSV-2, and VZV, but weakly effective against HCMV. HCMV does not encode a thymidine kinase, although a putative protein kinase, UL97, is capable of phosphorylating ACV at low levels. ACV is also ineffective against HSV and VZV strains that are deficient in thymidine kinase activity. The viral thymidine kinases are attractive drug targets because they are well studied, both functionally and structurally (reviewed in Ref. 281).

Additionally, ACV has a low oral bioavailability (15–30%). Increasing oral bioavailability of ACV would allow lower or less frequent doses. Prodrug forms of ACV and its analogs were developed that provide substantial increases in bioavailability. Valaciclovir (VACV) is a valyl ester of ACV that improves bioavail-

ability to greater than 70%. The currently available progeny of ACV will be discussed with regard to improving on these two limitations of ACV.

3.1.1.1 Range of Action-Modifications to ACV and Development of Unrelated Drugs. Relatively small changes to the acyclic side chain of ACV have resulted in two additional drugs, ganciclovir (GCV) (3) and penciclovir (PCV) (4) that differ in their effectiveness



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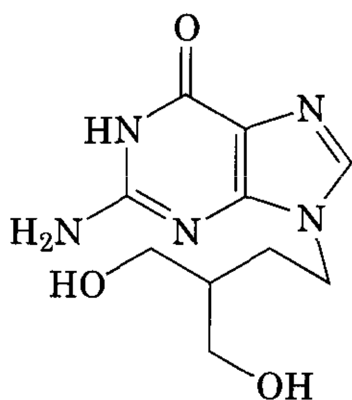


(4)

against herpeviruses. These differences result from changes in the efficiency of recognition by viral kinases and viral DNA polymerases.

Addition of a hydroxymethylene group to the acyclic side chain of ACV yielded GCV. GCV has antiviral activity against all herpesviruses, although its potency varies between viruses. GCV is more effective against HCMV than against HSV or VZV (282), the reverse of ACV. It is phosphorylated by both viral thymidine kinases (283) and by HCMV UL97 kinase (284, 285). HCMV phosphorylates GCV relatively efficiently, whereas it is very poor at phosphorylating ACV. HHV-6 and HHV-7 are β-herpesviruses with genetic similarity to HCMV. GCV is a potent inhibitor of HHV-6, but it is a weak inhibitor of HHV-7, indicating that there are differences within the class of β-herpesviruses (286–290). GCV does have efficacy against thymidine kinase-deficient VZV strains, because of the fact that VZV encodes a UL97 homolog (ORF47) (291). GCV-triphosphate has a half-life sixfold longer than that of ACV-triphosphate, which results in higher intracellular concentrations of GCV-triphosphate than of ACV-triphosphate (reviewed in Ref. 292). This difference in triphosphate concentrations gives GCV efficacy against HSV, even though ACV-triphosphate is recognized by HSV DNA polymerase with higher affinity. GCV is also active in inhibiting HBV replication. The mechanism of action of GCV differs somewhat from ACV. Whereas ACV terminates the growing DNA chain, GCV seems to slow down the rate of elongation, while at the same time increasing the amount of repair.

The factors limiting GCV efficacy are its low bioavailability (<10%) and its side effect profile. Maintaining serum levels effective for treatment by oral administration of GCV is difficult; therefore, the initial treatment course involves intravenous injections twice daily for 2–3 weeks, followed by daily injections or oral dosing. The prodrug form valganciclovir improves the oral bioavailability of GCV (see Section 3.1.1.2), although it still has the toxicity profile of GCV (293). Despite the existence of at least four treatments for HCMV, there is still an unmet need for an effective, orally available drug with a limited side effect profile.

Intravenous delivery of GCV has been of value in treating HCMV retinitis, a complication of AIDS that often results in blindness. GCV is also efficacious for infections of the gastrointestinal tract and central nervous system. For HCMV retinitis, an additional option is to treat by implanting a slow release pellet in the eye. The pellet maintains high concentrations of GCV in the eye for a period of 7–8 months, with very low systemic concentrations. This results in effective treatment of the retinitis with low side effects. However, because HCMV causes a systemic infection, the localized treatment of the implant leaves the patient at risk for retinitis in the other eye or for gastrointestinal or neural complications.

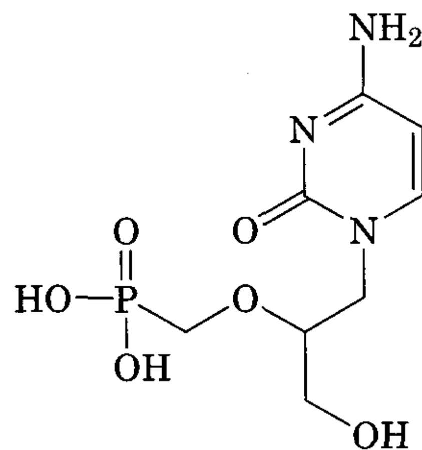
Thus, treatment with implants is often combined with oral GCV to limit systemic viremia. Treatment options for HCMV retinitis are reviewed by Hoffman and Skiest (294).

The trade-off for the larger number of treatable viruses is toxicity—GCV is more cytotoxic than ACV and must be managed more carefully when used as a maintenance therapy. This aspect is critical because effective treatment requires long-term administration of the drug. The cytotoxicity is a function of the fact that GCV is phosphorylated more readily by cellular kinases than is ACV.

Penciclovir (4) shares structural similarity with GCV but is closer to ACV in its antiviral spectrum (295). It is phosphorylated by HSV thymidine kinase, and thus is inactive in kinase-deficient strains. However, the triphosphorylated form retains activity against ACV-resistant HSV polymerase. HSV thymidine kinase has a much higher affinity for PCV than ACV (K_i = 1.5 versus 173 μM), but the triphosphate form is used about 40-fold less well by the HSV and VZV polymerases than ACV triphosphate (296–298). On the other hand, PCV triphosphate is approximately 10-fold more stable than ACV triphosphate, such that PCV-triphosphate levels are over 300-fold higher than ACV-triphosphate levels. In cell culture, the drugs were about equally inhibitory when present continuously [EC_{50} = 3.8 and 4.2 μg/mL against VZV (298)], but PCV retained antiviral activity for longer on withdrawal. The weak activity of PCV against HCMV suggests that it is not phosphorylated efficiently by the UL97 kinase.

PCV is activated at a low level by cellular kinases. This, combined with a greater than 4000-fold greater affinity for the HBV polymerase than the cellular polymerase, makes PCV an inhibitor of HBV replication. Oral bioavailability is about 5%; however, an effective prodrug form, famciclovir, has been developed (see below).

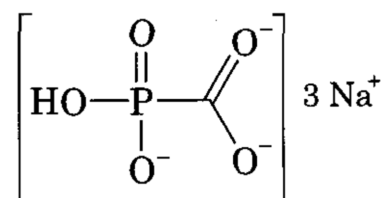
Cidofovir [9-(3-hydroxy-2-(phosphonomethoxy)propyl)cytosine, HPMPC] (5) is a pyrimidine phosphonate analog. Because it does not rely on a viral kinase to generate a monophosphate form, it is active against more viruses. In addition to HCMV and other herpesviruses, it is active against papillomaviruses, adenoviruses, and poxviruses. Because oral



(5)

bioavailability is less than 5%, intravenous injection is required for systemic treatment (293). This is countered somewhat by its slow turnover, which allows longer periods between dosing. The primary factor limiting treatment with cidofovir is renal toxicity, which can be ameliorated somewhat by treatment with probenecid, hydration, and longer dosing intervals (299, 300). In a phase I/II study of its anti-HCMV effect, cidofovir was effective at inhibiting HCMV replication at a dose of 3 mg/kg weekly. The dose-limiting toxicity was renal damage (301).

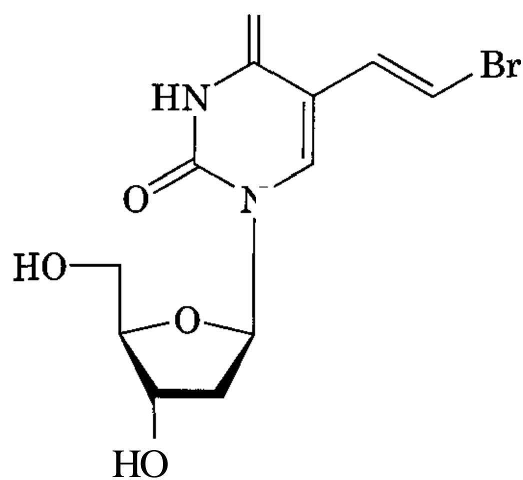
Foscarnet (phosphonoformic acid) (6) is not a nucleoside analog, but rather an analog



(6)

of the leaving group, pyrophosphate. It is active against all of the herpesvirus DNA polymerases but must be administered intravenously. Effectiveness against HCMV is equivalent to GCV, and the magnitude of side effects is similar. Side effects include renal toxicity and electrolyte imbalances (293). GCV has the advantage of a prodrug form, valganciclovir, with an oral bioavailability of 60%, whereas there is no prodrug from of foscarnet. However, foscarnet can be useful as a salvage therapy for HCMV infections that do not respond to GCV.

Brivudin (BVDU) (7) is a selective inhibitor of herpesviruses, with the selectivity deter-



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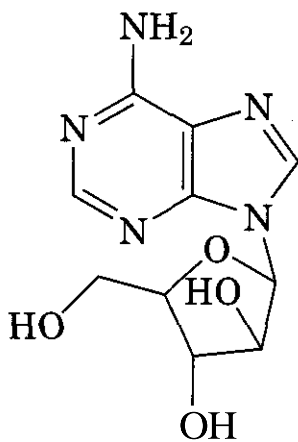
mined by the capacity of each viral kinase to convert it to the monophosphorylated form. BVDU is active against HSV-1, EBV, and is a very potent inhibitor of VZV, but is inactive against HSV-2, HCMV, HHV-6, and HHV-7 (302, 303). Orally administered brivudin was shown to be as effective as intravenous ACV in treating VZV in immunocompromised patients (304,305). However, metabolic instability of BVDU has led to further development of analogs, discussed later.

Vidarabine (araA) is an adenosine analog, with the D-ribose replaced by D-arabinose (8).



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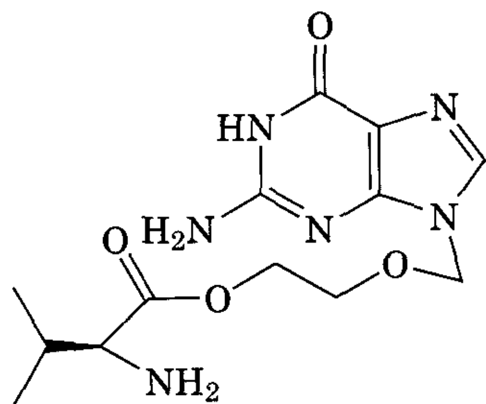
It is active against more viruses than ACV or GCV. However, it is also more toxic and less metabolically stable; it is prone to deamination by adenosine deaminase (303, 306–308). The deaminated product is at least 10-fold less potent than araA (309). Unlike ACV, araA is not preferentially phosphorylated in virus-infected cells. However, the half-life of the triphosphate form of araA (ara-ATP) is approximately threefold longer in HSV-infected cells compared with uninfected cells (310).

Resistance mutants of araA map to the DNA polymerase. AraA inhibits DNA polymerase activity through competitive inhibition with dATP. Ara-ATP is a substrate and can serve as an acceptor for the next base, but does so inefficiently, indicating that the mechanism of action of araA is both direct inhibition of viral DNA polymerase and a decrease in the rate of elongation by increasing the amount of excision repair needed (311). Because of its more rapid turnover and poorer toxicity profile, ACV and its derivatives have superseded ara A's use, but it remains a platform for further development.

Fomivirsen is a 21-nucleotide phosphorothioate oligonucleotide complementary to a region of the mRNA encoding immediate-early proteins of HCMV. Its mode of action is to bind to the mRNA and provide a substrate for a cellular RNase that destroys the mRNA. It has been approved for use in treating HCMV retinitis (312). In cell culture, it has an EC_{50} between 0.03 and 0.2 μM in fibroblasts. Using retinal pigment epithelial cells as the cell culture model rather than fibroblasts gave a sixfold increase in potency (313). Clearance studies in rabbits have indicated a half-life of 62 h; it is applied to patients intravitreally on a weekly basis. However, its use may be limited because of retinal toxicity (314).

3.1.1.2 Prodrugs that Increase Oral Bioavailability. The other limitation to ACV and the related drugs discussed earlier is their low oral bioavailability. A number of prodrug approaches have been used to increase oral bioavailability. Esterification of the acyclic side chain has been the most successful. For ACV, esterification with valine to give valciclovir (VACV) (9) resulted in a threefold improvement in oral bioavailability, with a spectrum of action and toxicity profile similar to ACV. Acetal derivatives have also led to efficacious compounds. The diacetal derivative of PCV, famciclovir (FCV) (10) is approved for treatment of HSV-1 and HSV-2 diseases. Oral bioavailability for FCV is 70%, compared with 5% for PCV. Other prodrug approaches are discussed in Section 3.1.2.

3.1.1.3 Resistance in Herpesviruses. Drug resistance of herpesviruses is not a significant



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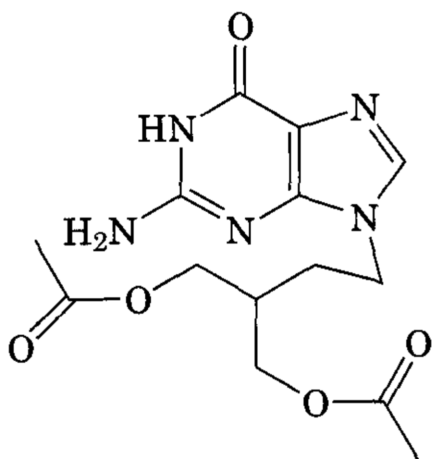


(10)

problem in immunocompetent hosts. Resistant strains do become a threat in immunocompromised patients, however, and this population is increasing in size because of suppression of the immune system in transplant patients and coinfection with HIV. This is particularly important for HCMV, where infection is largely asymptomatic except in immunocompromised patients.

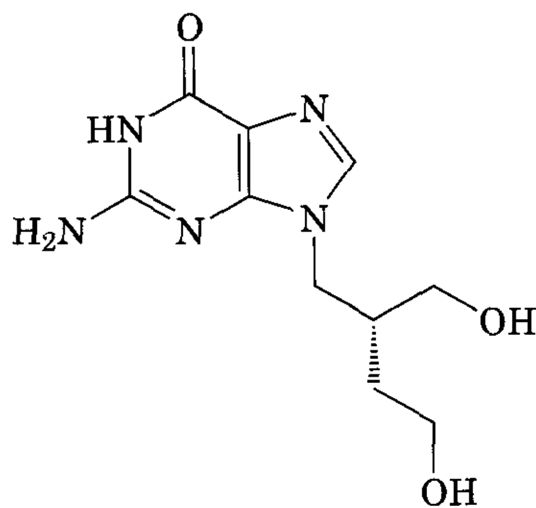
Patterns of resistance mutations generated by currently available drugs have been well documented (315–317). Phosphorylation of cidofovir and foscarnet by UL97 is not required, so mutations occur in the DNA polymerase. The majority of isolates of GCV-resistant mutants map to the UL97 kinase. However, with long-term use of GCV, or coadministration with cidofovir or foscarnet, mutations in the DNA polymerase are noted. The mutations tend to confer resistance to both GCV and cidofovir, and occasional resistance to foscarnet is also observed (317).

As with GCV in HCMV, resistance to ACV (and V-ACV) in HSV or VZV is most often a result of mutations in the viral kinase. This resistance takes two forms: mutations that alter the specificity of the kinase or loss of the expression of the enzyme. The same applies to PCV and FCV. ACV- and PCV-resistant strains are also generally cross-resistant, although the actual amino acid mutations selected by ACV and PCV differ (318).

Cross-resistance of drugs is an important issue in deciding salvage therapies when resistance occurs. Nucleoside analogs related to ACV (GCV, PCV, and their prodrugs) are cross-resistant, either because of a dependence on thymidine kinase for monophosphorylation or because they select for mutations in DNA polymerase that provide cross-resistance. Foscarnet is an alternative to these nucleoside analogs. Cidofovir also selects a different set of polymerase mutations, so that cidofovir-resistant strains remain sensitive to both foscarnet and ACV (319).

3.1.2 Compounds Under Development.

3.1.2.1 *Nucleoside Analogs.* (*R*)-9-(4-Hydroxy-2-(hydroxymethyl)butyl)guanine (H2G, omaciclovir) (11) is an acyclic guanosine analog



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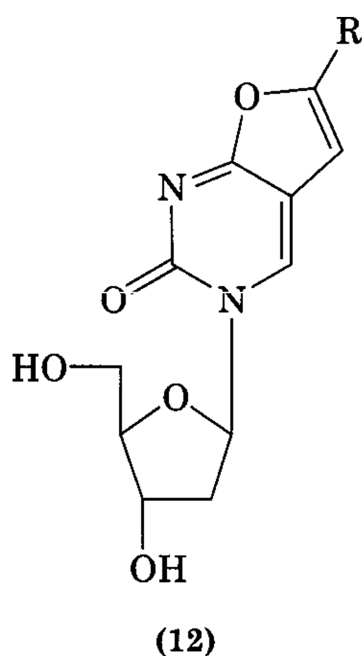
similar in structure to ACV. H2G is roughly comparable in activity to ACV against HSV-1, 20- to 400-fold more active against VZV, and somewhat less active against HSV-2 (320, 321), with a toxicity profile similar to ACV. H2G is efficiently phosphorylated by viral thymidine kinases (322), and the triphosphate form is maintained at levels more than 170-fold higher than for ACV in VZV-infected cells (321). The half-life of H2G ranged from 4 to 14 h, versus 1–2 h for ACV. H2G is a competitive inhibitor of dGTP incorporation by the

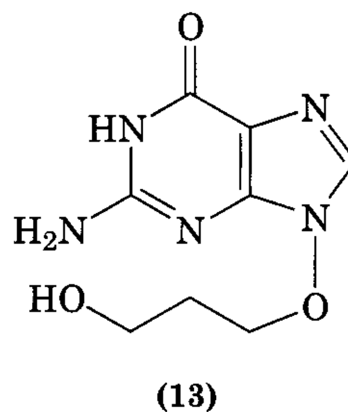
viral DNA polymerases and is poorly recognized by human **DNA** polymerase a. **H2G** seems to function by a mechanism analogous to GCV, in that it does not chain-terminate but rather inhibits progressive elongation by the polymerase. **H2G** is cross-resistant with ACV, and like **ACV,** resistance mutations accumulate first in the thymidine kinase **(321).** This compound is currently in phase **II** clinical **development.**

McGuigan et al. (323–325) constructed a potent and highly selective series of **VZV** inhibitors based on a bicyclic furopyrimidine base (12). When the R group was an 8–10 carbon alkyl group, these compounds were up to 100 times more potent in cell culture assays than ACV. A terminal halogen was tolerated with no loss of activity. Use of a phenyl group *para*-substituted with an alkyl chain increased potency a further 100-fold, with selectivity indexes of more than 10^6. The optimal chain length decreased from 8–10 to 4–7 with the insertion of the phenyl group. These compounds were inactive against other herpesviruses and required the VZV thymidine kinase for activity.



(12)

Modification of the side chain of **ACV** or GCV, forming the 9-alkoxy derivative (**13**), (**14**), produces isomers that retain activity against herpesviruses. The **ACV** isomer is three- to fivefold more active than **ACV** against HSV and VZV, whereas the GCV isomer is equivalent to ACV in activity (326). Bioavailability of these compounds is low, although it might be improved by using a



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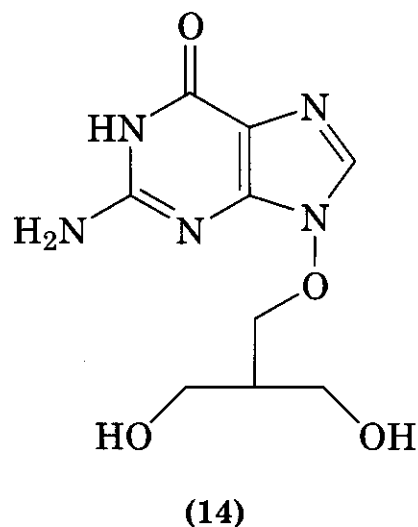
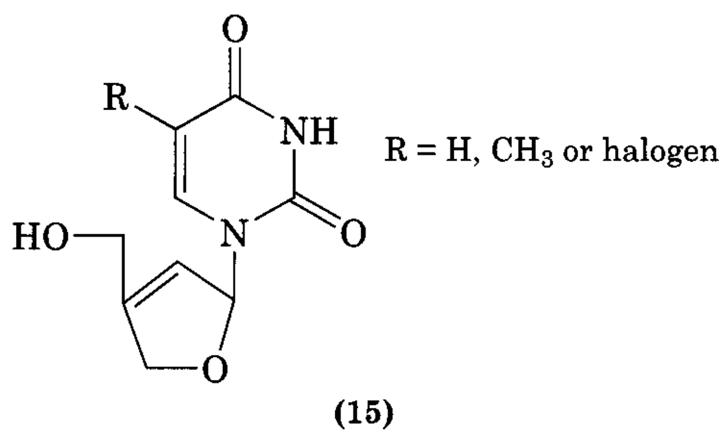


(14)

prodrug form. The length of the acyclic side chain is critical for recognition by the kinase, and 8-methyl purines are inactive, as are analogs containing substituents other than the hydroxymethylene group at the **2'** position (327).

In contrast to L-nucleoside analogs, which have been shown to have activity against HIV reverse transcriptase and **HBV** polymerase with useful therapeutic indices, **D-isomers** have demonstrated potent antiviral activity, but with excessive toxicity. Variations on this theme, apio dideoxydidehydronucleosides(**15**) have been evaluated as herpesvirus inhibitors.

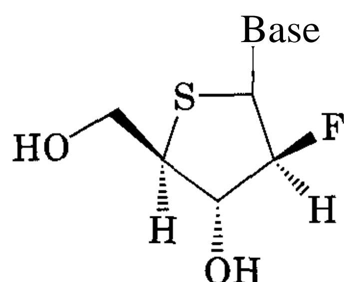


(15)

These compounds have the furanose oxygen and **C2** transposed. They showed moderate **ac-**

tivity against HCMV (EC_{50} = 3–44 vs. 0.9 μg/mL for GCV) and were inactive against HSV-1 and HSV-2. Cytotoxicity was minimal.

A series of **2′-deoxy-2′-fluoro-4′-thioarabino**furanosyl nucleosides (16) were tested as HSV



(16)

inhibitors (328). The **β-anomers** of pyrimidines were largely active against HSV-1 and HSV-2, with EC_{50} against HSV-1 ranging from 0.012 to 27 μg/mL (ACV EC_{50} = 0.32 μg/mL; Table 9.1). Of the purines, the **2,6-di**aminopurine and guanine derivatives were highly active (EC_{50} = 0.0041 μg/mL; HSV). The a-anomers were largely inactive. Except for the thymine analog, selectivity indices were greater than 100.

The D- and L-enantiomers of **cylohexenyl**guanine and -adenine (17) were synthesized as part of a study of how sugar analogs bind to nucleoside **kinase** and polymerase enzymes (329). The adenosine analogs were inactive against HSV, HCMV, and VZV. Both guanosine enantiomers had activity comparable with ACV against HSV and **VZV** and activity comparable with GCV against HCMV. Selectivity indices for the D-enantiomer were >**1000** for HSV and ranged from 7 to 40 for VZV and HCMV; the L-enantiomer showed **two-** to fivefold less **selectivity** than the **D-enantiomer.** Both compounds tended to be about fivefold less active against **thymidine kinase–deficient** strains, but they retained significant activity.

Borrowing from the observation of potency against HIV-reverse transcriptase of L-nucleoside analogs with lower toxicity than the D-isomers, synthesis of analogs of these compounds identified 2'-fluoro-5-methyl-P-L-arabinofuranosyluracil (**L-FMAU**) (18) as a compound with antiviral activity. It has been studied primarily as an anti-HBV inhibitor, but it is also an inhibitor of EBV (330, **331**), with an EC_{90} of 5 *μM*, equivalent to GCV (330). Its selectivity index was 180. L-FMAU showed no activity against HSV.

(1′*S*)(2′*R*)-9-{[1′,2′-Bis(hydroxymethyl)-cycloprop-1′-yl]methyl}guanine (A-5021) **(19)** is a competitive inhibitor of HSV DNA **polymerases** with respect to **dGTP** (332). It is more potent than ACV against HSV-1, HSV-2, VZV,

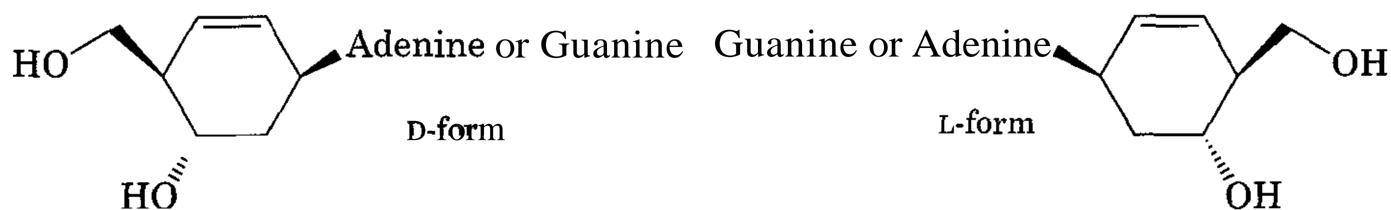


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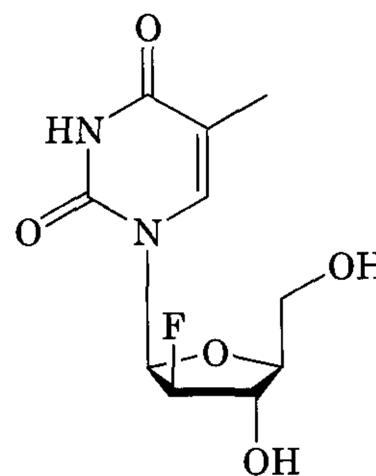
Table 9.1 Inhibition of HSV-1 by 2'-deoxy-2'-fluoro-4'-thioarabinofuranosyl Nucleosides

Base	EC_{50} (μg/mL)
Thymine	0.32
5-Ethyluracil	0.012
5-Iodouracil	0.037
5-E-bromovinyluracil	0.032
5-Chloroethyluracil	0.11
5-Flurouracil	>27
5-Iodocytosine	0.037
5-Ethyluracil	1.0
5-Hydroxyethyluracil	27
ACV	0.32



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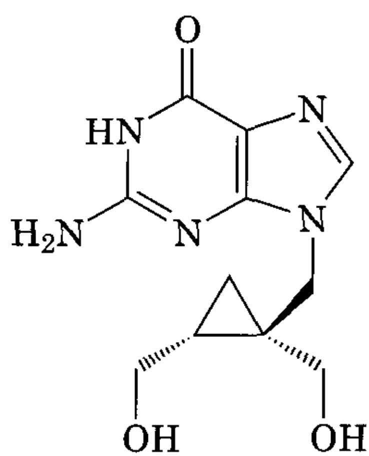
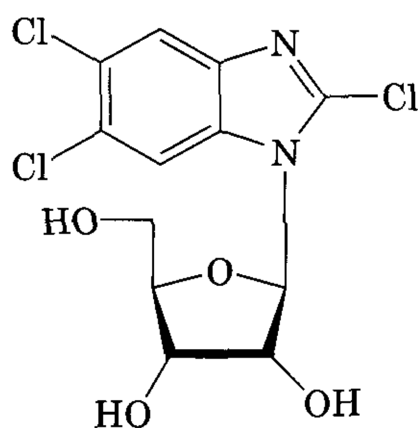


(19)

EBV, and HHV-6, but it is inactive against HHV-8. A-5021 was more effective than ACV at preventing intracutaneous and intracerebral HSV-1 infections in mice.

Modifications to the base of ACV have been made with varying degrees of success. Addition of a methyl group at N-1 of ACV reduces activity 5- to 20-fold. Etheno-ACV is inactive, but the 6-methyl- and 6-tert-butyl derivatives retain weak activity. Addition of a phenyl group at this position restores activity, particularly for the GCV analog, and results in a compound that shows activity against nucleoside kinase-deficient HSV and VZV. The biphenyl derivative show both reduced activity and narrower specificity, acting only on HSV. ACV analogs containing a 2-(3,5-dichloro anilino) substituent inhibit HSV-1 and HCMV replication but also inhibit cellular DNA polymerases at approximately the same concentration.

A series of benzimidazole and indole nucleosides have demonstrated activity against HCMV. 2,5,6-Trichloro-1-(β-D-ribofuranosyl) benzimidazole (TCRB) (**20**) and 2-bromo-5,6-dichloro-1-β-D-ribofuranosyl benzimidazole (BDCRB) (**21**) are potent inhibitors of HCMV (333), but have half-lives of less than 1 h because of cleavage of the glycosidic bond. Introduction of a fluorine on the ribose to act as a stronger electron-withdrawing group (**22**) led to an approximately 10-fold loss of potency and an increase in cytotoxicity (334, 335). Removal of 3-N to give the corresponding indole nucleosides also caused a 10-fold loss in activity (336). A trichloro-tricyclic analog (**23**) was about as active as TCRB, but more cytotoxic,



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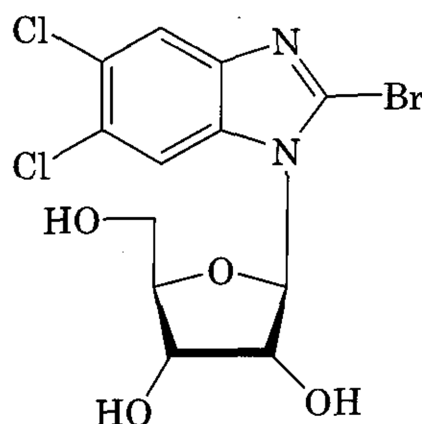

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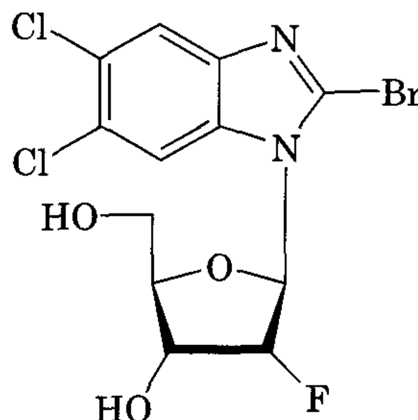

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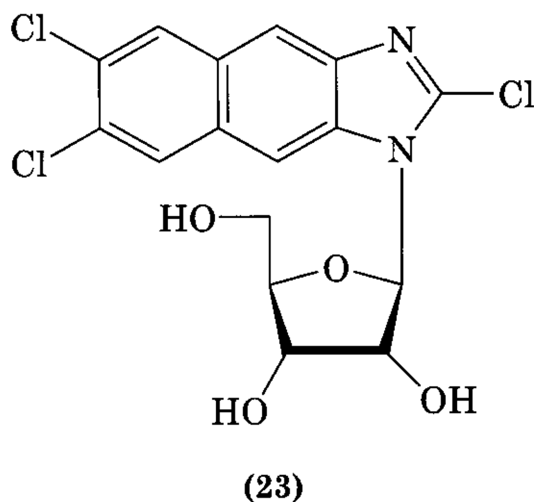
suggesting that the tricyclic structure resulted in a relative increase in affinity for cellular enzymes (337). Replacement of the D-ribofuranose with a D-erythrofuranose gave a 10-fold increase in activity relative to TCRB and 40-fold greater potency than GCV. **An** iodine or amino group at the 2 position resulted in considerably less activity. BDCRB was inactive against EBV, but the related compound, 1263W94 was active (**24**), with EC„ ranging from 0.15 to 1.1 μM (338) 1263W94 also showed ninefold greater potency against clinical isolates of HCMV than GCV and was ef-



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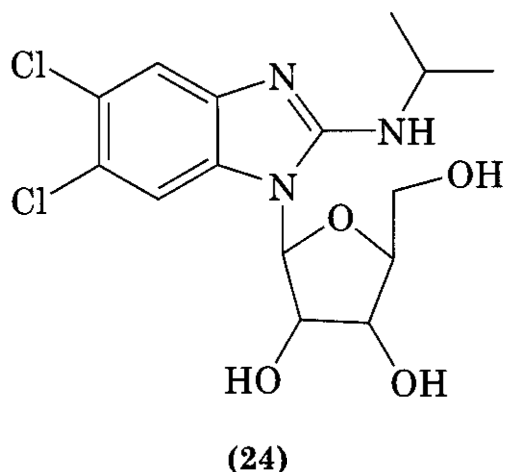

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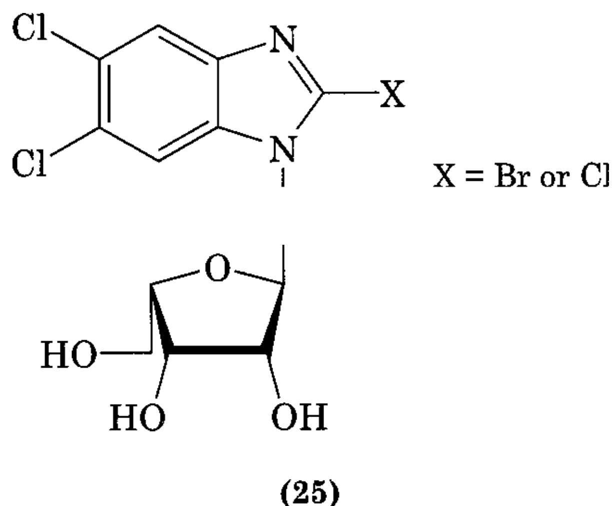
fective against GCV-resistant isolates (339). 1263W94 differs from BDCRB in having an L-ribose and a 2-isopropylamino group instead of a 2-bromine.

Several a-D-and a-L-lyxofuranosyl derivatives of 1263W94 and TCRB were synthesized and tested against HSV-1 and HCMV (340). None of the compounds were efficacious against HSV-1. The 2-halogen derivatives of both the a-lyxose and 5-deoxy-α-lyxose derivatives (e.g., 25) were active against HCMV, with the L-enantiomers being the most potent (EC_{50} = 0.2–0.4 μM).



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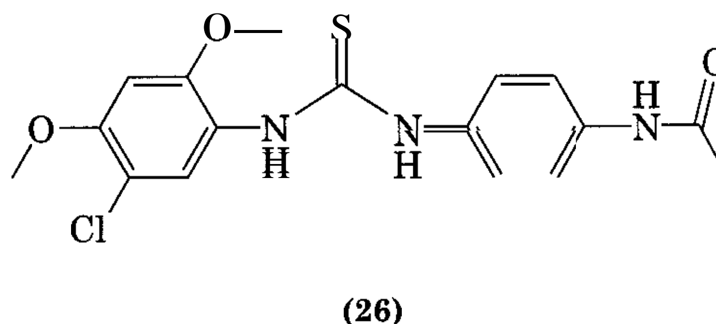
The 2-halogenated compounds act through a different mechanism than most nucleoside analogs, and a target for them has been identified. Selection of strains resistant to TCRB or BDCRB identified mutations in the UL89 and UL56 genes, and these strains were also resistant to the 2-halo-erythrofuranose compounds (341–343). None of these four compounds generated resistance mutations in UL97. The 2-isopropylamino and 2-cyclopropylamino derivatives acted on the UL97 kinase, indicated by resistance mutations in UL97. UL89 and UL56 are required for post-synthetic DNA processing before packaging into virions. Studies with BDCRB indicated that assembly was blocked at the packaging step because the rolling circle replication product was not cleaved at the viral temini (343). Thus, these compounds identify another potential drug target in herpesviruses. Because the UL89 gene is highly conserved across herpesviruses, effective drugs that target this protein may have broad activity within the herpesvirus family.

UL6 is an HSV-1 gene required for DNA cleavage and encapsidation. Two thiourea compounds, N-(4-(3-(5-chloro-2,4-dimethoxyphenyl)-thioureido)-phenyl)-acetamide (CL-253824) (**26**) and its 2-fluoro-benzamide



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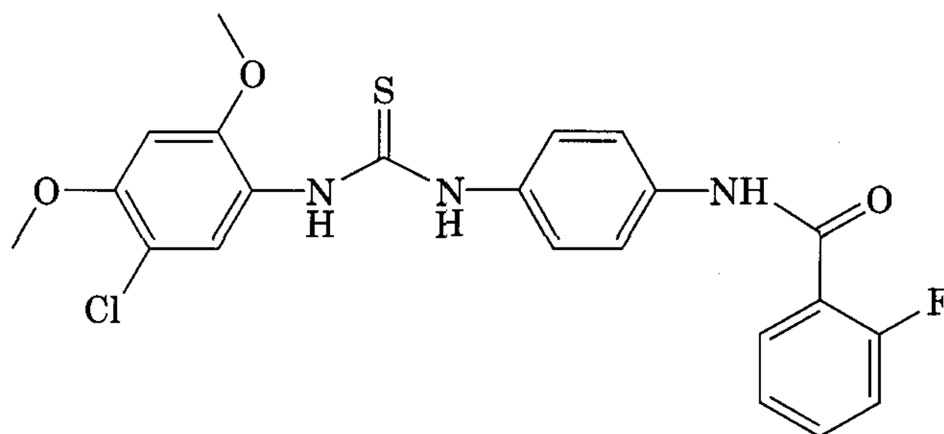
derivative (WAY-150138) (**27**), have been identified as inhibitors of cleavage and encapsidation (344). Resistance mutants generated with these compounds mapped to UL-6. CL-253824 is active against HSV-1 and weakly active against HSV-2 and HCMV (EC_{50} values of 8–25 μM against HSV-I). WAY-150138 is more active, with EC_{50} = 0.2–1.5 μM against HSV-1 and 6–19 μM against HSV-2 and HCMV. Both inhibitors are inactive against VZV.



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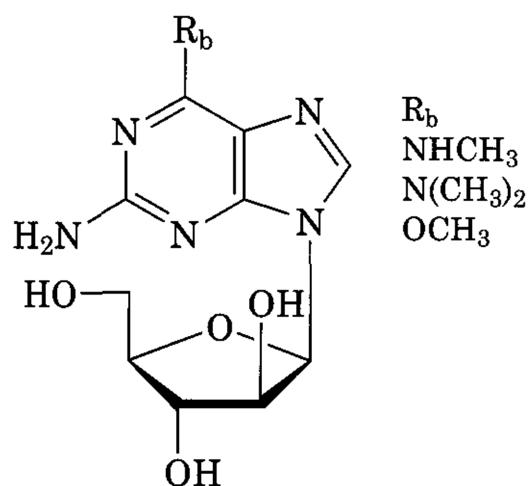
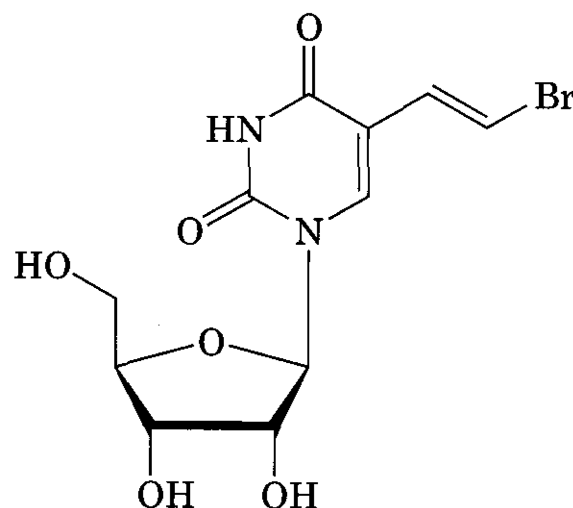
Vidaribine (araA) analogs are prone to metabolic degradation by deaminases. Modifications to the 6-N position (28) have proven partially effective at blocking deamination (345). Methylation provided a 10- to 30-fold increase in potency against VZV relative to vidaribine and a decrease of about the same magnitude in cytotoxicity. Replacement of the amine with a methoxy group (araM) gives about 10-fold greater selectivity than araA against VZV. araM is inactive against other viruses because of an inability to be phosphorylated. Whereas these 6-N-substituted compounds afford a significant improvement, they still undergo relatively rapid catabolism. Their development as drugs will require prodrug forms that block this metabolism.



(28)

(E)-5-(2-bromovinyl)-2′-deoxyuridine (bivrudin, BVDU) (7) and 1-(B-D-arabinofuranosyl)-(E)-5-(2-bromovinyl)uracil (BVAU) (29) were shown to be potent inhibitors of VZV, although their metabolic instability made them impractical as drugs. Additional bromovinyluracil analogs (30) were made to circumvent this catabolism (346). They contain a 2′-oxo moiety and one of three halogens on the vinyl group. These compounds were of equal or greater potency than ACV and PCV against VZV, with little toxicity. They were active in the order of Cl > Br > I. They also were active against EBV, but in the reverse order with regard to halogen. They showed activity against HSV-1 but not HSV-2.



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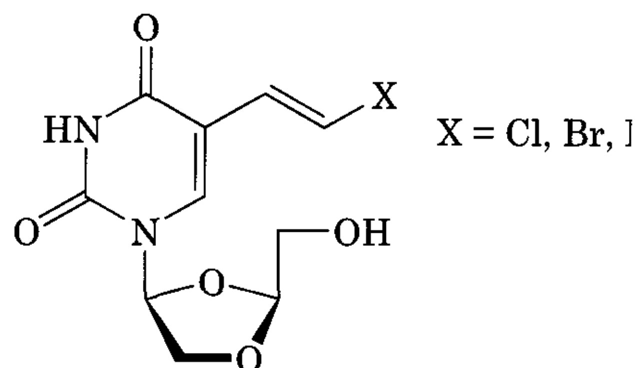

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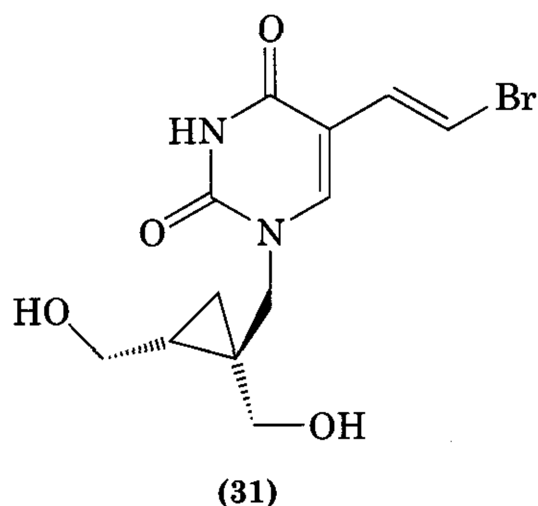
Out of a series of 5-substituted uracil nucleoside analogs containing a cyclopropyl side chain, bases with 5-halovinyl substitutions



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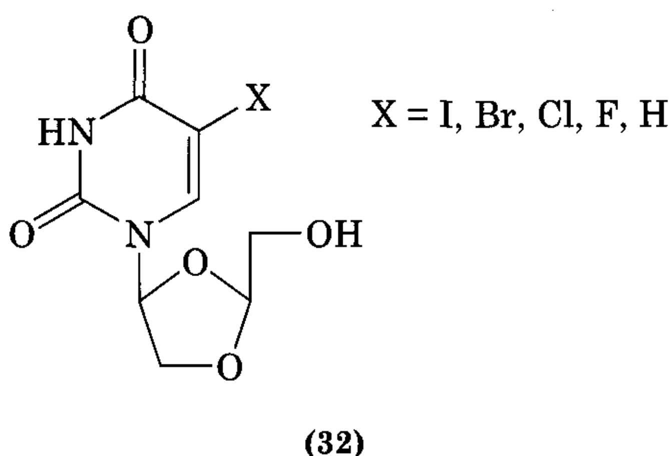
(31) gave activity up to 60-fold greater than ACV against **VZV** but were weaker than ACV against HSV-1 (347). The bromovinyl derivative had oral bioavailability of 69% in rats, and these compounds showed no detectable toxicity.

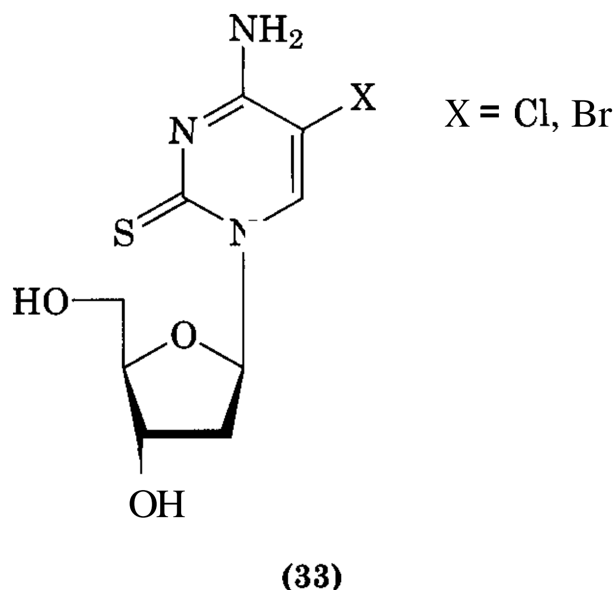
Similarly, L-dioxolane uracil nucleosides (32) were active against EBV, in the order X =



(32)

I > Br > Cl (IC_{50} = 0.033–0.6 μM), with little toxicity noted (348). Most L-nucleosides are phosphorylated by cellular kinases, but the activity of these dioxolane uracil analogs is dependent on the viral thymidine **kinase** to ***generate*** the monophosphorylated form (349, 350). Note that these compounds do not affect latent replication cycle of EBV, where the host cellular replication machinery replicates the viral DNA. **As** a result of this, virus DNA levels return to pretreatment levels by 50–60 days posttreatment because of reactivation of latent virions. The bromo- analog was 80-fold more active against VZV than was ACV, with no detectable toxicity (349).

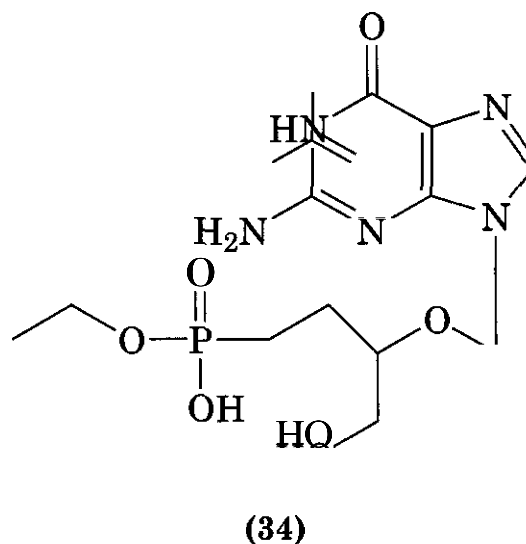
Shigeta et al. (351) synthesized 20 analogues of 2-thio-pyrimidines and found that the 2-thiouracil and 2-thiothymidine **arabino**side analogs were weakly to moderately active against HSV and **VZV** (>lo-fold less active than ACV). None of these compounds were active against HCMV or **thymidine kinase–deficient** HSV. 5-Chloro-2-thiocytosine and 5-bromo-2-thiocytosine (33) were relatively potent



(33)

inhibitors of VZV, with activities in the range of ACV.

9-[3-Hydroxy-2-(phosphonomethoxy)propyl] (HPMP)-purine and pyrimidine analogs have been synthesized for each of the four natural bases **(352)** (see (**5**) for **HPMPC**). HPMPG, the guanosine derivative, was about fivefold less active than ACV in cell culture but was more protective against HSV-1 infection in a mouse model. A related compound, 9-(3'-ethylphogphono-**1'-hydroxymethyl-1'propyloxymethyl)**-guanine **(SR 3727A) (34)** was inhibitory against

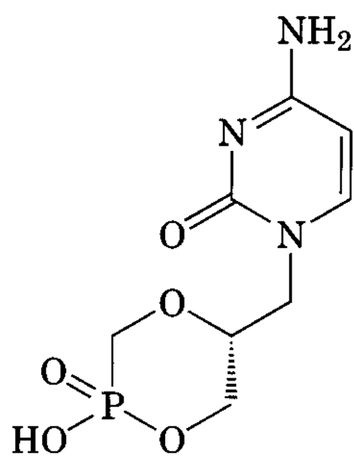


(34)

HCMV (EC_{50} = 6–17 μM), with no toxicity noted in cell culture. It also provided protection against a lethal dose of murine CMV in mice (353). Studies of the efficacy of ACV **phosphonate** and GCV phosphonate in protecting against MCMV infection in mice showed the

GCV phosphonate derivative to be equivalent to GCV in potency in cell culture (354), and more effective at lengthening the time to death than the parent, GCV (355). However, it was less effective in cell culture and showed more renal toxicity than HPMPC (cidofovir). HPMPC has become the focus of development for this class of compounds.

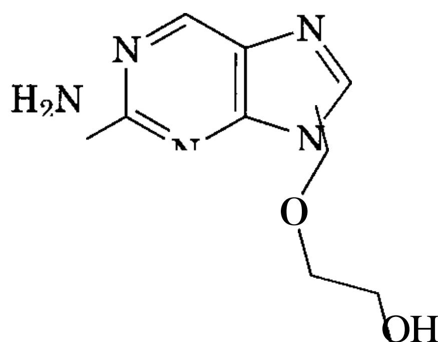
HPMPC (cidofovir) (5) has shown broad-spectrum and potent activity against several DNA viruses, but nephrotoxicity limits its use (299,300). Its cyclic congener (cHPMPC) (35) shows similar antiviral activity with reduced



(35)

renal toxicity (299, 356–358). cHPMPC is converted after cellular uptake to cidofovir and shows similar potency and cytotoxicity in cell culture. In 14- and 30-day studies in rats, cHPMPC was >10-fold less toxic than cidofovir. Similar results were observed in guinea pigs and cynomolgus monkeys. These observations have also been extended to immunocompromised guinea pigs, which are especially sensitive to cidofovir toxicity.

S-2242 is a guanine analog substituted at the N9 position (36). It has potent activity



(36)

against HSV-1 and-2, VZV, HCMV, HHV-6, HHV-7, and HHV-8. EC_{50} ranges from 0.01 to 0.4 μg/mL (359–361). Of 25 compounds tested, S-2242 was the most potent against HHV-7, with a selectivity index greater than 200. It also showed good potency and selectivity against HHV-8. S-2242 is about 10-fold less active against HSV than is acyclovir, but it is a good inhibitor of activity of thymidine-kinase-deficient strains of HSV and VZV. The toxicity profile in cell culture varied with cell type. In the majority of cell types it was comparable with or somewhat more toxic than GCV, though in HSB-2 and CEM cells it was more than 60-fold more toxic than GCV. Animal studies have shown toxicity profiles similar to GCV.

S-2242 has several characteristics that differ from currently available purine nucleoside drugs. Its activity against thymidine kinase–deficient HSV and VZV strains, its broad range of activity, and its toxicity profile indicate that phosphorylation does not involve a viral kinase. Instead, it is phosphorylated by a cellular deoxycytidine kinase (362). Blocking the phosphorylation of S-2242 by deoxycytidine kinase decreased its potency by 50- to 100-fold. S-2242 is not phosphorylated by HCMV UL97 (363), but infection with HCMV increased metabolites of S-2242 by 5- to 25-fold, indicating that HCMV either encodes an additional kinase capable of phosphorylating the compound or it activates a cellular kinase.

S-2242 resembles GCV and PCV in the stability of its triphosphate form, with a half-life of 3–6 h in CEM cells (362). When cells were treated for 24 h before infection with drug withdrawn at the time of infection, the apparent EC_{50} of S-2242 increased from 0.45 μg/mL with continuous treatment to 0.7 μg/mL after the drug was withdrawn. By contrast, the apparent EC_{50} of ACV increased from 0.03 to greater than 50 μg/mL when comparably treated (359).

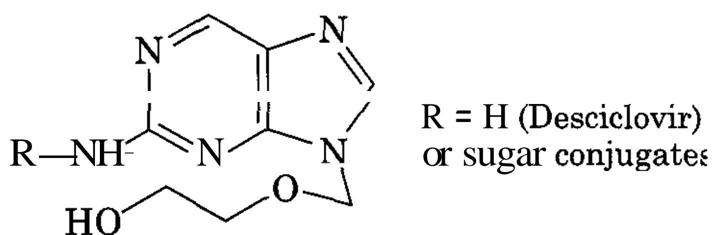
Because a viral kinase is not required for activation of S-2242, the bulk of strains resistant to ACV, 'GCV, or PCV are sensitive to S-2242 (364). S-2242 shows cross-resistance with drugs generating certain DNA polymerase mutations (e.g., foscarnet), but it is not cross-resistant with drugs targeting other

polymerase amino acid mutations, e.g., cidofovir or bromovinyl-deoxyuridine.

3.1.2.2 ***Prodrugs Under Development.*** There are three primary purposes that have driven prodrug development:

1. To increase absorption of the drug. Low oral bioavailability is a characteristic of most nucleoside analogs, and lipophilic prodrug forms have provided dramatic increases in bioavailibility, as discussed earlier for ACV, GCV, and PCV.
2. To provide the drug in the monophosphate form. The need for an initial phosphorylation step to be carried out by a viral kinase provides a large degree of selectivity, but this approach is limited to viruses encoding such a kinase. One way to extend treatment to viruses lacking a suitable kinase is to develop nucleoside monophosphate analogs. In this case, the selectivity is limited to the difference in affinity for viral versus cellular polymerases, but if sufficient selectivity can be attained, the trade-off may be worthwhile for viruses where viral phosphorylation to the monophosphate is not an option (e.g., HBV), or for nucleoside kinase–deficient or –resistant herpesviruses. In this case, increasing the lipophilicity or masking the charge are essential considerations in developing the prodrug, because phosphorylated nucleosides are impermeable to cellular membranes.
3. To target the drug to a specific tissue. This approach may be particularly useful for diseases affecting the liver, such as HBV, or for certain cell types of the immune system. An example of this approach is compounds targeting the liver asialoglycoprotein receptor, discussed later.

A prodrug form of ACV, desciclovir (37), is designed to increase uptake of the drug, which is readily processed to ACV by xanthine oxidase (365). However, desciclovir is more toxic than ACV. Modification of desciclovir by adding reducing sugars to the 2 position of adenine provided good water solubility, but poor adsorption after oral dosing in rats. They were also inefficiently processed to ACV (366).

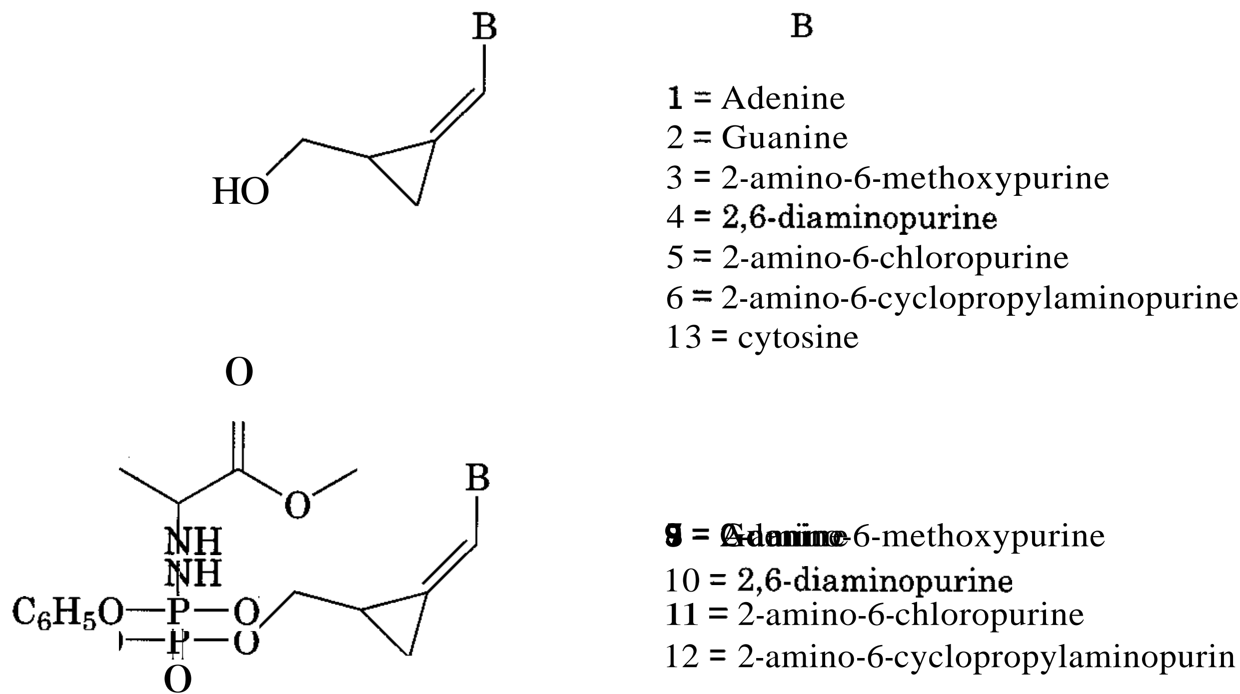


(37)

A series of methylenecyclopropane analogs of purine nucleosides and phosphoroalaninate prodrug forms of these compounds (**38**) have shown activity against several DNA viruses (367–370). Note that the prodrug does not need to undergo phosphorylation to the monophosphate. Most of these compounds were approximately as potent as GCV against HCMV, the exceptions being 3, 5, 8, and 10, which were 12- to 30-fold less active. Compounds 1–4 were also active against murine CMV in *vivo* (371). The 2,6-diaminopurine prodrug was equivalent to ACV in activity against HSV-1 and -2, and 100-fold more active than ACV against VZV. Compounds **13** and 8 were very potent against EBV. The diamino purine was moderately active against HBV (EC_{50} = 10 μM) but the potency increased substantially in prodrug (monophosphorylated) form (EC_{50} = 0.08 μM). Overall, these compounds showed good activity against the range of herpesviruses, but the potent compounds varied depending on the viral target, with no clear pattern emerging yet. Toxicity was minimal in a variety of cell types.

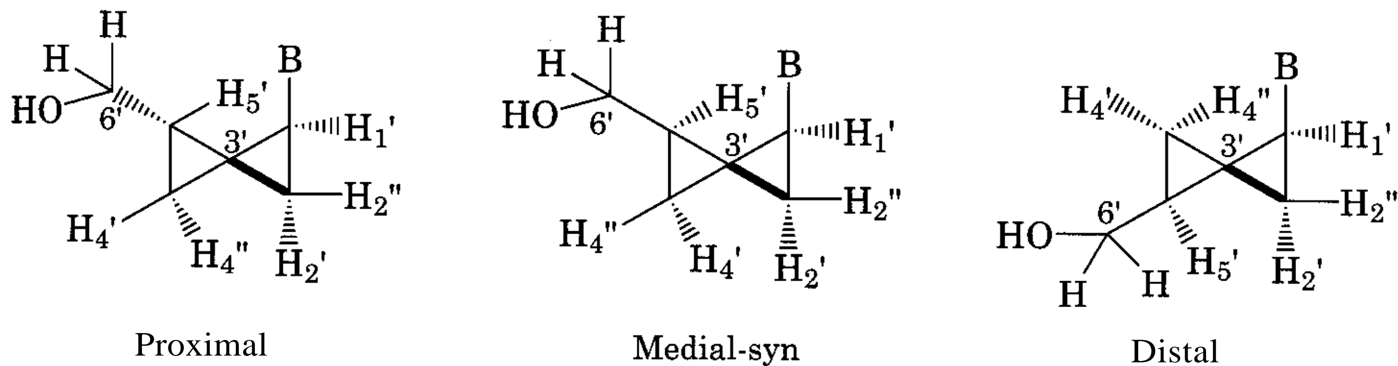
Related analogs of ribose, spiropentane derivatives (**39**), were synthesized in four conformations, and conformation-dependent activity was observed (**372**). The proximal and medial-syn adenosine analogs showed moderate activity against HCMV and EBV, although the proximal analog was relatively toxic in one cell line. The distal isomer of the guanosine analog was also active against EBV, with no toxicity noted. These compounds showed little activity against HSV or VZV.

When the proximal isomer of the adenosine analog was converted to the phenylphosphoalaninate prodrug form, it showed a 50-fold increase in activity against HCMV (EC_{50} = 0.4 μM), and gained 10-fold in potency against EBV, although it was still cytotoxic to Daudi cells ($EC_{50} = 2.8\ \mu M$, $CC_{50} = 7.8\ \mu M$). It was also active against HBV and VZV (EC_{50} = 3.1 and 9.3 μM).



(38)

B = adenine or guanine



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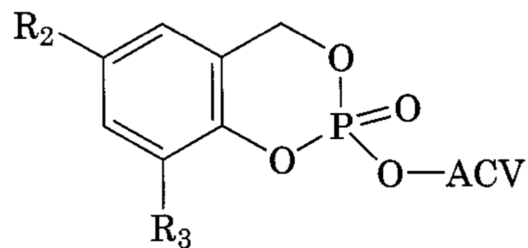
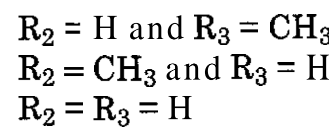
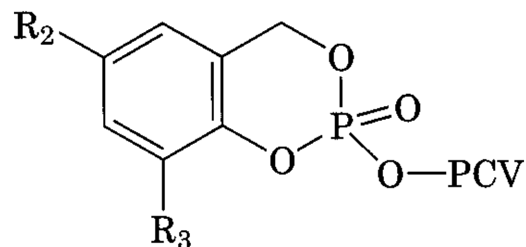
*cyclo*Saligenyl nucleoside monophosphate derivatives were designed as an attempt to create a more efficacious tool for making monophosphate derivatives sufficiently lipophilic to enter cells (373). Derivatives of ACV and PCV (40) have been tested against HSV and EBV. The ACV derivatives were about equivalent to ACV in activity against a thymidine kinase-competent HSV strain and against EBV. However, in a thymidine kinase-deficient HSV strain, the *cyclo*Saligenyl derivatives were 20- to 60-fold more active than ACV. This approach did not work with PCV derivatives, which were 32- to 95-fold less active than PCV.

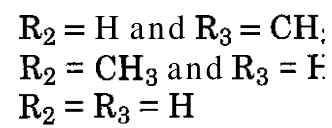
Borrowing from experience with HIV, ACV-5′-(phenyl methoxy alaninyl) phosphate (41) was synthesized as a kinase-independent lipophilic ACV prodrug (374). This approach worked successfully for the HIV drug d4T, which was non-toxic and a potent HIV inhibitor. However, whereas the ACV derivative was not toxic, it had weak antiviral activity.

A phosphonomethyl ether analog of ACV (42) was fivefold less active against HSV-1 than ACV (352). However, as mechanistic studies would predict, the difference in efficacy for HSV and HCMV narrowed, with the analog being eight times more active against HCMV than ACV. Phosphonate ethers can also have an effect on cytotoxicity; a phosphono-derivative of GCV (**43**) is less toxic than GCV.









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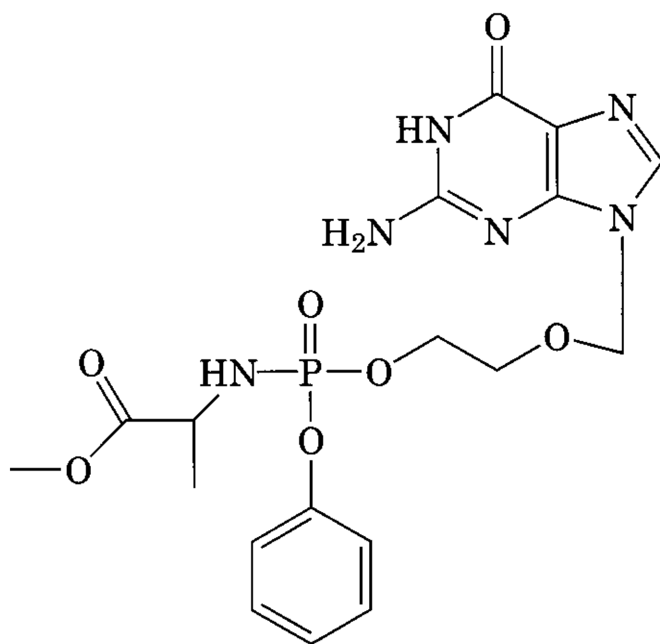

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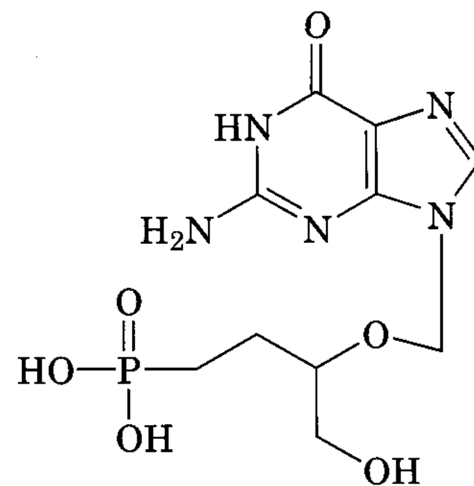

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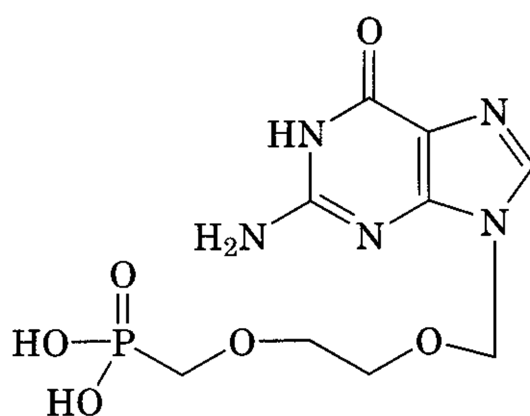

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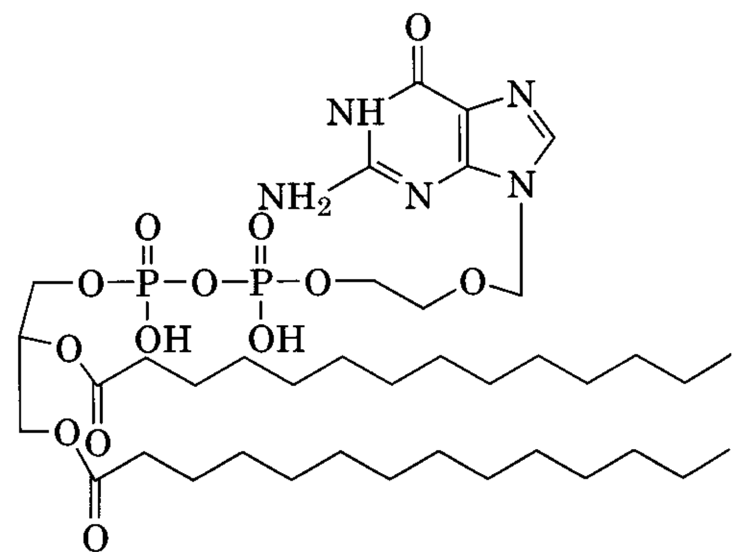

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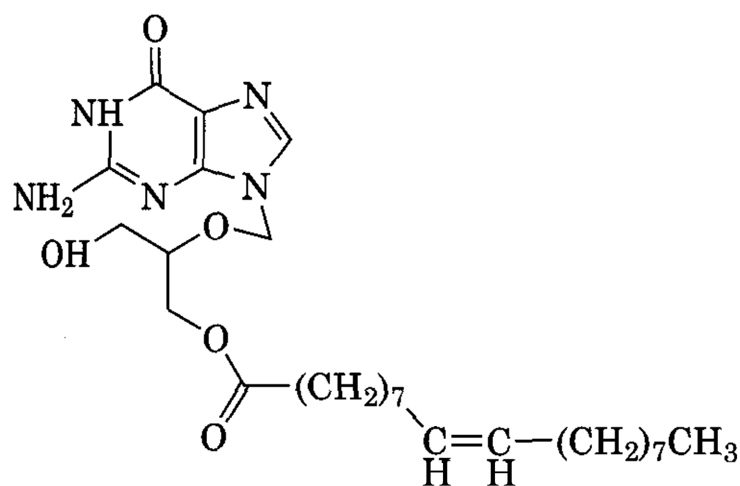
Another approach to treating thymidine kinase-deficient strains is to provide the phosphorylated form as a phospholipid, shown here for ACV **(44)** **(375)**. ACV-diphosphate-dimyristoylglycerol was equivalent in activity to ACV against wild-type HSV-1 and HSV-2 strains. With thymidine kinase–deficient strains, ACV had no detectable activity,

whereas the phospholipid **prodrug** was as active against them as against wild-type (EC_{50} = 0.25–1 μM). In uninfected W138 lung fibroblasts, the levels of intracellular **phosphorylated** ACV were 56-fold higher using the phospholipid **prodrug** than those for ACV.

GCV has oral bioavailability of less than 10% in humans and is given intravenously for acute treatment of HCMV disease. It is active against HSV and VZV, but the poorer toxicity profile relative to ACV limits its use. **An** elaidic ester of GCV (E-GCV) (**45**) was 5- to 30-fold



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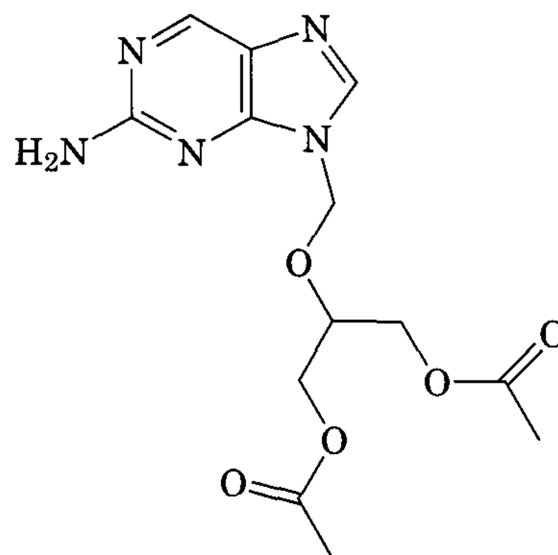
more potent in cell culture than GCV against HSV and 15- to 90-fold more potent than ACV (376).The ester was about equivalent to GCV in toxicity (less than twofold more toxic). E-GCV and GCV were of equivalent potency against VZV and HCMV (EC_{50} = 0.7–4.5 μM for both viruses). When E-GCV was used **intraperitoneally** to treat HSV-2 infection in mice, death rates were reduced to 10% under **conditions where 100% of the mice died when treated** with an equimolar amount of GCV. The activity profiles of GCV and E-GCV were **similar** with regard to potency in thymidine **kinase–deficient** strains or strains resistant to **foscarnet** or ACV.

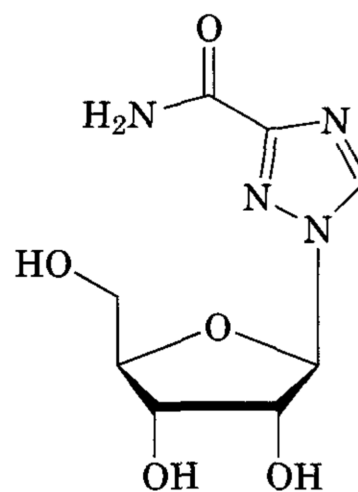
H961 (46) (277) is the **prodrug** form of S-2242 (36). The poor oral bioavailability of S-2242 necessitates delivery by injection (377). However, the **prodrug** was been tested in mice infected with vaccinia virus and was **effective when delivered orally. Oral administration** of **H961** at 100 **mg/kg** daily for 10 days completely blocked viral infection. The only toxic effect noted was atrophy of testicular



(46)

germinal epithelium, an effect that is also observed in mice treated with ganciclovir (277, 377).

3.1.2.3 Nonnucleoside Inhibitors and Other Targets. IMP dehydrogenase is the **rate-limiting** enzyme in the synthesis of guanine **nucleotides**, converting IMP to XMP. It is a target for two compounds, ribavirin (47) and **mycophenolic** acid.



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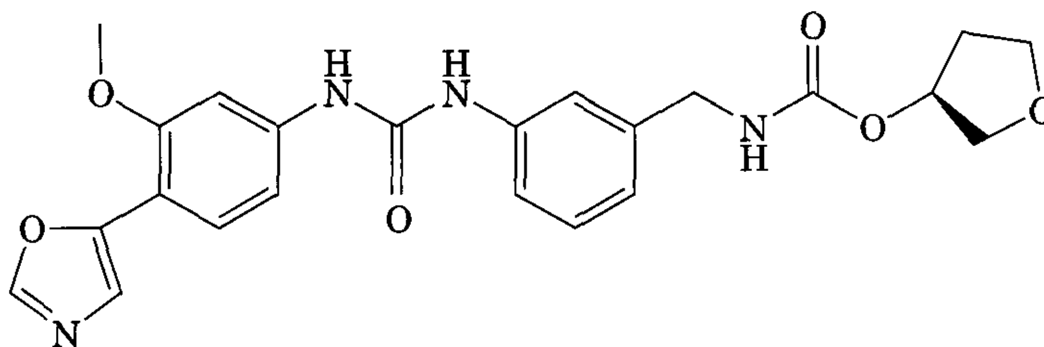
Mycophenolate mofetil (**MMF**) is an immunosuppresant used for kidney transplant recipients. MMF is hydrolyzed to mycophenolic acid. Ribavirin both inhibits the activity of some RNA polyrnerases and increases their rate of nucleotide **misincorporation,** thus decreasing the fitness of the resulting virions (378–380). However, a second effect of both of these compounds is a depletion of GTP and **dGTP** pools through inhibition of IMP dehydrogenase activity. **A** synergy



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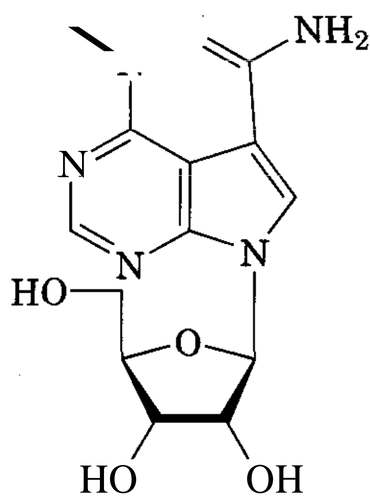
might be expected between these compounds and guanosine analogs, because a decrease in cellular **dGTP** would increase the rate of incorporation of the guanosine analog. This mode of action has been tested with MMF. A combination of MMF and acyclovir was more effective at preventing posttransplant lymphoproliferative disorders resulting from EBV reactivation or primary infection than was treatment with acyclovir alone **(381)**. The combination of MMF and **H2G** was particularly effective, with a 10- to 150-fold increase in potency observed against HSV-1 and HSV-2 and VZV in cell culture (382). With a thymidine **kinase–deficient** HSV-1 strain, potency increased more than 2500-fold, such that **H2G** with MMF was more potent in the thymidine kinase–deficient strain than **H2G** alone in the wild-type strain. The effect in **VZV** strains lacking thymidine **kinase** was smaller than with HSV, but activity still approached that of **H2G** alone in a wild-type strain. **Ribavirin** caused similar effects, although smaller in magnitude. This approach may have more general applicability; a preliminary study in AIDS patients indicates that MMF may increase the effectiveness of currently used **multidrug** treatment regimens used against HIV (383).

VX-497 (48) is an uncompetitive inhibitor of human IMP dehydrogenase (384) and **a** broad-spectrum antiviral agent. Of the DNA viruses, it inhibits HBV and HCMV at EC_{50} concentrations of 0.4–0.8 μM. HSV-1 is less potently inhibited ($EC_{50} = 6\ \mu M$). VX-497 was 25- to 100-fold more potent than ribavirin against these viruses but was also more toxic in some cell lines, with a selectivity index of about 10 in those cell lines. This might be anticipated for a compound that targets an enzyme that is essential in rapidly dividing cell types. The same constraint also applies to ribavirin, which had a smaller selectivity index in those cell types than did VX-497. Based on its mechanism of action, it is anticipated that VX-497 might share the synergies with guanosine analogs noted for MFF and ribavirin.

Hydroxyurea inhibits the activity of the cellular ribonucleotide reductase and potentiates the inhibitory effect of **2′,3′-dideoxynucleotide** analogs against **HIV-1**, particularly **didanosine (ddI)** (385–387). The combination of hydroxyurea and **ddI** is currently being investigated as a much less expensive alternative for HIV treatment in those populations where cost makes triple drug regimens prohibitive. By analogy with the mechanism outlined above for interaction of MMF, ribavarin, and possibly VX-497 with guanosine analogs, **hydroxyurea** might act synergistically with **nucleoside** analogs targeting DNA viruses. When tested in combination with a variety of **nucleoside** analogs (**ACV**, GCV, PCV, **H2G, cidofovir**, and adefovir) for activity against HSV-1 and HSV-2, hydroxyurea had a moderate **stimulatory** effect (388). In thymidine **kinase–deficient** strains, where the triphosphate concentration of the nucleoside analogs would be much lower, the potentiation was larger. In these strains EC_{50}s were lowered from 20–100 to 0.2–5 μg/mL by addition of hydroxyurea. The potentiating effect of hydroxyurea is not sufficient to warrant its general use for this purpose. However, given its potential for treating HIV infections, this combination of drugs could result when opportunistic herpesvirus infections are treated in AIDS patients.

Triciribine (TCN) (49) is a tricyclic **nucleoside** with antineoplastic and antiviral activity (389). It is phosphorylated by adenosine kinase to the monophosphate form but is not



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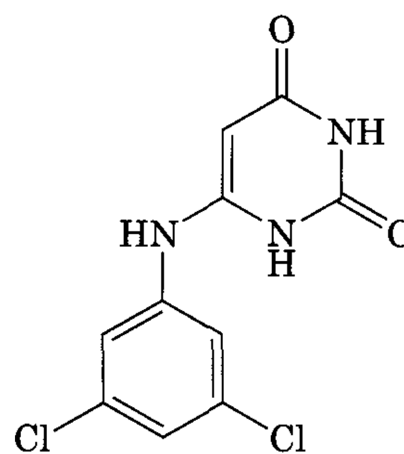

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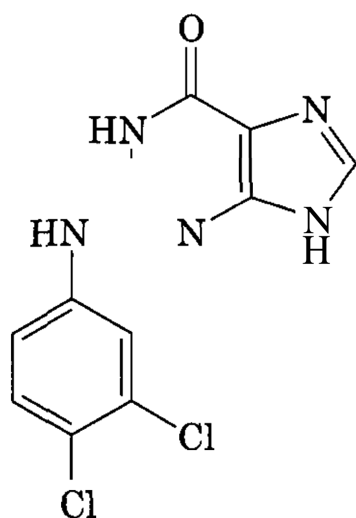
phosphorylated further, and it is not incorporated into DNA. In an effort to dissect its mechanism of action, analogs of TCN varying in the number of hydroxyl groups on the ribose were synthesized and tested for antiviral activity against HCMV. The parent TCN was active (EC_{50} = 2.5 μM), but all of the analogs were inactive, indicating that hydroxyls at the 2', 3', and 5' positions were essential for activity.

The helicase-primase complex of herpesviruses carries out two essential DNA replication activities: it provides a primer for DNA polymerase to initiate from, and it separates the two DNA strands. A series of dichloroanilino-purines and -pyrimidines (**50**), (51) were



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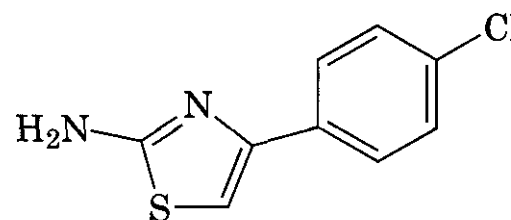
examined for their antiherpevirus activity, and several were found to inhibit not the polymerase but the helicase-primase complex (390, 391). They were weak inhibitors with poor selectivity, but they demonstrate that this enzymatic activity is a potential drug target. Helicase-primase has proven a much more difficult target than polymerases, and thus far, no drugs targeting helicase have been approved for any disease. However, as mutants accumulate in the currently used targets, it may be necessary to broaden our approach to include targets such as helicase.

More recently, a 2-amino thiazole compound, T157602 (52) has been identified that



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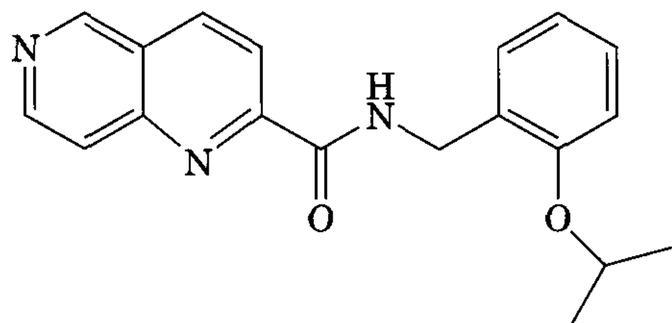
targets the UL5 protein of the HSV helicase-primase complex (392, 393). It has an IC_{50} against the purified complex of 5 μM, an EC_{50} in cell culture of 3 μM, and no toxicity at 100 μM. It is not active against helicase-primase complexes of VZV, HCMV, or EBV.

A 1,6 naphthyridine (53) and related 7,8-dihydroisoquinoline derivatives were identified as having potent activity against HCMV. Compound (**54**) was 39- to 220-fold more active than GCV. Strains resistant to GCV, foscarnet, cidofovir, and BDCRB were still sensitive to these compounds. In most cell lines, these compounds were substantially more cytotoxic than ACV or GCV, resulting in a poorer selectivity index than ACV and approximate equivalence to GCV.



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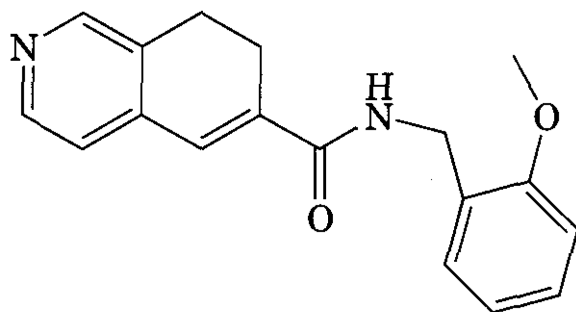

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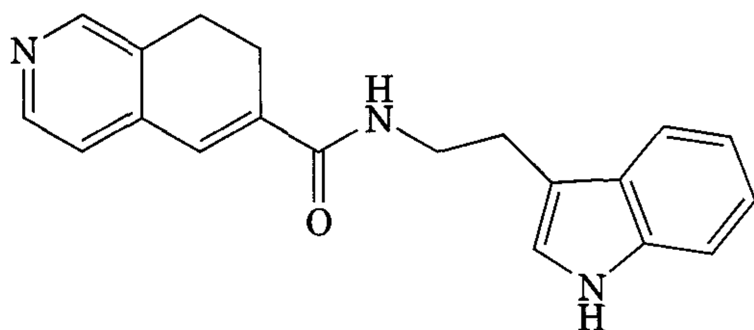

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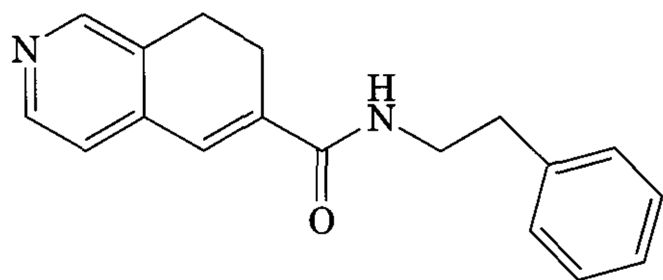

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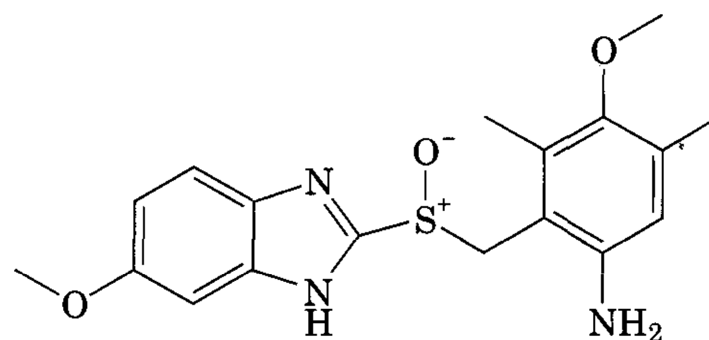
Dendrimers have been proposed as a topical microbicide, to be used to inhibit viral entry through mucosal cells (394). These molecules are highly branched polymers synthesized on a polyfunctional core, which are capped with a layer to give the desired surface properties. The choice of core, polymer, and capping group determine the shape, size, and charge characteristics of the molecule. Five dendrimers were tested for their capacity to inhibit entry of HSV-1 and HSV-2. All five dendrimers showed antiviral activity in cell culture when they were added to cells before the addition of virus. Two of three dendrimers tested were effective in a mouse model in preventing infection when applied topically to the vagina. The compounds were applied as 15 μL of a 100 mg/mL solution and protected 15 of 16 mice from infection (16 of 16 control mice were infected). No toxic effects were noted from a single application, although multiple applications were not tested.

3.1.2.4 ***Herpesvirus Protease Inhibitors.*** Proteolytic activity is required to process herpesvirus structural protein precursors during assembly of the virion. The protease responsible is a serine protease encoded by the HSV-1 UL26 gene and the HCMV UL80 gene (reviewed in Refs. 395, 396). These herpesvirus proteases seem to be a distinct subfamily of serine proteases, with minimal structural similarity and minimal crossreactivity with inhibitors to other serine proteases. A benzimidazolylmethyl sulfoxide (57) inhibitor of HCMV



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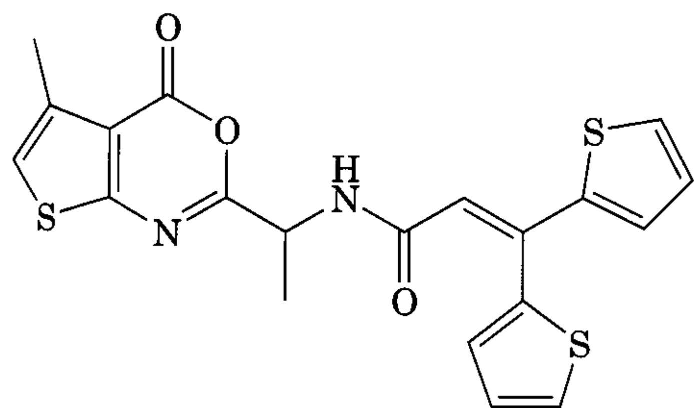
protease activity has been identified (397). This compound inactivates the protease through interactions with one or possibly two cysteines on its surface. It had an IC_{50} in a biochemical assay with recombinant protease of 1.9 μM and was functional in a cell culture assay, with an EC_{50} of 18 μM. No toxicity noted at 100 μM. It also showed no activity at 100 μM against chymotrypsin, trypsin, thrombin, factor Xa, plasmin, or kallikrein. Based on the lack of activity of this inhibitor against these other serine proteases and the amino acid sequence differences between the herpesvirus proteases and other serine proteases, the

herpesvirus proteases seem to represent a subclass of the serine **protease** superfamily, an important determinant of the likelihood of identifying inhibitors with a useful selectivity index.

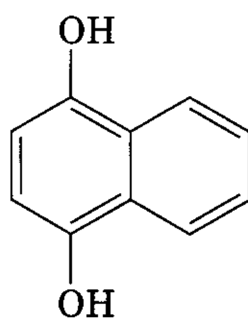
A series of **thieno(2,3-d)oxazinone protease** inhibitors (**58**) were demonstrated to have



(58)

good potency against HSV-2, HCMV, and **VZV** (**EC_{50}** from 0.02 to 0.9 *μM*) (398). Substitution of one or both of the thiophenes by phenyl groups had little effect on potency. These compounds were inactive at 100 *μM* against **elastase** and trypsin but were moderately toxic (CC_{50} = 20–100 *μM*).

A random screen for HSV-1 **protease** inhibitors yielded **1,4-dihydroxynaphthene** (59) and three related naphthoquinones (60) (399).



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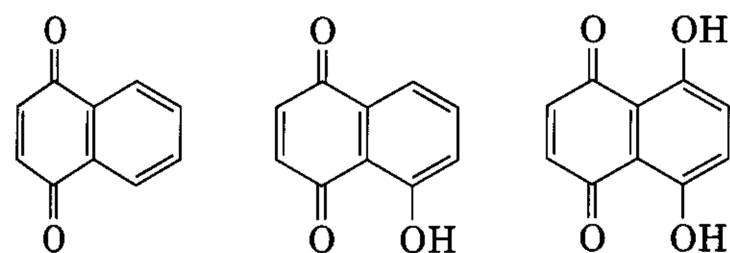

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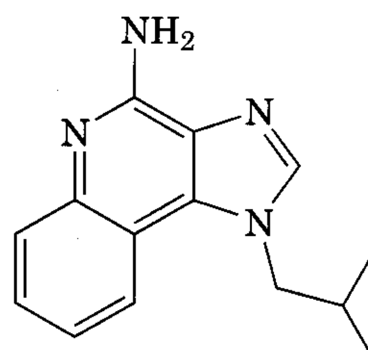
These compounds exhibited IC„ values against the **protease** ranging from 6.4 to 16.9 *μM*. They were more active against HCMV protease, with **IC_{50}** values of approximately **1** *μM*. While structurally similar, the **naphthoquinones** differed mechanistically from the naphthalene. Against HSV-1 protease, the naphthalene was a competitive inhibitor, whereas the naphthoquinones were noncompetitive. The reverse was the case against HCMV protease. These compounds were not active against trypsin, chymotrypsin, **kallikrein**, **plasmin,** thrombin, or factor Xa at 100 *μM*.

3.1.2.5 ***Immune Modulators.*** Imiquimod (61) has been tested as a topical treatment for herpesvirus and papillomavirus infection. **A** more potent analog, resiquimod (**62**), has been



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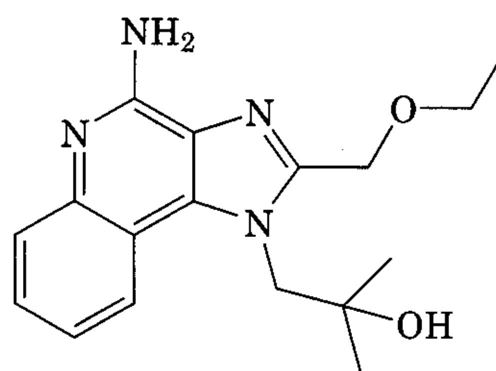

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tested for anti-HSV-2 activity in a guinea pig model, where the endpoint was the number of recurrences over the test period (400). The number of recurrences was decreased by **65–**75% by subcutaneous injection of resiquimod. Topical application of imiquimod also had a similar effect on recurrence rate (401). In a trial of 52 patients with frequently recurrent genital herpes, topical application of **re**siquimod one to three times per week (0.01% or 0.05%) for 3 weeks resulted in 32% of patients completing a 6-month observation **pe-**

riod without a recurrence, compared with 6% in the control group (402). These compounds are immunomodulators whose effect is to increase interferon-a levels. Resiquimod was demonstrated to activate B-cells by a mode that resembled B-cell activation by the CD40 ligand (403). Given a mode of action that is quite distinct from other HSV-2 inhibitors, it may prove to be useful in combination therapy with inhibitors of viral targets such as polymerase.

3.2 HBV

Replication of HBV requires first transcribing the cccDNA genomes in the nucleus into RNA, followed by copying the RNA into DNA by the reverse transcriptase activity of HBV polymerase. Effective treatment of HBV has two important components. First the replication process of the virus must be interrupted, and second, the reservoir of cccDNA genomes in the nucleus must be eliminated. The replicative cycle can be effectively broken with HBV polymerase inhibitors, preventing production of new virions. However, it is common for plasma virus levels to be decreased to undetectable levels with polymerase inhibitors, only to have the viral replication rebound after removal of the drug because of the availability of the DNA genomes in the nucleus for transcription. These genome copies are stable and are only effectively removed by destroying infected cells. Thus, the second arm of the treatment scheme is to boost the capacity of the T-cell–mediated immune response to viral antigens expressed in infected cells.

The immunomodulatory approach was actually the first employed, with the use of interferon-a (IFN-a). IFN-a is successful in stimulating T-cell–mediated removal of infected cells in a small proportion of patients (404). However, most patients do not respond and the side effects are often severe, so affecting the immune system alone has not proven a sufficient treatment (405,406).

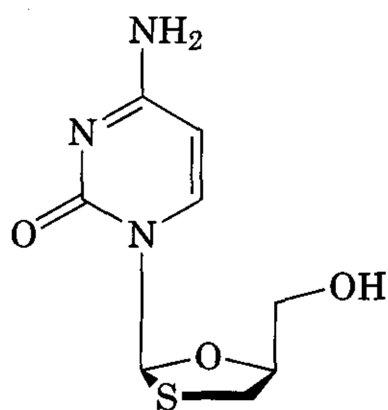
There is evidence that preventing reinfection with effective inhibitors of the replicative cycle is sufficient to allow eventual clearance of infected cells by the immune system, with or without immunomodulators. However. the course of treatment is long. A study of the clearance rate of virus showed a biphasic response. In the initial phase, virus was removed with a half-life of 1.1 days, whereas in the second phase, the half-life was 18 days (407). Another study estimated a clearance rate for the second phase of 10–100 days (121). These two phases likely reflect the clearance of free virus and infected cells, respectively. If infected cells are cleared with a half-life of 18 days, removal of infected cells from the liver would be likely to take in excess of 2 years. The implications of this need for prolonged treatment-are that drugs need to be well tolerated, with few side effects to keep patients on treatment, and that treatment regimens need to be able to deal with the inevitable accumulation of drug resistance mutations in the viral target.

Attempts to interrupt the replicative cycle have focused almost exclusively on the viral polymerase. Many of the compounds in use or under development as HBV polymerase inhibitors have their historical roots in either herpesviruses or HIV drug development. Most of the HBV inhibitors fall into two groups: (1) L-analogs of pyrimidine nucleosides or *(2)* purine analogs with modified sugar groups.

Before inhibitors of HBV polymerase are discussed, a note about nomenclature is needed. Historically, there has not been a universally accepted numbering system for HBV proteins. Mutations in HBV polymerase are reported by two numbering systems. For instance, the two most common mutations are M552I/V and M550I/V or I528M and I526M, depending on the source of the genome used. To standardize nomenclature. it has been recommended that a numbering system be used that starts with 1 at the consensus amino terminus of polymerase rather than at the beginning of the open reading frame, with each protein given a two-letter designation to identify it (408). With this system, the two mutations noted above become rtM204V/I and rtL180M. The numbering system used most commonly in the existing literature has been used in this chapter (M552I/V and I528M for the examples cited). Hopefully the proposed change in numbering will provide more clarity to the nomenclature in the future.

3.2.1 Pyrimidines. The most successful of the pyrimidine analogs in development thus far have been L-nucleoside analogs of active

D-nucleosides, including lamivudine (**63**), emtricitabine (**FTC**) (**64**), clevudine (L-FMAU) (**18**), and **Fd4C** (**65**). These L-nucleo-



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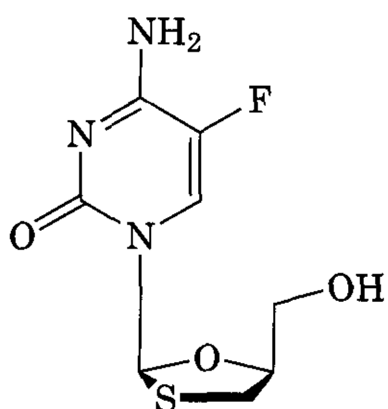

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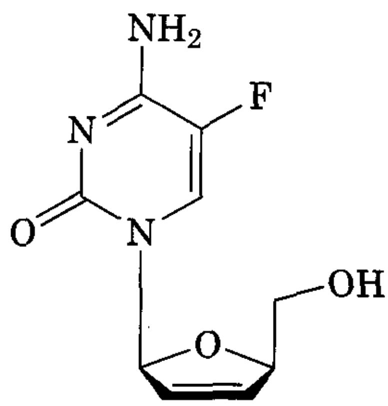


(65)

sides show potent activity while **lacking** the toxicity of the D-enantiomers. Some of them are quite specific for HBV, whereas other show activity against HIV and certain **herpesviruses** as well. The structure-activity relationship of these compounds as well as additional L-nucleosides have been elegantly examined by Bryant et al. (**409**), and the following conclusions can be drawn from their observations. (1)The specificity for HBV versus retroviruses derives primarily from the presence of a **3'** hydroxyl on the ribose. *(2)*The lack of a **3'** substituent resulted in retention of activity, but the specificity for HBV was lost. **(3)**Halogenating the 5 position on the pyrimidine ring reduced potency but did not affect specificity for HBV. (4) Retaining the 3' hydroxyl but changing its orientation (**L-xylo-nucleoside**) resulted in the loss of anti-HBV activity. These observations were made with cytidine and thymidine derivatives and were also extended to adenine derivatives.

Lamivudine is approved for treating hepatitis B patients. It is a chain terminator, and all three phosphorylation events to give the triphosphate are carried out by cellular enzymes. The first phosphorylation event is by a cellular deoxycytidine **kinase** rather than a viral **kinase**; despite this, cytotoxicity is low because all of the cellular polymerases have a very low affinity for lamivudine. Lamivudine triphosphate concentrations reach high levels, and the half-life of the triphosphate form is long, 17–19 h. Oral bioavailability is high (greater than 85%) (120).

Lamivudine is an effective initial treatment for HBV, but development of resistance is a major concern. HBV replicates through a genomic RNA intermediate, and the reverse transcriptase of HBV shares the characteristic of other reverse transcriptases of having poor fidelity. The incorporation of mismatches allows a rapid accumulation of mutations that result in resistance. For HBV polymerase, the most common mutation noted with **lamivudine** treatment is Met 552 to Val or Ile (**M552I/**V). This mutation changes a highly conserved motif (YMDD), and results in up to a 2-log increase in EC_{50} of lamivudine (410). The price that the virus pays for this protective effect is an approximate 100-fold decrease in replication efficiency, although there are additional mutations (**e.g., Leu**^{528} to Met) that partially compensate the loss of polymerase activity. Resistance is discussed further below.

Lamivudine has good potency against HBV in cell culture (EC_{50} = 0.008–0.116 *µM*) (411–415). The initial response to lamivudine at doses of 100–300 **mg/day** in *vivo* is good, with seroconversion and greater than 2-log loss in viral titers occurring over the first 6–12 months of treatment in greater than 80% of

the patients (416). However, the response rate starts declining at some point between 1 and 2 years of treatment. In one study, the response rate was 96% at 12 months of treatment, but by 30–36 months it had dropped to 43% (416). ALT levels rebounded, indicative of liver damage, and virus titers increased. These changes were associated with a mutation in the YMDD motif (M552I/V).

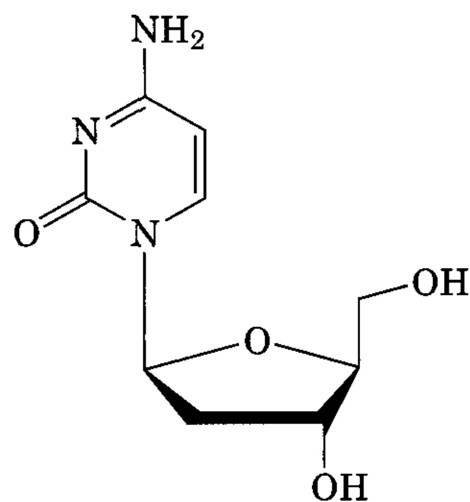
For patients that respond successfully to lamivudine, both the length of treatment and how treatment is withdrawn seem to be important. In about 15% of patients, "hepatitis flares" were experienced after cessation of treatment, with significant increases in ALT and virus titers (133,417). These flares may be a result of stimulation of clearance of infected cells carrying double-stranded DNA genomes (132, 406, 418, 419), and the majority of them resolve by themselves. However, in some cases, the flares may be severe enough to require readministration of lamivudine to limit the extent of the hepatitis. Possibly related to this phenomenon, an increase in the immune response to HBV was noted in 83% of patients treated with lamivudine during a time that corresponded with the decrease in viremia (420). Thus, whereas high levels of virus production have a depressive effect on T-cell–mediated HBV response, some level of virus synthesis may be needed to stimulate the immune system to remove infected cells.

The appearance of a mutation in the precore region that blocks expression of HBeAg is associated with a poor prognosis, presumably because lack of HBeAg results in less efficient T-cell–mediated destruction of infected cells. Lamivudine seems to select against precore mutants initially. In six patients that started lamivudine treatment expressing a precore mutant, all had reverted to wild-type by approximately 12 months of treatment. However, these mutants reappeared after prolonged therapy (421).

Organ transplantation poses an additional burden on treatment because of the immunosuppressive agents that are used to protect the new organ. Lamivudine has demonstrated potential as a prophylactic treatment before liver transplant. The extent of replication before a liver transplant is a predictor of recurrence of HBV (422). Lamivudine has been used to suppress HBV replication in liver transplant candidates both before and after transplantation. As an example, six patients with two to four times normal ALT levels were placed on daily 150-mg doses for 4 months, at which time all six were negative for HBV DNA and had ALT less than 1.2 times normal. After 6–15 months of treatment, no viral rebound had occurred (423). In another study, post-tranplantation treatment with lamivudine resulted in loss of serum HbsAg in 64% of patients (424). Sixty percent of HBV-positive renal transplant patients treated with lamivudine for 1–4 months after transplantation responded to the treatment (425,4261, but when patients were followed after cessation of treatment, almost all of them rebounded (426). This is not a surprising result, because immunosuppression should result in poor clearance of infected cells.

Phosphorylation of lamivudine to the triphosphate form (3TCTP) is enhanced by hydroxyurea, methotrexate, or fludaribine, such that pool sizes increase by up to 3.5-fold (427). These compounds decreased the pool size of the endogenous competitor, dCTP. The result may be a moderate improvement in the efficacy of lamivudine. However, levels of 3TCTP needed to inhibit wild-type virus are readily achievable in the absence of this treatment, whereas the EC„ against the M552V/I mutant is 50-fold higher than for wild-type. This is well out of the range of the effect of these compounds on 3TCTP levels.

L-dC (**66**), L-dT (**67**), and L-dA were tested for activity against a panel of 15 viruses, including herpesviruses, and were only active



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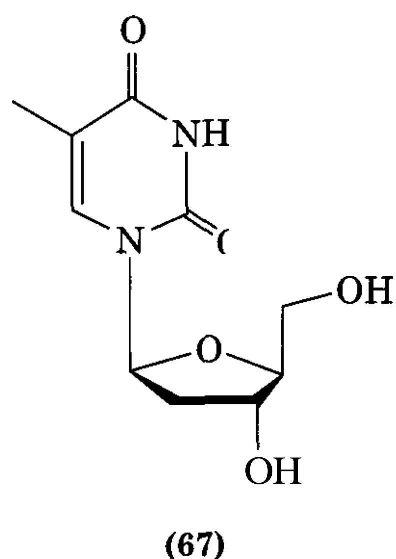

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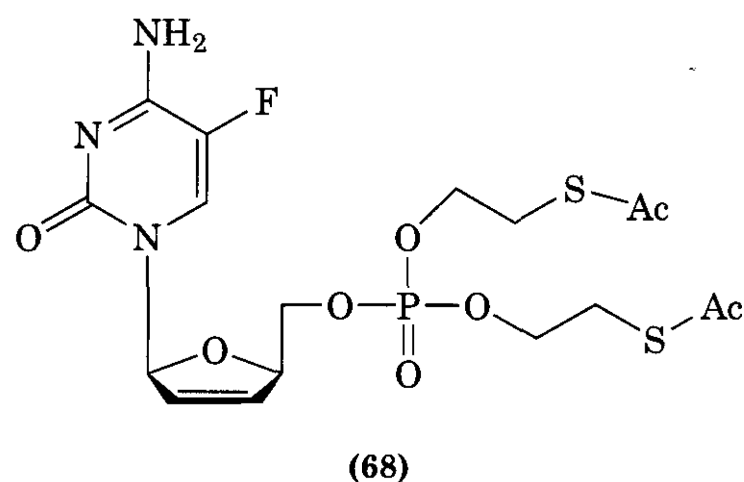
against HBV (409). They were also inactive against human DNA polymerases a, *β*, and y. Consistent with this observation, these nucleosides showed no **cytotoxicity** against a variety of cell lines. Finally, in infected woodchucks, **L-dC** and **L-dT** gave a 6- to 8-log reduction in serum virus titers after 3 weeks of treatment (10 **mg/kg/day**). No signs of toxicity were noted over 12 weeks of treatment. These pyrimidine analogs seem to be promising candidates for clinical development.

Another example of pursuit of an L-isomer of a D-nucleoside is 2',3'-dideoxy-2',3'-didehydro-*β*-L-5-fluorocyticine **(Fd4C)** (65) **(413)**. **Fd4C** has potency in cell culture about 15- to 30-fold greater than lamivudine **(EC_{50}** = 2-200 n*M*), with minimal cytoxicity **(CC_{50}** = **7-20** vs. **>50** μM for lamivudine) (413, **428**–430). The derivative lacking the flourine **(d4C)** was four- to fivefold less active than **Fd4C** (413)Similar results were noted in comparing lamivudine vs. **Fd4C** treatment of infected woodchucks (431). A 4-week treatment of HBV in a duckling model with **Fd4C** gave strong suppression of viremia and gave no evidence of toxicity (429). Higher levels of **Fd4C** triphosphate are maintained in cells, in part because conversion of **Fd4C** from the **diphosphate** to triphosphate form is more efficient than for lamivudine. Turnover of **Fd4C** metabolites is much slower than for lamivudine, and this is reflected in a longer time to reappearance of HBV DNA after removal of the drug for **Fd4C** (428). **Fd4C** has the potential for once daily dosing, which gives it an advantage over lamivudine. It suffers less loss of potency in viruses with mutations at amino acids 552 and 528 (432). The future of **Fd4C** as an HBV antiviral may hinge on whether its increased potency, higher intracellular **triphosphate** levels than lamivudine, and better potency against resistance mutants **allow it** to retain effectiveness against these mutants.

An S-acyl-2 thioethyl-monophosphate (SATE) **prodrug** form of **Fd4C** (68) was tested



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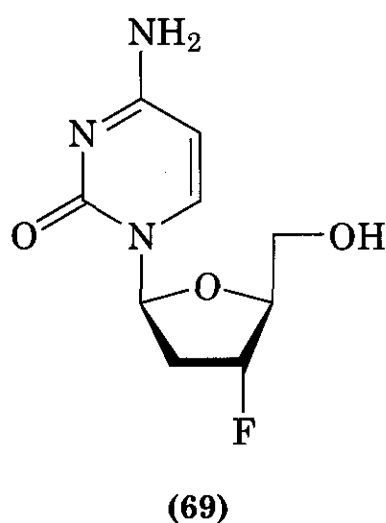
as a means of increasing the **intracellular** concentration of **Fd4C-monophosphate**. The **prodrug** was eightfold more potent than the parent and showed fourfold lower toxicity (**430**).

Additional members of this group include FTC (64) and L-FMAU (18). *β*-L-2',3'-dideoxy-5-fluoro-3'-thiacytidine (**emtricitabine, FTC**) has good potency against HBV and low toxicity (cell culture **EC_{50}** = 40 n*M*, TC_{50} > 700 **μM**) (433). In woodchucks, it showed potency equivalent to **lamivudine** when administered at a dose of 30 **mg/kg/day** and no evidence of toxicity (434). **2'-Fluoro-5-methyl-*β*-L-arabinofuranosyluracil** (L-FMAU) was a byproduct of the search for **HIV** antivirals. **It** was inactive against **HIV**, but inhibited HBV. Use of the D-enantiomer has been limited by both toxicity, primarily of the mitochondrial DNA polymerase (435) and by deamination (330). L-FMAU has 20-fold greater potency than the D-isomer (**EC_{50}** = 0.1 versus 2.0 **μM**) and a selectivity index of **>2000** versus 25 for the D-isomer (330, 436). In the duck model, oral administration of 40 **mg/kg/day** for 8 days gave a 72% decrease in peak viremia, with no short-term toxicity noted (437). Toxicity was also not noted in a 30-day study in mice or in 4-week or 3-month studies in woodchucks (438,439). A short-term study in woodchucks demonstrated that the reduction in viral DNA

synthesis was very rapid, with a 3-log reduction within 3 days of treatment. Viremia remained suppressed for a significant length of time after withdrawal of treatment, with one-half of the animals showing suppression of viral DNA at 10–12 weeks post-withdrawal (439). L-FMAU is a substrate for at least three cellular kinases, thymidine kinase, a mitochondrial deoxypyrimidine kinase, and deoxycytidine kinase (440). Related 2'-fluoro-5-methyl-β-L-arabinofuranosyluridine analogs were inactive, including 5 substitutions with halogens or alkyl groups. Cytosine and 5-iodocytosine showed weak to moderate activity, 15- and 50-fold less than FMAU, respectively (441).

2′,3′-Dideoxy-3′-fluoro-D-ribonucleosides have also been shown to have potent antiviral activity, but excessive toxicity. Because other L-enantiomers have shown less toxicity while maintaining potency, L-enantiomers of these fluoro compounds were tested. They were inactive with the exception of the cytosine analog (**69**), which showed moderate activity



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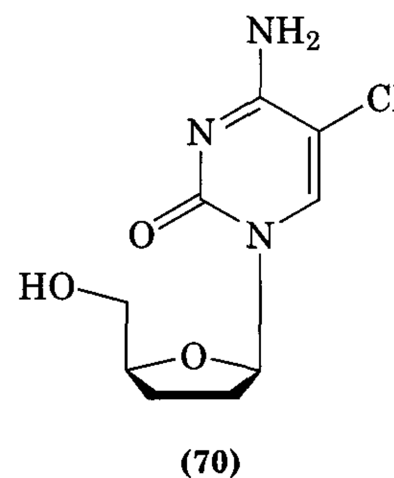
against HBV (442). Moving the fluorine to the 2' position gave a 5-fluorocytosine analog with potency against HBV about fourfold better than that of lamivudine (443). Several 2′-deoxy-2′,2′-difluoro-L-erythro-pentofuranosyl nucleoside derivatives were synthesized to try to combine the antiviral effect and lack of toxicity of the L-nucleosides and the antiviral effect of 2′-deoxy-2′,2′-difluoro nucleosides (444). While these compounds were not toxic, they did not show activity against HSV-1 or -2 or HBV.

Some β-D-2′,3′-dideoxy-5-chloropyrimidine compounds have efficacy as HIV inhibitors, but the corresponding L-enantiomers do not. However, selected L-enantiomers of this class of compounds have shown activity against HBV. As an extension of this work, β-L-2′,2′-dideoxy-5-chlorocytidine (70) and β-L-2′,2′-dideoxy-3′-fluoro-chlorocytidine (71) were tested against HBV. They are active,



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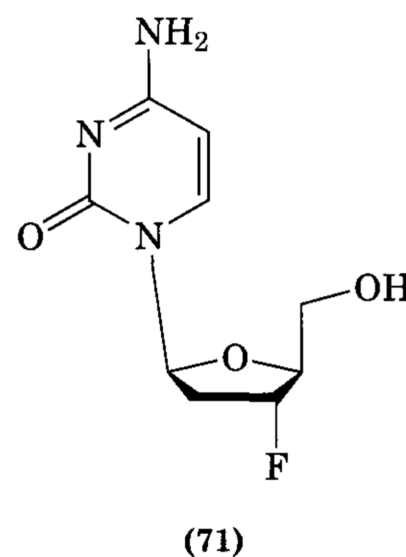

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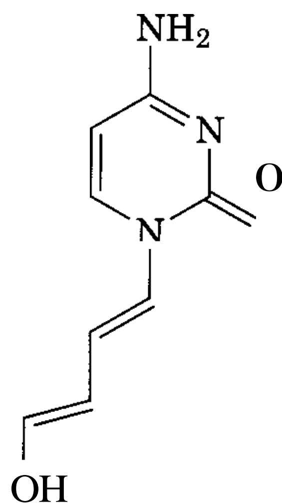
but were 100- to 800-fold weaker than lamivudine (445).

Cytallene (1-(4′-hydroxy-1′,2′-butadienyl)-cytosine) (72) is a potent HBV inhibitor (EC_{50} = 80 n*M*) (446). It is somewhat toxic (TC_{50} = 12 μ*M*), giving it a selectivity index of 150. It is efficiently phosphorylated by human deoxycytidine kinase (447). Cytallene is unique amongst these pyrimidines in being an acyclic analog.

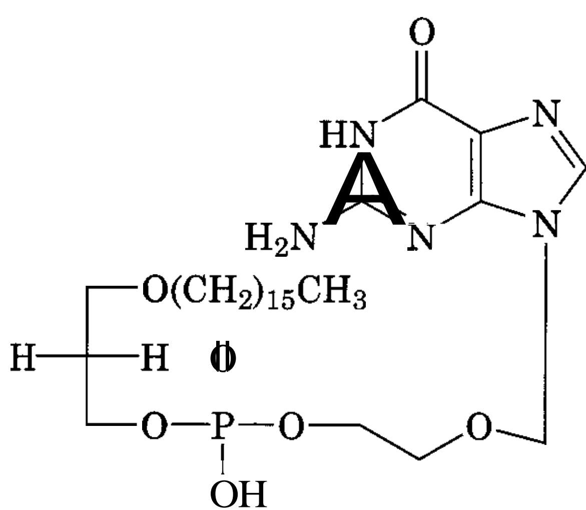
3.2.2 Purines. GCV, PCV, and famciclovir (the prodrug form of PCV) have some use against HBV (448, 449). Whereas their potency approaches that of lamivudine, they have several disadvantages that limit their use. GCV and PCV require intravenous administration, and all three have poor side ef-



(72)

fed profiles relative to lamivudine. Further, as discussed below, they show cross-resistance to lamivudine. As monotherapies, they have no significant advantages over the use of lamivudine, but they may have use as part of a combination therapy regimen; this is discussed later.

ACV-triphosphate is an effective inhibitor of the HBV DNA polymerase ($IC_{50} < 1\ \mu M$). However, ACV is ineffective against HBV because of the lack of phosphorylation of ACV to the monophosphate form. When ACV was supplied as a monophosphate, in the form of the orally bioavailable **1-*O*-hexadecyl** propanediol-3-P-acyclovir (HDP-P-ACV) (**73**), it



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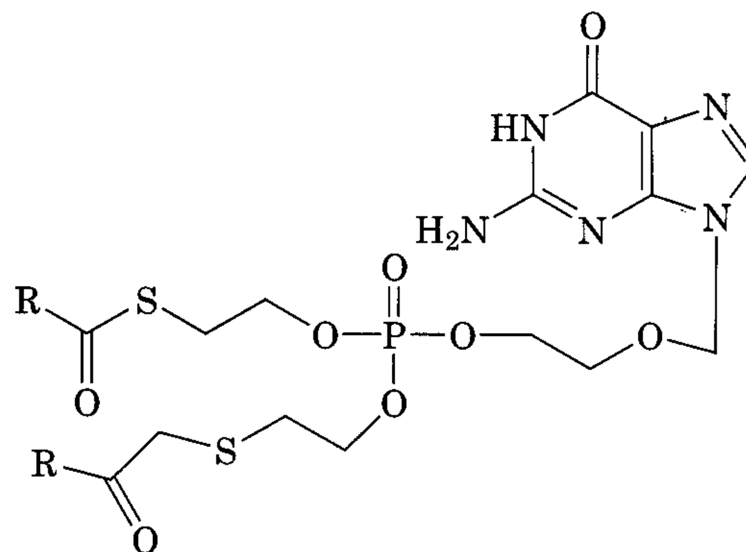
was effective in inhibiting virus production in woodchucks (450, 451). When HDP-P-ACV was given at 10 mg/kg twice daily, serum viral DNA was reduced by 95% after 4 weeks. ACV given at five times that molar dose had no effect on serum viral DNA levels. No evidence of toxicity was noted for HDP-P-ACV at doses up to the maximum dose of 30 mg/kg twice daily.

An S-acyl-2 thioethyl-monophosphate (SATE) derivative of ACV (74) was also shown



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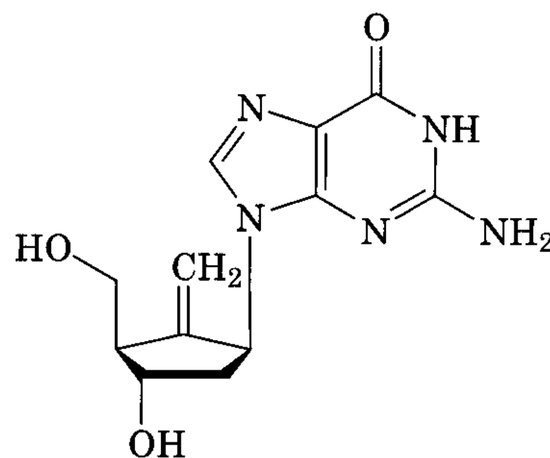
to have potent anti-HBV activity in the duck model *in vitro* and *in vivo* (452, 453). The methyl derivative exhibited an $EC_{50} = 30$ n*M* in infected primary duck hepatocytes, whereas the tert-butyl derivative exhibited an $EC_{50} = 0.6$ n*M*. The equivalent value for ACV was 100 n*M*. These compounds were more toxic than ACV, particularly the methyl derivative ($TC_{50} = 5\ \mu M$). The tert-butyl derivative was less toxic ($TC_{50} = 120\ \mu M$), and its high potency gave it a selectivity index of 200,000. The tert-butyl derivative was also effective in reducing viral titers *in vivo*.

Entecavir (BMS-200475) (75) is a guanosine analog that is approximately 10-fold more po-



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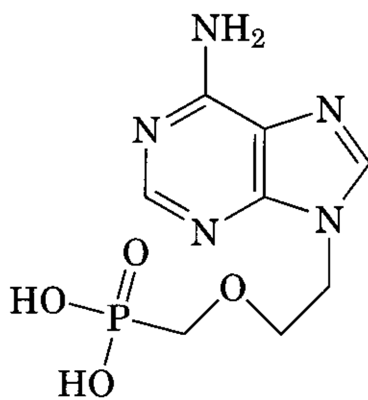
tent than lamivudine (454). It is a competitive inhibitor with respect to **dGTP** binding, and is capable of inhibiting the priming, reverse transcription, and DNA-dependent DNA synthesis steps of HBV polymerase. It is not a chain terminator; it causes termination two to three residues downstream of the site at which it is incorporated (454). In a 3-month study in woodchucks, doses of 0.1 or 0.5 mg entecavir/kg body weight reduced viral titers from 10^{10} to less than 10^3 virions/mL (455). Similarly, in a 21-day study employing ducklings infected with duck hepatitis B virus, entecavir treatment resulted in a 2- to 3-log decrease in viral titer compared with less than a 1-log decrease with lamivudine (456).

A recent clinical study of entacavir in hepatitis B patients has been reported by de Man et al. (457). The 28-day double-blind placebo-controlled study tested four daily doses of entacavir (0.05, 0.1, 0.5, 1.0 mg). All doses of entacavir show a greater than 2-log mean reduction in viral load. Approximately 25% of the patients in the study (42 patients) exhibited HBV DNA levels below the levels of detection. The drug was well tolerated by all patients and there was no detectable change in ALT levels before or after the study. Patients receiving the 0.5 and 1.0 mg doses showed a slower return to baseline HBV DNA levels than the lower doses.

Adefovir (9,(-2-phosphonomethoxyethyl)-adenine, PMEA) (**76**), 1-β-2,6-diaminopurine



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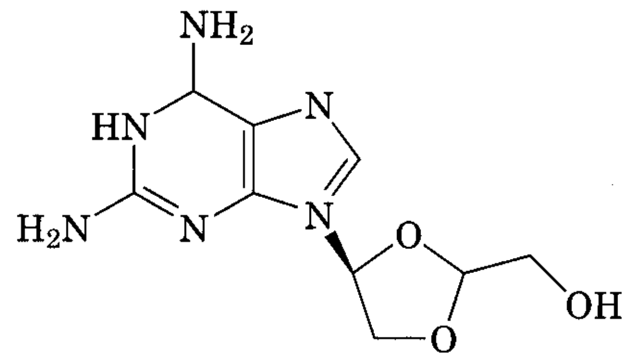
dioxalane (DAPD) (**77**), and 9,(3-hydroxy-2-phosphonomethoxypropyl)adenine (PMPA) (78) are purine analogs with potent anti-HBV activity (302,458). Adefovir is an acyclic phos-



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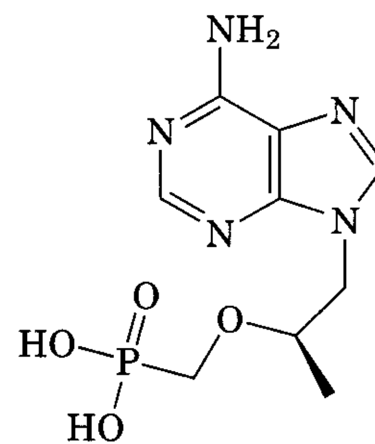

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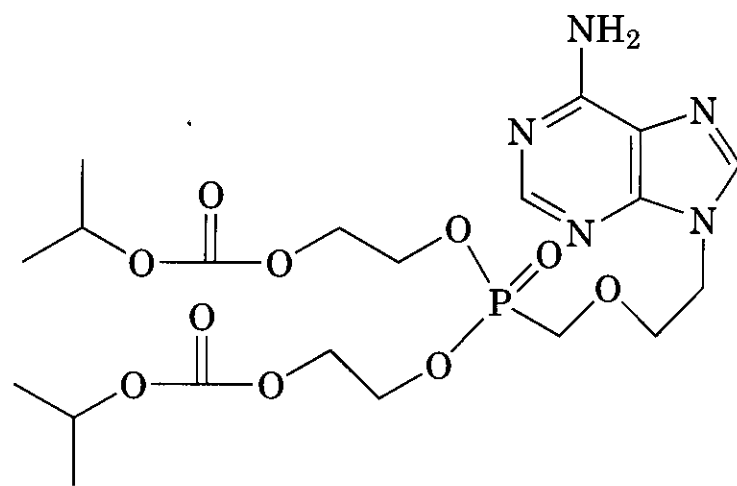
phonate analog. of adenine monolshoslshate, which shows activity against herpesviruses and retroviruses, in addition to HBV (459). Adefovir is not orally bioavailable, but a prodrug form, adefovir dipivoxil (**79**) has 40% bio-



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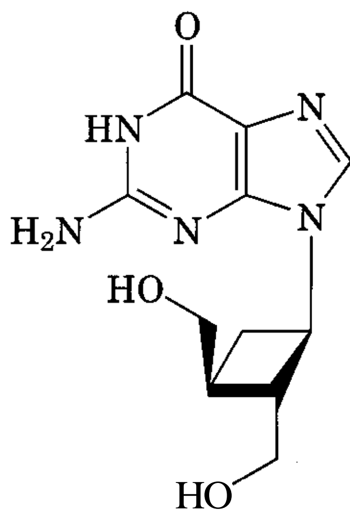
availability in humans (460). A 2-log drop in viral DNA secretion was noted from woodchuck hepatocytes infected with WHBV over 27 days of treatment (461). Treatment of woodchucks for 12 weeks with 15 mg adefovir

dipixovil per kg body weight resulted in a 2.5-log decrease in serum viral DNA (462). A study of HBV production and clearance with adefovir dipivoxil treatment indicated that virus production was reduced to 0.7% of its pretreatment levels (407). In short-term studies in humans, adefovir dipivoxil caused an 1.8- to 4-log decrease in serum HBV DNA within 2 weeks (407, 460). A 48-week study in lamivudine-resistant patients resulted in a mean 4-log decrease in serum HBV DNA titers (463). HIV-positive patients experienced some liver toxicity, with elevations in ALT occurring in 50–60% of HIV-infected patients and in none of the HIV-negative patients (460, 464). Side effects were primarily associated with the gastrointestinal tract: abdominal pain, diarrhea, nausea, vomiting, etc. These side effects were measured in a 4-week study, which was not long enough to assess whether patients adapted to the drug with time.

Lobucivar (**80**) is a potent inhibitor of HBV replication, with some characteristics of ad-



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efovir. It has a shorter half-life than adefovir (459) but was 10-fold more potent than lamivudine against purified polymerase (454), and it was effective in short-term studies in woodchucks at 10–20 mg/kg/day (465). It does not show cross-resistance with lamivudine (466). However, it was withdrawn from clinical trials because of the appearance of tumors during carcinogenesis studies in rodents (467).

3.2.3 Resistance in HBV. Resistance mutants are the factor limiting successful treatment with lamuvidine (405, 468–471). A study of 27 patients treated for 2–4 years showed the following pattern of response to lamivudine treatment (417): (*1*) a rapid decrease in viremia, with serum viral DNA levels decreasing 4–5 logs in the first year; (*2*) an improvement in liver histology over this time period; (3) appearance of resistant virus starting at about 8 months, which eventually appeared in one-half of the patients. The appearance of resistance was inversely correlated with the level of virus suppression-resistance mutants developed in 76% of the $HbeAg^+$ patients, but only 10% of the $HbeAg^-$ patients. The appearance of resistance mutations is also correlated with the phenotypic state of the liver, with high serum ALT levels being predictive of more rapid generation of drug resistance (472).

A mutation of Met^{552} to Val or Ile (M552V/I), alone or in combination with Leu^{528} to Met (L528M) increases the lamivudine EC_{50} by 2–4 logs (473–475). The M552I/V mutation also causes a 1-log decrease in viral DNA replication (476). Because of this loss of fitness, discontinuation of lamuvidine treatment leads to reappearance of the wild-type virus, although if lamivudine treatment is restarted the resistant virus rapidly returns (417). This decrease in virus fitness is largely compensated for by L528M (476). The cause of the resistance because of the M552I/V mutation is decreased binding affinity of lamivudine by HBV polymerase, resulting from steric hindrance by the side chain of Val or Ile (477).

An additional mutation conferring resistance to lamivudine, A529T, has been reported in 3 of 23 patients included in a study of resistant patients (469). This mutation also introduces a stop codon in the gene coding HbsAg, which impairs its secretion. The effect on HbsAg may limit the spread of this mutation.

One of the lessons of drug resistance in other viruses, particularly HIV, is that treatment with combinations of drugs is likely to be important. Therefore, it is important to understand which combinations of drugs generate cross-resistance. In addition to lamivudine, the combination of M552I/V and L528M mutations also provide cross-resistance to FCV, PCV, ddC, FTC, AZT, FMAU, and to a lesser extent, Fd4C (477,478). FCV generates an overlapping, but not identical set of resis-

tance mutants; I528M, V521L, V555I, and T532S, but not mutations at M552, have been reported from both FCV- and lamivudine-resistant patients, (471, 479–481). It is likely that treatment of FCV-resistant patients with lamivudine would result in a more rapid appearance of M552V/I.

Adefovir is not cross-resistant with lamivudine (410, 432, 466, 482), thus providing an alternative therapy for resistant cases. A comparison of inhibition by lamivudine of wild-type HBV polymerase activity vs. mutant derivatives showed an increase in inhibition constants (K_i) of 8.0-, 19.6-, and 25.2-fold relative to wild-type for M552I, M552V, and M552V/L528M mutants, respectively. Increase in K_i for adefovir was twofold or less for these mutants (410). Potency of lamivudine was decreased in cell culture by 100- to 10,000-fold by these mutants, whereas adefovir was only affected by 4- to 16-fold (476). In a study involving five patients who had developed resistance to lamivudine after 9–19 months of treatment, all responded to adefovir dipivoxil, with a 2- to >4-log decrease in viral titer (483). These decreases were maintained over the 11- to 15-month treatment period and were correlated with improvements in liver function.

The experience of HIV treatment indicates that additional therapies will be necessary to counteract developing resistance. HBV and HIV are similar with regard to the amount of virus that is produced per day and the fidelity of their polymerases. Combination therapy for HIV now typically involves three drugs, with alternative drugs available for patients who fail their initial therapy. The relatively limited selection of options currently available to treat HBV is unlikely to provide sufficient treatment.

3.2.4 Combination Treatments. Given the difficulty of maintaining effective long-term monotherapy, several combinations of drug treatments have been investigated. Treatment of patients that failed interferon-a monotherapy with interferon-a plus ribavirin showed improvements in a fraction of patients, but greater than one-half of the patients did not respond and the side effect profile was poor (484). When interferon-a was combined with lamivudine, there was a modest but statistically insignificant improvement in the proportion of HBeAg seroconverters (485). The benefit of this combination seemed to depend on the extent of damage to the liver, because patients with lower baseline ALT levels showed greater improvement than those with higher ALT levels. Thus, it is possible that a subgroup of patients could be identified for which this combination of drugscould provide benefit.

In contrast to the previously mentioned beneficial effect of adding mycophenolate mofetil (MMF) in treatment of HSV and HIV, adding MMF to the treatment regimen of HBV-infected liver transplant patients who had failed lamivudine treatment did not provide any benefit (486). However, when mycophenolic acid or ribavirin were used in cell culture in combination with guanosine analogs, PCV and lobucavir, their potency was improved by as much as 10-fold (487). This effect could be reversed by adding exogenous guanosine, indicating that the increase in potency was a result of a decrease in dGTP pool size by mycophenolic acid or ribavirin. Because liver transplant patients are often treated with MMF, the prodrug form of mycophenolic acid, they represent a subset of HBV-infected patients that may derive more benefit from guanosine-based drugs than non-MMF–treated patients.

Relatively few studies have examined the effects of combination therapy in patients who were not already resistant to one of the treatments (412, 452, 488–490). A comparison of PCV, lamivudine, and adefovir in naive infection of duck hepatocytes showed that all combinations of the three treatments were at least additive (488). Cytotoxicity with all combinations was insignificant. Whereas PCV is not a useful follow-up to lamivudine in resistant patients caused by cross-resistance, the combination of PCV and lamivudine against drug-sensitive virus is more effective than either alone. Adefovir has the added advantage of suppressing the appearance of viruses resistant to either PCV or lamivudine, which should increase the length of time that those drugs will be useful. In a related study, PCV and lamivudine in combination were more effective at reducing the copy number of cccDNA than were either separately (490).

Similarly, the combination of famcyclovir (FCV) (the prodrug form of PCV) and lamivudine in woodchucks was at least additive relative to the individual drugs (489). As an example, treatment with 5 mg/kg of lamivudine reduced viremia by 75-fold, whereas 50 mg/kg of FCV showed a 10-fold decline. Combining the two treatments reduced viremia 10,000-fold.

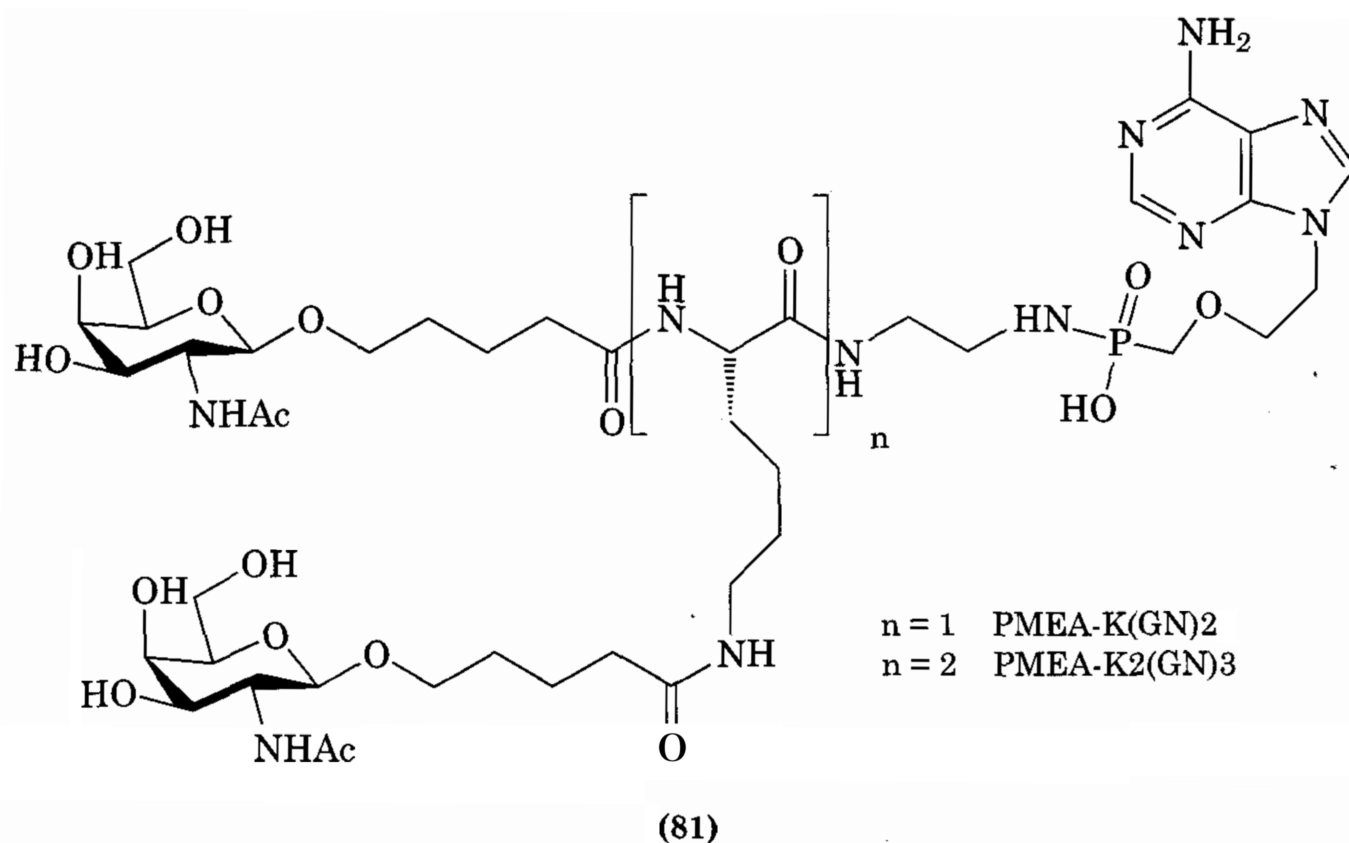
In a short-term human trial, the combination of lamivudine and FCV was shown to be superior to lamivudine alone (491). After 12 weeks of treatment, the combination provided greater viral clearance than lamivudine alone. Viral DNA levels returned to pretreatment levels in 44% of the patients 16 weeks after termination of treatment but in none of the patients given the combination therapy. These combinations need examination in more long-term trials.

3.2.5 Targeting Drugs to the Liver. Two traits of the liver present an unusual opportunity for increasing potency and limiting the side effects of HBV treatments. First, hepatocytes have receptors capable of recognizing relatively simple molecules, which can be attached to make prodrug forms of drugs. Second, the liver is one of the first organs to which orally delivered compounds are exposed; thus, increasing uptake by the liver reduces the exposure of other organs to a drug. Several attempts have been made to take advantage of these features in designing anti-HBV drugs.

The most commonly used hepatocyte-specific receptor is the asialoglycoprotein receptor. Molecules with carbohydrates that terminate with galactose are recognized and internalized by this receptor. This subject is reviewed by Fiume et al. (492); several examples follow. Adenine arabinoside monophosphate (ara-AMP, vidariabine-MP) has been conjugated to human serum albumin (HSA) and used to treat patients for 1 and 4 weeks in separate studies. It was administered at a dose equivalent to 1.5 mg ara-AMP/kg/day, a dose that is 10- to 20-fold lower than is used for free ara-AMP. At this dose it was as effective as free ara-AMP at the higher concentration. However, with the free drug, by 4 weeks of treatment, neurotoxic and other side effects appeared; these side effects were absent with the conjugated drug (493,494). Similar results were obtained by conjugating ara-AMP to polylysine (495, 496). This conjugate was tested in the woodchuck model. Free ara-AMP administered at 2.5 mg/kg/day had no effect on viremia, whereas the conjugate delivered at the same molar concentration of drug decreased viremia to undetectable levels. Polylysine conjugates have the advantages over albumin conjugates of being synthetic rather than derived from a blood product and of being administered intramuscularly rather than intravenously. Both have the disadvantage of no oral bioavailability, however.

Adevofir is an effective drug with a slightly less favorable side effect profile than lamivudine. For treating HBV, it has the additional disadvantage that it is taken up poorly by hepatocytes. An approach conceptually similar to the one just discussed for targeting it to the liver was taken (497). Adefovir was derivatized with glycosides (81) with a high affinity for the asialoglycoprotein receptor. This modification resulted in a 10-fold increase in the amount of drug taken up by the liver (52–62% versus 5% for adefovir). This caused a decrease in the amount of drug taken up by extrahepatic tissues. The kidney removes about 10-fold more adefovir from serum than does the liver. However, the ratio of liver to kidney uptake shifted by 30- to 45-fold toward the liver with the prodrug forms. The end result was a fivefold increase in potency for the smaller glycoside and a 52-fold increase for the larger.

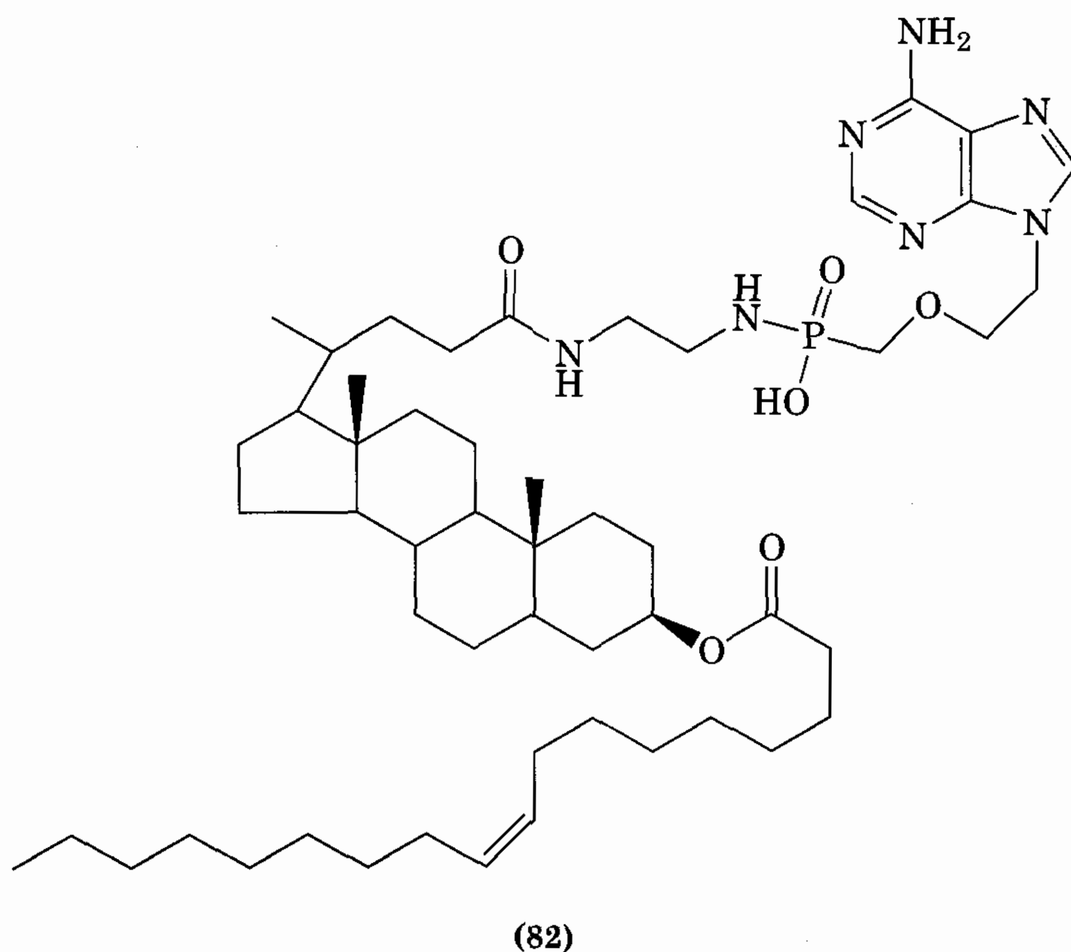
Another approach to targeting adefovir to the liver employed a lactosylated high-density lipoprotein (498). This protein, in combination with phospholipids, can bind lipophilic drugs. Adefovir is not sufficiently lipophilic, so it was derivatized by addition of a lithocholic acid-3α-oleate (referred to as PMEA-LO) (82). This derivative was mixed with the protein and phospholipids and injected intravenously into rats. It was taken up efficiently by the liver, with approximately 70% of the dose localized to the liver and less than 2% found in the kidneys. This ratio is 500 times greater than that noted with free adefovir. Of the liver dose, 88% was found in hepatocytes. The adefovir was efficiently released by acid hydrolysis in lysosomes.



(81)

A phosphatidyl derivative of lamivudine was synthesized that might be expected to be delivered preferentially to the liver (499). This lamivudine analog was comparable in activity in cell culture with the parent compound. Because lamivudine shows few toxic effects and an effective dose is attainable orally, there may be no advantage to such an approach with lamivudine. However, this compound may provide a model for targeting other compounds with less favorable characteristics.

If this liver-targeting approach can be combined with oral bioavailability, it may provide a powerful way to expand the selection of drugs that could be used without encountering harmful side effects. This drug delivery



(82)

feature has a negative side, in that other cell types that can be infected by HBV may not recognize the prodrugs. These cells would then provide a reservoir of virus that is not eliminated. This aspect of these compounds merely provides another impetus to develop good combination therapies.

3.2.6 Antisense Oligonucleotides. Oligonucleotides that are complementary to a stretch of an RNA can, on binding to that RNA, either promote its degradation by a cellular RNase H or prevent movement of polymerases or ribosomes through that area. As an experimental tool, this approach has been used in dozens of model systems. This approach has also been applied by a few groups to the inhibition of HBV replication. The oligonucleotides face two hurdles. First, they are not orally bioavailable; second, they must avoid degradation by cellular nucleases. The first hurdle is currently cleared by intravenous injection and the second by modification of the oligonucleotide to make it more resistant to degradation.

A study of a series of antisense phosphorothioate oligonucleotides to the duck hepatitis virus genome identified two that showed effective inhibition of viral replication in isolated duck hepatocytes, with a 1- to 2-log decrease in viral DNA after 10 days of treatment (500). The most potent oligo was administered to infected ducks for 10 days to examine its potency in *vivo*. At a dose of 20 mg/kg body weight, viral DNA was reduced to nearly undetectable levels. No side effects were noted, although long-term treatment with phosphorothioate oligonucleotides can lead to cytoxicity. Treatment with the oligonucleotide before infection was able to block infection.

In an attempt to improve the membrane permeability and nuclease resistance of the oligonucleotides, oligoribonucleotides modified by addition of 2'-*O*-(2,4-dinitrophenol) (DNP) at a 0.7 ratio of DNP to nucleotide were tested in the duck model (501). At ratios less than 0.5, oligonucleotides were easily degraded, whereas ratios >0.8 interfered with hybridization. The oligonucleotide inhibited purified polymerase with an IC_{50} of 8–20 n*M*. The oligonucleotide was tested in ducklings with a 45-day treatment of 0.5–1 mg/kg/day. Nine of nine treated ducks had undetectable viremia by day 25 of treatment. One of six control ducks spontaneously cleared the virus during this time. None of the treated ducks showed a reappearance of the virus during a 30-day follow-up period after treatment.

Antisense oligonucleotides offer some significant advantages. Their synthesis is routine and does not have to be reinvented for each new oligonucleotide. The number of potential targets is large, in that whereas the sequence and location in the genome affect the potency of the oligonucleotide, many sequences are likely to work. Thus, resistance can be overcome by moving to a new location; combination therapy could simply mean multiple oligonucleotides. Nevertheless, delivery of the oligonucleotide to the interior of the infected cell, especially in an orally bioavailable fashion, is still an imposing barrier to their use as drugs.

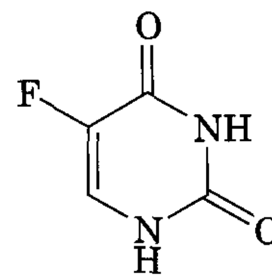
3.3 Papillomaviruses

Papillomaviruses have proven difficult to treat because of the paucity of targets in the latent phase. Thus, treatment regimens have focused on eliminating the infected cells rather than treating the virus directly. Methods for doing this have included surgery, cryotherapy or caustic agents applied to warts, and topical application of cytotoxins such as podophyllin (502,503). These approaches have low success rates because of a high recurrence rate of the warts. Whereas the bulk of the wart can be removed by this method, the infected basal cells that provide the reservoir for continued viral genome replication are difficult to remove. One approach to overcoming this barrier comes from the observation that the warts may clear without treatment, presumably because of an immune response (504). This assertion is bolstered by observations that HPV-associated warts in HIV-positive patients responded to antiretroviral treatment (505, 506). Attempts to bolster the immune response led to treatment with interferon-a by injection into the infected tissue, but analogous to the response to interferon-α as an HBV treatment, interferon induction of an immune response has seen only limited success (507–509).

Induction of an interferon-mediated response by small molecule inducers of interferon has shown more success. Imiquimod (1-(2-methylpropyl)-1H-imidazo(4,5-*c*)quinolin-4-amine) (**62**) and the related compound S-28463 (63) induce an interferon-a-mediated response (510,511). They have shown efficacy as immunomodulators against lesions caused by both herpesviruses and papillomaviruses (512,513). In a placebo-controlled trial, 40% of patients experienced complete clearance of warts when treated with a 5% imiquimod cream for 8 weeks; no patients receiving placebo showed complete clearance (514). Three-quarters of the treated patients had a 50% reduction in wart area versus 8% with placebo. Of the patients who cleared the warts, 80% remained free of warts during a 10-week follow-up period. Greater than 50% of the patients reported mild-to-moderate localized side affects as a result of treatment, with no systemic effects reported. Similar results were noted in a 16-week study with 5% imiquimod, but 1% imiquimod was ineffective (14% versus 52% complete clearance) (515). Note that the improved results with imiquimod versus direct injection of interferon could be a result of an indirect effect of imiquimod on interferon induction. This effect is mediated by cytokines, which may also result in regulation of other factors that promote regression of the lesions. These trials demonstrated that a local interferon response is beneficial in a subset of papillomavirus-infected patients, but that it is not universally applicable. Factors that identify patients for whom this treatment is beneficial have yet to be identified.

A combination of vidaribine (**8**) and podophyllin (a DNA polymerase inhibitor and a cytotoxin) were tested in a 6-week topical application to 28 patients with cervical intraepithelial neoplasia (516). This treatment resulted in regression of the lesions and loss of detectable HPV DNA in 80% of the patients. However, 30% of patients for whom treatment resulted in regression of lesions relapsed during a 13-month follow-up period, indicating that longer treatments or additional combinations of drugs were needed for effective elimination of the proliferating cells.

5-fluorouracil (5-FU) (83) has been used as a topical application for treatment of warts



(83)

with mixed results. In two small studies, the majority of patients cleared the warts, with a low recurrence rate (517,518). However, in a placebo-controlled trial of 40 subjects with weekly application of a cream containing the treatment for 4 weeks, fewer of the 5-FU treated patients showed regression of HPV at 4–6 months post-treatment than the placebo-treated patients, indicating that the 5-FU may have actually exacerbated the disease (519).

Photodynamic therapy mediated by 5-aminolevulinic acid (ALA) has been tested as a treatment for papillomavirus-induced low-grade cervical intraepithelial neoplasia (520). The basis of this approach is that the ALA accumulates in the proliferating tissue and sensitizes those cells to visible light. At 9 months post-treatment, 95% of the patients showed improvement in PAP smears, and 80% had no detectable virus. Side effects were minimal and transient.

Cidofovir (5) has shown efficacy in selectively blocking proliferation of HPV-infected cells. This is an attractive application of cidofovir because in treating papillomavirus infection it can be applied topically, which limits its negative side effect profile (521, 522). However, the mechanism for this inhibition is not immediately obvious because HPV does not encode a polymerase. Selectivity for HPV-infected cells implies a mechanism whereby the cellular polymerase of infected cells is more affected by cidofovir than that of uninfected cells. One mechanism has been identified by Johnson and Gangemi (523). In cell culture studies designed to determine the metabolic fate of cidofovir, the authors noted that in infected cells, cidofovir was readily converted to its fully phosphorylated form. However, in uninfected cells, most of the cidofovir was in the form of a choline adduct. Cidofovir inhibited proliferation of infected cells with an EC_{50} =

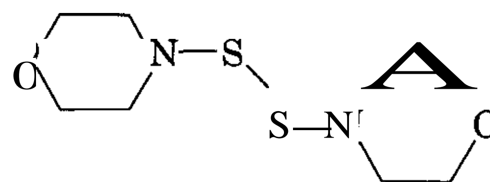
200 n*M*, whereas **1000** n*M* inhibited normal cell growth by about 15%. This loss of proliferative capacity was maintained when drug was removed after a 1-week treatment. In uninfected cells, greater than 75% of the cidofovir was in the form of a choline adduct. In infected cells, greater than 75% of the cidofovir was phosphorylated. Thus, the concentration of cidofovir converted to polymerase substrate was considerably higher in infected cells. This factor, plus the ability to treat warts topically, may limit the toxic effects of cidofovir sufficiently to make it a practical treatment.

Using a rabbit model of papillomavirus infection, a 1% cidofovir cream applied for 18 days delayed the appearance of warts and decreased their size when applied within 1 week of infection (524). Its effectiveness decreased as the delay after infection increased. On the other hand, a related study where treatment was twice daily for 8 weeks found complete regression of the warts when treatment was delayed until 4 weeks after infection (525). Adefovir (76) also gave moderate activity. In about 50% of the warts treated with cidofovir, recurrences were noted with longer monitoring times. When this treatment was combined with vaccination with DNA coding for papillomavirus proteins E1, E6, and E7, the recurrence rate dropped from 53 to 15% (526). Vaccination alone was not an effective treatment in this study.

Treatment of 15 women with stage III cervical intraepithelial neoplasia with 1% cidofovir three times every other day resulted in removal of histological signs of the lesion in 7 of 15 patients. Four of those patients had undetectable levels of papillomavirus DNA. Two patients did not respond, and the remainder showed limited responses (527).

Respiratory papillomatosis is a rare but often debilitating and difficult to treat disease. A case has been reported where cidofovir alone was insufficient at reversing the growth of lesions in an advanced case (528). However a combination of treatment with cidofovir (5 mg/kg every 2 weeks) and interferon a-2b led to a significant reduction in pulmonary lesions and complete regression of endobronchial lesions after 12 months of treatment.

The above studies concentrated on elimination of infected cells; few attempts to take on the virus directly have been reported. One such approach has been to identify compounds that cause release of zinc from the zinc binding sites if E6 (529, 530). Loss of zinc is associated with loss of capacity of E6 to bind to cellular proteins with which it interacts. In this case, the goal is not to block viral propagation but to interfere with the progression of the infected cell to malignancy. An initial screen identified compounds that were able to eject zinc from E6 and block binding of E6 to two of its cellular cofactors. One of these compounds, 4, 4'-dithiodimorpholine (84) was



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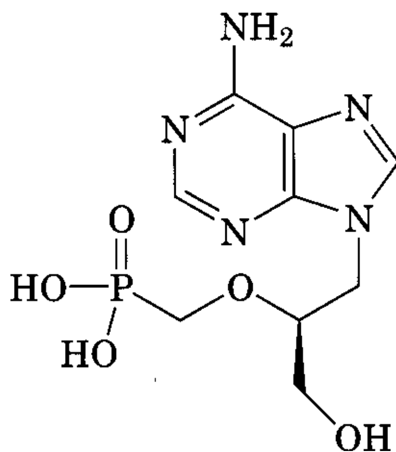
weakly active in cell culture (EC_{50} = 50–100 μM). No toxicity was noted, indicating that this compound showed specificity for E6 rather than having a general effect on zinc binding proteins. Further structure-activity relationship studies indicated that the monocyclic amines with the dithiobisamine moiety were important for activity. Decrease in ring size, moving the nitrogens out of the rings, or acyclic derivatives caused loss of specificity for E6, measured by increase in cytotoxicity. Modification of the N-S-S-N moiety resulted in loss of activity. Compounds that were incapable of electrophilic attack on the sulfur atoms coordinating the zinc or incapable of cleaving to generate a radical were also inactive.

3.4 Polyomaviruses

Whereas the disease states are different, the life cycles of polyomaviruses and papillomaviruses are similar. They both depend on the cellular DNA replication machinery, and they use similar mechanisms to stimulate cellular proliferation. These similarities are reflected in their currently available inhibitors. Murine polyomavirus and SV40, which are closely related to JC virus and BK virus, were inhibited in cell culture by cidofovir, and weakly inhib-

ited by HPMPA ((*S*)-9(3-hydroxy-2-phosphonylmethoxypropyl)adenine) (85) and PMEG (9-(2-phosphonylmethoxyethyl)-guanine) (**86**) (531). Other drugs tested in this study included



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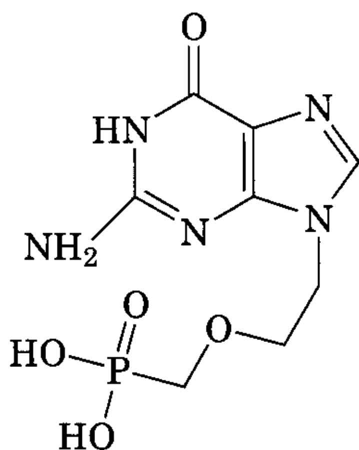

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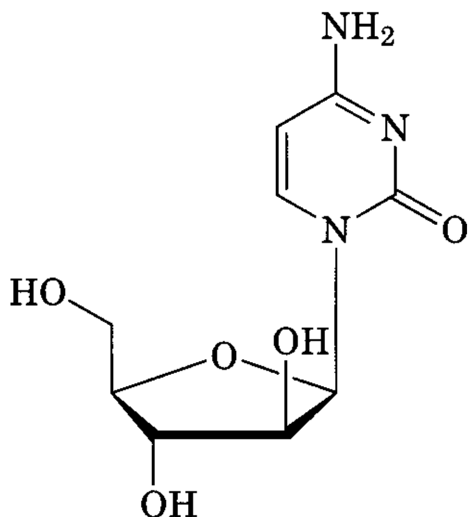
ACV (**2**), GCV (**3**), **brivudine** (**7**), **ribavarin** (**47**), and **foscarnet** (**6**), all of which were inactive. A neuroglial cell line persistently infected by JC virus was used to test ara-C (87) and cidofovir. ara-C had an antiviral effect at 10 μg/mL. Cidofovir showed only a minimal effect, but it was only tested up to 1 μg/mL (532). For comparison, the EC_{50} for cidofovir against murine polyomavirus was reported as 4–7 μg/mL (531).



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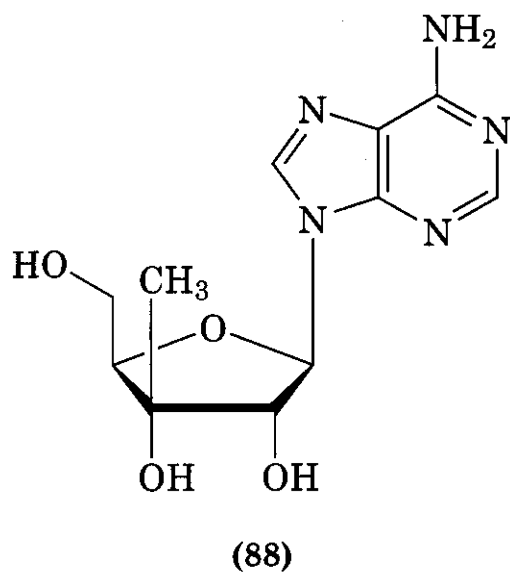
A hemangiosarcoma cell line derived by infection with mouse polyomavirus provided evidence that cidofovir was not exerting a direct antiviral effect (533). These tumor cells do not produce virus, yet they are susceptible to cidofovir. When injected into SCID mice, tumor formation could be decreased to less than 5% of controls by injection of 100 mg/kg of cidofovir three times weekly. The metabolic fate of cidofovir in these cells was not examined, but it possible that its selectivity for the tumor cells is caused by an increased conversion to the active form as described for papillomavirus-infected cells (523). The case for the antipolyomaviral activity of cidofovir is bolstered by observations of improvements in the outcome from AIDS-related multifocal leukoencephalopathy when cidofovir is included in the treatment regimen (534, 535).

3.5 Adenoviruses

Adenoviruses have received relatively limited study as drug targets. Again, cidofovir shows antiviral activity. Tested in a rabbit ocular model, topical application of 1% or 5% cidofovir cream significantly reduced adenoviral titers (536). A small clinical study of seven pediatric bone marrow transplant patients with clinical signs of adenovirus infection showed efficacy for systemic cidofovir treatment (537). The patients were treated with 5 mg/kg weekly for 3 weeks and then every other week. Five of the seven responded to treatment with loss of detectable viral DNA and improved clinical symptoms. Comparison of cidofovir to S-2242 (36) and HPMPA (**88**) in a cell culture model showed that S-2242 was 30-fold more active than cidofovir, and HPMPA was sixfold more active (538).

3.6 Poxviruses

Poxviruses encode more of the machinery that they need to complete their life cycle than any of the other viruses discussed here. They therefore encode a wide variety of potential drug targets, but extensive efforts to develop anti-poxvirus drugs have not occurred. De



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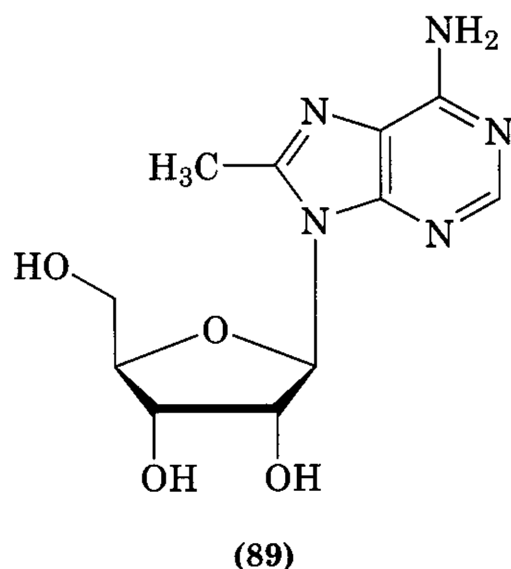
Clercq (539) has extensively reviewed the classes of known targets for which inhibitors exist. Whereas potent inhibitors of **poxvirus** replication have been identified, most of them validate their targets but are not practical drug candidates. The focus here will be on established drugs or likely candidates. In brief, some of the targets of effective inhibitors include IMP dehydrogenase, S-adenosyl **homo**cysteine hydrolase, OMP decarboxylase and CTP synthetase, thymidylate synthase, and DNA polymerase. Detailed discussions of these inhibitors and their mechanism of action are given in Ref. 539.

Inhibition of IMP dehydrogenase by **ribavirin** effectively inhibits vaccinia virus replication (EC_{50} = 4–20 μg/mL) (540). Ribavirin has shown efficacy in rabbit and mouse models applied topically to treat keratitis or injected in combination with cidofovir to treat systemic infection (541,542). Nucleoside analogs showing activity include **araA** (**543**), 3'-C-methyl adenosine (88)(**544**), 8-methyl adenosine (89) (545), S-2242 (**36**), HPMPA (**546**), and **cidofo**vir (547). HPMPA inhibited vaccinia virus replication in cell culture with an EC_{50} = **0.3**–0.7 μg/mL (546). Cidofovir was 10-fold less potent; however, its improved side effect profile relative to HPMPA has resulted in its development and licensing as a drug. Cidofovir was also effective against parapoxviruses that infect humans and domestic animals (**548**).



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HPMPA and cidofovir were both active in preventing mortality caused by cowpox infection in mice when given subcutaneously at doses of 1–20 **mg/kg/day** (547,549–551). In an infection regimen where untreated mice die between 6 and 8 days post-infection, cidofovir had some efficacy as a prophylactic treatment. When a single dose was given as much as 16 days before infection, survival rates increased from **0** to 50%. When treatment was given from day −4 to day 2, survival rates were greater than 90% (**550**). Another study designed to identify an easier delivery route for cidofovir showed that intranasal administration of 10–40 **mg/kg** of the drug was sufficient to provide greater than 90% protection from viral infection (551).

S-2242 showed potency equivalent to HPMPA in cell culture (EC_{50} = 0.4 μg/mL) (359) and was more effective in infected mice. HPMPA and cidofovir were able to limit infection in normal mice, but were unable to **pre**vent mortality in SCID mice. However H-961 (**46**), the **prodrug** of S-2242, provided complete protection against vaccinia virus infection in SCID mice (277).

3.7 Parvoviruses

The most common clinical manifestation of **parvovirus** infection is anemia caused by erythrocyte hypoplasia. Direct treatments of the virus have not been pursued, but the disease is usually self-limiting if the anemia is treated. The most common treatment is provision of intravenous immunoglobulin (244, 245). There is one report as well of treatment with erythropoietin to increase red blood cell counts (552). From the point of view of medicinal chemistry, the most effective treatments for **parvoviruses** have been treatments for HIV. Parvovirus-induced anemia in AIDS patients can be chronic rather than acute. Restoring an immune response by effective treat-

ment of the HIV infection is often sufficient remediation for the anemia (553–556).

4 CONCLUSIONS

A great deal has happened since the last edition of this review. Most of the developments have been in the expansion of useful nucleoside analogs, although other biological targets such as protease and accessory replication proteins are starting to come into their own. At the same time, the bar for treatment continues to rise higher with the appearance of resistance mutants to current effective treatments. Nucleoside analogs with novel binding modes (e.g., adefovir as an adjunct to lamivudine for HBV treatment) provide a partial answer to this problem. However, effective treatment is ultimately going to require additional targets other than the polymerases and nucleoside kinases, and such compounds are starting to appear. Even within the well-established class of nucleoside analogs, side effect profiles still curb the use of many of these compounds, so additional work remains to be done to remove this limitation.

For almost one-half of the virus families discussed here, few to no treatments have been devised that target the virus directly. These viruses (parvoviruses, papillomaviruses, polyomaviruses) pose major technical challenges because of the paucity of virus-encoded activities that they present as targets. Their treatment has benefited from unexpected modes of action (or unexpected metabolic fates) of nucleoside analogs, particularly cidofovir. However, attacking virally encoded targets is likely to be an important key to treating these virus families. Hopefully expansion of the investigation of drugs with novel modes of action for herpesviruses and hepatitis B virus will provide a boost to research on these viruses as well.

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CHAPTER TEN

Antiviral Agents, RNA Viruses (Other than HIV), and Orthopoxviruses

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Burger's Medicinal Chemistry and Drug Discovery
Sixth Edition, Volume 5: Chemotherapeutic Agents
Edited by Donald J. Abraham
ISBN 0-471-37031-2 © 2003 John Wiley & Sons, Inc.

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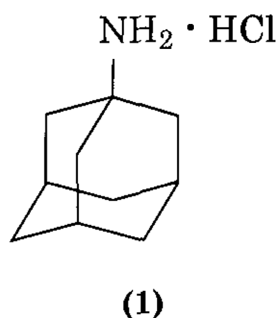
1 FDA APPROVED ANTIVIRAL AGENTS FOR SELECTED RNA VIRUS INFECTIONS

1.1 Introduction

The discovery and development of safe and effective antiviral therapies is inherently more difficult than the comparable effort to develop antibacterial agents. The primary difficulty is that viruses replicate inside the cells of their host and actually hijack host metabolic and replication processes and use them to replicate progeny viruses. Consequently, most compounds that inhibit viral replication are also toxic to the host. Most of the approved antiviral drugs target a specific viral function, such as amantadine's interaction with the influ-

enza M2 ion channel. Accordingly, unlike antibiotics, most antiviral therapies are narrow spectrum.

Although there is an extensive and promising amount of new drug candidates in preclinical development, only nine compoundls have been approved by the FDA for the treatment of infections caused by RNA viruses other than HIV. These are amantadine (1) and rimantadine (2) that interfere with the influ-



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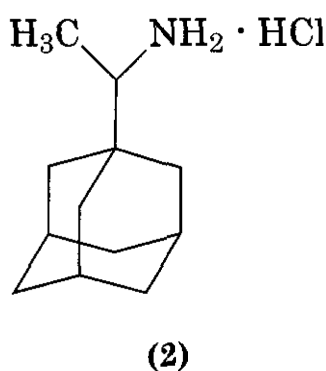

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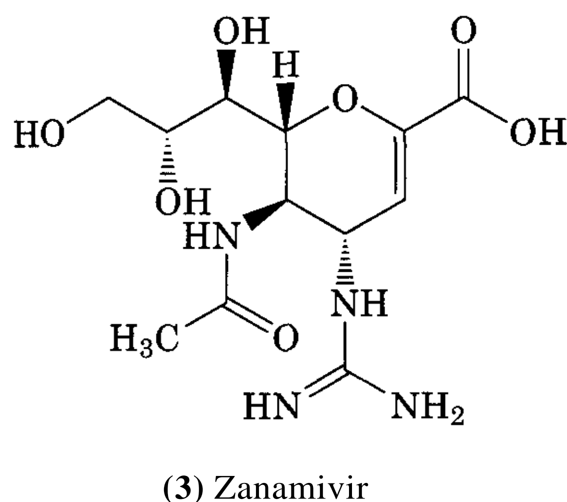
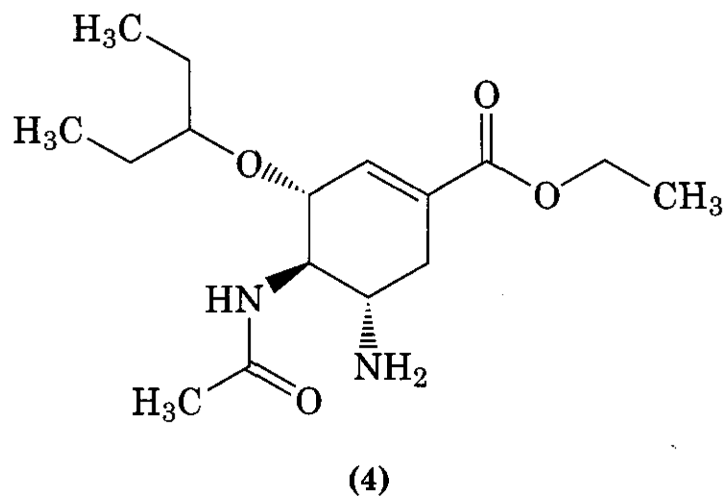
enzaion channel protein, and the newer inhibitors of the influenza neuraminidase, zanamivir (3) and oseltamivir (4). Respiratory



(3) Zanamivir

syncytial virus can be life threatening to infants, especially those with other underlying heart or lung problems. Aerosolized ribavirin (5) is approved as a therapy for this disease, and both a polyclonal antibody preparation, Respigam, and a monoclonal antibody, Syner-



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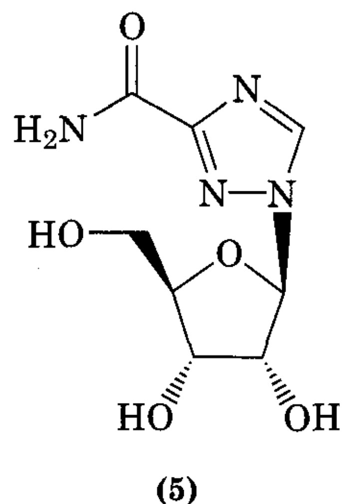


(5)

gis, are approved for prophylaxis in high-risk infants. Finally, several forms of interferon-α, both natural and pegylated, and the combination of these interferons with ribavirin have been approved for the treatment of chronic hepatitis C infection.

This chapter will review the clinical data supporting the development of therapies for hepatitis C. In addition, the chapter includes a discussion of the historical efforts to discover a treatment for smallpox, a disease that has been eradicated from the world. However, interest in the development of treatments for this disease has been revived because of its potential for bioterrorist use.

1.2 Hepatitis C Virus

Hepatitis C virus (HCV) a member of the flavivirus genus, is a negative-stranded RNA virus that is estimated to have infected 170 million people globally and 4 million in the United States. The current HCV-associated mortality rate in the United States is almost 10,000 per year and is expected to triple in the next decade (1). With the implementation of screening blood products for HCV in the 1990s, the

rate of new infections has decreased, but the virus is still spread as a consequence of intravenous drug use and blood to blood exposure (2, 3). The acute period of infection is rarely recognized, but in most people, progresses to an asymptomatic chronic infection that may last 20–30 years. Virus replicates at very high levels during this time, in the range of 10^{12} new particles per day (4), and eventually about 70% of chronic infections result in hepatitis and fibrosis and about one-fifth further progress to cirrhosis (1, 5). Those who develop cirrhosis are at high risk for further progression to hepatocellular carcinoma.

This high rate of viral turnover, combined with the high error rate of RNA-dependent RNA polymerases, results in the generation of genetically diverse quasispecies. This degree of diversity is correlated with outcome in that greater diversity in the acute phases is associated with progression to chronic disease (5) and response to interferon therapy is associated with a decrease in diverse quasispecies (2). Other prognostic factors that have been identified that generally are associated with a good outcome to therapy include the following: genotype 2 or 3, low baseline viral RNA, female gender, Caucasian race, age less than 40 years, and lack of cirrhosis (2, 6–10).

1.2.1 Interferon-α

Discovery and Preclinical Findings. Although the original report of the discovery of interferons and their antiviral potential was published in 1957 (11), and they have been actively studied between then and now, hepatitis B and C are the only viral infections for which they are widely used clinically. (Interferon is also approved, but not widely used, for the treatment of condyloma acuminata—a type of external genital wart). Interferons are a family of multiple low-molecular-weight cellular proteins that include the four types of a-interferon commercially available and in current clinical use. These are recombinant interferon alfa-2b (Intron A, Schering-Plough), recombinant interferon alfa-2a (Roferon, Hoffmann-La Roche), natural interferon alpha-nl (Alferon N, Purdue Frederick), and recombinant consensus interferon (Infergen, InterMune).

Mechanism of Action. Interferons are thought to act at both immunomodulatory and antiviral levels. The classic antiviral activities of interferons are mediated through the interferon-induced cellular proteins 2'-5'-oligo adenylate synthetase and protein kinase R (PKR). The first activates an RNAse and thus causes the consequent degradation of viral and cellular RNAs. The PKR inhibits protein synthesis. A recent report demonstrated that interferon also inhibits HCV RNA translation through a PKR-independent pathway (12). Interferon's immunomodulatory actions are thought to involve enhancement of HLA class I antigen expression and signaling as well as stimulation of a Th-1 type immune response with production of y-interferon and interleukin-2 (13).

Interferon seems to reduce the HCV viral load by decreasing the production of new virus from infected cells rather than by blocking the infection of new cells (4). The speed with which this effect is exerted, a 0.5–2.0 log reduction in 24 h, is astonishing (14, 15). This effect is most pronounced in patients infected with genotypes 2 or 3 as opposed to 1 and is likely to be correlated with their ability to respond to interferon treatment (6). Interestingly, the resistance of type 1 genotype HCV to interferon may be a property of the nonstructural NS5A viral protein that may somehow elude the interferon-induced inhibitory pathways. Another report similarly implicates the HCV E2 (16, 17).

Monotherapy. Two controlled clinical trials used interferon alfa-2b at 1–3 million units three times a week for 6 months. Complete response was defined as a return of liver enzyme levels to the normal range and was achieved by 50% of the enrolled patients. They also showed improvement in liver histology. However, virtually all patients relapsed after therapy was discontinued (18, 19). The strong association of response to therapy with the reduction of the viral load has led to the achievement of a sustained viral response, the reduction of viral load to undetectable for a period after cessation of therapy as the most easily studied reliable endpoint for clinical trials.

A standard course of therapy with interferon-a consists of 3 million units three times a week for 12–18 months. This usually results

in a loss of virus load to the undetectable level in 40% while the patient is on therapy. However 50–90% of responders relapse and retreatment is usually not successful.

1.2.2 Interferon Combined With Ribavirin. The sustained viral response rate is improved if the nucleoside analogue ribavirin is combined with interferon-a. One group reported a sustained rate of 35% after 4 weeks of therapy and 40% after 48 weeks (10).

Ribavirin is a purine nucleoside analog synthesized in 1970 with broad-spectrum antiviral activity in vitro. It was FDA approved in 1985 for aerosol treatment of RSV pneumonia in hospitalized infants and young children and for use as an oral medication in combination with injected interferon as a treatment for HCV in 1998. Other clinical targets of past or ongoing investigation include the arenavirus Lassa, the hanta bunyaviruses that cause hemorrhagic fever with renal syndrome and hantavirus pulmonary syndrome, and the encephalitis caused by the West Nile flavivirus.

Mechanism of Action. Ribavirin has been shown to have diverse activities in experimental systems and it is not clear which are relevant to its enhancement of interferon's activity against HCV (20). Ribavirin is a known inhibitor of inosine monophosphate dehydrogenase (IMPDH), an essential enzyme in the synthesis of guanosine triphosphate (GTP). IMPDH inhibition results in decreased intracellular GTP pools and a general inhibition of RNA synthesis. A second mechanistic possibility is that ribavirin may directly inhibit the HCV RNA polymerase. Ribavirin is phosphorylated by cellular kinases and ribavirin 5'-triphosphate, an analog of purine nucleotides, present in large amounts, would likely inhibit viral RNA synthesis. Consistent with this theory is the recent demonstration that ribavirin triphosphate is incorporated into RNA products synthesized by the HCV RNA-dependent RNA polymerase in cell-free systems (21). A variation on this proposed mechanism is derived from the demonstration that growth of poliovirus in high concentrations of ribavirin resulted in a high mutation rate with the generation of significant genomic diversity. Although viral replication was only moderately inhibited, viral fitness as measured by infectivity was significantly decreased (22). The authors referred to this phenomenon "error catastrophe." Another hypothesized mechanism is interference with capping, the process in which a GTP is added to the 5' end of many viral and host mRNAs to provide protection from host nucleases. In another flavivirus, dengue, RTP competed with GTP for binding the viral enzyme responsible for capping (20). Finally, ribavirin has long been known to have immunomodulatory activity. Recent studies have suggested that the mechanism of this proposed mechanism is the stimulation of a Th-1 type cytokine response (2, 23).

Ribavirin is not effective as a monotherapy, although it did result in some improvement in liver enzymes and inflammation (2, 24). Side effects of ribavirin include anemia and exacerbation of cardiac disease. The anemia may be sufficiently severe as to require dose reduction in up to 10% of patients (2, 25). Furthermore, teratogenicity has been demonstrated in multiple animal species, and ribavirin is therefore contraindicated in both male and female partners to a pregnancy. The mechanism of ribavirin causing anemia is believed to be the result of the lack of ability of red blood cells to hydrolyze ribavirin-triphosphate. Consequently, ribavirin is concentrated in red blood cells leading to a depletion of ATP and damage to cellular membranes, culminating in removal by the reticuloendothelial system (26).

1.2.3 Pegylated Interferon. Recombinant interferon alfa-2b is linked to polyethylene glycol (PEG) a non-toxic water-soluble polymer to create PEG-Intron, the first pegylated interferon product approved by the U.S. FDA. A second pegylated product, Pegasys, is now available from another manufacturer and both are more effective clinically than their unmodified parents. Pegylation increases the half-life of interferon by increasing its molecular weight, which usually reduces elimination. In addition, pegylation stabilizes interferons to temperature and pH variation, protects them from the immune system and from degradation. For example the elimination half-life of the pegylated interferon alfa-2a is 77 h compared with 9 h for the unmodified interferon. Because of increased stability, pegylated interferons can be given once

a week, instead of the thrice-weekly doses that are standard for unmodified interferon. This both improves compliance, and efficacy as serum levels are steady state rather than a fluctuating series of peaks and troughs (27). The PEG component of peginterferon alfa-2b is a 12-kDa linear molecule and the modified interferon is excreted renally. Peginterferon alfa-2a includes a 40-kDa branched-chain PEG component and is primarily cleared by the liver. Pegylation usually decreases the antiviral potency of interferon so determination of the optimum amount of modification requires balancing antiviral activity and the kinetics of elimination.

A large phase III study of pegylated interferon alfa-2b for 48 weeks showed a sustained viral response rate of 25% in patients receiving the pegylated form as opposed to 12% in patients receiving the non-pegylated parent interferons (28). This led to FDA approval of pegylated interferon alfa-2b for monotherapy in the United States. A similar study comparing peginterferon alfa-2a with its unmodified parent showed respective virologic response rate at 48 weeks of 69% versus 28% and final sustained viral response rates at week 72 of 39% versus 19% (27, 29). Commonly encountered side effects of pegylated interferons are the same as those of unmodified interferons. and include flu-like symptoms, injection site reactions, and psychiatric side effects, although neutropenia may be increased.

In general pegylated interferons are twice as effective as their unpegylated parents but do not decrease the relapse rate or alter genotype response sensitivities. Their future clinical role is likely to be as a component of combinations with ribavirin or as a monotherapy for ribavirin intolerant patients.

1.2.4 Pegylated Interferon-α Combined With Ribavirin. After the demonstration of the superiority of pegylated interferons to their unmodified parents as monotherapies, yet their inferiority to unmodified interferons combined with ribavirin, the next step was clearly to evaluate pegylated interferons in combination with ribavirin. This has been done with both interferon alfa-2b and alfa-2a. In the alfa-2b study, treatment for 48 weeks led to sustained viral response rates of 54% with the pegylated combination compared with 47% for either the unpegylated combination or a lower dose pegylated combination (30). In this study, the response rate of patients infected with genotype 1 was increased from 33% with the non-pegylated combination to 42% with the pegylated combination. The rate of sustained viral response for non-group 1 genotypes was 82%. Compliance was demonstrated to be important, as the response rate of those who received more than 80% of both their pegylated interferon and ribavirin doses was 63% compared with 54% who did not. Pegylated alfa-2a combined with ribavirin was compared with pegylated alfa-2a alone or pegylated alfa-2b combined with ribavirin. The sustained viral response rates was 56% for the alfa-2a combination, and it seems clear that the combination of ribavirin and a pegylated interferon is currently the best therapeutic option for the treatment of chronic HCV infection (3, 31).

2 DISCOVERY AND DEVELOPMENT OF INHIBITORS OF RNA VIRUSES OTHER THAN HIV

This section provides updates on antiviral compounds that were published in the literature from 1996 to early 2002. Relevant reviews of earlier research are available and will be cited throughout the section.

2.1 Influenza A and B Viruses

2.1.1 Inhibitors of Influenza Neuraminidase (Sialidase). The approval of Relenza (zanamivir for inhalation) and Tamiflu (oseltamivir phosphate) by the U.S. FDA in 1999 (http://www.fda.gov/cder/approval/index.htm) marked the advent of clinically effective anti-influenza therapies achievable by structure-based drug design. Both drugs are inhibitors of influenza neuraminidase (NA). A third structure-based NA inhibitor, RWJ-270201 (32) (also known as BCX-1812), has also been in phase III trials in Europe (BioCryst News, August 10,2001). There have been several excellent reviews of the work in this area published in recent years (33–39).

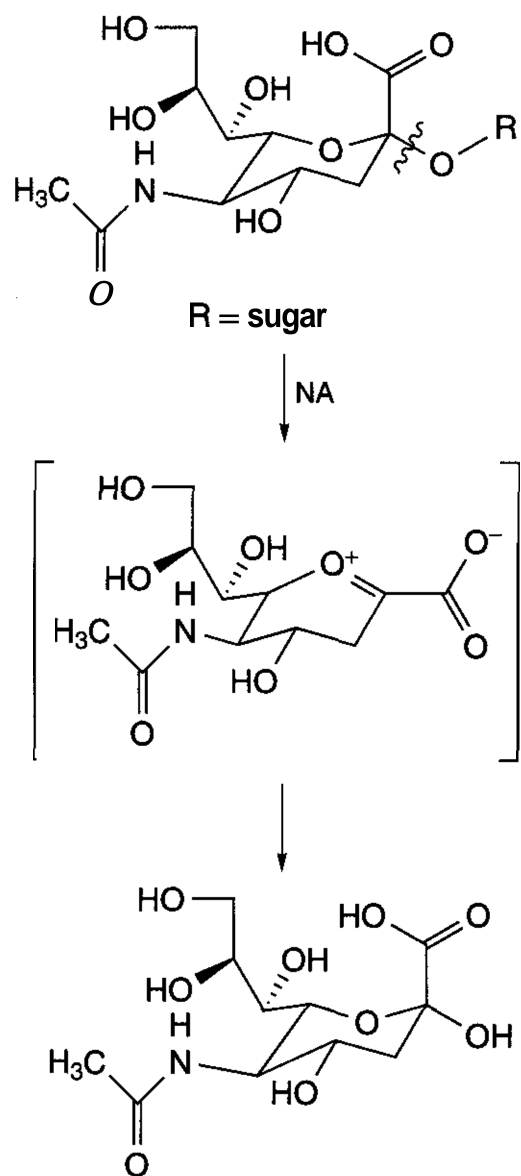


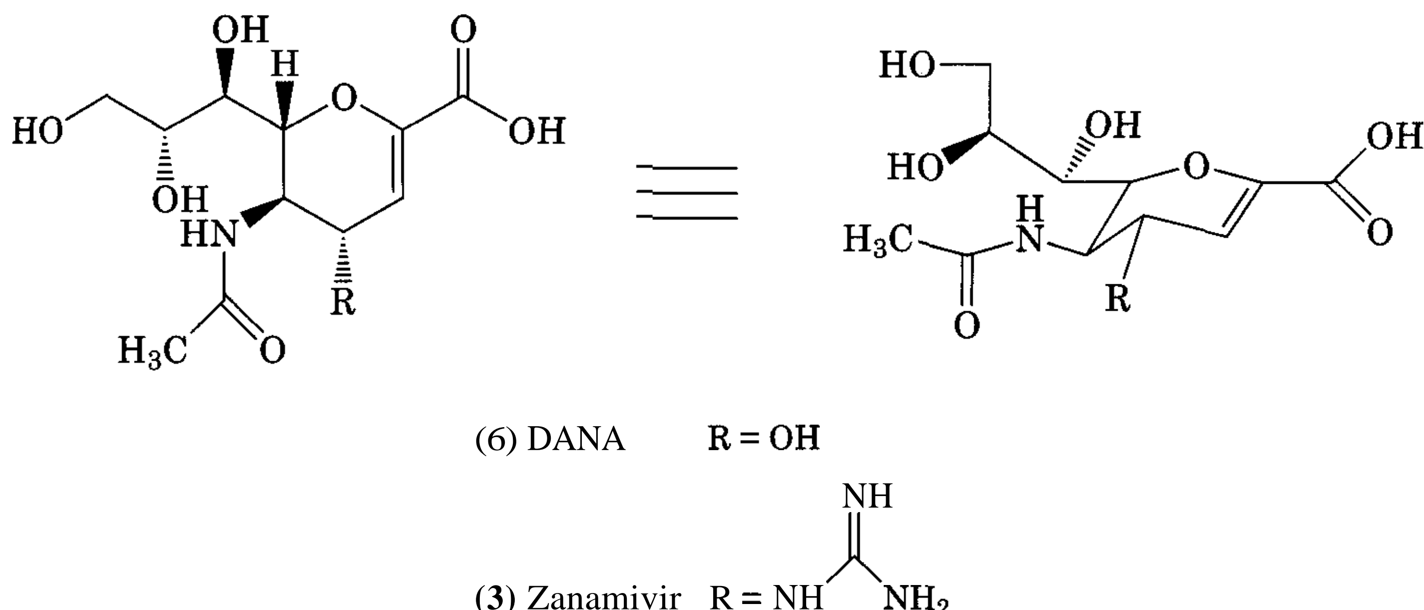
Figure 10.1. The catalytic mechanism of the neuraminidase-mediated cleavage of sialic acid.

The premise for targeting NA (40) for anti-influenza chemotherapy is based on the principles that (1) the active site amino acid residues are completely conserved across all known strains of influenza A and B NAs, suggesting that NA inhibitors can be broad-spectrum antiviral agents against both influenza A and B viruses, and (2) mutations of these conserved amino acids inactivates the enzyme, suggesting that the virus may not easily escape therapeutic intervention through mutation (41,42).

Although there is no consensus on the mechanism of action of NA, it is accepted that the sialic acid cleavage by NA might proceed through an oxonium cation transition state intermediate adopting a half-chair conformation (Fig. 10.1) (38, 42, 43). It has been shown that a transition state mimic can be an inhibitor of a particular enzymic reaction. In this case, 2-deoxy-2,3-dehydro-N-acetylneuraminic acid (**6**) (Neu5Ac2en, also known as DANA) was the first mechanism-based analog synthesized, of which the pyranosidic ring adopts a similar planar structure to the putative sialosyl cation transition state intermediate. DANA shows potent NA inhibitory activity with K_i in the micromolar range; however, this compound inhibits various viral, bacterial, and mammalian neuraminidases with similar affinity. It also failed to protect animals in experimental therapies against influenza virus (see the review articles cited above).

For convenience of discussions and comparisons, we use the numbering system of N2 subtype of influenza virus type A to denote the active site amino acid residues.

2.1.1.1 Zanamivir (GG-167). With the availability of the X-ray three-dimensional molecular structures of the NA active site with and without binding with DANA and sialic acid



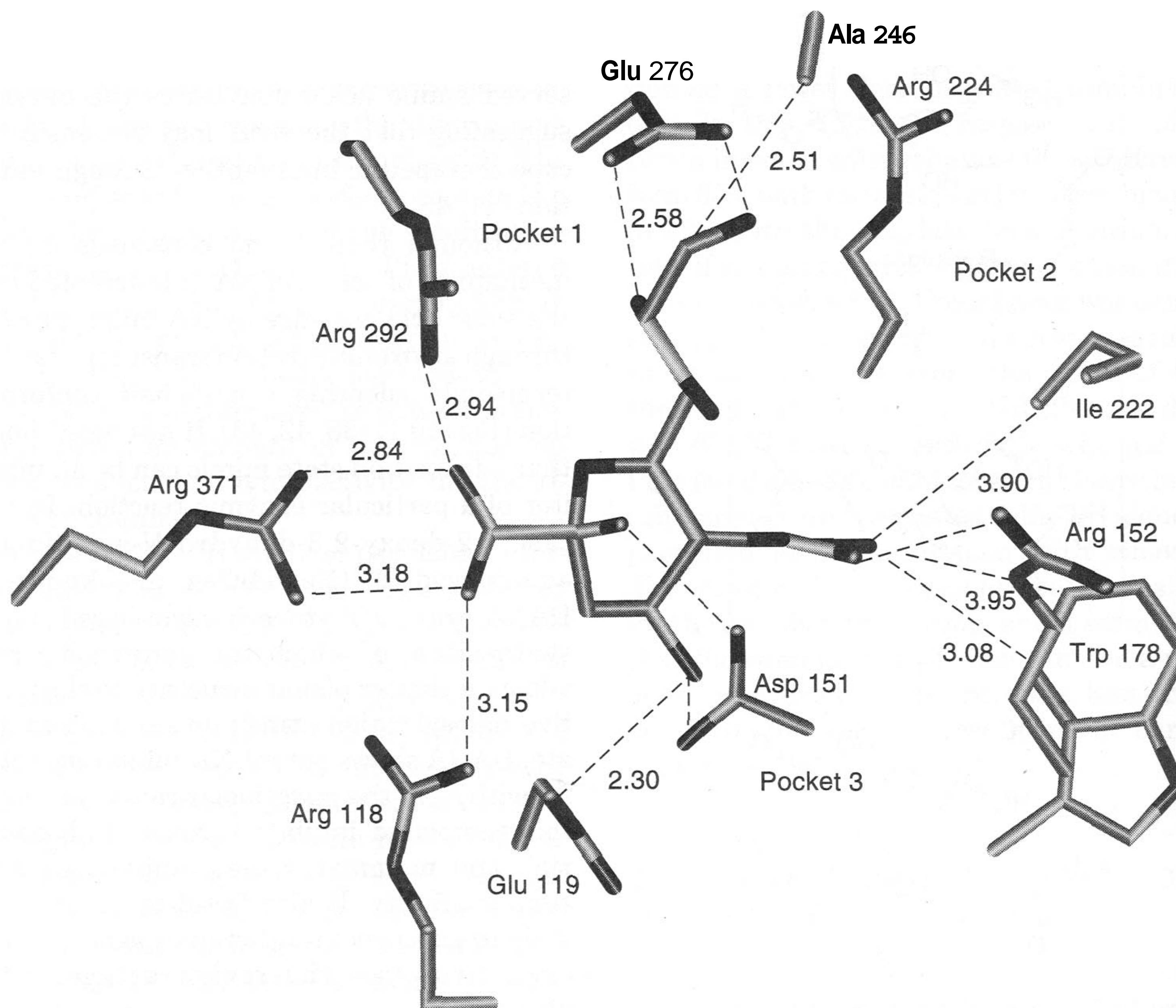


Figure 10.2. Complex structure of NA and sialic acid (dashes indicate H-bonding [red] and hydrophobic [black] interactions; some active site residues are omitted for clarity). Reproduced with permission of Dr. C. U. Kim (Gilead Sciences). See color insert.

(the natural substrate of NA), further drug design assisted by using the program GRID resulted in a rational-designed, potent transition state analog, 4-guanidino-2,4-dideoxy-N-acetylneuraminic acid **(3)** (zanamivir, also known as **GG167**) with binding affinity (K_i = 10^{-11} M) more than 100-fold tighter than that of DANA (43). It has been proposed that modifications at the C_4, C_5, and C_6 positions of sialic acid would lead to function-specific interactions with the NA active site; thereby, this might be the reason that the 4-guanidino group renders zanamivir influenza-specific (36). On the other hand, it might be because DANA has the same functional groups at these positions as that of sialic acid; DANA is a non-selective inhibitor.

The crystallographic structure of zanamivir complexed with NA is very similar to that found in the X-ray crystal structures of sialic acid and DANA complexed with NA (44). Figure 10.2 shows the key interactions between NA active site residues and sialic acid (37). The dihydropyran ring of zanamivir adopts a half-chair, near-flat conformation, and all C_4, C_5, and C_6 substituents on the ring are all equatorial on the same plane (45). The structures suggested strong charge-charge interactions of the carboxylate functional group with three arginine residues (Arg-292, -371, and -118). A quantitative structure-activity relationship (QSAR) model derived by COMparative BINding Energy (COMBINE) analysis, which concluded that the triarginyl cluster is the predominant factor for orienting and stabilizing inhibitor molecules (46), further supports this observation. A negatively charged group (e.g., carboxylate or phosphonate) that

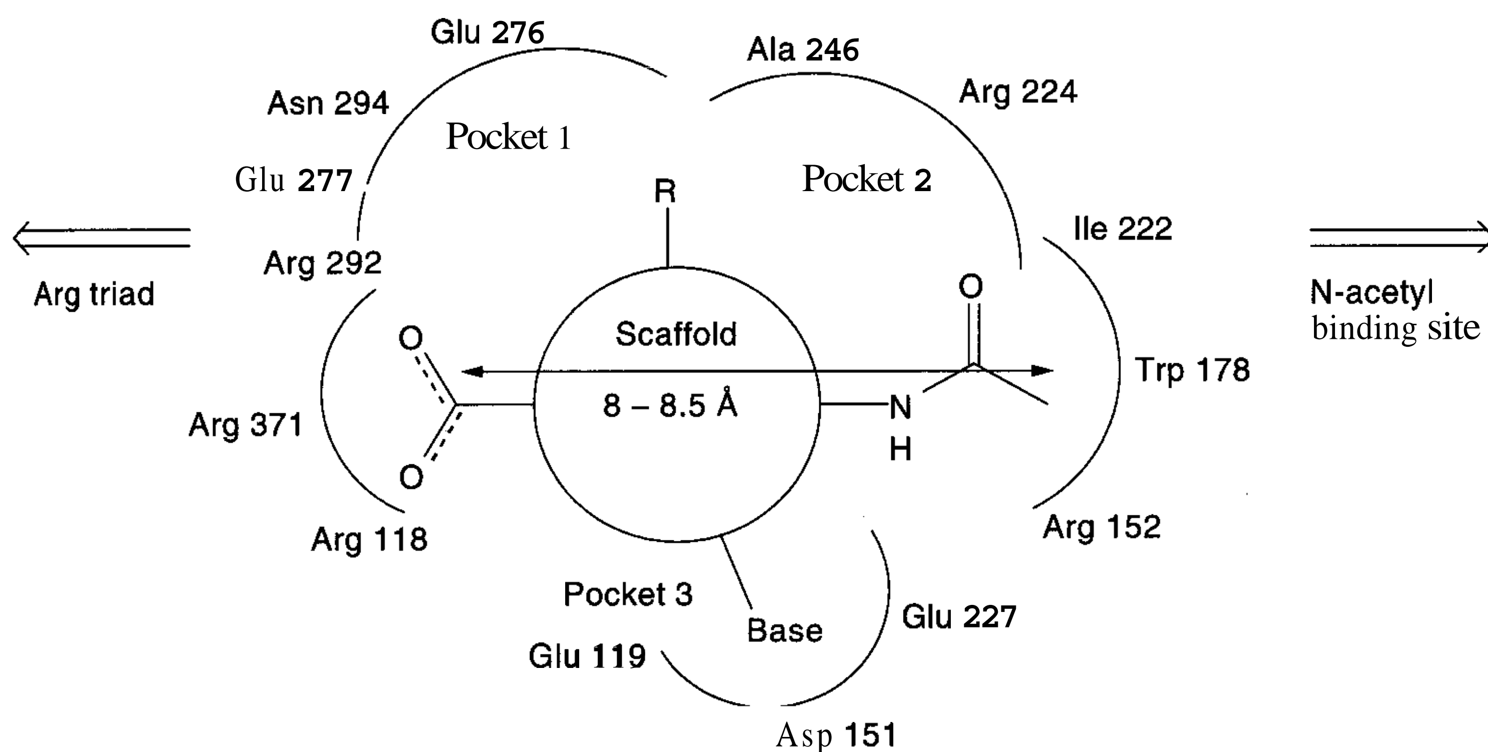


Figure 10.3. Basic principles for NA inhibitor design. Reproduced with permission from Dr. C. U. Kim (Gilead Sciences).

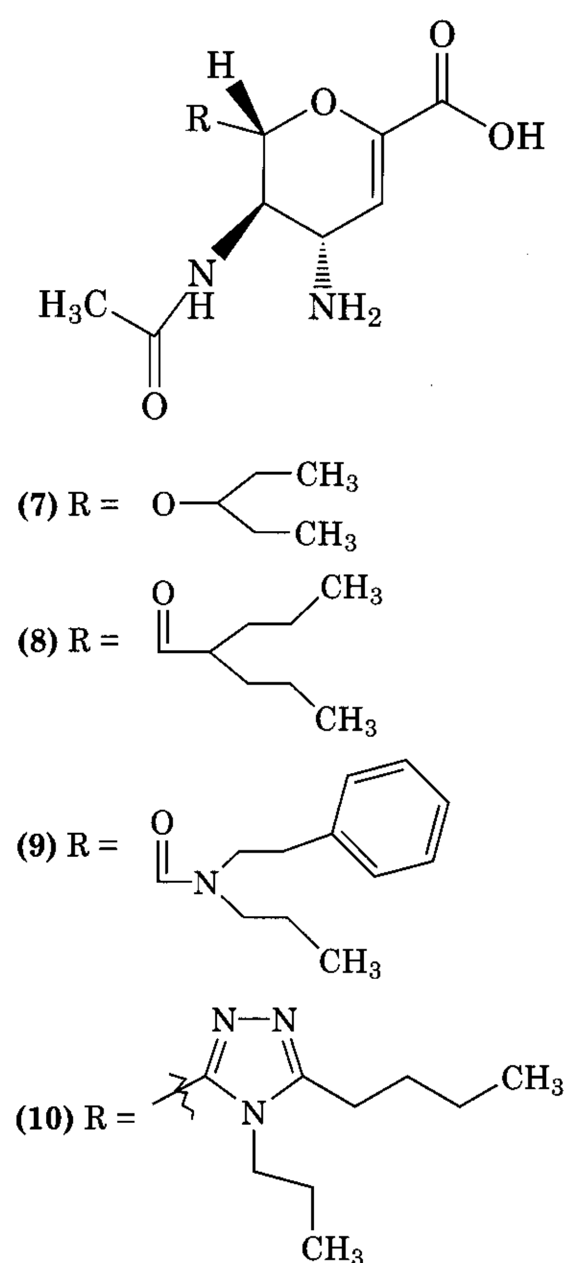
makes strong charge-charge interactions with the triarginyl pocket is highly favorable for binding (46, 47). These strong interactions and the interactions of the N-acetyl methyl group with the hydrophobic region formed by residues Ile-222 and Trp-178 and of the amide carbonyl oxygen with Arg-152 through a hydrogen bond (46, 48) are likely important for orientation of zanamivir and other inhibitors in the binding site (49, 50). Based on these observations, a model for anti-influenza NA inhibitor design has been suggested as shown in Fig. 10.3 (44, 51).

The X-ray crystal structures also show that the two terminal hydroxyls of the glycerol moiety form a bidentate hydrogen bond donor-acceptor interaction with the carboxylate of Glu-276 (Glu-276 re-orients on binding with inhibitors containing aliphatic substituents as discussed in the following sections) and the C_8 of the glycerol side-chain makes hydrophobic interactions with the hydrocarbon chain of Arg-224 (48). It is of interest to note that the C, hydroxyl of the glycerol moiety does not involve in the binding, suggesting that, in the sense of drug design, the C, hydroxyl could be eliminated and replaced with other functional group without compromising the affinity to the enzyme (48, 52).

Computational analysis predicted that replacement of the C_4 hydroxyl group in DANA by a positively charged amino group would be beneficial for binding affinity by the formation of a salt bridge with negatively charged Glu-119 (43). The analysis further predicted that even higher affinity could be achieved with a larger and more basic guanidino group, because the terminal nitrogens of the guanidino group seem to exhibit lateral binding to both Glu-119 and Glu-227 (43). In the crystal structure of zanamivir bound to NA, the predicted binding between one of the primary guanidinyl nitrogens and the carboxylate of Glu-227 does occur, whereas Glu-119, although slightly further removed than predicted, is found within a distance close enough for electrostatic interaction with the secondary guanidinyl nitrogen (34, 36), which also found to interact with the carboxylate of Asp-151. When the guanidino group occupies this binding pocket, it expulses the existing water and this replacement may contribute a favorable

entropic factor to the binding (53). Because it needs to expulse a water molecule from the C_4 guanidino-binding pocket of the active site, zanamivir is a slow binding inhibitor (33, 40).

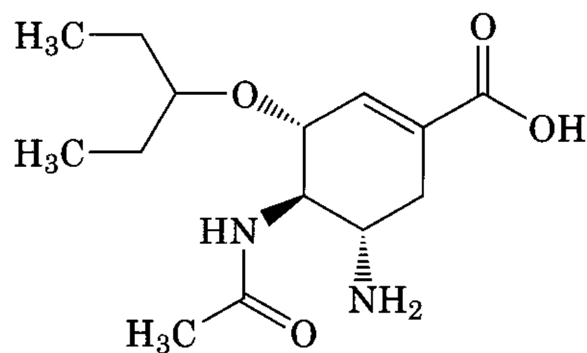
2.1.1.2 Zanamivir Analogs. In the search for further clinical candidates, a number of zanamivir analogs have been reported. Substitutions on the guanidino nitrogens generally resulted in much weaker inhibitors (34). 5-Trifluoroacetamidoand 5-sulphonamide derivatives of zanamivir remained the activity approaching to that of zanamivir (54). The C_6 glycerol moiety has also been replaced by ether (7)(55), ketone (8) (55), carboxamide (9)



(53, 56–58), or a heterocycle such as triazole (10) (59). Interestingly, all of these C_6 modified compounds showed a strong selectivity against influenza A, with much worse activity against the type B virus. In the mouse model, despite the similar enzyme affinity and in *vitro* activity, the carboxamide analog (9)exhibited a much lower intranasal efficacy compared to zanamivir in reducing viral titers in the infected animals (58).

It has been noticed that the carbohydrate and the dihydropyran rings have no direct interaction with the active site amino acids. In addition. unlike the active site of most other enzymes, the NA active site contains an unusually large number of polar or charged residues (37), implying that the ring structure might merely act as a structure frame (scaffold) to correctly orient the substituents to have proper electrostatic interactions with the active site amino acid residues. Therefore, novel inhibitors can be designed by constructing novel frame structures to place the interacting substituents in correct relative positions in the enzyme active site (32, 46, 50). A number of potent influenza NA inhibitors based on the ring structure of cyclohexene [e.g., oseltamivir (48)], benzene [e.g., BANA-206 (41)], cyclopantane [e.g., RWJ-270201 (32)], or pyrrolidine [e.g., ABT-675 (60)] have been reported. These compounds will be discussed in the following sections.

2.1.1.3 Oseltamivir (GS-4104). Taking into consideration that transition state forms the rather flat oxonium cation, which could be considered as an isostere of a double bond, the cyclohexene scaffold was selected as a replacement for the oxonium ring in the design of oseltamivir (4) (also known as GS-4104) and its related analogs. In addition, the carbocyclic system was expected to be more chemical and enzymic stable than the dihydropyran ring. A series of articles on the discovery and development of oseltamivir have been published by Kim at Gilead Sciences (37, 48, 61, 62). Oseltamivir is the prodrug of GS-4071 (11).



(11) GS 4071

A/PR IC_{50} = 1 nM

B/Lee IC_{50} = 3 nM

To treat influenza infection, oral administration is considered more convenient and economical for patient care. However, because of its extreme hydrophilicity, zanamivir is not orally bioavailable; it has to be given intranasally or by inhalation to treat patients. In the design of orally bioavailable drugs, balancing lipophilicity and water solubility could be critical for their absorption from the intestinal tract (37, 48). Gilead investigators decided to use a less polar amino group to replace the highly polar guanidino group, because this change might be beneficial for increasing oral absorption.

As does zanamivir, the cyclohexene-based inhibitors also contain the carboxylate and the acetamido moieties, located at C_1 and C_4 positions of the cyclohexene ring, respectively, which are anticipated to interact with the Arg triad (Arg-292, -371, and -118) on one end and with the amide recognition region (Trp-178, Ile-222, and Arg-152) on the opposite end, and thereby, help anchor the inhibitors into the NA binding side (Fig. 10.3).

It has been noticed that although the glycerol chain of zanamivir is polar in nature overall; the C_8 of the glycerol chain makes hydrophobic interactions with the hydrocarbon chain of Arg-224 (37, 48), suggesting that the optimization of this hydrophobic interaction would lead to new inhibitors with increased lipophilicity (48). The C_7 hydroxy group makes no direct interaction with the enzyme and is exposed to bulk solvent, suggesting that it may be replaced with other functional group (48, 52). These two notions provided the theoretical base for the design of GS-4071 (11) and its analogs by replacing the whole glycerol chain with a variety of alkoxy groups (at the C_3 position of the cyclohexene ring). Because the C_3 linker atom may not participate in the interaction with the enzyme, it can be replaced with nitrogen (63, 64), sulfur (65), or oxygen (37). The use of oxygen as the linkage in the design of GS-4071 was based on the need to reduce the electron density in the double bond in the cyclohexene ring, because the double bond in the sialosyl oxonium transition state intermediate is electron deficient. as well as the versatility in the synthesis of a variety of substituents. SAR studies showed that the changes in length, size, and branching of the alkyl chains profoundly influence the NA inhibitory activity (48, 61,621.

X-ray crystallographic analysis of GS-4071 (11) and its analogs bound to NA confirmed the existence of a hydrophobic region, which is corresponding to the glycerol-binding region for zanamivir, to accommodate bulky lipophilic groups (Fig. 10.4) (62). On binding of GS-4071 and related carbocyclic inhibitors, Glu-276 is forced to rotate to adopt an alternative conformation, which is stabilized by a strong charge–charge interaction with the nearby guanidino group of Arg-224, and expose its hydrophobic atoms to the aliphatic side chain of the inhibitor (Fig. 10.5). This re-orientation enlarges the binding site and creates a much less polar environment, making this site possible to accommodate one branch of the hydrophobic pentyloxy group of GS-4071. The other branch of the pentyloxy group makes hydrophobic contacts with a larger, pre-existing binding region lined by hydrocarbon chains of Ile-222, Arg-224, and Ala-246 (Fig. 10.4) (48, 62). This latter pocket is significantly large enough to accommodate larger functional groups, such as a cyclohexyl ring (66).

Interestingly, the Glu-276 of type B NA undergoes a much smaller conformational change on binding to GS-4071 (Fig. 10.5) (62). It has been noticed that the region around Glu-276 is hydrophilic in type A NA, whereas it is hydrophobic and more crowded in type B NA (53). Therefore, the re-orientation creates distortions in the protein backbone near Glu-276 and in the second amino acid shell, which contains non-conserved amino acids compared with type A (67). As a consequence, the conformational rearrangement of type B NA residue Glu-276 occurs with energy penalties (53, 67), resulting in small changes in size and the polar nature of the pocket and decreased affinity for aliphatic side-chains (53, 62). Because of this, type B NA depends more likely on the second region, a larger hydrophobic pocket formed by Ile-222, Arg-224, and Ala-246, for inhibitor binding (32, 62); however, this region is very sensitive to the size of inhibitor bound and binding affinity is significantly affected by the increased bulk of the C_3 side-chain, suggested by the SAR studies with GS-

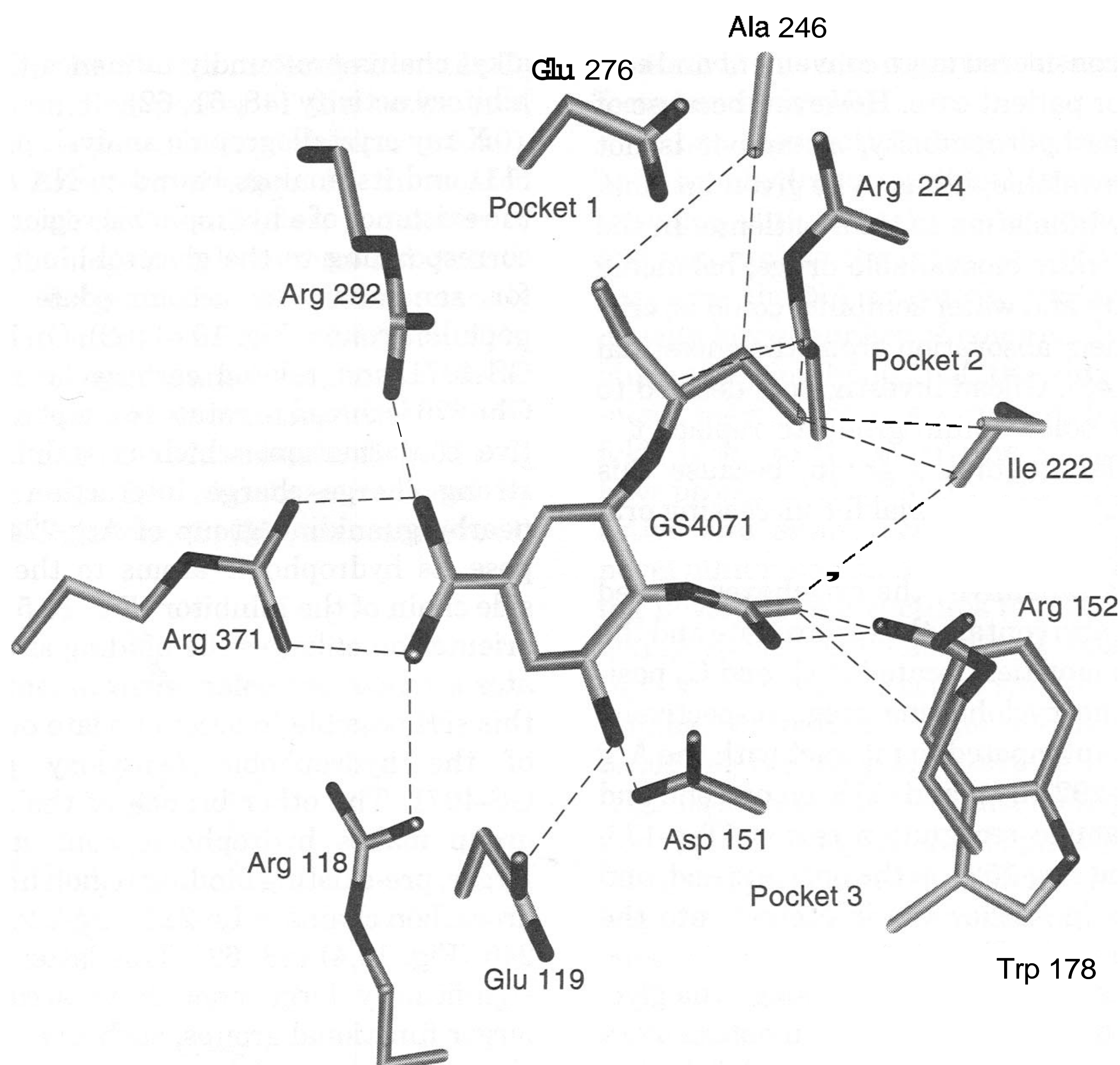


Figure 10.4. Complex structure of NA and GS 4071 (dashes indicate H-bonding [red] and hydrophobic black] interactions; some active site residues are omitted for clarity). Reproduced with permission from Dr. C. U. Kim (Gilead Sciences). See color insert.

4071 analogs (61–63). In type A, the reorientation of Glu-276 resulted in minimal disruption of the second amino acid shell (41).

The NA of a clinical isolate (A/H1N1) recovered from a patient treated with oseltamivir was shown to be 400-fold more resistant to the drug than that of the wild-type virus (68). This variant carried a His-274-Tyr substitution. It was postulated that the large side-chain of Tyr at position 274 could interfere with the re-orientation of the side-chain of Glu-276 and, as a consequence, the successful binding of oseltamivir to the NA active site. Because no rearrangement of Glu-276 is required for zanamivir binding to the NA, this variant remained susceptible to zanamivir (68).

A similar rearrangement of Glu-276 has also been reported for the aforementioned carboxamide analogs of zanamivir when bound to NA (53). This might explain the strong selective affinity for the NA of the type A virus.

GS-4071 (11) has a similar low bioavailability [–5% in rats (69)] compared with zanamivir. However, GS-4104 (3) improves the oral bioavailability after rapid conversion to the active form during gastrointestinal absorption. A high bioavailability was found in mice (~30%), dogs [–70% (69)], and humans [–80% (37, 70)]. Oral administration of GS-4104 results in high and sustained systemic absorption in animal tests with a half-life of 5 h in most tissues. In rats, a metabolite was isolated (71).

2.1.1.4 Oseltamivir Analogs. The location of the double bond in the cyclohexene ring was found to be critical (37, 48, 62). Although molecular modeling analysis showed that (11) and (12) overlap quite well, their enzyme in-

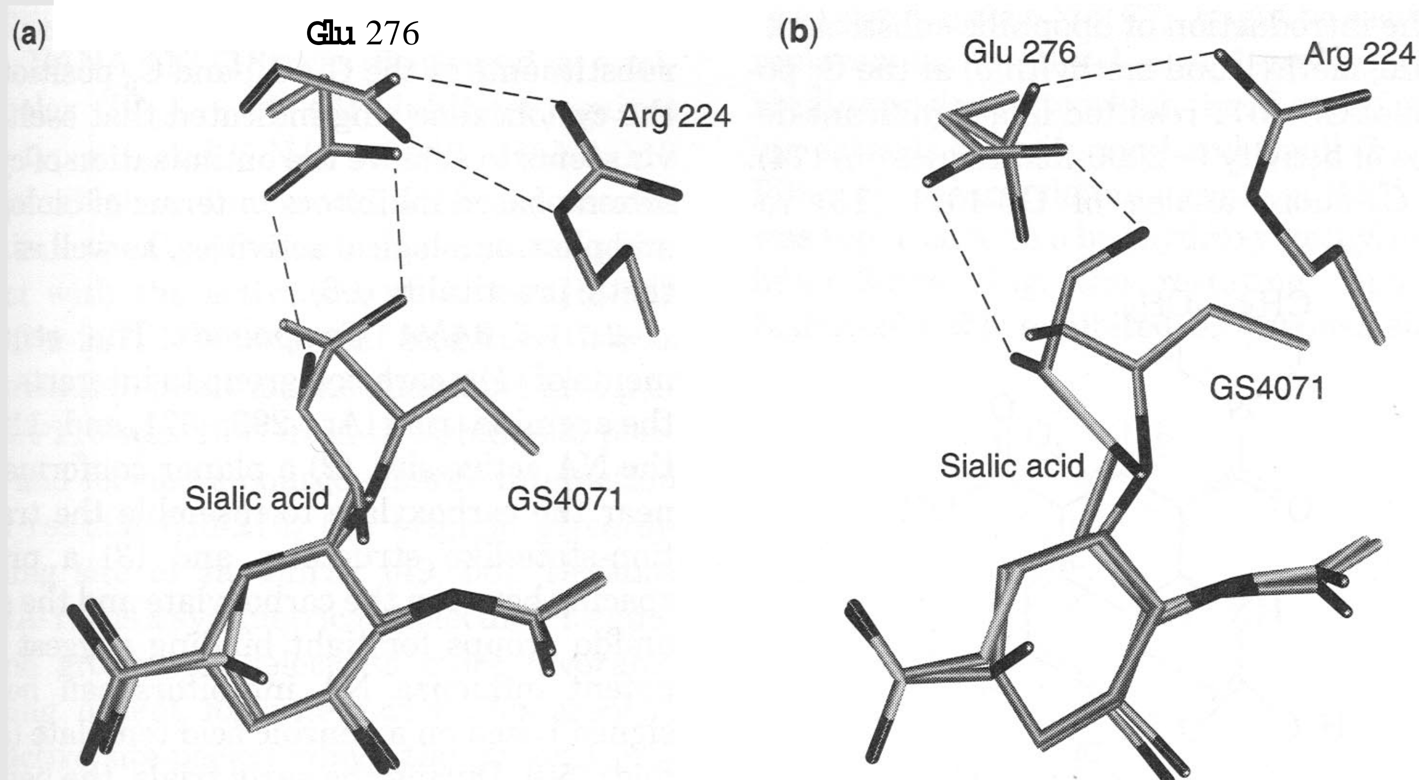
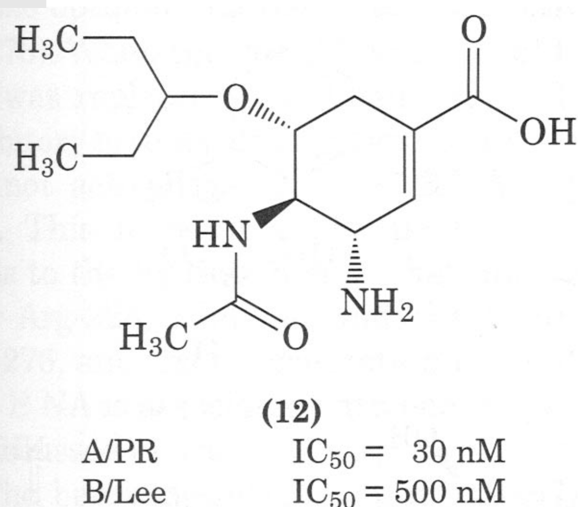
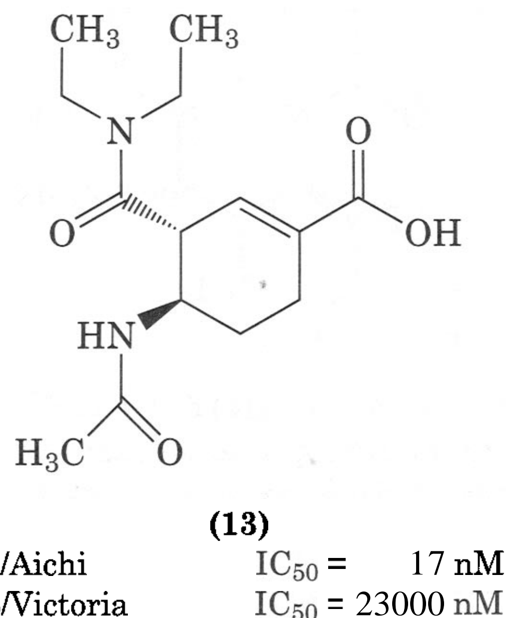


Figure 10.5. NA Glu-276 side-chain (a for type **A** neuraminidase complex; b for type B neuraminidase complex) can adopt alternative conformations on binding of sialic acid or GS4071 (dashes indicate H-bond; atoms are colored as following: blue for nitrogen, red for oxygen, brown for carbon in sialic acid complex, and green for carbon in GS4071 complex). Reproduced with permission from Dr. C. U. Kim (Gilead Sciences). See color insert.

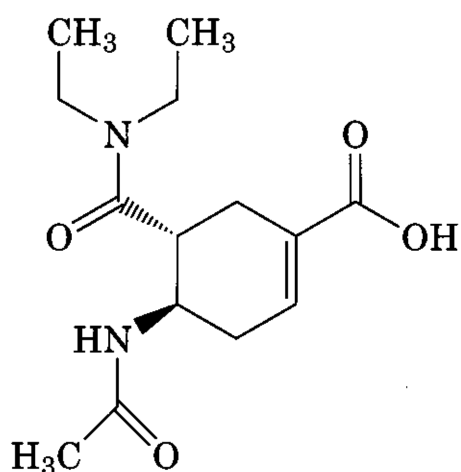




hibitory activities were clearly different. For GS-4071 (**11**) the double bond is in a position analogous to the position of the oxonium ion double bond in the transition state. GS-4071 exhibited a significantly better activity than (**12**), especially against influenza B (62). Similarly, (13) was much more inhibitory than (14) (72).

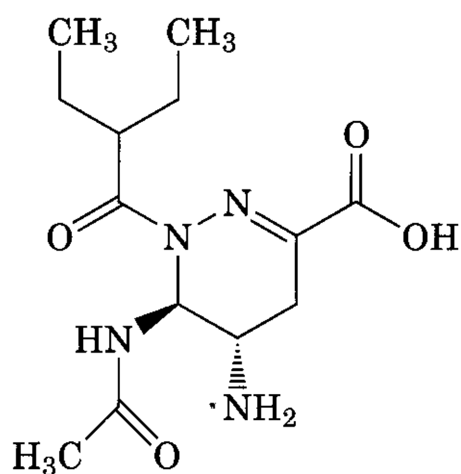
The GS-4071 analog based on a tetrahydropyridazine ring, compound (**15**), was reported to have IC_{50} values of 6 and 62 μM against NA A/PR (H1N1) and B/Lee/40, respectively (73). Structural analyses of the GS-4071 analog revealed that the amino and acetamido groups are in the pseudo-axial positions, rather than the preferred pseudo-equatorial positions as in the case of GS-4071, and that the 3-pentyloxy group points to the small hydrophobic pocket formed by the rearranged Glu-276, whereas in the case of GS-4071, the two ethyl moieties of the 3-pentyl side-chain bind in two different pockets as discussed above. The partial planar nature of the amide bond of the tetrahydropyridazine ring might cause the poor fit and energy penalty on binding to the enzyme (73).

The introduction of lipophilic substituents (chloro, methyl, and methylthio) at the C, position of GS-4071 resulted in a significant decrease of activity (–2000-fold reduction) (74). The C_2-fluoro analog of GS-4071 (16) remained as potent as GS-4071 against NA A with an IC_{50} of 3 n*M*; however, the activity against NA B was reduced by approximately 30-fold (IC_{50}, 90 n*M*) (61).



(14)

A/Aichi IC_{50} = 210 nM
B/Victoria IC_{50} = 150000 nM



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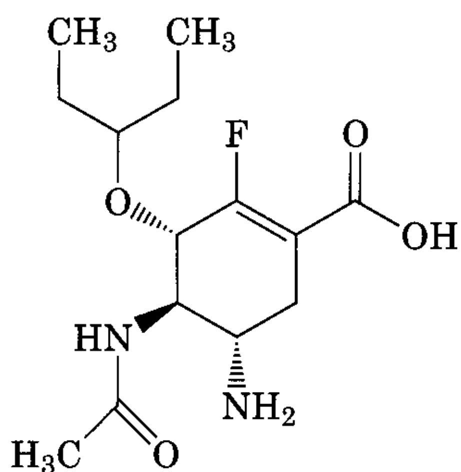
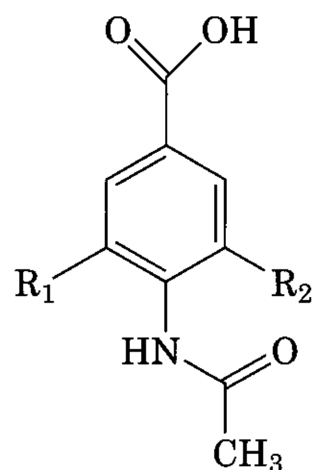
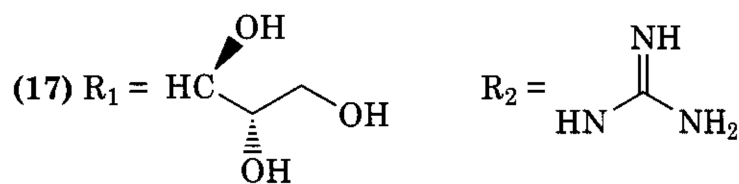


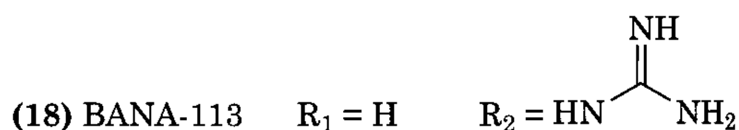
(16)

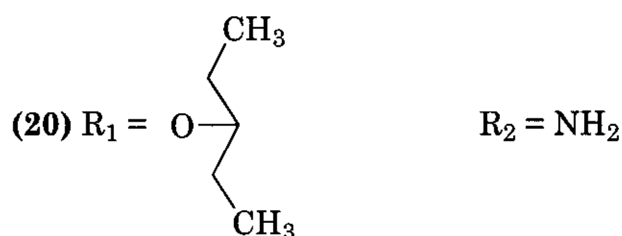
SAR studies by systematic modifications of substituents at the C, C, and C_5 positions of the cyclohexene ring indicated that oseltamivir seems to achieve the optimization of cyclohexene-based inhibitors in terms of biological and pharmacological activities, as well as synthetic practicality (66).

2.1.1.5 BANA Compounds. The requirements of (*1*) a carboxyl group to interacts with the arginine triad (Arg-292, -371, and -118) in the NA active site, (*2*) a planar conformation near the carboxylate to resemble the transition-state-like structure, and (*3*) a proper spacing between the carboxylate and the acetamido groups for tight binding suggest that potent influenza NA inhibitors can be designed based on a benzoic acid template (scaffold) (50). During the early trials, the benzoic acid analog of zanamivir (17) was made, and unfortunately, was found to be devoid of NA





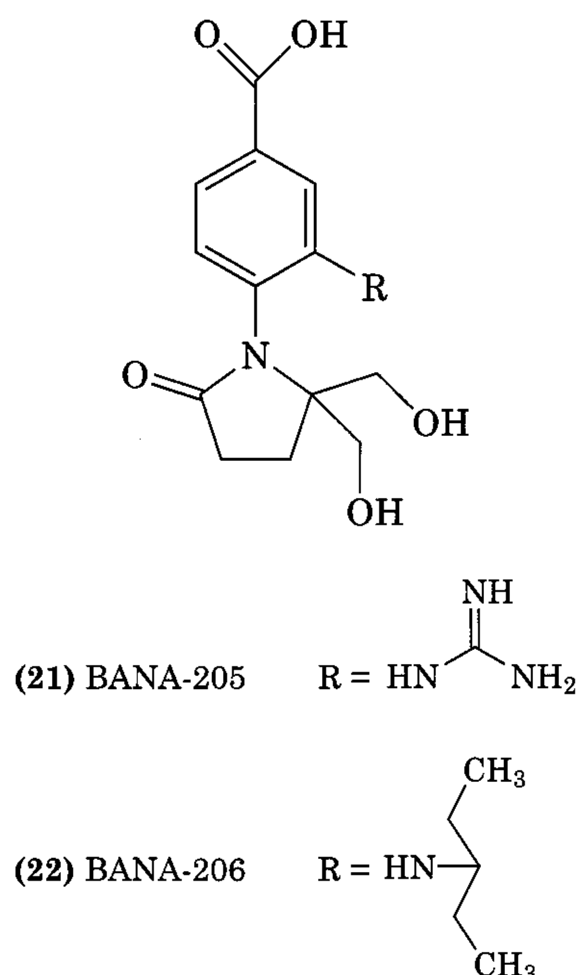




inhibitory activity (45, 75). Around the same time, BANA-113 (18)was discovered as a micromolar (IC_{50} - 10 @) inhibitor against both types A and B **NAs** (45, 49). BANA-113 contains a guanidino group, which is intended to mimic the **C_4-guanidino** of zanamivir to interact with the active site residues Glu-119 and Glu-227. In the crystal structure, it was surprising to find that the guanidino group of BANA-113 was 180" from the predicted position and formed a charge–charge interaction with residue Glu-276, the original **glycerol-binding** site of zanamivir (49, 50). Because BANA-113is a symmetrical molecule, the **guanidino** group can select its more favorable binding pocket for interaction. Apparently, the active site pocket where the glycerol **substituent** of zanamivir bonds is the preferred binding pocket for the guanidino group. This might be the reason why the benzoic acid analog of zanamivir (17) was not active **(75)**, because the benzene scaffold might not be able to provide right orientation for all substituents for the optimal binding to the NA active site **(37, 76)**. When the guanidino group of BANA-113 was replaced with 3-pentyloxy (**19**), the inhibitory activity against type A NA remains, but not active against type B NA anymore **(67)**. This is because the pentyloxy group binds to the hydrophobic pocket lined by **Ile-222,** Arg-224, Ala-246, and the re-oriented Glu-276, and the re-orientation of Glu-276 of type B NA is associated with energy penalties as discussed above.

The benzoic acid analog of GS-4071 **(20)** has also been reported (67). This compound showed similar inhibitory activity as that of **BANA-113** against influenza A NA (–10 *μM*), but it was essentially inactive against **influenza** B NA (67). These observations suggest that the individual substituent contributions to overall binding to the active site cannot be considered to be additive; each substituent influences the overall interaction of the compound with the active site (66, 75). Therefore, aglobal consideration of binding energy is necessary to more reliably predict the binding energy of a designed compound **(50)**.

Assisted with **LeapFrog** software, a de *novo* design program, Brouillette et al. postulated that for benzoic acid-based inhibitors, a small cyclic substituent containing side-chains, such as a pyrrolidinone **(76, 77),** might be a suitable replacement for the N-acetyl grouping [this binding pocket, into which the N-acetyl grouping extends, is quite rigid and small (51, **76**)]. When the N-acetylamino group of BANA-113 was replaced with a bis(hydroxymethyl)pyrro**lidine-2-one** ring, the resulting compound BANA-205 (21) exhibited **IC_{50}** values similar



to that of BANA-113 **(41, 77)**. Moreover, when both N-acetylamino and guanidino groups of BANA-113 were replaced with **bis(hydroxymethyl)pyrrolidine-2-one** and 3-pentylamino groups, respectively, the resulting compound BANA-206 (22) showed dramatic improvement in activity against influenza A NA reaching an **IC_{50}** of 48 n*M*. However, its inhibitory activity against influenza B NA was significantly reduced (**IC_{50}**, 104 *μM*) (41, **77**). For both BANA-205 and BANA-113, the **guanidino** group could interact with the active site residue Glu-276 in its native conformation; therefore, both compounds work equally well on both type A and B NA, whereas, for BANA-206, Glu-276 needs to adopt an alternative high-energy conformation to accommodate the hydrophobic pentylamino functional group, resulting in poorer inhibition constant (Fig. 10.6) (41, 77). The X-ray crystallography

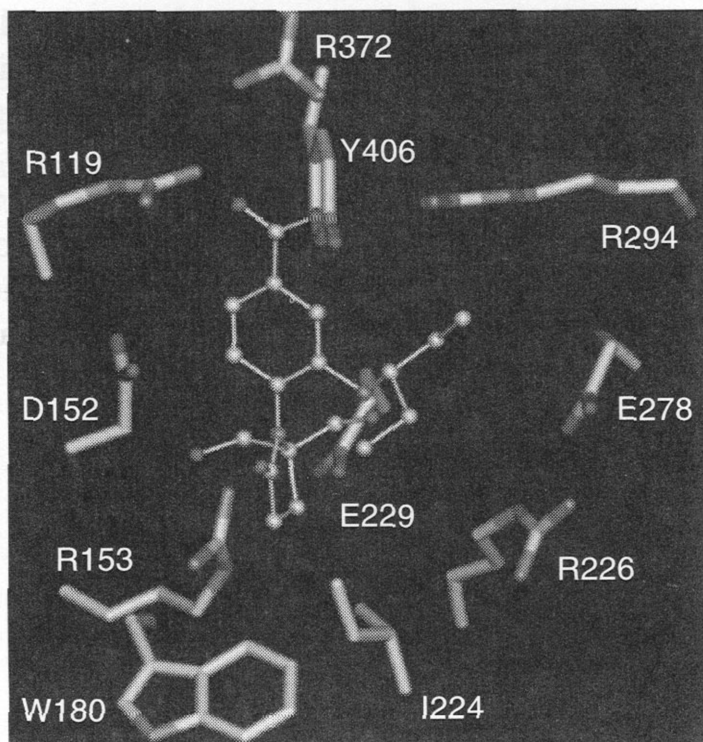
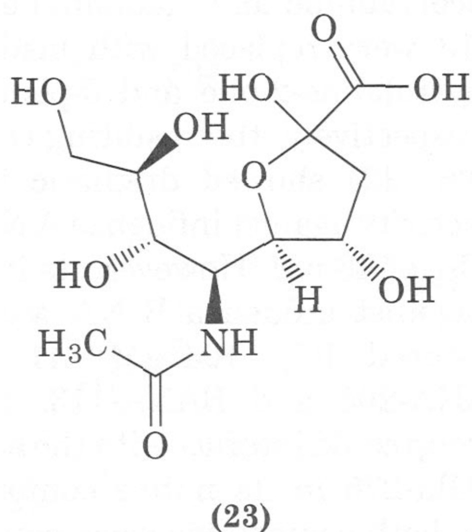


Figure 10.6. Complex structure of NA and BANA-206. Reproduced with permission from Dr. W. Brouillette (University of Alabama at Birmingham). See color insert.

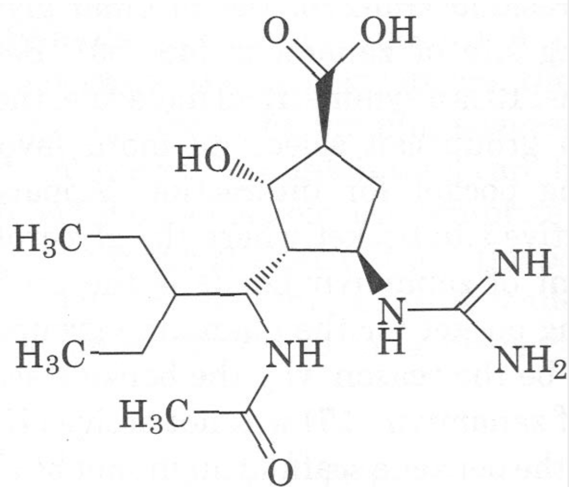
also showed that the 2-pyrrolidinone and benzene rings are perpendicular to each other (76, 77). In conclusion, BANA-206 is a simple achiral benzoic acid derivative that has achieved nanomolar activity as an inhibitor of influenza A NA.

2.1.1.6 RWJ-270201 (BCX-1812, Peramivir). The investigators of BioCryst Pharmaceuticals compared the crystal structure complexes of α/β-6-acetyl-amino-3,6-dideoxy-D-glycero-altro-2-nonulofuranosonic acid **(23)** (a



(23)

micromolar inhibitor) and DANA **(6)** and found that, regardless of their very different positions on the rings, the main functional groups (carboxylic acid, glycerol, acetamido group, and C_4-hydroxy group) in both complexes have the same relative positions in the active site and have similar interactions with the enzyme (32). This finding suggested that a cyclopantane ring might be a suitable scaffold for novel NA inhibitors. Based on the literature data, simultaneous occupation of all binding regions by the four functionalities—carboxylate, guanidino, acetamido, and 3-pentyl—seems to be the basic requirements of a potent NA inhibitor. RWJ-270201 **(24)** was de-



(24) RWJ-270201

signed based on the premise that a cyclopantane ring could position these functionalities for optimal interaction with the four NA binding sites (32, 78).

Because this compound has five chiral centers, no efforts were made to fix the stereochemistry during the initial synthesis. Instead, it was synthesized as a mixture of isomers, followed by soaking a crystal of influenza NA in a solution of isomers to select the most active isomer from the mixture. RWJ-270201 was identified as the right isomer that bound to the active site (32).

X-ray crystallographic studies of RWJ-270201 bound to NA revealed that the carboxylic acid and 1′-acetylamino-2′-ethylbutyl group are trans to each other, whereas the guanidino and carboxylic acid groups are cis to each other (32). The guanidino group is bound to the same binding pocket as the guanidino group of zanamivir; however, they are oriented differently in the binding site. These differences in the orientation of the guanidino group might render RWJ-270201 active against the zanamivir-resistant strains containing Glu-119-Gly and Glu-119-Ala, which

Table 10.1 Comparison of NA Inhibitory Activities of Zanamivir, Oseltamivir, and RWJ-270201 Against Various Influenza NAs (79)

	Mean IC_{50} (n*M*) (range)			
	A/H1N1	A/H2N2	A/H3N2	B
Zanamivir	0.47 (0.3–0.8)	1.23 (0.76–1.38)	1.40 (0.68–2.32)	4.75 (1.53–17.0)
Oseltamivir	1.41 (0.69–2.24)	0.48 (0.01–1.45)	0.31 (0.21–0.56)	9.68 (5.0–24.3)
RWJ-270201	0.35 (0.09–0.81)	0.49 (0.17–1.39)	0.36 (0.14–0.83)	3.84 (0.6–10.8)

IC_{50}, concentration of the compound required to inhibit enzyme activity by 50% in an acellular assay

were selected by *in* vitro passages (32, 68, 79). In a matter similar to GS-4071, one of the two ethyl terminals of the 1′-acetylamino-2′-ethylbutyl functionality point to the induced hydrophobicpocket created by the rearrangement of the Glu-276 side-chain, whereas the other ethyl moiety toward the pre-existing hydrophobic surface, which was formed by the hydrocarbon chains of Arg-224 and Ile-222 (32).

RWJ-270201 seemed to have a better *in vitro* activity against influenza A viruses than the other two drugs (Tables 10.1 and 10.2). In addition, all NA subtypes (N1-N9) of avian influenza viruses were also sensitive to RWJ-270201 (80–82). Exposure of cells to RWJ-270201 caused most of the virus to remain cell associated, with extracellular virus decreasing in a concentration-dependent manner (83). This seems in accordance with its effect as a neuraminidase inhibitor, which cause viral particles to be unable to release from cells but remain clumped at the cell surface.

Orally administered RWJ-270201 has been shown to be highly effective against experimentally induced influenza A (H1N1), A (H3N2), and B virus infection in mice (84, 85). When comparing the effects of the same doses, RWJ-270201 seemed to be more often efficacious than oseltamivir, although such differences were not seen in every experiment. Similar to oseltamivir, oral treatment could be delayed for up to 60 h post-infection, and the drug was still able to protect infected animals from death (84, 85). When administered intranasally, RWJ-270201 demonstrated better protection against lethality than oseltamivir and zanamivir at the same dose (79). Moreover, oral RWJ-270201 and oseltamivir protected mice against lethal challenge with A/Hong Kong/156/97 (H5N1) and A/quail/Hong Kong/G1/97 (H9N2); both viruses have been responsible for the 1997 Hong Kong outbreak (80, 81). Furthermore, pharmacodynamic evaluation of RWJ-270201 in mice predicted efficacy for once-daily dosing (82, 86). Indeed, in controlled clinical trials, oral, once-daily RWJ-270201 was well tolerated and effective in the treatment of experimental human influenza A and B infections (87).

Although RWJ-270201, oseltamivir, and zanamivir are structurally similar, they interact differently with residues of the NA active sites. In cross-resistance studies, RWJ-270201 was fully active against certain zanamivir-resistant enzymes and partially active against oseltamivir-resistant enzymes (68). A point mutation (Lys-189-Glu) in the hemagglutinin

Table 10.2 *In Vitro* Antiviral Activities of Zanamivir, Oseltamivir, and RWJ-270201 on Influenza Virus Replication in MDCK cells (83)

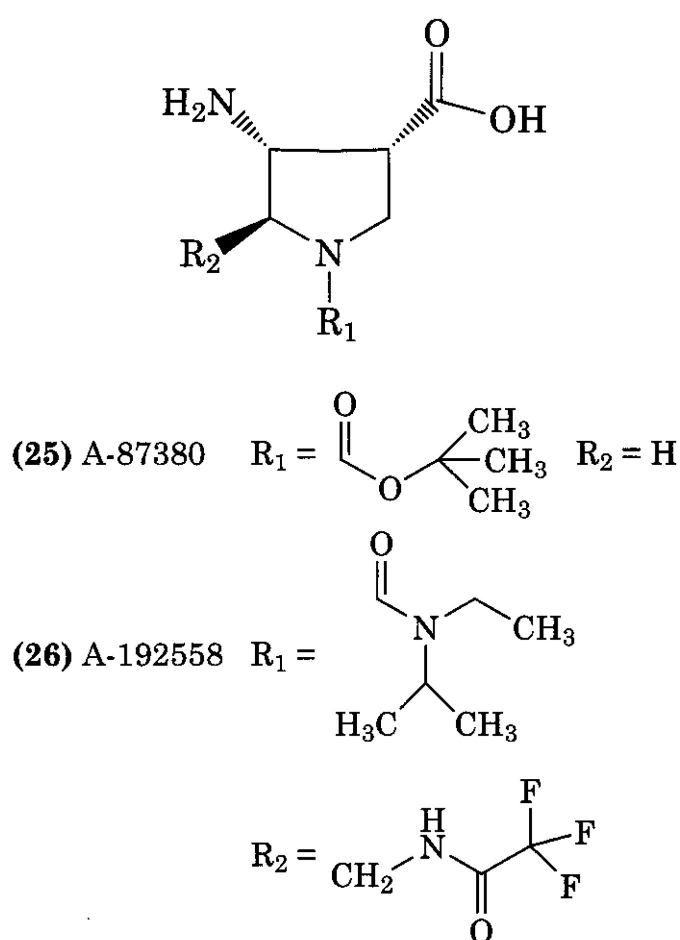
	EC_{50} (μM)			
	A/H1N1 (4 strains)	A/H3N2 (12 strains)	A/H5N1 (2 strains)	B (5 strains)
Zanamivir	0.22–3.4	<0.01–0.65	0.20–0.22	0.03–1.3
Oseltamivir	0.17–2.7	<0.01–0.5	0.22–0.26	0.11–3.0
RWJ-270201	0.09–1.5	<0.01–0.19	0.01–0.02	0.06–3.2

EC_{50}, concentration of the compound required to inhibit viral-induced effect (cytopathic effect or plaque formation) or virus yield by 50% in cell culture.

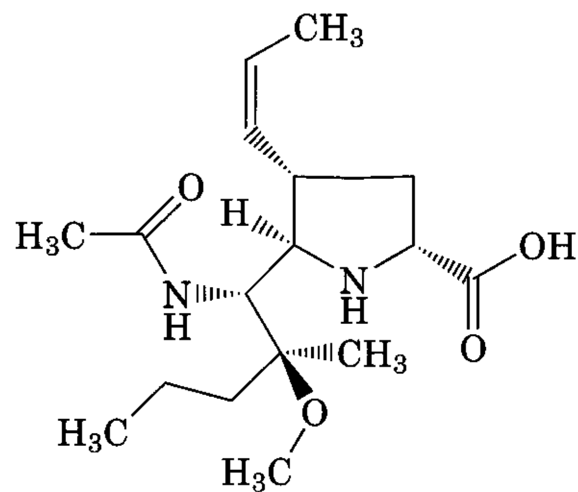
The data on A/NWS/33 have been excluded from the table because both zanamivir and oseltamivir were inactive, and RWJ-270201 was only moderately active against this virus.

(HA)gene of A/Shangdong/H3N2 was selected in the presence of RWJ-270201 in cell culture (88). Based on virus challenge dose to infect mice, the resistant virus was approximately 10-fold less virulent than the wild-type virus. Mice infected with a lethal dose of the resistant virus could still be effectively treated with RWJ-270201 (88).

2.1.1.7 Pyrrolidine-Based Inhibitors. The investigators of Abbott Laboratories recently discovered that A-87380 (25) (IC_{50}, 50 μM versus type A NA) could serve as a NA inhibitor lead after comparing it with zanamivir and by computer modeling (51, 89). A series of tri-substituted and tetra-substituted pyrrolidine analogs were then synthesized using high-throughput parallel synthetic combinatorial chemistry for SAR studies. Of all compounds synthesized, A-192558 (26) was the most potent inhibitor, with IC_{50} values of 0.28 and 8 μM against NA A and B, respectively (51). The preferential activity against NA A over NA B might be caused by the interaction between the urea functionality with the hydrophobic site by inducing a conformational change of Glu-276, and this change is known to be energetically less favorable for NA B. As anticipated, the carboxylate interacts with the positively charged arginine triad formed by Arg-118, Arg-292, and Arg-371 when the compound bound to the enzyme. The C_2 trifluoroacetamido group occupies the small hydrophobic pocket consisting of Ile-222 and Trp-178. Unexpectedly, the exocyclic amino group does not make close contact with all three acidic amino acid residues, Asp-151, Glu-119, and Glu-227.



Subsequently, it was found that this traditional amine-binding pocket contains a previously unrecognized hydrophobic portion formed by Asp-151 and Leu-135 (90–92). This portion could be occupied by a cis-propenyl functional group through van der Waals force to achieve excellent affinity (90, 93). Moreover, studies with substituents pointing to the hydrophobic sub-site formed by Arg-224, Ile-222, and Ala-246 showed that a tertiary amine N-oxide (94) or ether (95) group could enhance a molecule's inhibitory activity. The enhanced activity comes from an intra-molecular H-bond involving the oxygen and the pyrrolidine nitrogen; this interaction serves to direct the aliphatic side-chains toward the hydrophobic surface in the active site of the enzyme. After an iterative structure-based method, the Abbott scientists identified ABT-675 (A-315675) (27) as a new potent broad-spectrum inhibitor of influenza NA, with K_i values ≤0.3 nM (60). In MDCK cells, ABT-675 displayed comparable nanomolar activity as that of RWJ-270201 on the replication of both type A and B viruses. In the same study, GS-4071 was slightly less potent compared to ABT and RWJ compounds (60). The ethyl or isopropyl ester oral prodrug of the Abbott compound was equal or more



(27) ABT-675 (A-315675)

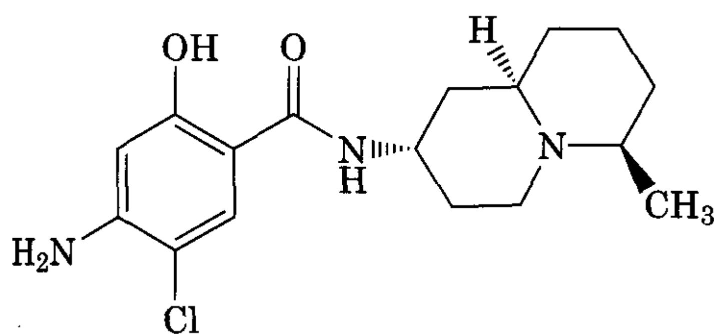
active than GS-4104 against B/HK/5/72 in a BALB/c mouse model. For A/N2/Tokyo/3/67, the ethyl ester prodrug was more efficacious than GS-4104 (96). In vitro passage of A/N9/NWS/G70c, using high concentrations of A-315675, selected novel NA and HA mutations different from those selected by GS-4071. Variants selected with each drug were not highly cross-resistant (97).

In addition, ABT-675 dissociates from the enzyme about 18-fold more slowly than does GS-4071 ($t_{1/2}$ ~ 10 h for ABT-675, –0.5 h for GS-4071) (98). The slower rate might mean a prolonged therapeutic effect, because the drug will stay bound to NA even if circulating drug has been removed from the site of influenza infection (98).

2.1.2 Inhibitors of Influenza Hemagglutinin

2.1.2.1 Monomeric Inhibitors of Influenza *Hemagglutinin*

2.1.2.1.1 BMY-27709 and Derivatives. In a series of publications, Bristol-Myers Squibb investigators have identified BMY-27709 (28)



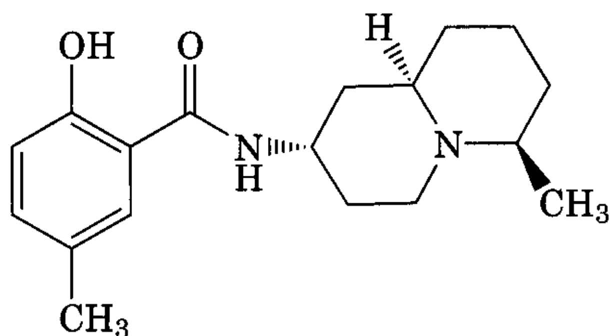
(28) BMY-27709

as a new lead for the development of influenza fusion inhibitors (99–101). In cell-based assays, this compound inhibited the growth of both H1 and H2 subtypes of influenza A virus (only when added at the early stage of infection) with EC_{50} of 3–8 μM (99). However, it is inactive against H3 subtype (99). To better understand the mechanism of action of the compound, 21 independent resistant viruses were selected (100). Two hot spots are identified. One is the methionine at position of 313 of the HA1. Another hot spot is the phenylalanine at position 110 in the HA2 subunit, which is mutated to either a serine (>100 resistance level) or a leucine (15–25 resistance level). Both H1 and H2 HAs contain a Phe-110, while H3 HAs code for a Leu at this position. This may explain why H3 subtype viruses are not sensitive to BMY-27709 (99).

Most of the amino acid substitutions in the HAs of the resistant viruses are located in a region near the N-terminus of the HA2 subunit, suggesting that a binding pocket for BMY-27709 exists near this fusion peptide (100). It is known that HA2 subunit encodes the hydrophobic peptide believed to play a pivotal role in membrane fusion. A simulated H1 HA structure, constructed based on the known crystal structure of H3, also revealed a crevice in the region of Phe-110, in which BMY-27709 could be docked (100). Further photoaffinity-labeling experiments identified the covalent attachment site to be within HA2 amino acid residues 84–106, a region corresponding to part of the pocket proposed through molecular modeling (101). An interesting feature of the model is that HAs of H1 and H2 subtypes contain amino acid residues Glu-105 and Arg-106, which could form tight H-binding with the inhibitor. In contrast, these two respective positions in H3 are neutral Gln-105 and His-106 (100–102).

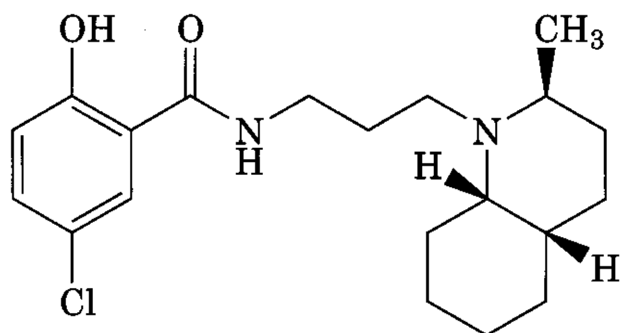
Through the use of reassortant viruses, drug-resistant variants, monoclonal antibody specificity, susceptibility to tryptic digestion, and transfectant viruses, it was concluded that BMY-27709 inhibits influenza virus infection by inhibiting the HA-mediated membrane fusion through blockage of the low-pH-induced conformational change of the native HA, which is a prerequisite for entry of the virus into host cells through membrane fusion (99–101).

A series of derivatives of BMY-27709 were synthesized in an attempt to illuminate the SARs associated with these quinolizidine salicylamides (103, 104). Variation of the substituents of the salicylic acid moiety suggested that the phenolic hydroxy group is essential for activity. This seems to be in agreement with the model, which shows the acid surrogate ketophenol moiety of BMY-27709 interacts with Arg-106 (100,101). For substituents at the 5-position, small and non-polar groups are preferred, with optimal activity residing in the 5-halo and 5-methyl derivatives. The most active one is the 5-methyl phenol derivative (29), with an EC_{50} of 0.25 μg/mL, which is



(29)

fivefold more potent than BMY-27709 (103). The quinolizidine ring was then replaced with structurally simpler heterocycles–piperidines [closely resembles the structure of CL-61917 (102) discussed in the following section] or decahydroquinolines–to explore optimization of potency and spectrum of activity. The 2-methyl-cis-decahydroquinoline (30) showed

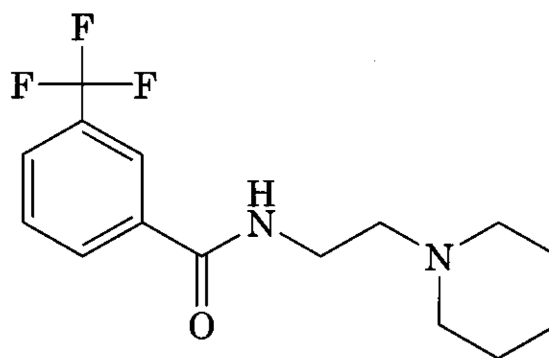


(30)

potent activity, with an EC_{50} of 0.09 $\mu g/mL$ in a plaque reduction assay against A/WSN/33 (H1N1) virus. However, this compound was moderately toxic to the MDCK cells (104). Disappointingly, none of the compounds in the series demonstrated significant activity against H3 subtype influenza virus type A.

H1 and H2 subtype HAs share nearly 70% overall sequence homology. However, H1 and H3 or H2 and H3 subtypes are with about 40% similarity overall (99).

2.1.2.1.2 CL-61977 and Related Compounds. By screening of a chemical library, Wyeth-Ayerst investigators also have identified several compounds that specifically inhibited replication of the H1 and H2 subtypes of influenza virus type A, particularly CL-61917 (31)(102). This compound showed EC_{50} = 1 $\mu g/mL$ against replication of H1 and H2 subtypes; it was much less effective against H3 subtypes and virtually ineffective against influenza B virus. Interestingly, both CL-61917



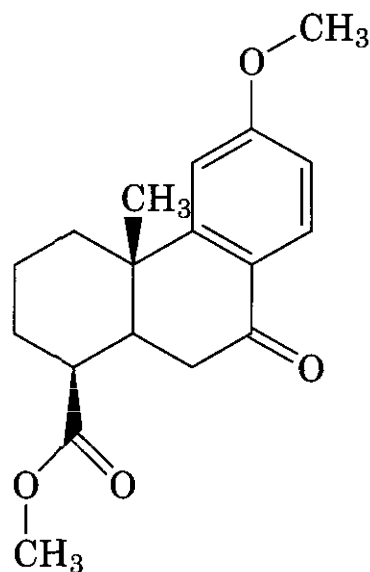
(31) CL-61917

and BMY-27709 are composed of a substituted benzamide linked to a nitrogen-containing heterocyclic ring structure. Both compounds demonstrate the ability to inhibit various manifestations of fusogenic activity of the representative strains of influenza A virus.

Computer-aided modeling and mutagenesis analysis suggested a putative docking site for CL-61917 in the middle of the stem region of the HA near the *HA2* fusion protein. The docking surrounded by three (Phe-3, Asn-50, and Phe-110) of the four HA2 amino acid residues that are altered in the resistant mutants. The computer model illustrated two acid residues, Glu-105 and Asp-109, from one of the monomer chains of HA2, form charge–charge interaction with the piperidines nitrogen, whereas Arg-106, from a second HA2 chain, participates in a H-bonding with the amide carbonyl oxygen. The trifluorophenyl group points to a hydrophobic pocket lined partially with Phe-110 from the second monomer chain of HA2. In contrast, X-31 (a representative of H3 subtype) HA contains Gln-105, His-106, and Leu-110, yielding a significant poorer fit for CL-61917 (102).

Although there are close similarity between the CL and BMY compounds, each compound selects for some different mutations in different viral HAs, illustrating the need for caution in making generalization between these two families of compounds. Ultimately, clarification of the precise interactions between the inhibitors and HAs must await the outcomes of co-crystallization studies (102).

2.1.2.1.3 Podocarpic Acid Derivative. A group of Lilly investigators reported that a compound related to podocarpic acid, designed as 180299 (32) was identified as a specific inhibitor of influenza A viruses in tissue culture (105). Genetic analysis of reassortants be-



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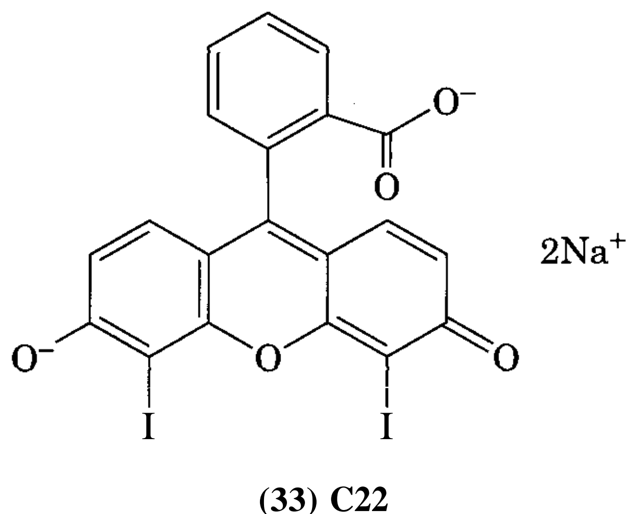
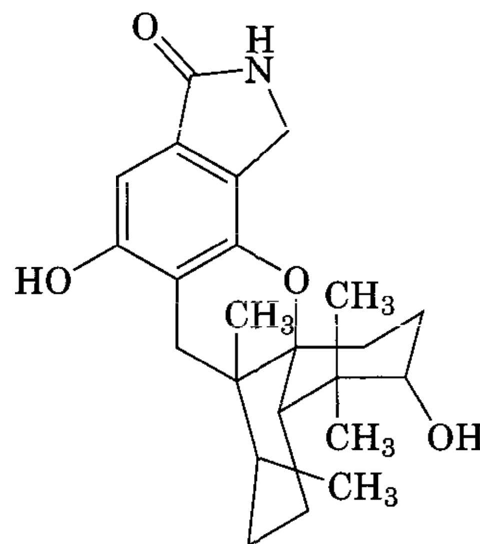


(33) C22

tween sensitive and resistant viruses, as well as independent isolates of mutant strains, showed that mutations are dispersed throughout the HA primary amino acid sequence and cluster in the interface between HA1 and HA2 and in a region near the fusion domain of HA2. Fusion of human erythrocytes and pH-of-inactivation studies suggested that, like the aforementioned CL and BMY compounds, 180299 interacts with the neutral pH conformation of influenza A HA and prevents the low-pH-induced change of HA to its fusogenic conformation (105). However, unlike the CL and BMY compounds, 180299 displayed no in vitro activity against S/WSN/33 (H1N1), a strain that displays an elevated pH-of-inactivation. It was found that naturally resistant influenza viruses [e.g., A/Aichi/68 (H3N2) and B/Lee/40] generally have an elevated pH-of-inactivation. In contrast, the most sensitive strain, A/Kawasaki/86 (an H1 subtype), has the lowest pH-of-inactivation (105).

2.1.2.1.4 Diiodofluorescein. White et al. used a computer-searching algorithm known as DOCK to conduct a series of structure-based inhibitor searching by targeting two sites surrounding HA2 54-81, the region of HA2 that undergoes conformational change at low pH (106). Of 12 new compounds selected by DOCK, diiodofluorescein (33) (also designated as C22) was identified as a new lead. This compound facilitates the conformational change with 50% effective concentration of approximately 8 μM, yet inhibits viral infectivity with an EC_{50} of 8 μM. In the case of BMY-27709, the compound acts to stabilize HA during the course of an infection and this stabilization impedes infectivity (100). In contrast, C22 inhibits viral fusion and infectivity by destabilizing HA, it acts as an irreversible facilitator of the conformational change (106).

2.1.2.1.5 Stachyflin and Derivatives. Stachyflin (34) a novel sesquiterpene derivative isolated from fungus, was shown as having H1 and H2 subtype-specific anti-influenza A virus activity by a group of investigators of Shionogi Labs (107). One-step virus growth experiment suggested that this compound interfered with the HA-mediated virus-cell membrane fusion process through the inhibition of the physiological HA conformational change induced by low pH.

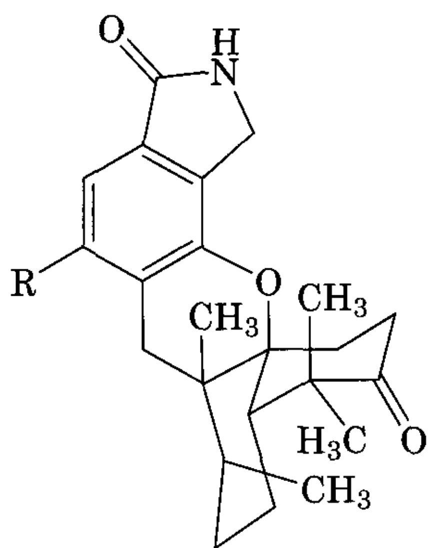


(34) Stachyflin

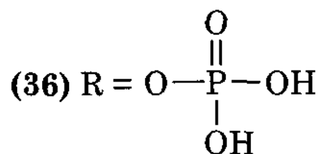
Stachyflin is lipophilic and insoluble in water; therefore, it cannot be given orally to treat experimental infection in animals. However, when given intraperitoneally at doses 2–8 mg per mouse, twice a day, stachyflin showed approximately 70% reduction of virus [A/Kumamoto/5/67 (H2N2)] titers in the lungs of the

infected animals compared with that of the control animals (108). Oral administration used either an aqueous solution of the phosphate ester prodrug or a solution of stachyflin in polyethylene glycol (PEG), PEG 400 or PEG 4000, could also achieve 60–70% reduction in the pulmonary virus titers (108). Unfortunately, concerns about its lack of activity against human clinical isolates and mutagenicity preclude stachyflin from further development (109).

The keto derivative of stachyflin III (**35**), as well as its phosphate prodrug III-Phos (**36**),



(35) R = OH

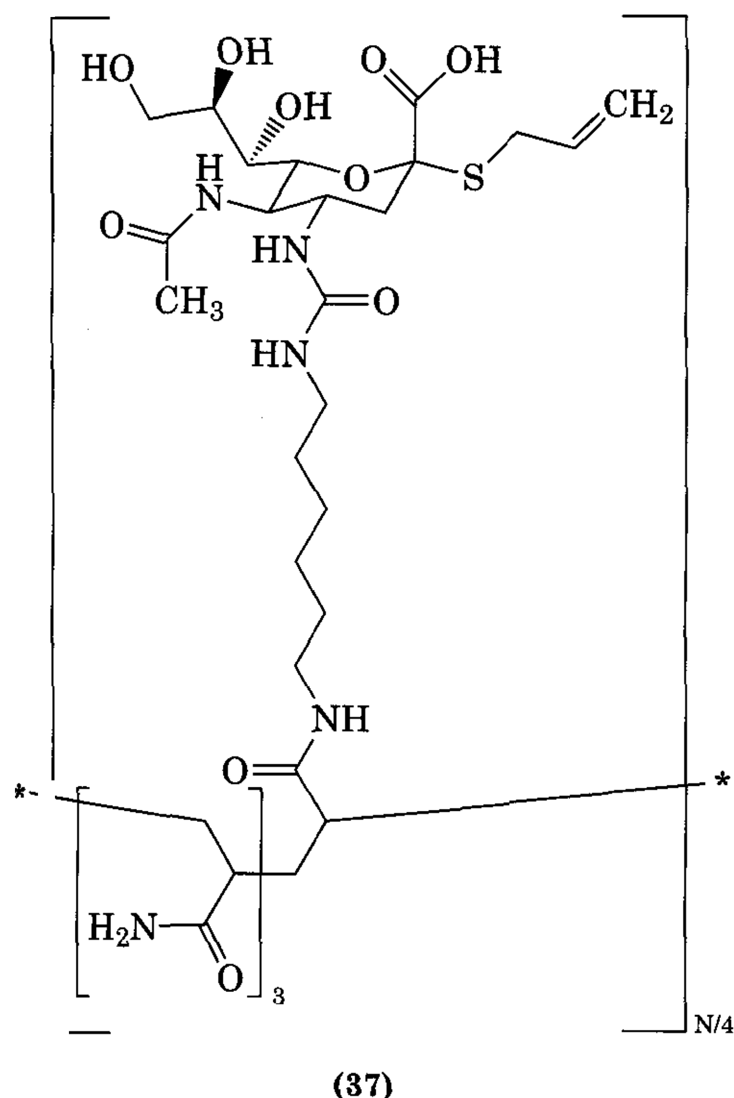


has been chosen for further development (109). Mice infected with mouse-adapted A/Kumamoto/5/67 (H2N2) were orally administered with III dissolved in PEG 4000 or with III-Phos dissolved in water (4 mg per mouse), twice a day for 2 days, resulting in approximately 85% virus inhibition in the lungs. Surprisingly, no effect was observed in ferrets infected with a fresh clinical isolate, A/Sendai/808/91 (H1N1), after oral administration of III, irrespective of the long-lasting high concentrations of the compound in the plasma. Nevertheless, intranasal administration of III-Phos could still inhibit viral replication in the nasal cavity and suppress fever. It was postulated that the accumulation of III and III-Phos on the surface of nasal membrane and good nasal absorption of III-Phos contribute to the *in vivo* efficacy after intranasal administration of III-Phos to the infected ferrets (109).

Both mouse and ferret are good animal models for the evaluation of experimental therapies of influenza infection. In mice, mouse-adapted influenza virus replicates in the lung and causes pneumonia without fever (110). In comparison, ferrets are susceptible to human influenza, which replicates in the upper respiratory tract and causes illness similar to humans (109,111).

2.1.2.2 Polymeric Carbohydrate-Based Inhibitors of Influenza Hemagglutinin. Multivalency (polyvalency) is the simultaneous binding of multiple ligands on one molecule to multiple receptors on another (112). An influenza virion presents approximately 200–300 copies of HA trimeric units on its surface; each subunit contains a binding site for sialic acid (SA; also termed Neu5Ac) at its outmost portion (113). Because the binding pocket is small and shallow, the interaction of a single HA binding site with a single SA is weak. Nevertheless, the binding of a viral particle to the surface of a cell is strong. This strong interaction reflects the interaction of multiple copies of HA on the viral surface simultaneously with multiple SA groups on the surface of the cell. Therefore, in principle, highly effective prevention of the attachment of influenza virus to the cell can be achieved with multivalent (polyvalent) inhibitors that present multiple copies of SA to the virus (114). Multivalent sialosides bearing multiple sialyl moieties tethered to various synthetic backbones of polymers, liposomes, or dendrimers have been reported (112, 113, 115).

2.1.2.2.1 Sialic Acid-Containing Polymers. In the X-ray crystal structures of HA complexed with SA derivatives, it has been shown that the 4-hydroxyl of SA does not interact with the enzyme (116, 117). Thus Watson et al. reported the synthesis of a multivalent SA-containing polymer (37) in which a sialoside has been coupled to polyacrylamide through a 4-N-linkage, as well as a 2-linked conjugate (38)(117). Both compounds showed potent activity (<0.5–4 μM of SA units) against an influenza H3 subtype (X-31) and two H1 subtypes (A/Tokyo and G70C) by an HAI assay.



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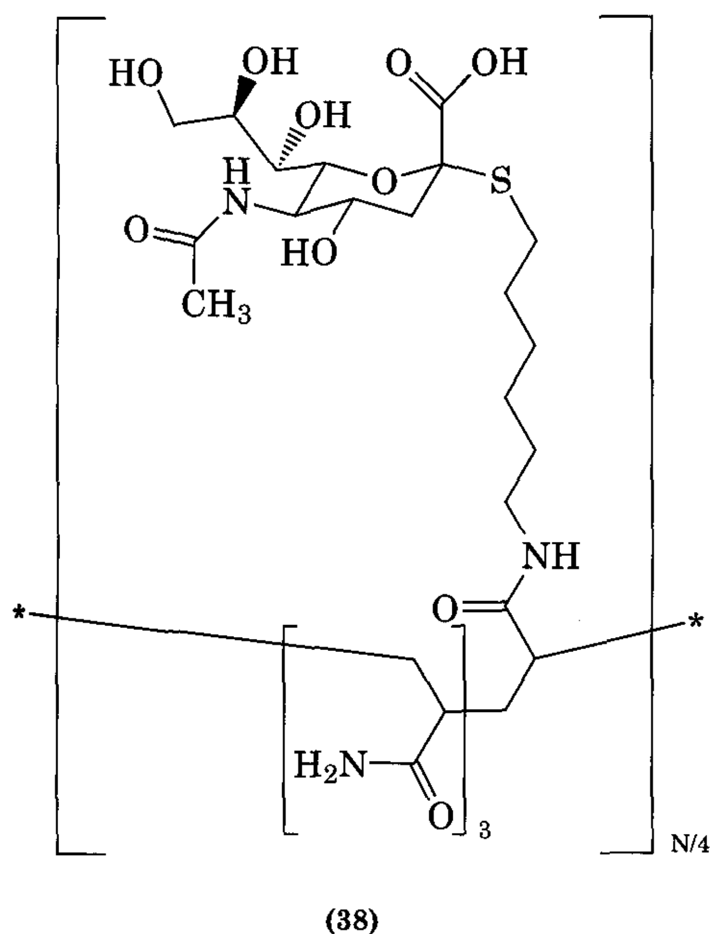
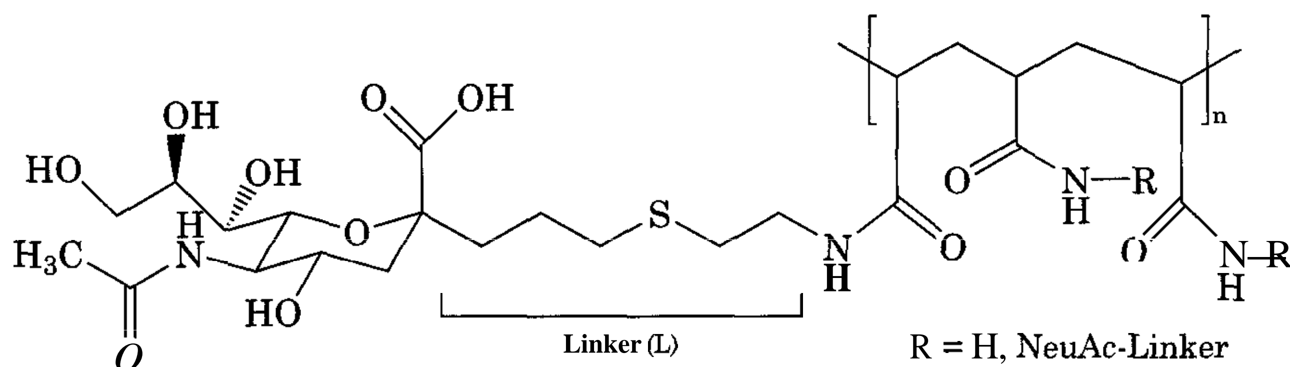


(38)

Generally, **hemagglutination** inhibition (HAI) assay is commonly used to measure the ability of a SA-containing polymer (**glycopolymer**) that inhibits the attachment of influenza virus to erythrocytes (which serve as surrogate target cells). HAI assay is easy to perform, but limited to inhibition constants greater than 1 n*M* (112). Therefore, inhibitors with the lowest concentration that inhibits **hemagglutination** smaller than 1 n*M* seem to be equally effective in the HAI assay. Recently, Whitesides et al. reported a new method based on dual optical tweezers, termed OPTCOL, which is able to measure a single cell (**e.g.**, erythrocyte) and a single microsphere coated with viral particles. The lowest limit of measurable inhibition constants is less than 10^{-18} M (112, 118). By using OPTCOL, a derivative of **poly**acrylamide **pA(NeuAc,** χ) (39) with 35% of the side-chains (χ = 0.35) tethered to SA by a short flexible linker was shown to prevent hemagglutination at concentration of **35 p*M***— 10^8 times more effective than most monovalent derivatives of SA (**e.g.**, a-methylsialoside inhibits hemagglutination at concentration 2.5 m*M*) (118). Whitesides' group also reported, by using an enzyme-linked **immu**nosorbent assay (ELISA), affinities of **polyac**rylamides bearing pendant a-sialoside groups for the surface of influenza virus **A** X-31 (**H3N2**) ranged between 10^3 and $>10^6$ greater than that of a-methyl sialoside, on the basis of total sialic acid groups in solution (**114**).

The significantly enhanced ability of **glyco**polymers over monovalent SA derivatives at preventing the agglutination of red blood cells by influenza virus is thought as a result of high-affinity binding through polyvalency (because of the cooperative binding of multiple SA groups per inhibitor molecule) and steric stabilization (because of the steric prevention of virus from close approach to the cell by a water-swollen layer of the polymer) (119, 120). The latter mechanism has been further supported by studies that showed that the efficacy of SA-containing polymers in inhibiting **hem**agglutination got enhanced (by 2- to 20-fold) by adding potent monomeric NA inhibitors (121). The SA groups on the polymers bind the HA-binding pocket as well as the NA active site. The enhancement of inhibition by the polymers in the presence of NA inhibitors is probably caused by expansion of the adsorbed



(39) pA(NeuAc); χ = mole fraction of NeuAc per side chain
= 0.05, 0.2, 0.35, 0.6, 1.0

polymer layer after the competitive release of SA groups originally bound to the NA active sites.

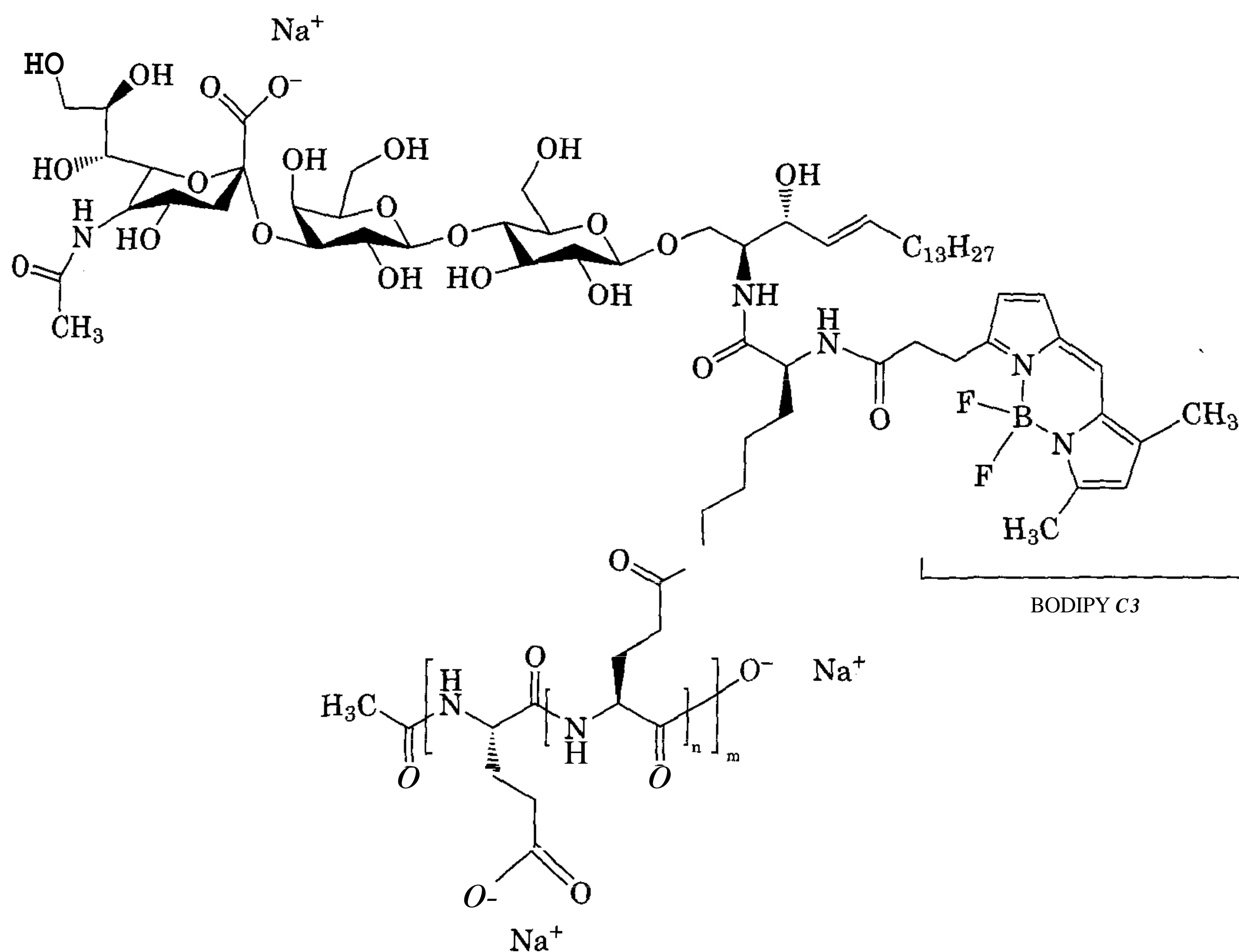
Furthermore, Whitesides et al. carried out both synthesis and the HAI assay, in the wells of microtiter plates, of libraries of polymers [poly(acrylic acid) (pAA)] bearing both sialoside and largely hydrophobic non-sialoside groups in random sequence (113). The *in situ* bioassay showed that new polymers containing both SA and certain non-sialoside groups [pAA(NeuAc-L; R)] were up to 10^4-fold more potent than the parent polymer bearing only the SA residue [pAA(NeuAc-L)] (113). It is postulated that the non-sialoside groups R may be involved in non-specific binding to hydrophobic sites on the surface of virus, resulting in enhanced affinity of the new polymers for the viral surface. This strategy may serve generally for screening and obtaining leads in biological systems that involve multivalency.

Conceptually, if the non-sialoside groups can interfere with the conformational change of the membrane-fusion domain of HA, a polyvalent polymer can inhibit influenza virus not only during initial binding, but also later in the course of HA-mediated membrane fusion. To test the hypothesis, Wong et al. reported the synthesis and evaluation of a polymer of poly-L-glutamic acid conjugated with lysoganglioside GM_3 (40) (122). A fluorescent tag BODIPY was also attached. Using an ELISA assay, the polymer showed EC_{50} against A/PR/8/34 (H1N1) at 7.5 p*M* based on the sialic acid content. The influenza inhibitory activity of the polymer is enhanced by 10^3-fold compared with that of lysoganglioside GM_3. Wong et al. thus proposed that the enhanced activity is a result from the formation of a stable polymer/HA complex by a "chain lock" mechanism, which suggests that after the sugar groups bind to the active sites on the top of HA, the hydrophobic substituents (e.g., the sphingosine moiety) of lysoganglioside GM_3 wrap around the hydrophobic sites on the stem area providing further interaction with HA.

2.1.2.2.2 Liposomes. It has been reported that anti-influenza activity can be enhanced with a multivalent display of the sialoside on liposome. Wong et al. synthesized a series of conjugates of 3- OH or 3-F-substituted SA derivatives with distearoylphosphatidylethanolamine (DSPE) as the liposome (41) (123). As determined in an HAI assay against A/Aichi/2/68 (H3N2), the DSPE conjugates showed a 10^3-fold increase in the inhibitory activity when compared with monomeric SA. However, the activity against A/PR/8/34 (H1N1) was not significantly enhanced.

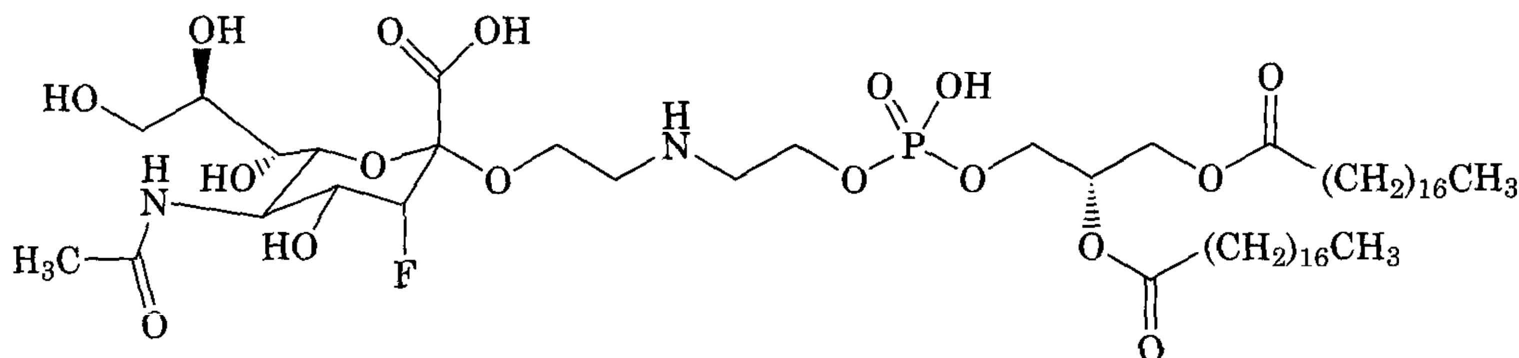
Suzuki et al. demonstrated that selective activity for H3N2 subtype over H1N1 subtype was also observed with synthetic sialylphosphatidylethanolamine (sialyl PE) derivatives (42)(124). Because H3N2 subtype influenza A viruses (e.g., A/Aichi/2/68 and A/Memphis/1/71) preferentially bind to Neu5Acα2–6Gal and H1 subtype (e.g., A/PR/8/34) binds most effectively to Neu5Acα2–3Gal linkage of sialo-sugar chains on the cell membrane, Suzuki et al. speculated that the structural assembly of sialyl PE derivatives might exhibit some similarity to the Neu5Acα2–6Gal linkage.

2.1.2.2.3 *Dendritic Polymers.* Baker et al. have reported the synthesis of several SA-conjugated dendritic polymers with various architectures that included spheroidal polyamidoamine (PAMAM) dendrimers, comb-

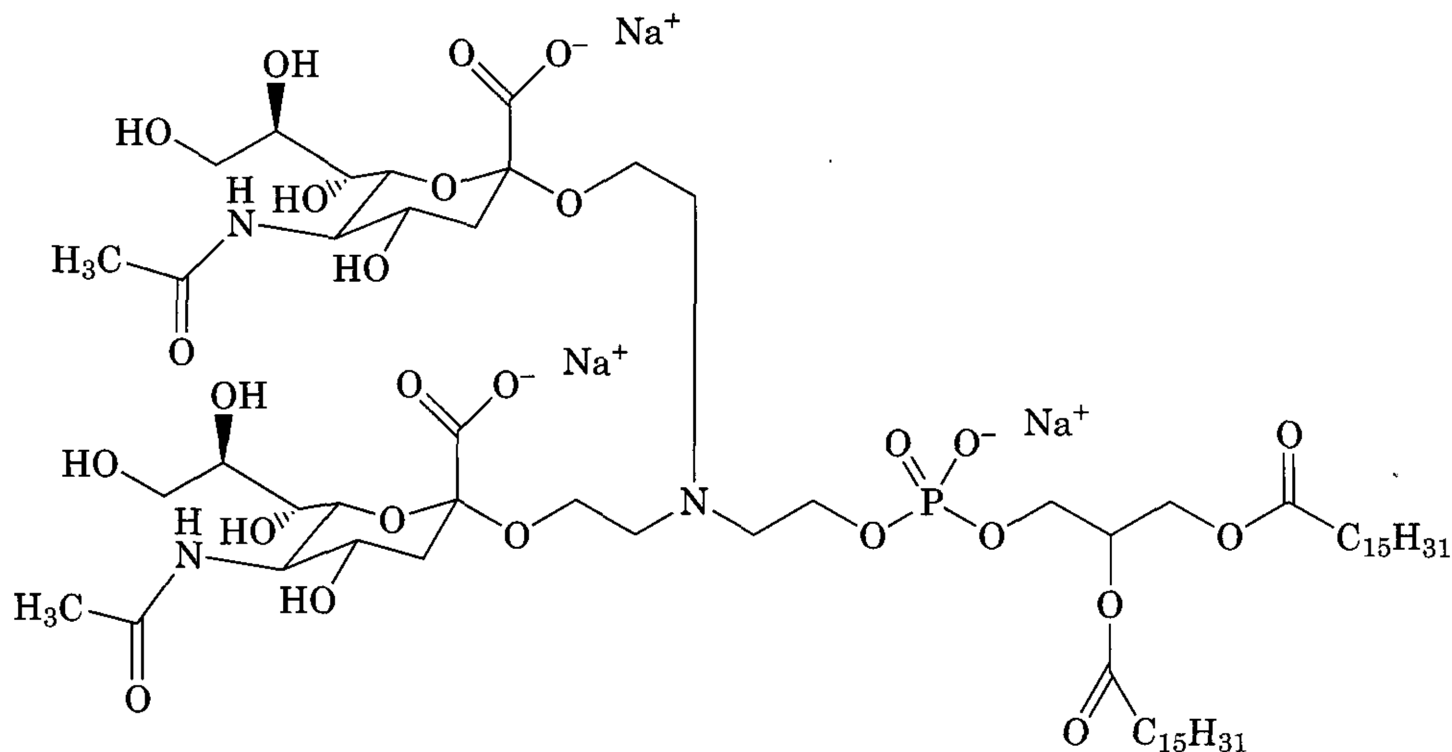


(40) m = 270
n : m ≈ 1.5 : 100

branched and dendrigraft polymers, and linear-dendron architectural copolymers **(125).** Linear polyethyleneimine **(PEI)** was involved in the construction of the last three structures. Significant variation in susceptibility to these polymeric compounds was observed when tested for their ability to inhibit virus hemagglutination (HAI assay) and to block infection of MDCK cells (ELISA assay). Generally, the larger and more flexible linear-dendron architectural copolymers, as well as scaffolding-type comb-branched polymers and dendrigrafts are more efficient than the spheroidal dendrimers, which have a fixed rigid size and shape. Both the degree of SA conjugation and the polymer size seem to influence the bioactivity through polyvalent binding and steric hindrance. Influenza virus **X-31** (H3N2) was shown to be more sensitive to these compounds than **A/AA/6/60 (H2N2).**



(41)



(42)

The most effective comb-branched and **dendrigraft** PEI polymers were up to **5** × lo^4-fold more effective than monomeric SA at inhibiting HA of the X-31 and sendai viruses.

A review on **PAMAM** dendrimers and their biomedical applications has been published recently (126).

2.1.3 Inhibitors of RNA-Dependent RNA Polymerase (RNA Transcriptase)

2.1.3.1 Antisense Oligonucleotides. It has been known that influenza viral **mRNA** synthesis is catalyzed by viral nucleocapsids, which consist of the individual viral **RNAs** (**vRNAs**) associated with four viral proteins: the nucleocapsid protein (**NP**) and the three P proteins (**PB1**, PB2, and PA). In a series of reports, Takaku et al. have demonstrated that antisense phosphodiester and **phosphorothioate** oligonucleotides that are complementary to the viral RNA polymerase (**PB1**, PB2, PA) and the nucleoprotein (**NP**) genes, specifically inhibited influenza A virus replication in MDCK cells (127, **128**), enhanced survival of mice infected with influenza A virus (129, **130**), and inhibited chloramphenicol **acetyltransferase** (CAT) protein expression in the clone 76 cell line (131–133). The clone 76 cell line is designed to express the influenza virus RNA polymerase (**PB1**, PB2, PA) and **nucleoprotein** (**NP**) genes in response to treatment with dexamethasone. The in vitro activities of these oligonucleotides on the expression of the viral gene products were assessed on the basis of their inhibition of CAT protein expression with a CAT-ELISA method (131,132).

The results revealed high inhibitory effects shown with the antisense oligonucleotides complementary to the sites of the PB2 AUG, PA AUG, and NP AUG initiation codons, with the best activity seen with the antisense oligonucleotides (**ATATAAGTTATACCTTTCTT**) targeting PB2 AUG. The antisense oligonucleotides targeted to the PB2 loop-forming site did not lead to efficient inhibition, and those targeted to **PB1** AUG initiation **codon** and the loop forming sequence were considerably less effective. In addition, the inhibitory activities the free oligonucleotides could be increased significantly with liposomal encapsulation. It was shown that the endocapsulated antisense phosphorothioate **oligonucleotides** accumulated in the nuclear region of dexamethasone-treated clone 76 cells (132) or virus-infected cells (128); the endocapsulated antisense phosphodiester oligonucleotides were found within the cytoplasm. Intravenous administration of antisense phosphorothioate oligonucleotides containing the PB2 AUG initiation **codon** encapsulated with liposome to the mice infected with influenza **A/PR/8/34** (**H1N1**) significantly inhibited viral growth in the lungs, prolonged the mean survival time in days and increased the survival rates of the

infected mice (134). DMRIE-C liposome was more effective than Tfx-10. Because the PB2 mRNA sequences around the AUG initiation codons of influenza A and B viruses share very low homology, not surprisingly, these oligonucleotides failed to inhibit influenza B both in vitro and in *vivo* (130).

Treating dumbbell RNA/DNA chimeric oligonucleotides with RNase H can also generate antisense phosphodiester oligonucleotides, which in turn is bound to the target mRNA (133).These new class of oligonucleotides are consist of a sense RNA sequence and its complementary antisense DNA sequence, with two hairpin loop structures.

2.1.3.2 ***DNAzymes, Ribozymes, and External Guide Sequences.*** In a recent report published by Toyoda et al., it was demonstrated in *vitro* that influenza virus replication was inhibited by RNA-cleaving DNA enzymes (135). Two oligonucleotides [DNzPB2 (14) and DNzPB2 (16)], which contain the DNA enzyme carrying the 10–23 catalytic sequence (136, 137), flanked with complementary sequences around the PB2 AUG initiation codon, were shown to be more effective than the same amount of antisense phosphorothioate oligonucleotides, which target AUG initiation codon sequences of PB2 mRNA. DNzPB2 (16) [TCTTTCCA***GGCTAGCTACAACGA***ATTGAATA (sequence of 10–23 DNA enzyme is shown in bold italic)] was more efficient than DNzPB2 (14) (CTTTCCA***GGCTAGC***TACAACGAATTGAAT). One concern with these DNA enzymes is that their RNA-cleaving activity has not been able to optimize under physiological conditions.

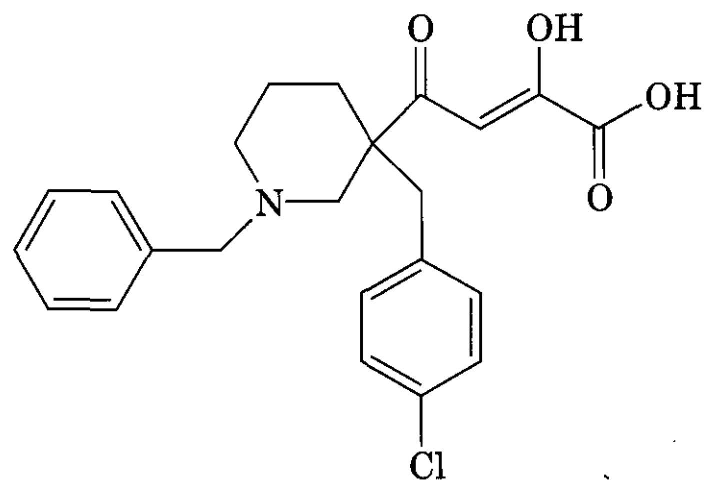
Instead of targeting the ***PB2*** gene, Lazarev et al. selected a ribozyme gene directed at a specific cleavage of mRNA coding for PB1 protein (138). Because the *PB1* gene is one of the least variable influenza A virus genes, PB1-directed ribozyme may be expected to cleave mRNA of widely different virus strains. Oligodeoxyribonucleotides were synthesized corresponding to the hammerhead ribozyme gene (containing a 24-nucleotide catalytic domain), flanked with antisense sequences (12 nucleotides on both sides of the catalytic domain) complementary to a GUC site at position 1568 in PB1 mRNA of A/Kiev/59/79 (H1N1). Plasmids containing the oligodeoxyribonucleotides were used to create ribozyme-expressing CV-1 cells, which along with the original CV-1 cells, were then infected with influenza A/Singapore/1/57 (H2N2) or A/WSN/33 (H1N1) for antiviral susceptibility. High levels (=90%) of inhibition of viral NP and NS1 proteins and influenza virus reproduction (by plaque assay) were noted in the cell lines expressing the functional ribozyme. Defective recombinant adenoviruses were also constructed carrying the genes of functional and non-functional ribozymes under the control of human cytomegalovirus promoter. Again, greater than 90% level of inhibition of the replication of influenza A/WSN/33 virus in CV-1 cells pre-infected with the recombinant ribozyme-expressing adenoviruses was observed compared with that in the non-infected cells. Analyzing the results with the cell line expressing the non-functional ribozyme suggested that the inhibition of influenza A virus reproduction with these ribozymes results mostly from the effect of RNA cleavage, and only to a small extent from the antisense effect of the flanking complementary sequences.

Ribonuclease P (RNase P) is an enzyme that cleaves tRNA precursors to generate the 5' termini of mature tRNAs. Research conducted by Altman has shown that RNase P might target any RNA for specific cleavage provided that the RNA is associated with a custom-designed external guide sequence (EGS) RNA (139,140). When the target RNA is complexed with EGS through hydrogen bonds, the resulting structure resembles a tRNA precursor and, therefore, is susceptible to cleavage by RNase P. Recently, Plehn-Dujowich and Altman reported that EGSs targeted to the influenza mRNA PB2 and NP genes effectively inhibited viral protein and particle production in vitro (141).Such inhibition was postulated as a consequence of a lowering of the amounts of the target mRNAs by the combined functions of EGSs and RNase P. In their design of EGSs, three sites in the PB2 transcript and two in the NP transcript that were assessable to digestion with RNase T1 were chosen to provide a G at the 3' side of the putative cleavage site by RNase P in the target mRNA:EGS complex and a uracil 8 nts downstream, as is found in all tRNAs.

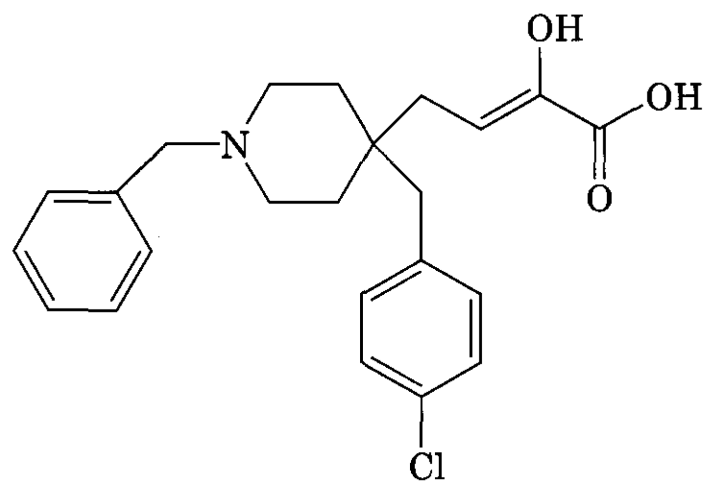
2.1.3.3 Short Capped Oligonucleotides. To initiate influenza viral mRNA synthesis, the viral associated RNA-dependent RNA polymerase binds to the cap structure at the 5' ends of host cell RNA polymerase II transcripts and then a virally encoded endonuclease cleaves the capped 5'-termini to provide caps for the 5'-termini of the viral mRNAs and to serve as primers for transcription by the viral RNA-dependent RNA polymerase (RNA transcriptase). The PB2 polymerase protein mediates both the binding and the endonucleolytic cleavage of capped mRNAs. Conceptually, the 5'-capped short RNA fragments by design are potential decoys of cap-dependent transcription. Takaku et al. reported the synthesis of short RNA molecules (8–13 ntds long) with a 5'-capped structure (m7GpppGm) using T7 RNA polymerase (141a). These short RNAs were tested, with or without liposomal encapsulation, for their inhibitory effect by a CAT-ELISA assay using the clone 76 cells, showing that the 9-ntd-long RNA molecule (m7GpppGmAAUACUCA) had the highest inhibitory activity. Furthermore, these RNA molecules exhibited higher inhibitory activity than that of the antisense phosphorothioate oligonucleotide complementary to the AUG initiation codon of PB2 mRNA.

Because the influenza virus employs the cap embezzled from the host cell, the virus is not sensitive to the inhibitory effect of *S*-adenosylhomocysteine (SAH) hydrolase inhibitors, which interfere S-adenosylmethionine (SAM)-dependent methylation reactions, leading to inhibition of a broad range of DNA and RNA viruses (142) (see Section 2.9.1).

2.1.3.4 2,4-Dioxobutanoic Acid Derivatives. As discussed above, an intrinsic property of influenza virus RNA-dependent RNA polymerase (RNA transcriptase) is its cap-dependent endonucleolytic cleavage activity. Through a random screening, Tomassini and a group of Merck investigators have identified L-735882 (43) a 4-substituted 2,4-dioxobutanoic acid, as a specific inhibitor of cap-dependent endonuclease activity of the transcriptase and with antiviral activity against both influenza A and B viruses in cell culture (143). Compound (**44**), a synthetic analog of (43) with sub-micromolar antiviral activity, was found to be the most soluble in water and readily adsorbed into the nasopharyngeal track in a mouse challenge model. When instilled intranasally into infected mice, (**44**), at its highest water-soluble dose, caused a 3.9-log reduction of the virus titers in nasal washes (143).

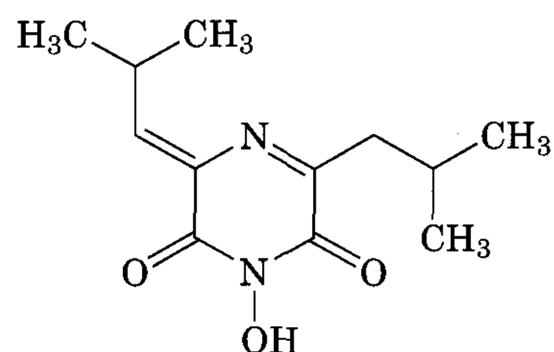


(43) L-735882



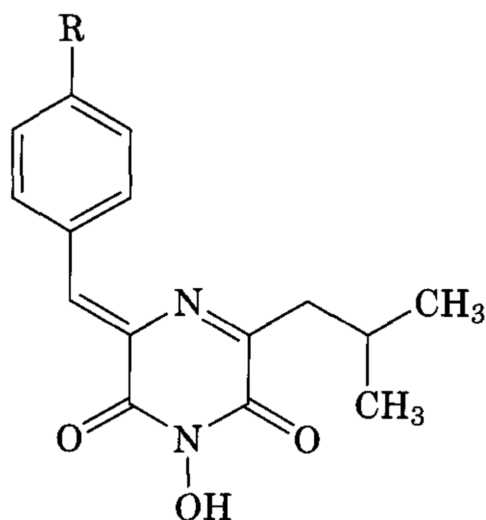
(44)

2.1.3.5 2,6-Diketopiperazine Derivatives. Tomassini et al. also reported the finding of flutimide (**45**), a natural product isolated from a fungus, which inhibited the cleavage of capped RNA by influenza virus endonuclease, with an IC„ of 6.8 μM (144). The SAR analysis with several synthetic analogs indicated that both the N-hydroxy and olefin groups were re-



(45)

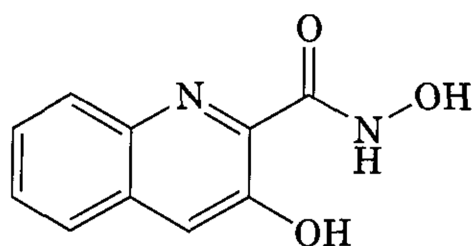
quired for activity (145). The most potent analogs were compounds (46) and **(47)**, both having IC_{50} of −0.9 μM. Flutimide inhibited



(46) R = OCH_3
(47) R = F

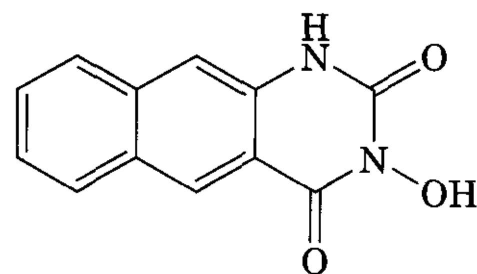
influenza virus infection of MDCK cells with an EC_{50} of 5.9 μM without any toxicity at 100 μM concentrations. Compounds (46) and (47) seemed to be more potent in the antiviral assay; however, they showed cytotoxicity to the cells at >10 μM concentrations (145).

2.1.3.6 BMY-26270 and Analogs. Krystal et al. of Bristol-Myers Squibb identified BMY-26270 (48) through a high-throughput *in vitro*

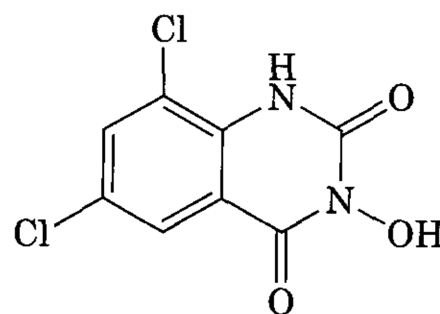


(48) BMY-26270

transcription assay from the company's chemical collection (146). This compound selectively inhibited influenza transcriptases of both A and B viruses with an IC_{50} �= 40 μM. When compared with related compounds selected from the chemical collection, it was suggested that the hydroxamic acid and phenol moieties, as well as their topological relationship, are essential for the activity. Two related N-hydroxy-imides, BMY-183355 (49) and BMY-183021 (**50**) where the relatively acidic **amine** NH presumably functions as an **isostere** of the phenolic hydrogen, also demonstrated an IC_{50} �= 50 @. Notably, these



(49) BMY-183355



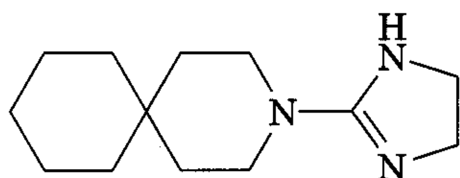
(50) BMY-183021

compounds inhibited endonuclease activity preferentially over capped RNA binding activity. However, they showed significant **cytotoxicity** in cell culture.

2.1.3.7* MI *Zinc Finger Pepfides. The matrix protein (M1) is a major structural component of the influenza virion. M1 can bind to RNA directly and inhibit its own polymerase; it was proposed that this activity might be because of the presence of a zinc finger motif. Judd et al. reported that **peptide** 6, a synthetic **peptide** based on the zinc finger region of the M1 protein sequence of influenza A/PR/8/34 (H1N1) centered around residues 148–166, was 10^3-**fold** more effective in polymerase inhibition than was M1, and greater than 10^3-fold more effective, on a molar basis, than ribavirin and amantadine again virus A/PR/8/34 by measuring the inhibition of viral **cytopathic** effect in MDCK cells (147). Little or no *in vitro* antiviral activity could be seen if the **peptide** was added later than 1 h after virus challenge, suggesting that the **peptide** inhibits virus at an early stage in viral replication, presumably through inhibition of the polymerase. Pre-treatment with the **peptide** also significantly protected the cells from viral challenge. Because the M1 sequence representing **peptide** 6 is highly conserved among type A influenza viruses, **peptide** 6 exhibited *in vitro* antiviral activity against a wide range of type A influenza viruses representing

H1N1, H2N2, and H3N2 subtypes. Interestingly, it was also active against two type B influenza viruses (B/Lee/40 and B/Shanghai/4/94). Because **peptide 6** shows therapeutic effect, it is possible that, with coordination of zinc, the **peptide** can assume a compact size and readily enters the cells. Derivatives with alternations in the finger loop, tail length, or residues involved in coordination of zinc showed reduced or abolished antiviral activity. When tested in a mouse model of influenza infections, **peptide 6,** administered **intranasally** beginning 4 h pre- or 8 h post-virus exposure to an H1N1 virus (A/PR/8/34) or an H3N2 virus (A/Victoria/3/75), was effective in preventing death, reducing the arterial oxygen decline, inhibiting lung consolidation, and reducing virus titers (titer reduction not seen with A/Victoria/3/75) in the lungs of infected animals (148).

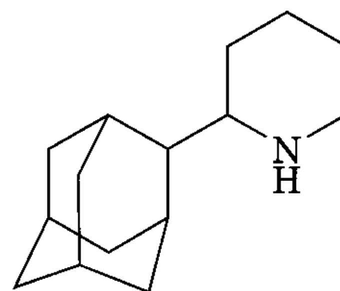
2.1.4 Inhibitors of Influenza M2 Protein. By employing a screen format that observes M2 expression in yeast cells, BL-1743 (**51**), a



(51) BL-1743

spirene-containing lipophilic **amine,** was identified from the Bristol-Myers Squibb chemical collection as an inhibitor of influenza replication (149). In a plaque reduction assay, it showed a similar profile to that of amantadine in that it was active against an amantadine-sensitive strain (EC_{50} ~ 2 μM against A/Udorn/72) but not against **amantadine-resistant** A/WSN/33. The majority of BL-1743 resistant strains were also amantadine resistant. Inhibition of ion channel activity by **BL-1743** differs from that with amantadine in that the inhibition with BL-1743 was reversible within the time frame of the experiment. The experimental results with amantadine on BL-1743-resistant strains indicated that two compounds interact differently with the M2 protein trans-membrane pore region. Because of the overlapping resistance profile of the two compounds and the higher apparent K_i (4.7 μM for BL-1743 and **0.3** μM for amantadine), BL-1743 should not be regarded as a potential replacement of amantadine for the prophylaxis or treatment of influenza virus infections in humans.

Kolocouris et al. have also reported new derivatives of amantadine that exhibit antiviral activity presumably by inhibiting the M2 ion channel of influenza virus type A (150). Using a CPE assay in MDCK cells, compound (**52**),

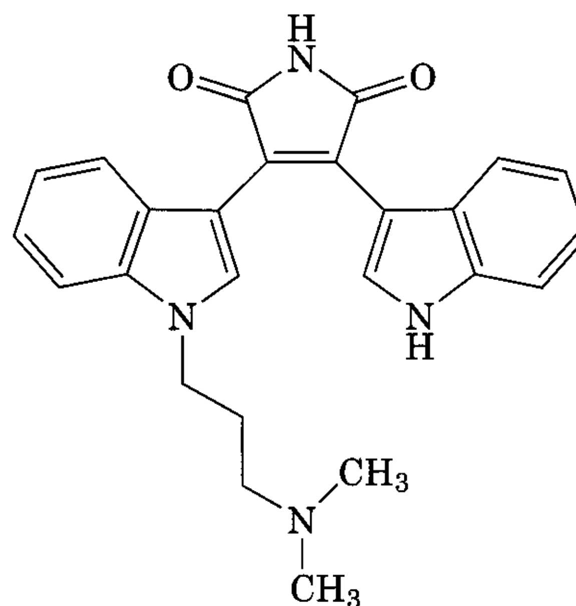


(52)

like amantadine and rimantadine, demonstrated antiviral effect on influenza virus A/Japan/305/57 (H2N2).

2.1.5 Other Inhibitors of Influenza Viruses

2.1.5.1 ***Bisindolylmaleimides.*** The effects of bisindolylmaleimide I (**53**), a potent inhibitor of protein kinase C (PKC), on the entry and

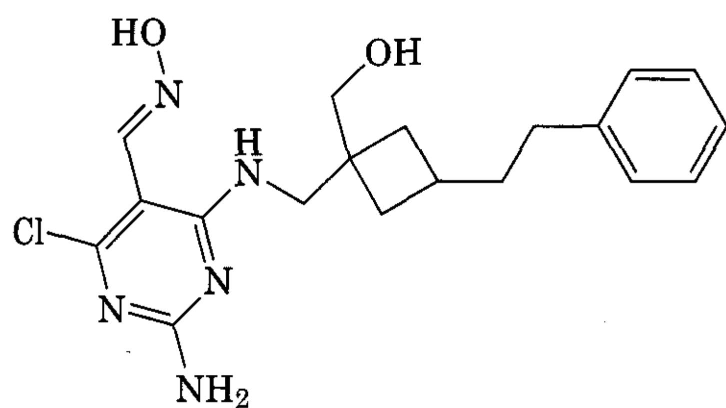


(53)

replication of influenza viruses were reported recently by **Whittakar** et al. (151). This compound inhibited in *vitro* replication of both influenza A and B viruses at micromolar concentrations in a dose-dependent and reversible manner. Further experiments showed that

this compound blocked influenza virus entry within the first 60 min of infection, at some point (probably endocytosis) before entry of viral ribonucleoproteins (vRNPs) into the nucleus. Although this compound seemed to be acting during influenza virus entry, it was shown being not acting as a weak base. Therefore, it would be interesting to see if other pH-dependent viruses such as vesicular stomatitis virus and Semliki Forest virus are also sensitive to such PKC inhibitors.

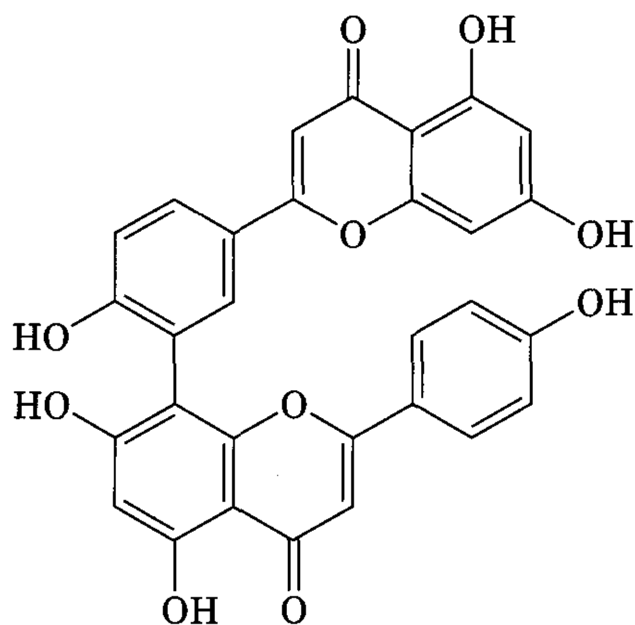
2.1.5.2 ***Pyrimidine Derivatives.*** Efficient synthetic routes of **2-amino-4-(ω-hydroxyalkylamino)pyrimidine** derivatives (54) were



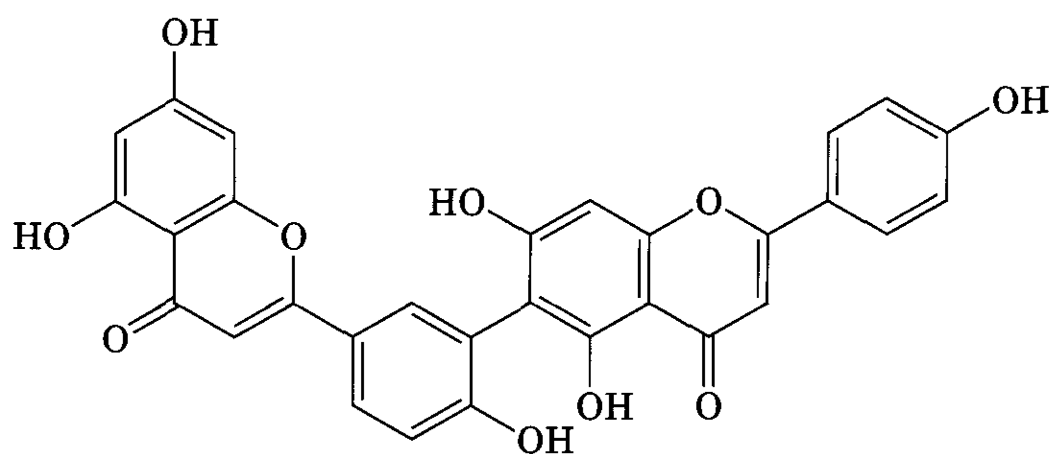
(54)

recently described (152). The compounds in which cyclobutyl group, which was further substituted by a phenylalkyl group at the 3′-position, were introduced to the β-position of the aminoalkyl side-chain were shown to be highly active in inhibiting both types of A and B influenza virus, with average EC_{50} in the range of 0.1–0.01 μM. Although their toxicity for the stationary cells was not notable, they were extremely cytotoxic for the proliferating MDCK cells, suggesting that these compounds might only be used for the topical treatment of influenza virus infection.

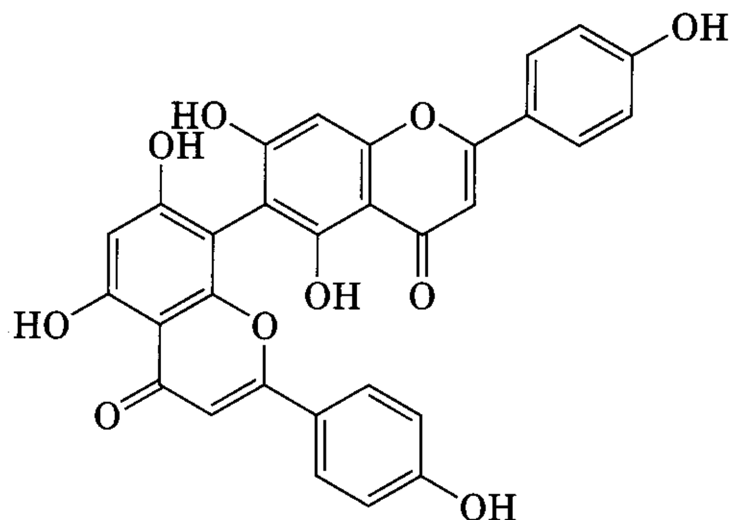
2.1.5.3 ***Natural Products.*** Certain biflavonoids isolated from Rhus succedanea and *Garcinia* multiflora exhibited strong inhibitory effects against influenza A and influenza B viruses through cell-based screens. These include robustaflavone (55) amentoflavone (**56**), and agathisflavone (57) (153). **Robustaflavone** was specifically active against influenza **B/Panama/45/90,** with an EC_{50} of 0.23 μg/mL and an SI of –435. From the medicinal plant Rhinacanthus *nasutus*, both **rhinacanthin-E** (58) and rhinacanthin-F (59) were found to have in vitro activity against influenza type A virus, with EC_{50} values of 7.4 and 3.1 μg/mL, respectively, in a CPE assay (154). Hirsutine (**60**), a Corynanthe-type **monoterpenoid indole** alkaloid found in the original plant of the Chinese **"Kampo"** medicine, was



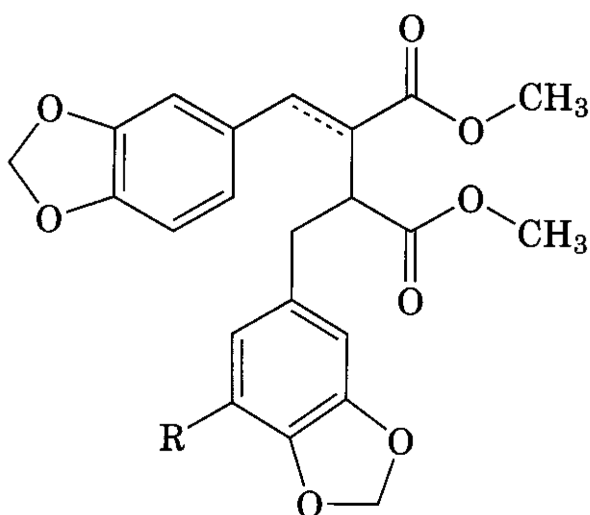
(56) Amentoflavone



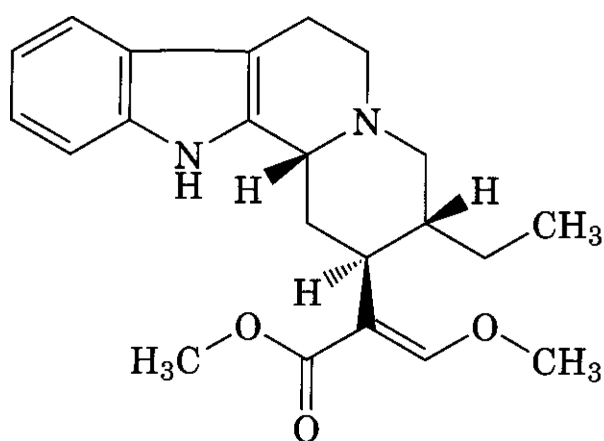
(55) Robustaflavone



(57) Agathisflavone



(58) Rinacanthin-E R = OCH_3, Δ 7E
(59) Rinacanthin-F R = OCH_3



(60) Hirsutine

selectively active against strains of H3N2 viruses (EC_{50} = 0.5 μg/mL) (155). In a separate report, amentoflavone (56) from *Selaginella sinensis* displayed potent *in vitro* activity against respiratory syncytial virus (RSV) (156).

The following compounds are from microbial origins. Fattiviracin A1 (61) isolated from the culture filtrate of *Streptomyces microflavus,* showed EC_{50} of 2.05 μg/mL against an H1N1 subtype of influenza A virus measured by a plaque reduction assay (157). FR191512 (**62**), originated from a fungus, exhibited potent anti-influenza virus [A/PR/8/34 (H1N1)] activity *in vitro* using plaque inhibition assay (158, 159). Its activity was slightly less potent than that of zanamivir on a molar basis. When administered intranasally to mice infected with influenza A/PR/8/34, FR191512 prolonged the survival of infected mice. The ED_{50} of FR191512, ribavirin, and zanamivir at day 7 after viral challenge was 3.2, 16.2, and 1.16 mg/kg, respectively.

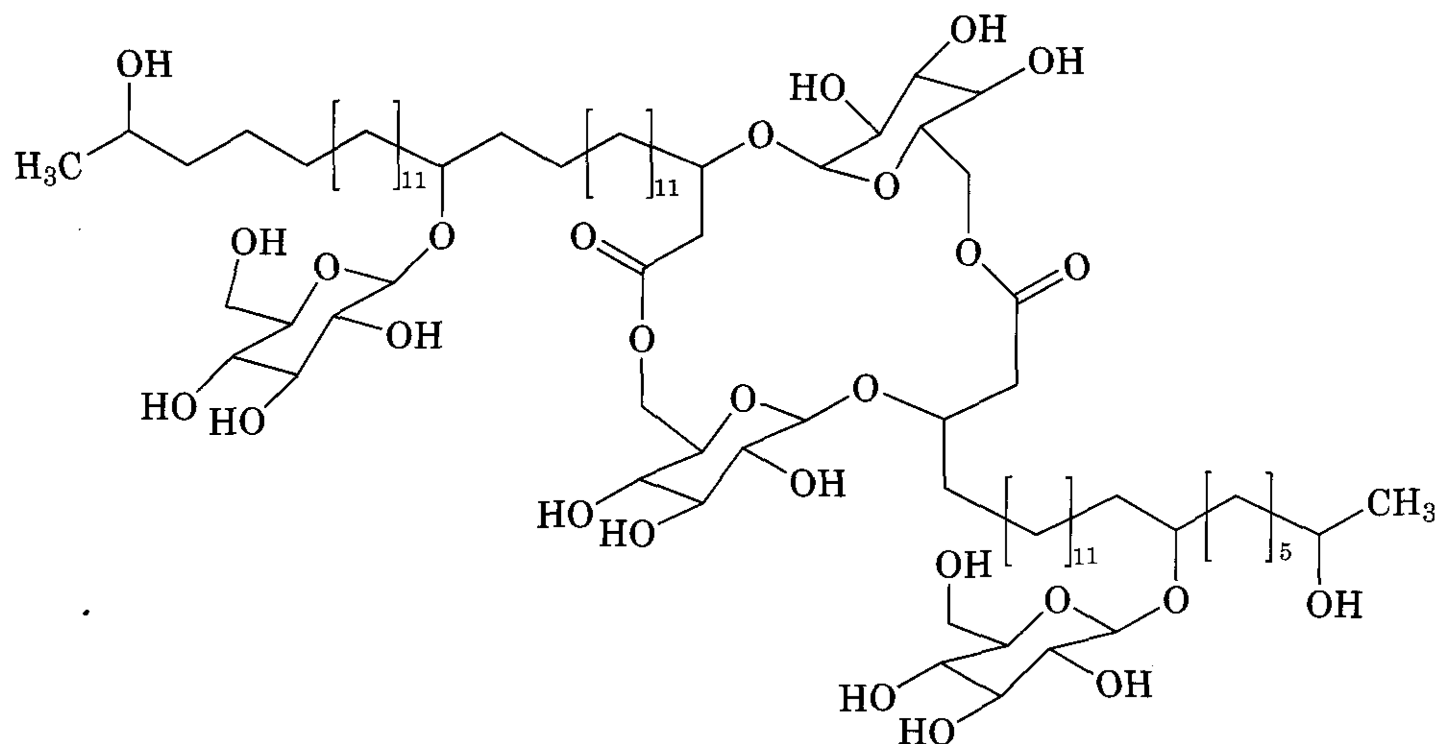
***2.1.5.4 Other* Compounds.** A potent inhibitor of all types of influenza A, B, and C viruses as well as the neuraminidase-resistant virus, termed T-705 (**63**), was reported at the 40th ICAAC (160). In an *in vitro* plaque reduction assay, the EC_{50} ranged from 0.02 to 0.6 μg/mL (without showing cytotoxicity up to 500 μg/mL) in MDCK cells. In influenza A/PR/8/34 virus-infected mice, oral dosing of 100 mg/kg/day for 5 days significantly reduced both the mortality rate and the virus titers in the lungs. T-705 exhibited more potent therapeutic efficacy than oseltamivir in low and high dose infection.

Fullerene C_{60}, presented as complex with poly(*N*-vinylpyrrolidone) (PVP) in water, inhibited the reproduction of influenza A/Victory/35/72 (H3N2) *in vitro* in concentrations of 500 μg/mL or higher (161). The C_{60}-PVP complex caused possibly multiple effects in the viral replication cycle, because its efficacy was the same after its addition at different stages of the infection. Of interest, C_{60} also was shown to inactivate Semliki Forest virus (an alphavirus) and vesicular stomatitis virus when illuminated with visible light (162).

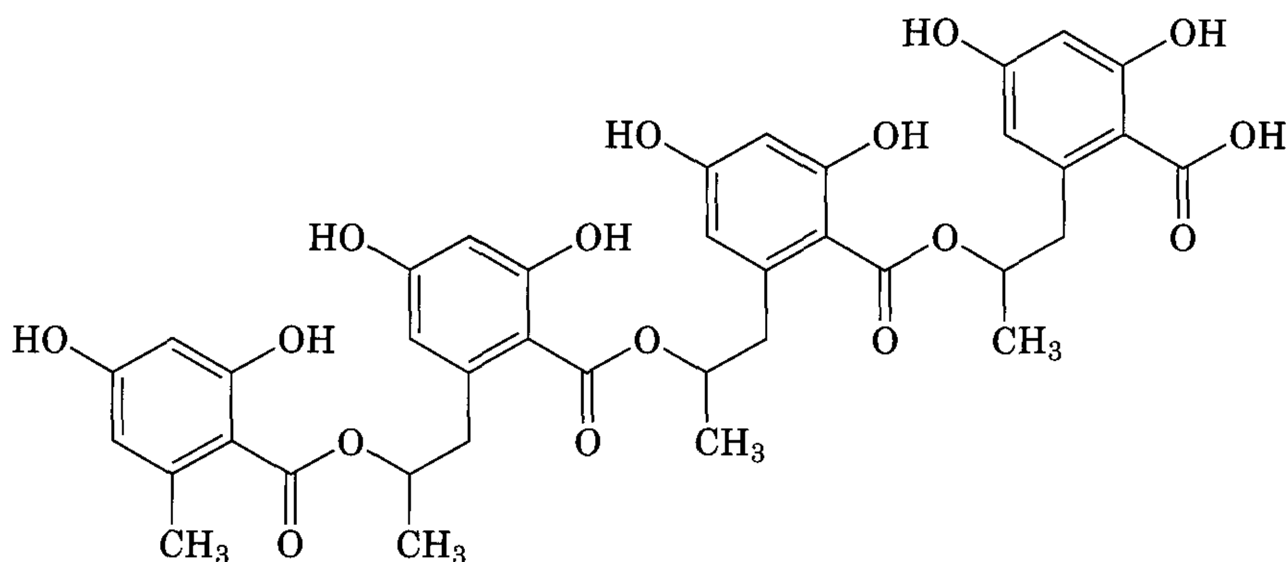
2.2 Respiratory Syncytial Virus, Parainfluenza Virus, and Measles Virus

2.2.1 Inhibitors of RSV and Paramyxoviruses Fusion Proteins

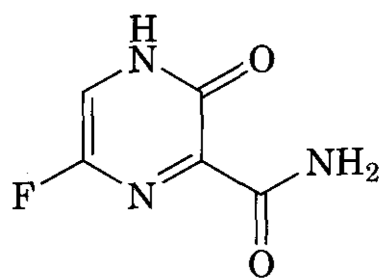
2.2.1.1 RFI-641 (WAY-154641). In a series of reports, investigators at Wyeth-Ayerst Research described the discovery of a family *of* novel anti-RSV agents including CL-309623 (**64**), CL-387626 (**65**), and RFI-641 (**66**) (163–



(61) Fattiviracin A1

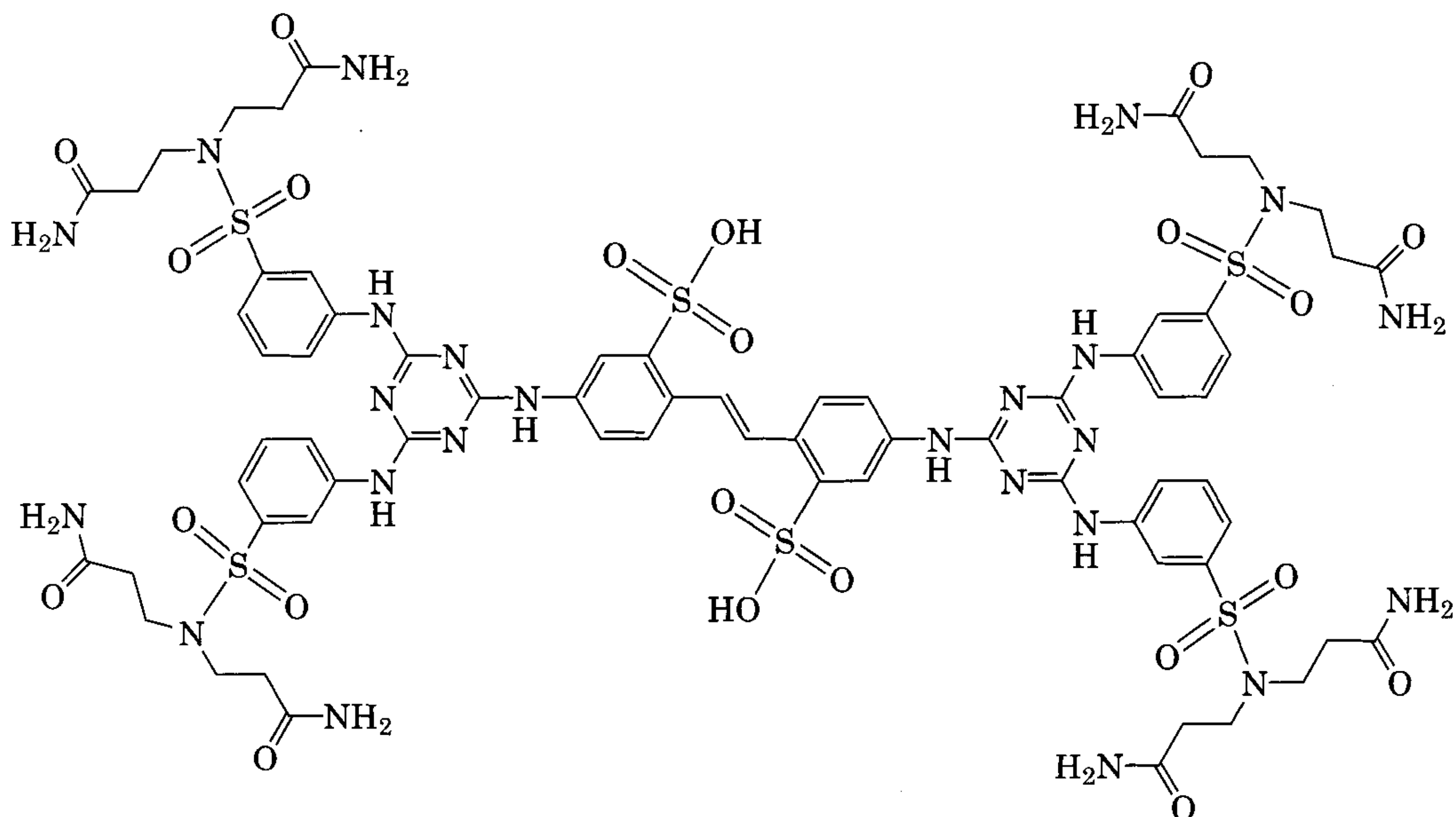


(62) FR191512



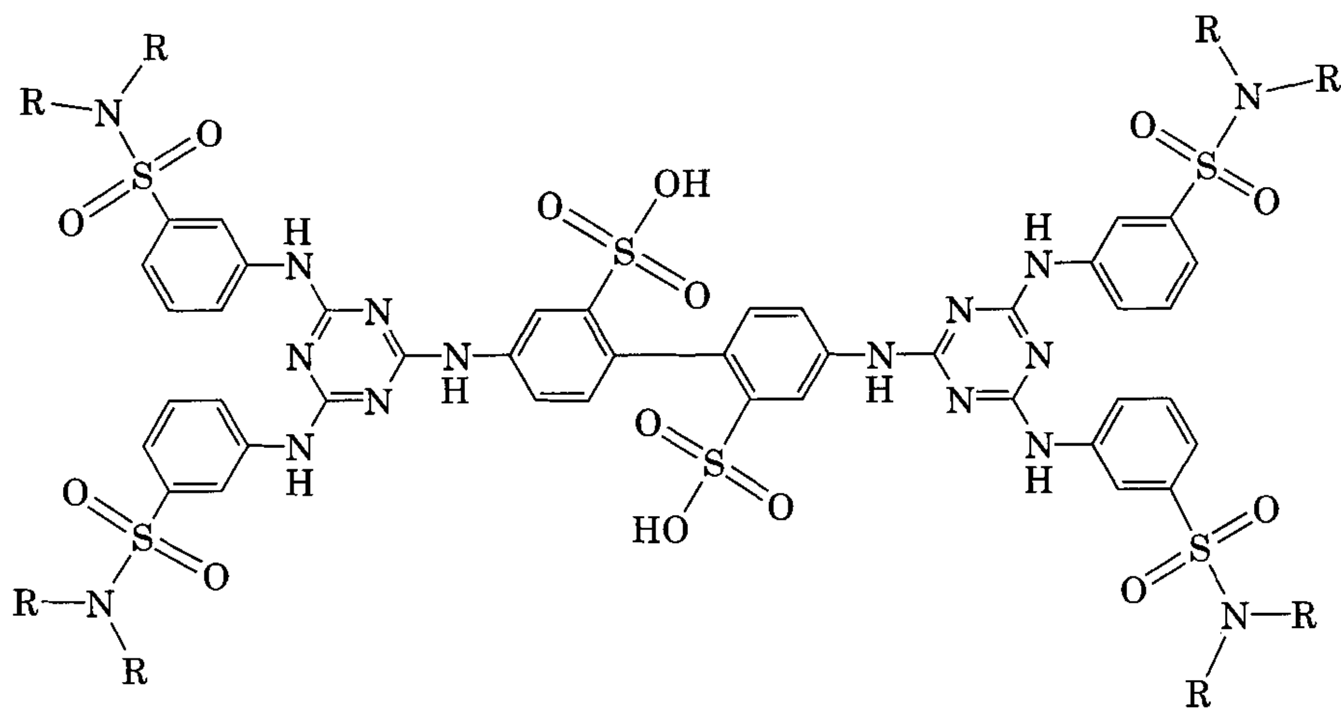
(63) T-705

166). The discovery began by screening of a compound library against a panel of viruses, and it turned out that CL-309623 was the only one to be remarkably effective and specific in inhibiting RSV infection. Interestingly, this compound was synthesized some 40 years ago as a brightener for industrial applications (165). Further refinement resulted in the biphenyl analogs CL-387626 and RFI-641, with improved anti-RSV potency (163, 166–169). Both of the biphenyl analogs inhibited laboratory and clinical isolates of RSV subtypes A and B in vitro in the range of sub-micromolar concentrations (e.g., 0.008–0.11 μM for RFI-641) without notable cytotoxicity, and the antiviral activity was independent of the cell line used in the assay (167). By using a fluorescence-dequenching assay against a wild type (A2 strain) and a mutant (cp-52) virus that contains only the fusion (F) protein on its surface, it was shown that RFI-641 inhibited both



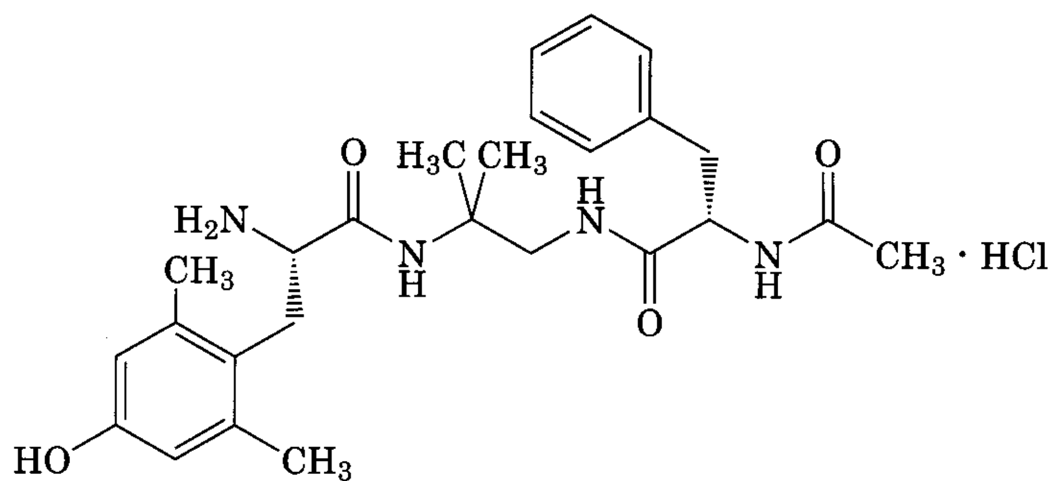
(64) CL-309623

virus-cell attachment and fusion events. Moreover, the fusion event is more sensitive than the attachment to the inhibitory effects of RFI-641 (170). Furthermore, because of heterogeneity of the aggregated F protein, multiple binding events happened when compound bound to the protein—an initial tight binding event followed by several weak binding events (163). Photoaffinity labeling experiments further suggested that the F1 subunit



(65) CL-387626 R = $CH_2CH_2C(O)NH_2$

(66) RFI-641 R = $CH_2C(O)NH_2$



(67) VP14637 CAS#235106-62-4

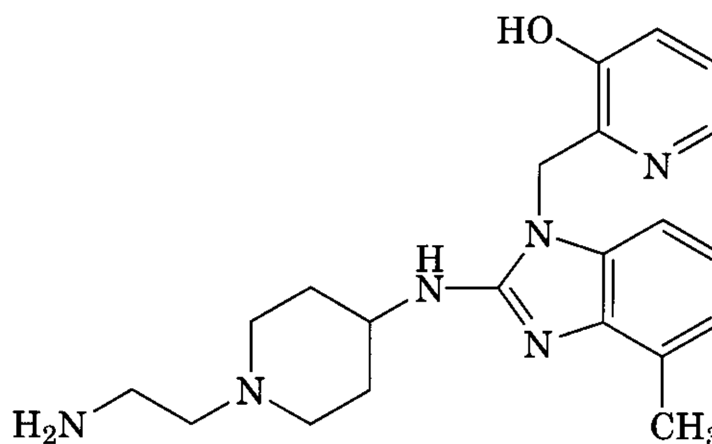
of the fusion protein is the primary target for this series of compounds (170). The convergent chemical synthesis of these compounds involved the last step coupling of disubstituted monochlorotriazines with the biphenyl core by heating in the microwave at 105°C for 1 h (166).

The following structural features are required for strong antiviral potency (163, 165): (*1*) the core should be rigid and must bear two negatively charged groups, preferably in the 2,2′-positions; (*2*) sulfonic groups are better than carboxylic; (3) a molecule needs at least three aminobenzenesulfonamido fragments to show activity and the substituents on each of the fragment should be meta to each other for optimal activity; and (4) the outmost side-chains should have hydrogen bonding groups and two side-chains are better than one. Given the fact that multiple functional groupings are needed for activity by these rather bulky and symmetrical compounds, their interactions with F protein seem to involve multivalency.

When CL-387626 was given intranasally to RSV-infected cotton rats, it demonstrated significant prophylactic activity (164). Although the protection could last for 5–8 days after a single administration, the compound seemed to be more effective when it was administered closer to the time of virus challenge. The in vivo efficacy of RFI-641 was further determined in three animal models of RSV infection (mice, cotton rats, and African green monkeys) (166, 169). Prophylactic intranasal administration of RFI-641 significantly impacted on the establishment of infection and subsequent spread of RSV to the lungs. In addition, RFI-641 also showed therapeutic effect because it could reduce viral loads in nasal and throat samples collected from infected monkeys after the compound was administered once daily, by the intranasal route, beginning 24 h after infection. RFI-641 has entered phase I clinical trials.

2.2.1.2 VP-14637. VP-14637 (67) was a sub-nanomolar fusion inhibitor of RSV reported by ViroPharma Inc. recently (171–173). In *vitro*, this compound specifically inhibited RSV and displayed a remarkable anti-RSV potency against RSV A and B strains and a panel of clinical isolates as seen by using viral cytopathic effect (syncytial formation), antigen detection, and virus yield (virus production) assays. The plausible mechanism of action involves functions associated with the viral F protein, a highly conserved RSV protein that is essential for virus reproduction. In October 2000, ViroPharma initiated clinical trials with VP-14637. A special inhalation drug delivery device is used to administer the drug to the lungs (ViroPharma Press Release).

2.2.1.3 R170591. Jansen Research Foundation recently identified R170591 (**68**), a

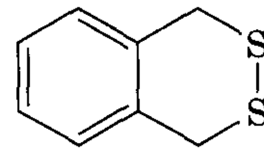


(68) R170591

benzimidazole derivative, through a cell-based assay as being capable of inhibiting fusion of RSV-infected HeLa cells (174,175). Its in vitro efficacy (EC_{50}, 0.15 nM) was about 10,-fold more potent than that of ribavirin. It was active against human (subgroup A and B) and bovine RSV. Time-of-addition and mutagenesis studies suggested that it might interact with the F protein, leading to the inhibition of both virus–cell fusion early in the infection cycle and cell–cell fusion at the end of the replication cycle. Pretreatment of cotton rats by local (inhalation) or by systemic (intraperitoneal) application resulted in >90% reduction of pulmonary virus titers.

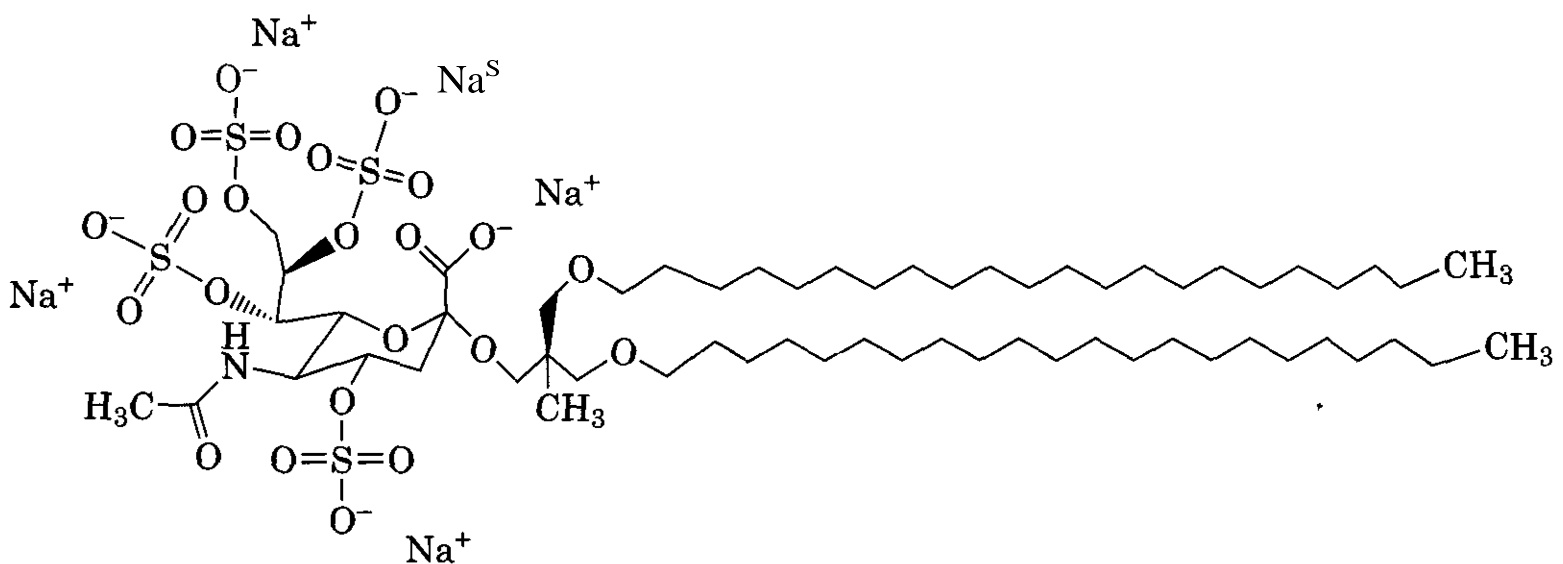
2.2.1.4 *NMSO3.* NMSO3 ***(69)*** is a nontoxic sulfated sialyl lipid that had been used to smooth the surface of instant noodles (176). This compound was found active in inhibiting RSV infection in vitro and in *vivo* (177). The compound did not show significant toxicity at the highest testing concentration in four different cell lines. Using ELISA, it was determined that the average EC_{50} of NMSO3 against several selected laboratory strains and clinical isolates was 0.23 μM and that of ribavirin was 12.3 μM (in Hep-2 cells). However, the best efficacy was observed when it was added 0–1.5 h after the viral inoculation, during the time of virus adsorption and penetration. Therefore, NMSO3 might target RSV-F glycoprotein. This preliminary conclusion was further supported by the results of a temperature shift study with NMSO3 and anti-RSV (F) monoclonal antibodies. Nevertheless, it was still active to some extent against virus replication, suggesting that NMSO3 might also inhibit some other later processes of RSV infection. Intraperitoneal administration of NMSO3 to RSV-infected cotton rats from 1 day before or 1 h after to 3 days, once a day every day, showed a significant reduction of RSV titer in the lungs. Its therapeutic effect against RSV growth in the lungs of infected animals was greater than that of ribavirin.

2.2.1.5 RD3-0028. Active anti-RSV benzodithiin derivatives were discovered through a random screening assay (178). Of these compounds, RD3–0028 (**70**) was the most active



(70) RD3-0028

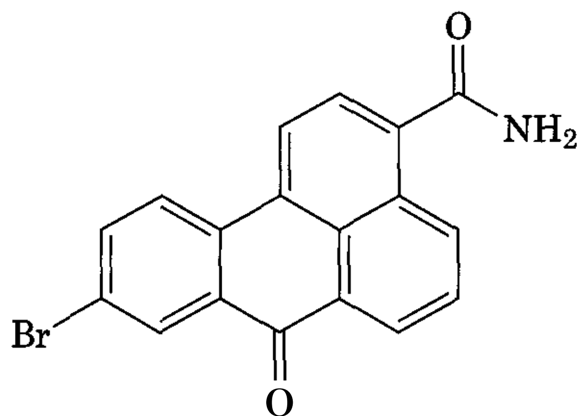
one, which showed activity against RSV subgroups A and B and clinical isolates, with EC_{50} values in the range of 4.5–11 μM in HeLa cells. This compound inhibited RSV-induced syncytium formation even added to the culture up to 16 h post-infection, suggesting that it might target a late stage of viral replication (179). By further analysis of drug-resistant mutants, it was concluded that RD3–0028 treatment resulted in the production of defective viral particles by interfering with the intracellular synthesis or processing of the RSV F protein, or a step immediately thereafter (179,180). When delivered by aerosol to virus-infected, cyclo-



(69) NMSO3

phosphamide-treated immunosuppressed mice (181), RD3–0028 significantly reduced the pulmonary titers and protected the lungs of against tissue damage. RD3–0028 was not toxic for the mice at the therapeutic doses, and the minimal effective dose seemed to be much less than that of ribavirin (178).

2.2.1.6 Benzanthrone Derivatives. Trimeris, Inc. has filed a patent (WO9839287) describing a series of RSV fusion-targeting inhibitors based on a benzanthrone skeleton (180, 182). Compound (71) displayed an EC_{50} of 0.04 μg/mL and a CC_{50} of 4.15 μg/mL.



(71) Benzathrone

2.2.1.7 Immunoglobulins. During the past few years, a wealth of data generated from prophylactic passive immunization on both experimental animals and humans have shown that parenteral administration of RSV-neutralizing antibodies could reduce the severity of RSV disease [see reviews by Mills (183) and Prince (184)]. Currently, there are two products manufactured by MedImmune—RespiGam (approved by the FDA in 1996) and Synagis (approved in 1998)—for RSV infections. RespiGam, a polyclonal respiratory syncytial virus immunoglobulin (RSVIG) derived from human plasma, is administered through intravenous infusions for the prevention of serious lower respiratory tract infection caused by RSV in high-risk human infants. Synagis (also known as palivizumab and MEDI-493), a humanized mouse IgG monoclonal antibody (mAb) directed against the RSV fusion protein, can be administered by intramuscular injections for use in a broader patient population than RespiGam, being suitable for prophylactic administration to pediatric patients at risk of RSV disease. In a recent study with cotton rats undergoing prolonged immunosuppression with cyclophosphamide, both prophylaxis and therapy with RSVIG significantly reduced pulmonary RSV replication. In addition, the use of multiple therapeutic doses of RSVIG was able to prevent rebound viral replication, though virus was not completely eliminated (185).

A number of other mAb preparations directed against the F protein of RSV have also been actively pursued, including HNK20 (a mouse IgA mAb developed by OraVax) and RSHZ19 (a humanized mouse IgG mAb, also known as SB 209763, licensed from Scotgen to SmithKline Beecham for development). In preclinical studies in rodents and rhesus monkeys (186), intranasal delivery of HNK20 showed significant protective effect. However, in an international control trial conducted with 600 high-risk infants, HNK20 did not result in a significant decrease in the incidence of hospitalization associated with RSV lower respiratory tract infection (187). Similar to the case with HNK20, RSHZ19 showed prophylactic efficacy in mice and cotton rats (188) but failed to protect infants at risk for severe RSV disease (189).

Palivizumab (MEDI-493) and RSHZ19 recognize distinct neutralizing epitopes on the F protein of RSV. In series head-to-head experiments (190), it was clear that the F protein. affinity of palivizumab was severalfold tighter than that of RSHZ19. Using ELISA to measure viral replication, palivizumab was approximately 5-fold more potent than RSHZ19 and 20-fold more potent than RSVIG to neutralize RSV [drugs were added either before (microneutralization assay) or after (fusion inhibition assay) attachment to Vero cells]. In a cotton rat prophylaxis model, palivuzumab was two- to fourfold more potent than RSHZ19 in inhibiting RSV replication in the lungs. Therefore, the difference in clinical efficacy seemed to relate to the greater potency of palivizumab compared with RSHZ19.

2.2.1.8 ***Peptides.*** The F protein of paramyxoviruses and the transmembrane (TM) protein of retroviruses facilitate the fusion of the viral envelope or infected cell membranes with uninfected cell membranes. By recognizing that the fusion domains at the amino termini of RSV F1 subunit and HIV-1 TM show a high degree of sequence homology, Lambert et

al. at Trimeris, Inc. have used a computer-searching strategy, based on the secondary structure characteristics to the DP-107 and DP-178 peptides of HIV-1 gp41, to identify conserved heptad repeat domains analogous to the DP-107 and DP-178 regions of HIV-1 gp-41 within the glycoproteins of paramyxoviruses, leading to the discovery of non-cytotoxic peptides T-118, T-205, and T-257 as nanomolar inhibitors against RSV, parainfluenza type 3 (PIV-3), and measles virus (MV), respectively, *in vitro* (191). Although these peptides were from domains that near the membrane anchor, the antiviral activity of these peptides was specific for the virus of origin. The sequence of T-118: Ac-$_{488}$FDASISQVNEKINQSLAFIRKSDELLHNVBAGKST$_{522}$-NH_2.

In separate studies, Yao and Compans showed that synthetic peptides containing the heptad repeat regions derived from the F proteins of human PIV-2 and PIV-3 could inhibit virus-induced cell fusion, virus entry, and spread of virus infection (192). Moreover, the inhibitory effects of these peptides were found to be virus-type specific. Similarly, Wild and Buckland reported that a peptide corresponding to the leucine zipper region (amino acids 455–490) of the MV F protein could block both MV entry and cell-to-cell fusion (193).

Graham et al. demonstrated that RhoA, a small cellular GTPase of the Ras superfamily, interacts with RSV F protein and facilitates virus-induced syncytium formation. This group further reported that $RhoA_{77-95}$; a peptide comprising amino acids 77–95 of RhoA, showed activity in inhibiting syncytium formation induced by RSV and PIV-3, both *in vitro* by inhibition of cell-to-cell fusion and *in vivo* by reduction of pulmonary virus titers in RSV-infected mice, when the peptide was administered intranasally to the animals immediately before or 2 h after RSV challenge (194). The authors suggested that when illness is mediated by the T-cell response and is not directly related to virus-induced cytopathology, antiviral therapy must be given early or combined with immunomodulators. The sequence of $RhoA_{77-95}$: TDVILMCFSIDSPDSLENI.

2.2.2 Oligonucleotides as Inhibitors of RSV.

The RSV genome encodes 10 viral proteins, which are, as shown in 3' to 5' order, NS1, NS2, N, P, M, NS3, G, F, M2, and L. Recently, Hybridon, Inc. reported the antiviral effects, by means of inhibiting RNA replication and transcription of NS2 mRNA, of an antisense phosphorothiolate oligonucleotide, v590 (5'-AAAAATGGGGCAAATAAATC-3'), which is complementary to two same 20-base sequences at the start of the *NS2* gene and the P gene, respectively (195). This oligonucleotide was 4- to 20-fold more potent than ribavirin in inhibiting RSV antigen and infectious virus yield. Treatment of cells with the compound specifically decreased the region of RSV RNA containing the v590 target sequence, suggesting that sequence-specific cleavage of RNA might have occurred (195). This cleavage might involve cellular ribonuclease H (RNase H), because RNase H has been shown to cleave RNA strand in oligonucleotide/RNA duplexes (195). Yet the all-phosphodiester-backbone and the G quartet–containing structural motifs might give rise nonspecific effects (196).

Targeting RNase L [(2–5A)-dependent endoribonucleasel to RSV RNA with 2',5'-oligoadenylate (2–5A)-antisense chimeric oligonucleotides has been shown as an interesting antiviral approach (197). (For a comprehensive review of the 2–5A system, see Ref. 198.) The premise of this approach is that the 2–5A moiety of the chimeras attracts (or recruits) and activates ubiquitous intracellular latent RNase L, which causes the degradation of the RNA target bound to the antisense domain (197, 199).

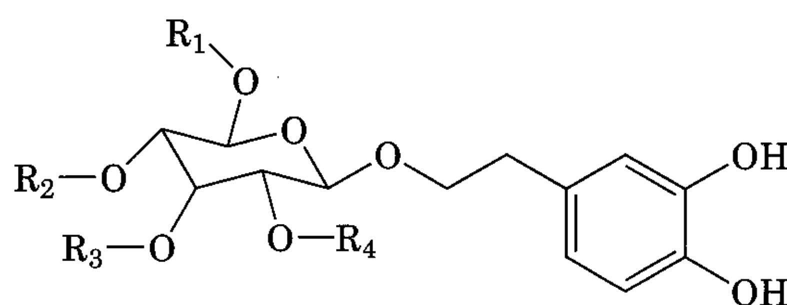
Silverman and Torrence synthesized a variety of (2–5A)-antisense chimeras through covalent linkage of 3',5'-antisense oligodeoxyribonucleotides (ODNs) and 2',5'-oligoadenylate molecules through a linker (butanediols). Both termini were also chemically modified to protect the chimeras from enzymatic (e.g., 3'-exonuclease and phosphatase) degradation. Using a computer-assisted analysis of the secondary structure of the RSV RNA sequence, they identified several regions in the M2 and L mRNAs as the targets for the (2–5A)-antisense chimeras (200, 201). The chimeras, sp(5'A2'p)$_3$A-Bu_2-5'-ATGGTTATTTGGGTTGTT-3'-3'T5' [called spA_4-antiRSV3'-3'T/(8281–8299) or NIH8281, where sp = 5'-monothiophosphoryated], which targeted the sequence 8281–8299 in the 0RF2 region of the

M2 mRNA, had the greatest antiviral potency in 9HTE cells. An approximately 75% reduction in viral yields (after 24 h post-infection) was observed when the compound was added twice to RSV-infected cells at 3.3 μM per dose (201). This oligonucleotide was remarkably selective for the RSV M2 mRNA. Moreover, the selective and specific degradation of the *M2* gene was dependent on the presence of the tetrameric 2–5A moiety, thereby confirming the involvement of RNase L (201). Further studies with NIH8281 showed that this oligonucleotide inhibited several representative strains of both A and B RSV serotypes as well as bovine RSV with low cytotoxicity in a variety of antiviral assays (202). The compound was inhibitory only when added within 2 h of virus infection, and the activity was multiplicity of infection (MOI)-dependent. The compound did not inhibit measles or parainfluenza viruses (202).

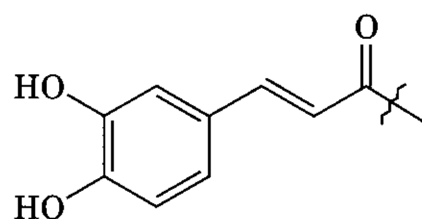
In separate studies, Torrence et al. chose the conserved consensus sequences that occur in gene-start, intergenic, and gene–end signals within the RSV genome for inhibitor design. Of particular interest was the antisense 17-mer, 5'-AAAAATGGGGCAAATAA-3', which could potentially target 10 sense targets, but with different hybridization efficiency, simultaneously (196). (This 17-mer was related in sequence to a 20-mer antisense phosphorothioate oligonucleotide v590 mentioned above.) To minimize the potential nonspecific effects associated with the all-phosphorothioate, Torrence et al. used a "gapmer" approach, in which only three internucleotide linkages at the 5' and 3' termini of the antisense 17-mer were thiophosphorylated. The resultant (MA)-antisense gap-mer, NIH351 [(2–5A)-Bu_2-AsAsAsAATGGGGCAAAsTsAsA] inhibited RSV strain A2 virus yields (EC_{50} and EC_{90} were 0.3 and 1 μM, respectively, when added once, immediately before virus adsorption) 100-fold more potent than did ribavirin in Hep-2 and in MA-104 cells. This chimeric oligonucleotide was not toxic to cells and showed the most potent *in vitro* effects when given once a day for 3 days or twice a day for 2 days. Beside RSV A2 strain, NIH351 was a potent inhibitor of a number of representative members of both A and B strains of RSV whether assayed in human, monkey, or bovine (against bovine RSV) cells. When NIH351 was compared with NIH320, which has the same antisense design but without the 2–5A moiety, it was clear that NIH351 owed its 30-fold enhancement in antiviral activity to the involvement of the 2–5A system's RNase L (196).

2.2.3 Other Inhibitors of RSV

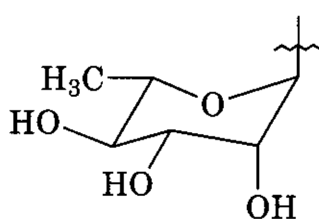
2.2.3.1 Natural Products. Phenylpropanoid glycosides **(72–75)** from the medicinal plant *Markhamia lutea Verbascoside* (203) and iri-



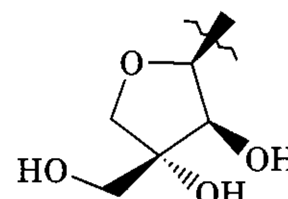
	R_1	R_2	R_3	R_4
(72)	H	Caf	Rha	H
(73)	Caf	H	Rha	H
(74)	$COCH_3$	Caf	Rha	Api
(75)	Caf	H	Rha	Api



Caffeoyl

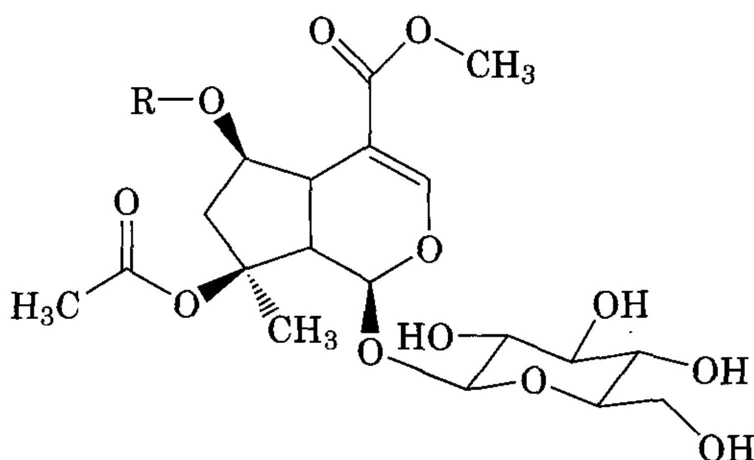


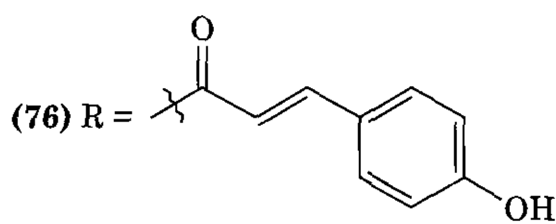
Rhamnosyl

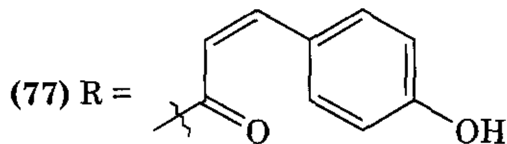


Apiosyl

doid glycosides (76 and 77) from the medicinal plant Barleria prionitis (204) have been reported as antivirally active against RSV in



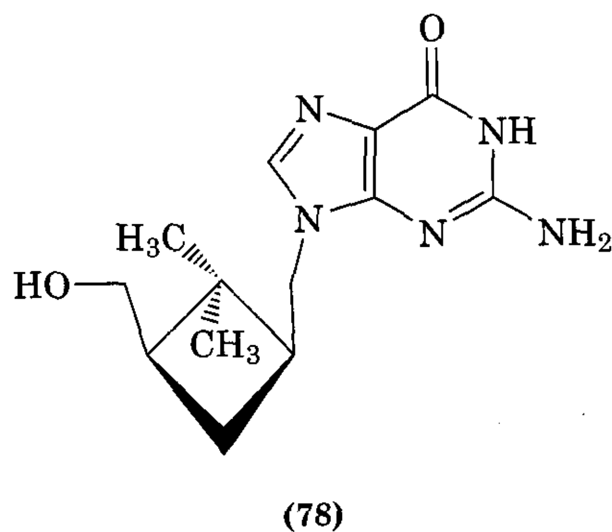




vitro in sub-micromolar concentrations, which were largely separated from their cytotoxic concentrations. The phenylpropanoid glycosides were active when added 3 h after virus infection of the cells.

2.2.3.2 **Purines and Pyrimidines.** Derivatives of purine and pyrimidine (e.g., EICAR, pyrazofurin, and cyclopentenyl cytosine) have been known for their potent anti-RSV activity both in vitro and in *vivo*. For a review of these and other anti-RSV compounds pre-dating 1996, see a recent publication by De Clercq (205).

There was a recent report showing that a synthetic guanine derivative (**78**) and its 2,6-diaminopurine analog both containing a common N9 cyclobutyl substituent, exhibited noteworthy in vitro activity against RSV with reasonable selectivity (206).



(78)

2.3 Picornaviruses

2.3.1 Inhibitors of Picornaviral Attachment and Uncoating

***2.3.1.1 Long-Chain* Compounds.** Many picornaviruses share a common icosahedral capsid architecture constructed from 60 copies of four proteins (VP1, VP2, VP3, and VP4) revealed by crystallographic studies of several human enteroviruses (namely, coxsackievirus B3, echovirus 1, poliovirus types 1, 2, and 3) and rhinovirus types 1A, 2 (207), 3, 14, and 16 (see references cited in Ref. 208). VP1, VP2, and VP3 compose the viral surface, whereas VP4 lays interior at the capsid/RNA interface (209).

In all of these structures, the virus surface reveal broad depressions, or canyons, formed by the junctions of VP1 and VP3. The canyon has been shown to be the site of receptor attachment for major group rhinoviruses (e.g., ICAM-1 for HRV-14) and for poliovirus. At the base, or floor, of the canyon there is a pore, which opens into the hydrophobic core, or pocket, within the VP1 protein (210). In most of the enteroviruses (comprise >60 serotypes) and rhinoviruses (>115 serotypes), the pocket is either empty (e.g., in HRV-14 and HRV-3) or occupied by a fatty acid-like pocket factor, of which the chemical identify remains unknown. A variety of diverse long-chain hydrophobic capsid-binding antiviral compounds (e.g., pleconaril) have been shown to displace the pocket factor and bind in the hydrophobic pocket. Drug binding has shown to lead to the inhibition of viral infectivity by stabilizing the viral particles and/or by preventing receptor attachment. The binding of antiviral drug not only causes local conformational changes in the drug-binding pocket but also stabilized the entire viral capsid against enzymatic degradation (209).

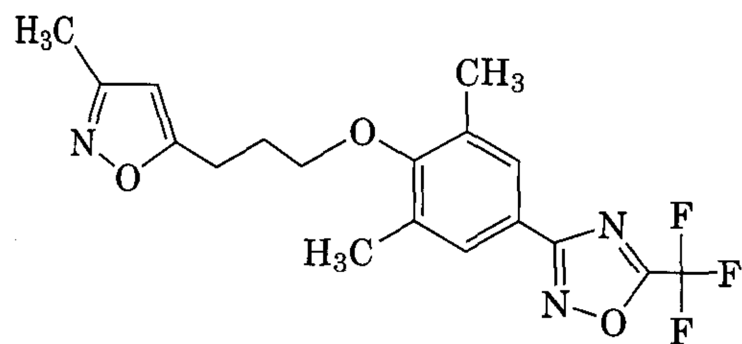
The drug binding-induced stabilization prevents the virion undergoes an irreversible conformational change (required for uncoat-

ing for RNA release) from the native 160S (or N) particle to the 135S (or A) particle, which facilitate cell entry. This stabilization effect has recently been shown to arise from higher entropy, and not through rigidification of the capsid as has been previously suggested (211–214). Binding of the antiviral compound increased the entropy (greater flexibility) of the HRV capsid and therefore reduced the free energy for uncoating. Further studies in the presence of soluble poliovirus receptor showed that capsid-binding compounds inhibited receptor-mediated N-to-A conversion through a combination of enthalpic and entropic effects (215).

Nevertheless, the variation in size and amino acid composition of the pockets, particularly, variations in the more hydrophobic end (the toe end) of the pocket among serotypes, might affect the pocket fit of these capsid-binding compounds. This factor might contribute, in part, to the different sensitivity of many of these serotypes to the compounds. In general, HRV-14, HRV-3, and poliovirus are more sensitive to longer compounds. Shorter compounds tend to be more effective against HRV-16, HRV-1A, and HRV-2 (216).

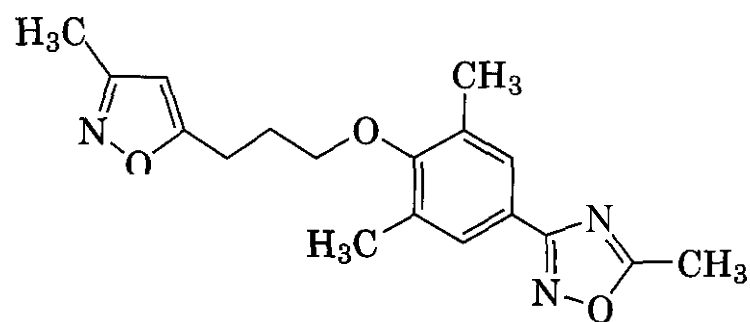
Several capsid-binding compounds have been investigated in clinical trials; only ViroPharma's pleconaril has advanced to phase III clinical studies (for a review of pleconaril and other drugs, see Refs. 217–223).

2.3.1.1.1 Pleconaril. Pleconaril (79) (registered as Picovir, also known as VP 63843) be-

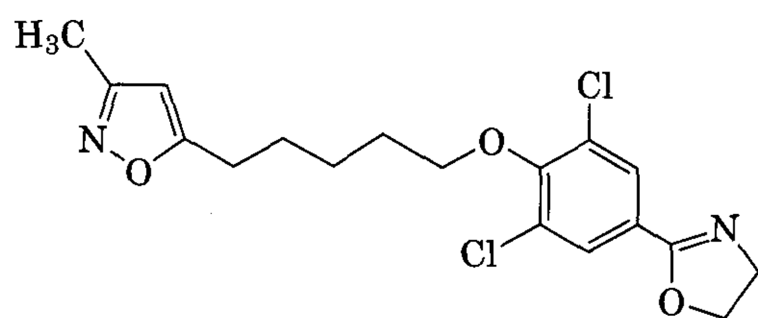


(79) VP 63843 Pleconaril

longs to WIN series of compounds. It possess much improved potency, chemical and metabolic stability, pharmacokinetics (224, 225), and safety than its predecessors in the series. In a monkey liver microsomal assay, pleconaril was found to produce two minor metabolic products. In comparison, WIN 61893 (80) formed at least eight and WIN 54954 (81) formed 18 metabolic products. It was further



(80) WIN 61893

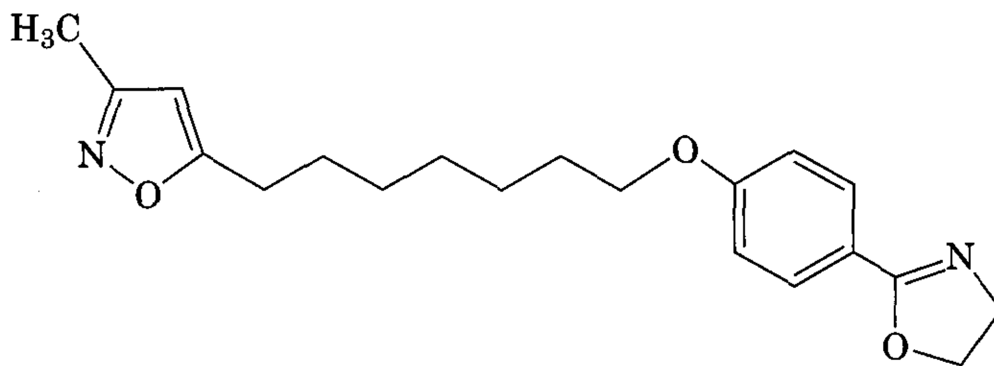


(81) WIN 54954

determined that the methyl groups on either end of WIN 54954 and WIN 61893 were the major sites of metabolism. Replacement of the methyl group on the oxadiazole ring of WIN 61893 with trifluoromethyl creates the metabolically stable and orally bioavailable (–70% in humans) pleconaril (221).

In preclinical studies, pleconaril has demonstrated a broad spectrum of activity against a wide range of rhinoviruses and non-polio enteroviruses both *in vitro* and *in vivo* (226). ViroPharma reported that pleconaril effectively inhibited the laboratory replication of 96% of the rhinovirus and enterovirus isolates from 322 human patients (http://www.viropharma.com/pipeline/pleconaril.htm). Significantly, echovirus 11, the most commonly isolated enteroviruses in the United States between 1970 and 1983, was the most sensitive serotype to pleconaril (226). This compound has also demonstrated excellent penetration into the central nerve system, liver, and nasal epithelium.

To date, pleconaril has shown clinical benefits for both adult and pediatric patients with enteroviral meningitis, viral respiratory infections, and potential life-threatening enteroviral infections in at-risk patient populations (e.g., patients with antibody-deficiency and



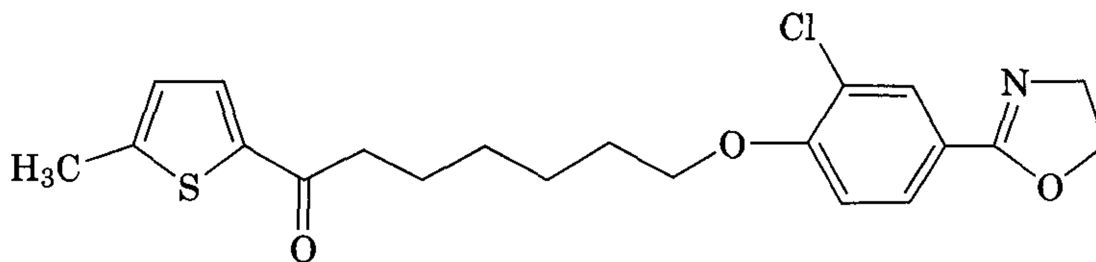
(82) WIN 51711 Disoxaril

bone marrow transplant recipients) (227, 228). ViroPharma issued two press releases in April 2000 and March 2001, respectively, reporting encouraging results from several phase III clinical studies of pleconaril in two disease indications: viral respiratory infection (common cold) in adults and viral meningitis in adults and children.

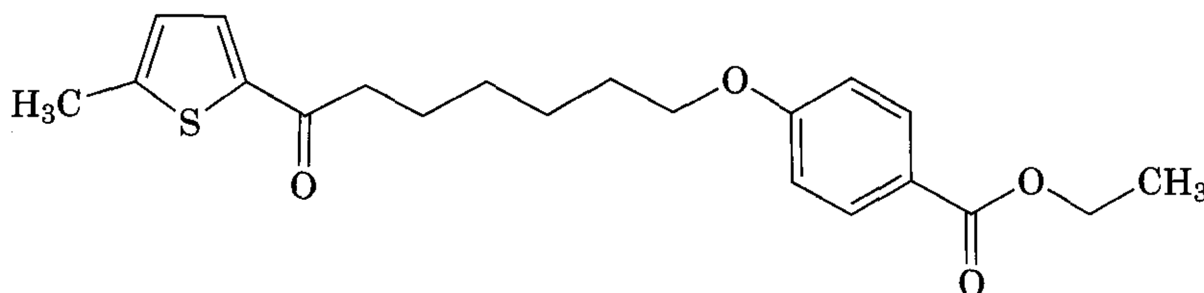
2.3.1.1.2 Disoxaril Analogs. In an attempt to search improved antipicornaviral compounds, based on the structure of disoxaril (WIN 51711) (**82**), Artico et al. synthesized a series of disoxaril analogs containing a terminal thiophene ring and a carbonyl group bound to the position 2 of thiophene (229). Although most of the analogs were equivalent to or more potent than disoxaril against HRV-14 and HRV-2, they were not broadly inhibitory to various **HRVs** as does by disoxaril. However, there were two broad-spectrum inhibitors. Compounds (83)and (84) were more potent than disoxaril when assayed against HRV-2, and as potent as disoxaril against HRV-14 and the other 14 selected serotypes. When thiophene was replaced with benzene, the anti-rhinoviral activity deteriorated (230).

2.3.1.1.3 Pirodavir, SCH 48973, and SDZ 880–061. Pirodavir (R 77975) (**85**), developed by Janssen, is another capsid-binding inhibitor that has been in clinic evaluation. It was efficacious in experimentally induced HRV infection when the drug was administered intranasally before or after infection, but before onset of symptoms (219). However, no clinical benefit was seen in treating naturally occurring HRV colds by intranasal administration (231).

SCH 48973 (86)was identified at Schering-Plough through molecular modeling and in an assay designed to detect compounds that stabilize poliovirus to heat inactivation (232). When tested in cell culture, this compound demonstrated significant activity against **a** wide range of enteroviruses; it inhibited 80%



(83)



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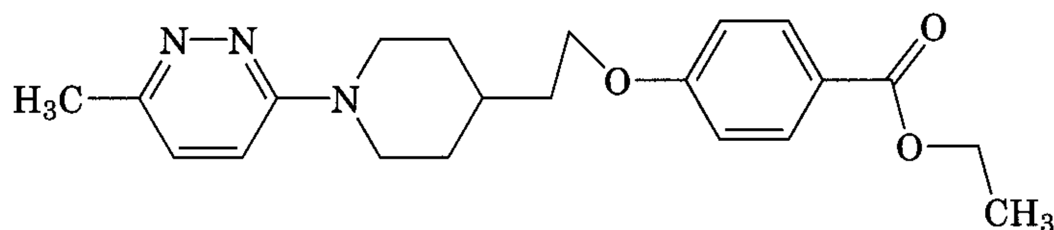


(85) Pirodavir (R 77975)

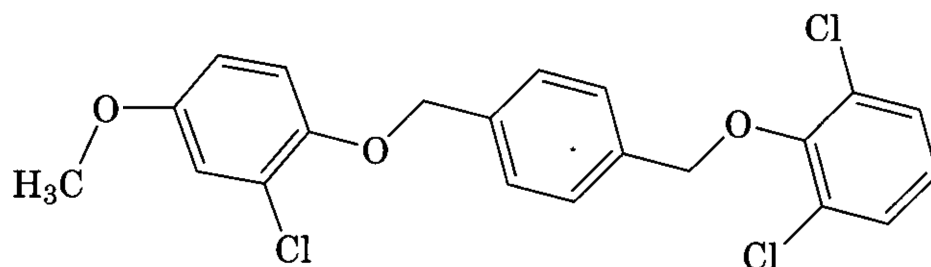
of 154 recent clinical isolates representing 15 common enterovirus serotypes with an average EC_{50} of –1 μg/mL. However, it was a poor inhibitor of HRV-14 (233).

Polioviruses exist in only three serotypes that are pathogenic for humans. Only poliovirus type 2 Lansing (PV2L) is neurovirulent in mice when injected intracerebrally (233). In poliovirus type 2–infected mice, therapeutic treatment with oral SCH 48973 (at dosages as low as 3 mg/kg/day) significantly reduced the viral titers in the brains and increased the survival of infected mice (232).

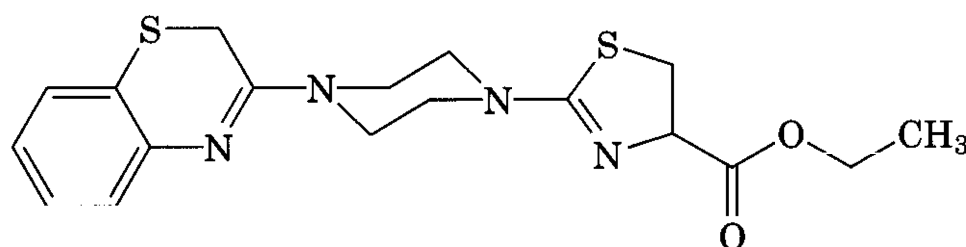
The crystal structure of PV2L complexed with SCH 48973 revealed that the compound was bound in a pocket within the β-barrel of VP1, in approximately the same position where the natural pocket factor binds to the virus. The structure also showed a surface depression located at the fivefold axis of PV2L capsid that is not present in the other two serotypes of poliovirus. In addition, unlike the other structures of enteroviruses, the entire PV2L VP4 is visible in the electron density, and bases of the genomic RNA are observed stacking with conserved VP4 aromatic residues (233).

Based on the structural features of earlier SDZ series of compounds, investigators of Sandoz synthesized SDZ 880-061 (87) which possessed a relatively broad antiviral spectrum (234). In contrast to SCH 48973, SDZ 880-061 inhibited HRV-14 in the nanomolar concentrations. It inhibited 85% of 89 HRV serotypes tested at a concentration of ≤3 μg/mL. HRV-42 and HRV-68 were among those being most sensitive to SDZ 880-061, but were refractory to inhibition by pirodavir.

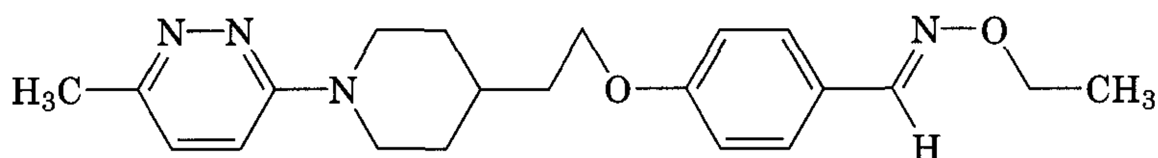
SDZ 880–061 was shown to bind to the same pocket, lying in the outer portion of the cavity (occupying 16 Å of the 21 Å length of the pocket) and cause similar, but less extensive, alternations of the HRV-14 VP1 backbone conformation compared with other capsid-binding antiviral agents. It might be that because it does not completely fill the hydrophobic pocket, SDZ 880-061 primarily interferes



(86) SCH 48973



(87) SDZ 880-061



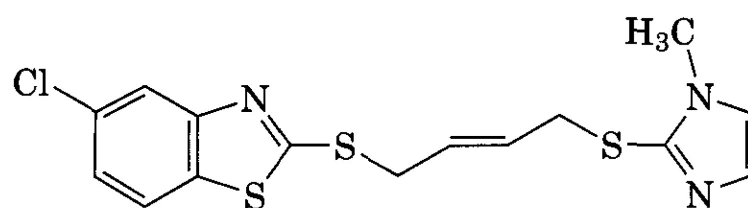
(88) BTA-188

with HRV-14 cellular attachment, and has only a marginal effect on uncoating (234).

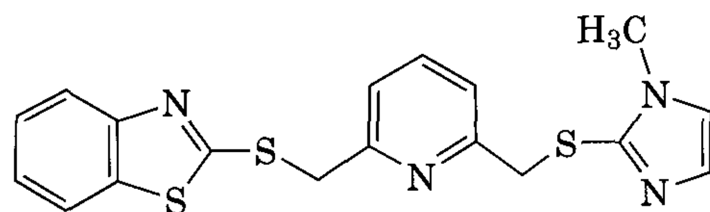
2.3.1.7.4 BTA-188. Biota Holdings has recently developed BTA-188 (88) as a new potential candidate for the treatment of HRV disease. This compound, a capsid-binding inhibitor, was discovered through molecular modeling and SAR analyses (235). When tested in cell culture, BTA-188 inhibited 87 of 100 numbered HRV serotypes (median EC_{50}, 0.01 μg/mL, ranging from 0.0003 to >0.1 μg/mL) and all 40 clinical isolates (median EC_{50}, 0.004 μg/mL, ranging from <0.001 to 0.05 μg/mL) (236). In comparison, BTA-188 inhibited HRV-14 with an EC_{50} of 1.0 ng/mL, whereas the EC_{50} values were 30 and 3.2 ng/mL for pleconaril and pirodavir, respectively (235). BTA-188 and pirodavir are closely related to each other structurally. BTA-188 showed good oral bioavailability in rodents and dogs, and could be detect in the nasal epithelium of dogs with levels several times above the *in vitro* EC_{50} for rhinoviruses. Its serum half-life was about 3 h (237).

2.3.1.1.5 Other Long-Chain Compounds. Hogle et al. have used a computational ligand design method called multiple copy simultaneous search (MCSS) to produce functionality maps of the drug binding sites of P3/Sabin poliovirus and rhinovirus-14 for the de *novo* design of new classes of picornavirus capsid-binding compounds (238). By simultaneously subjecting thousands of randomly placed copies of small molecular fragments, MCSS determined where specific functional (chemical) group have local potential energy minima in the binding site. Selected minima were clustered and connected with linkers [e.g., —$(CH_2)_n$—] to form candidate ligands. Their preliminary studies with fragment maps centered on the VP1 pocket suggested a template for a class of compounds that contain fused aromatic rings (e.g., benzimidazole) (238).

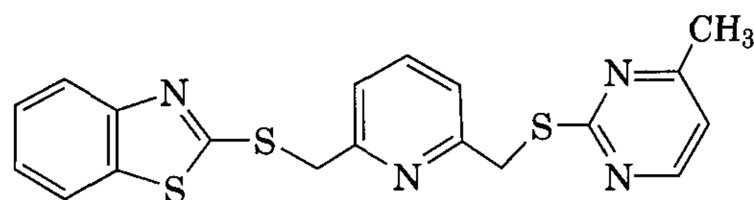
However, MCSS has its limitations because of the approximate methods being used. The same authors have recently reported a structurally biased combinatorial approach (a combination of structure-based design and combinatorial chemistry) to overcome this difficulty (208). A small set of combinatorial libraries of ligands resembling the template suggested by MCSS (based on both P3/Sabin and P1/Mahoney poliovirus) were synthesized and screened by using a novel assay in which virus is incubated with crude libraries and the components that bind are identified by mass spectrometry. Potential binders were re-synthesized as members of smaller sub-libraries, which, in turn, were re-screened with the mass spectrometry assay and tested for reduction of rate constant for uncoating (N-to-A transition) with an immunoprecipitation assay. Promising leads were individually synthesized and re-tested. This iterative method identified three promising leads [compounds L367 (**89**), L383 (90) and L396 (**91**)] from a crude library containing 75 compounds (208, 239). All three compounds were micromolar



(89) L367



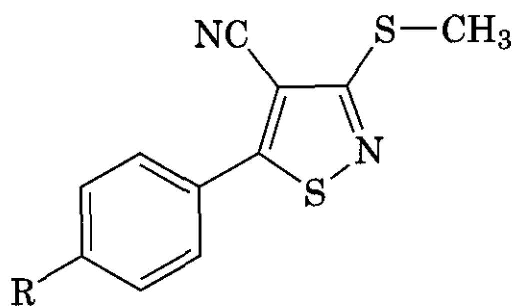
(90) L383



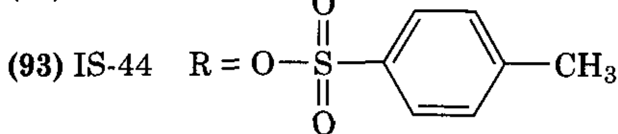
(91) L396

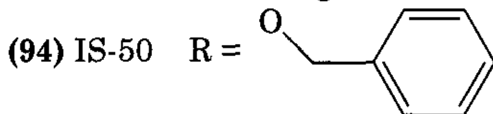
inhibitors against Mahoney strain of type 1 (Pl/Mahoney) poliovirus uncoating and infectivity. However, they failed to show activity against the Sabin strain poliovirus in a ViroPharma assay system. L383 also inhibited HRV-14 and HRV-3 infectivity in cell culture with EC_{50} of 0.8 and 0.55 μM, respectively. This work illustrates an advantage of designing ligand libraries, instead of individual compounds, as a means for drug design and discovery, because only enough information will be needed from the computational method, such as MCSS, to serve as a structural bias to guide (focus) the library design.

2.3.1.2 Isothiazoles, Dibenzofurans, and Dibenzosuberanes. The aforementioned compounds have a long-chain structure in common. The need to identify other chemical entities with activity against picornaviruses still exists. Among a series of 3,4,5-trisubstituted isothiazoles reported recently, IS-2 (92) showed the highest in *vitro* activity against poliovirus-1 (EC_{50}, 0.045 μM) and echovirus-9 (EC_{50}, 0.25 μM), if added to the cells within 1 h after poliovirus adsorption (240, 241). However, it was inactive against coxsackie B1 and rhinoviruses (242). In contrast, whereas IS-44 (93) and IS-50 (94) were inactive against both poliovirus-1 and echovirus-9, both compounds exhibited activity against rhinoviruses (242).



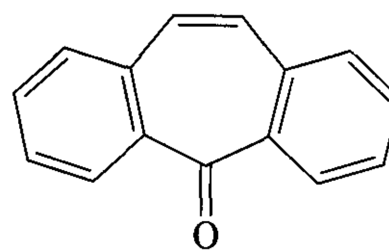
(92) IS-2 R = H





Of the 17 serotypes screened, 15 (88%) were sensitive to IS-50 (EC_{50}, 1–30 μM), including all of the group B serotypes screened. IS-44 was active against some group B rhinoviruses, with the lowest EC_{50} values for HRV-2, -85, and -89 (0.3, 0.3, and 0.1 μM, respectively). SAR showed that the active structural features seemed to have a short thioalkyl chain in the 3-position; a cyano or methylester group in the 4-position; and a not-substituted phenyl ring in the 5-position (241). Like the WIN compounds, IS-44 was shown to stabilize HRV-2 against thermal inactivation, suggesting that these isothiazoles might target the viral capsid.

Dibenzosuberenone (95) and 2-hydroxy-3-dibenzofuran carboxylic acid (96) are representatives of other structurally distinct compounds that have shown to block rhinovirus (e.g., types 14 and 16) replication in *vitro* (243). Time-of-addition experiments showed that compounds work during an early stage of the viral infection cycle, probably on adsorption or uncoating. Although these compounds are not as extremely potent against rhinoviruses as many of the compounds discussed above, they can serve as leads for novel therapeutic agents because they also have an additional anti-inflammatory property. Combination antiviral and anti-inflammatory therapies may be of significant benefit for intervention of common cold; an illness may not result from direct virus-induced tissue damage, but rather from release of inflammatory mediators (243–245).



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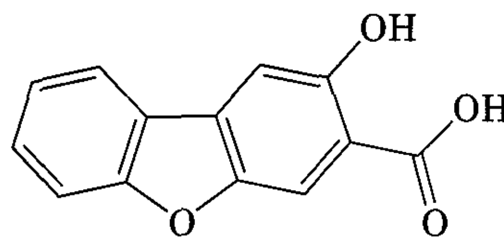


(96)

2.3.1.3 Soluble Intercellular Adhesion Molecules-l (sICAM-l). Intercellular adhesion molecule 1 (ICAM-1, CD54) is a cytokine-inducible cell surface receptor that has also been shown to be the receptor for nearly 90% of the human rhinoviruses (the major group of hu-

man rhinoviruses). Truncated soluble ICAM-1 molecules could inhibit a broad spectrum of rhinovirus serotypes in a variety of different cell lines (222), both by acting as a competitive inhibitor and by irreversible disruption of the capsid with release of the viral RNA (246). A truncated from of sICAM-1, $tICAM_{453}$, was recently tested as an intranasal spray in preventing HRV-16 infection in chimpanzees (247). As chimpanzees do not show clinical manifestations of diseases, measuring anti-rhinovirus serum antibody responses and virus shedding were used to detect infection. By both of these measures, intranasal application of $tICAM_{453}$ was efficacious as prophylaxis against rhinovirus infection (247). Efficacy of tremacamra, a recombinant sICAM-1 developed by Boehringer Ingelheim, has been tested in controlled trials in humans (248). Tremacamra, as an inhaled solution or as a powder, was given intranasally either before or after inoculation with HRV-39 (but before onset of symptoms). The results indicated that tremacamra was effective in reducing the symptoms of experimental common colds, regardless of whether the drug was given before of after the challenge with virus. The mean virus titer and the concentration of interleukin-8 (IL-8) were significantly reduced in the lavage fluid from treated volunteers. This drug seemed to be well tolerated. It could neither penetrate through the nasal mucosa nor interfere with development of neutralizing antibody (248). It remains to be determined if tremacamra would be effective if given after onset of symptoms.

2.3.2 Inhibitors of Picornaviral Proteases

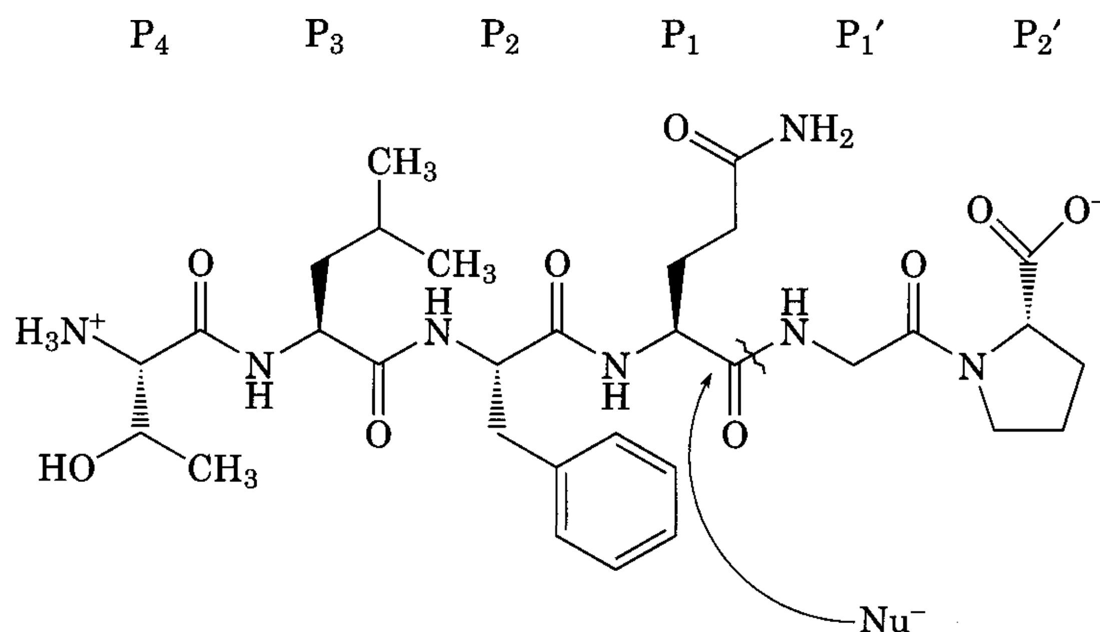
***2.3.2.1* Peptidic Inhibitors.** The genome of HRV contains a single open reading frame that can be translated into a large polyprotein, which undergoes further processing by two virally encoded proteases, designated 2A and 3C, to produce structural and functional proteins required for viral replication. In human rhinoviruses, the 2A protease separates the structural from the non-structural protein precursors, followed by the 3C protease, or its 3CD precursor, which carries out eight of the remaining nine proteolytic cleavage reactions, of which six cleavages occur at Gln-Gly bonds (249). The 2A and 3C proteases are a cysteine protease, which contain a nucleophilic cysteine residue at the active site [Cys-172 in hepatitis A virus (HAV) 3C (250), Cys-147 in both human rhinovirus (HRV) 3C and poliovirus 3C (251, 252), or Cys-146 based on HRV-14 numbering (253)]; their tertiary structures are similar to the trypsin-like serine proteases.

Because the 3C protease (1) plays a critical role in replication and maturation of HRVs, (2) has high selectivity for substrates containing Gln-Gly bonds, (3) has conserved active site among the known HRV serotypes (254), and (4) has no known cellular homologs; this viral enzyme seems to be an attractive antiviral target.

The hexapeptide H_2N-Thr-Leu-Phe-Gln-Gly-Pro-CO_2H (97) has been determined to be the minimal composition as an effective substrate for the HRV 3C protease, where Gln-Gly represents the scissile P_1-$P_{1'}$ bond (255). Peptide derivatives, where the scissile amide carbonyl was replaced with an electrophilic functionality (e.g., aldehyde, ketone, or Michael acceptor) that allows nucleophilic attack by the thiol group of the active site cysteine, have shown to be potent inhibitors of the HRV 3C protease (256, 257).

2.3.2.1.1 AG7088 and Peptidyl Michael Acceptors. A series of peptide-derived Michael acceptors, based on the X-ray structures of HRV-2 3C/inhibitors complexes, have been reported by investigators of Agouron Pharmaceuticals (257–263). Their investigations have led to the development of AG7088 (98), a potent and broad-spectrum anti-HRV compound currently in human clinical trials (administered as intranasal spray) for the treatment of common colds caused by the rhinovirus infection (Agouron press release, November 4, 1999).

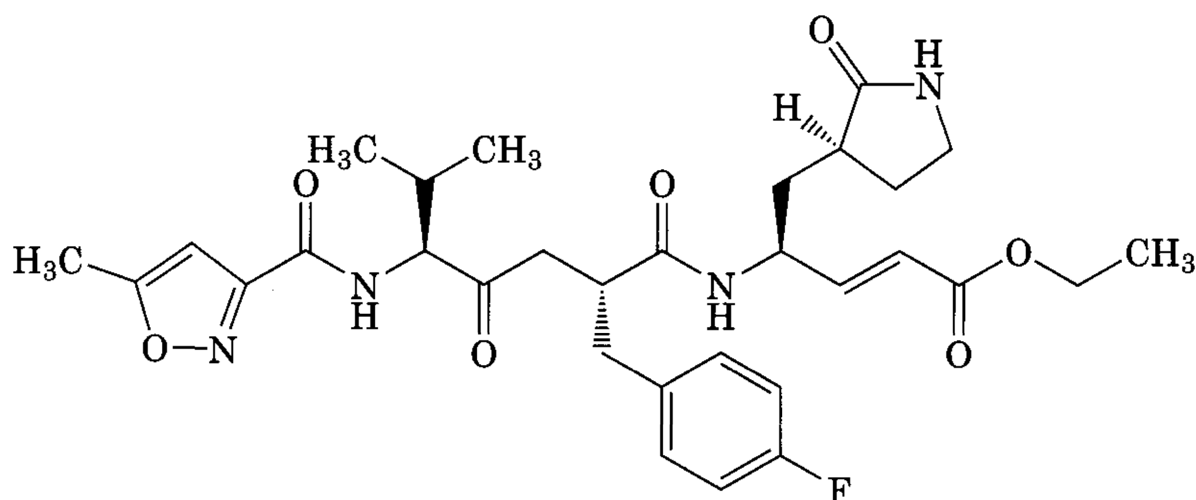
The drug design by Dragovich et al. at Agouron began with Cbz-protected tripeptides containing a P_1-$P_{1'}$ equivalent *trans*-α,β-unsaturated ester moiety (258). The representative methyl ester displayed relatively potent irreversible inhibition of HRV-14 3C protease; however, it exhibited moderate antiviral activity in H1-HeLa cells and was non-cytotoxic to the limits of its solubility. More importantly, it would not react readily with ubiquitous biological thiols (e.g., glutathione), suggesting



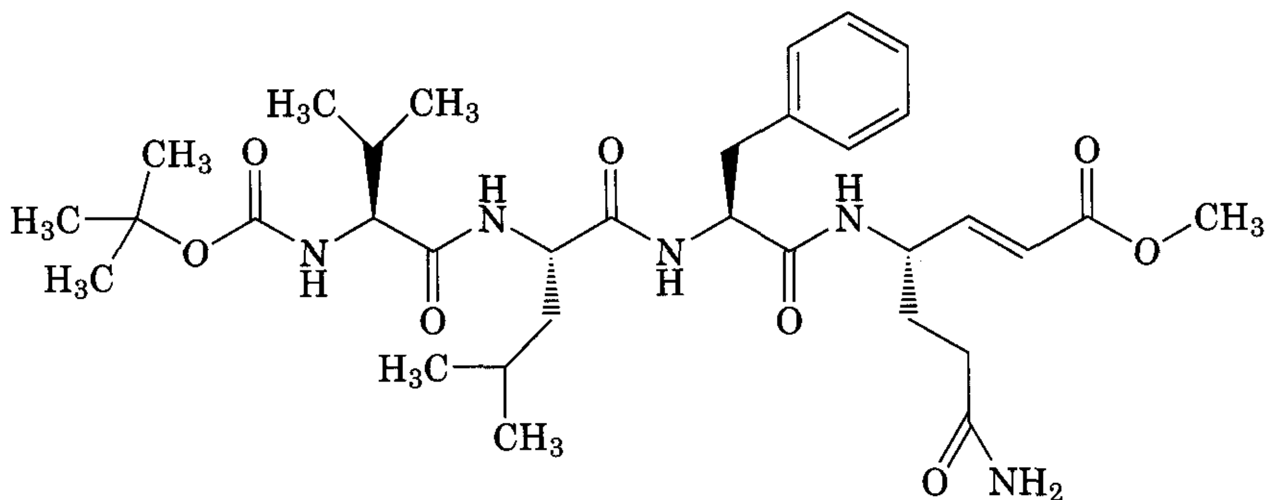
(97)

that peptidyl Michael acceptors of this type could be developed into useful anti-HRV agents (258). Crystallographic analyses of enzyme-inhibitor complexes showed a covalent bond was formed between the 3C **protease** active site cysteine residue (Cys-147) and the **β-carbon** of the Michael acceptors, confirming the binding orientation of these compounds (**258**). Systematic **SAR** studies suggested the following criteria for bioactivity.

- At least three amino acids were required for effective binding of the inhibitor to the enzyme (261,263).
- ***trans*-α,β-Unsaturated** esters were the optimal choice as the Michael acceptors (258, 261).
- The presence of a P_1 glutamine was essential (255, 263). Although a primary amide might not be required (**264**), cis-amide geometry was required for the y-carboxamide side-chain, and incorporation of an (*S*)-γ-**lactam** moiety could impose the proper **stereochemistry** (260,261).
- **P,** side-chains might be large and hydrophobic (**255**). Substitution of the **P,** phenyl ring with **4-fluorophenyl** moderately enhanced the activity; other modifications generally resulted in reduced activity (261,263).
- Replacement of the P_2-P_3 **peptide** bond with ketomethylene isostere slightly compromised the enzyme inhibitory activity but resulted in much improved antiviral properties (259,261).
- Substitutions with a wide variety of functionally at P_3 were generally tolerated (255, 258, 261). Replacement of P_3 **leucine** with valine improved both enzyme inhibitory and antiviral properties (259).



(98) AG 7088



(99)

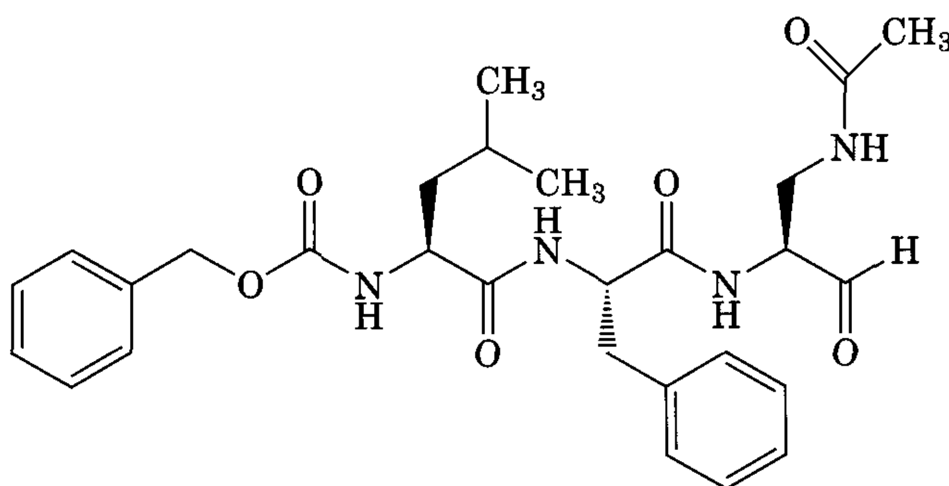
P_4 should be small and hydrophobic (255). Replacement of N-terminal P_4 amide with a thiocarbamate or 5-methyl-isoxasole-3-carboxamide also improved both enzyme inhibitory and antiviral properties (259,261,263, 265).

Interestingly, the structural features that made individual improvement in bioactivity could be combined in an additive manner (263), resulting in AG7088 (260,261).

AG7088 is a specific, potent, and irreversible inhibitor of HRV 3C protease (266). In cell cultures, it was non-toxic to the cells and inhibited a wide range of HRV serotypes, with a mean EC_{50} and a mean EC_{90} of 0.023 and 0.082 μM, respectively, and related echoviruses and enteroviruses tested (254, 266). Moreover, AG7088 seemed to be more potent and to have a broader anti-HRV spectrum than pleconaril when tested against HRV clinical isolates (267). AG7088 could be added up to 26 h after viral infection and still resulted in significant reduction of infectious virus and the levels of inflammatory cytokines, IL-6, and IL-8 (267a).

Another series of potent anti-3C protease peptidyl Michael acceptors have also been reported by Kong et al. (255) These compounds, typified by (**99**), showed a very rapid, 1:1 stoichiometric, covalent inactivation of the enzyme as determined by electrospray mass spectrometry.

2.3.2.1.2 Peptidyl Aldehydes and Ketones. Peptide aldehydes in which an aldehyde moiety (e.g., a glutaminal) serves as the glutamine isostere at the P_1 position have been reported as reversible inhibitors 3C proteases of both HRV and HAV [(256, 264) and references cited therein]. For obtaining enzyme-inhibitor cocrystal structures for guiding structure-based drug design, Agouron investigators reported the synthesis of a series of tripeptide aldehyde inhibitors (represented by compound 100) and their X-ray structures when they were covalently bound to HRV-2 3C protease (264).



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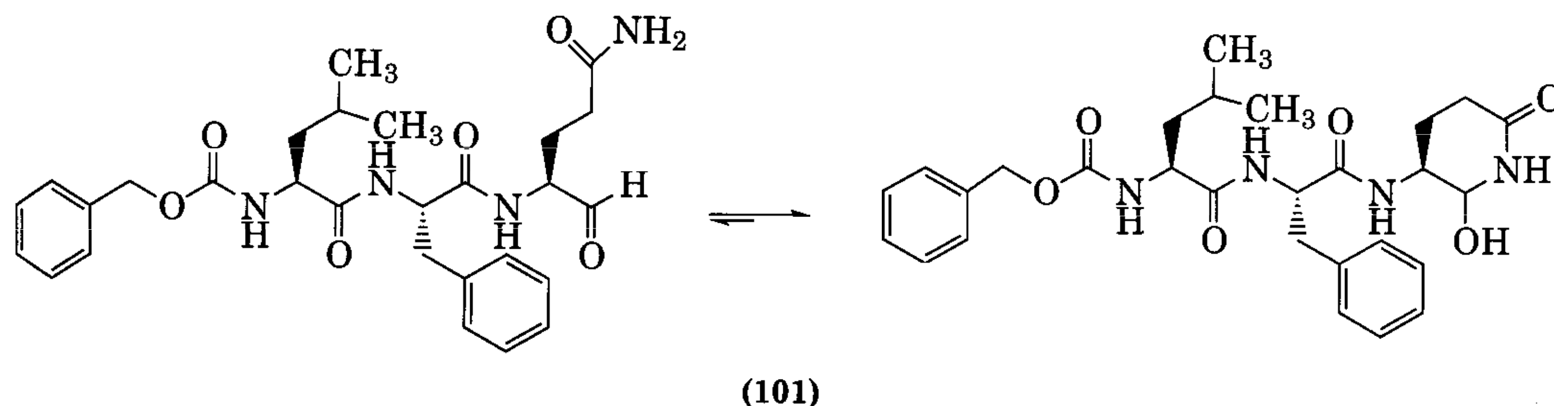
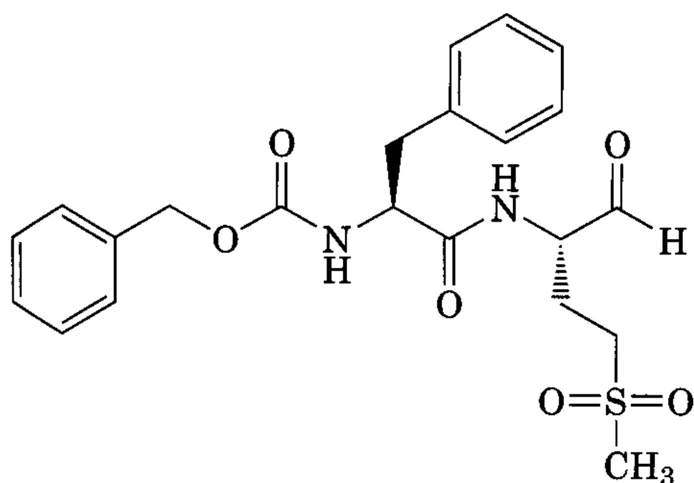


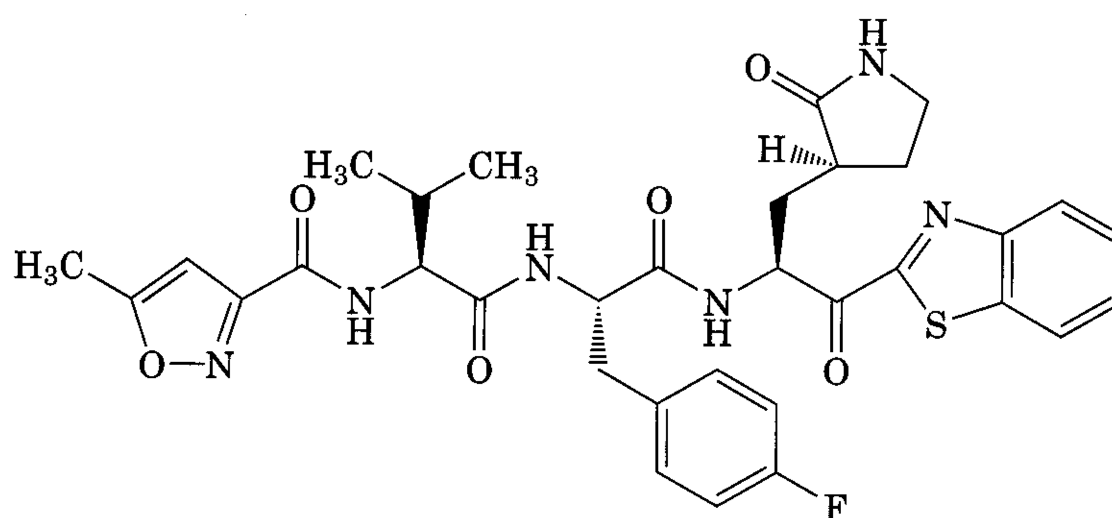
(101)

Only the *re* face of the aldehyde inhibitor was accessible to **Cys-147**. Isosteric replacement of P_1 **glutaminal** with N-acetyl-amino-alaninal not only prevented compound (100) from forming cyclic aminal, as in the case of compound **(101),** but also significantly improved the compound's enzyme inhibitory and **anti-**HRV properties over that of (101)(264). Independently, Lilly investigators reported that methionine sulfone residue could mimic the natural P_1 glutamine and demonstrated **LY338387** (102) as the first dipeptide **alde**hyde with low micromolar enzyme inhibitory (reversible inhibition) and *in vitro* antiviral (HRV-14) activity (268).



(102) LY 338387

Because an aldehyde-containing compound is prone to have higher toxicity as well as less selectivity and stability, Dragovich et al. recently reported the preparation of a **ketone-**containing tripeptide (103) and showed that his compound displayed very potent levels of reversible 3C **protease** inhibition along with low *in vitro* **cytotoxicity** and sub-micromolar antiviral activity against HRV serotypes 14, **1A,** and 10 **(269)**. The benzothiazole nitrogen atom seemed to be important for hydrogen bonding interaction with the enzyme active site, because drastically reduced enzyme inhibitory activity was seen with an analog containing a 2-benzothiophene moiety **(269)**. Independently, Vederas and Malcolm reported their work on a peptidyl monofluoromethyl ketone **(104)** as an irreversible inactivatior of **HAV** 3C **(270)**. Enzyme inactivation might involve the formation of a **(alkylthio)methyl** ketone accompanied by liberation of fluoride ion



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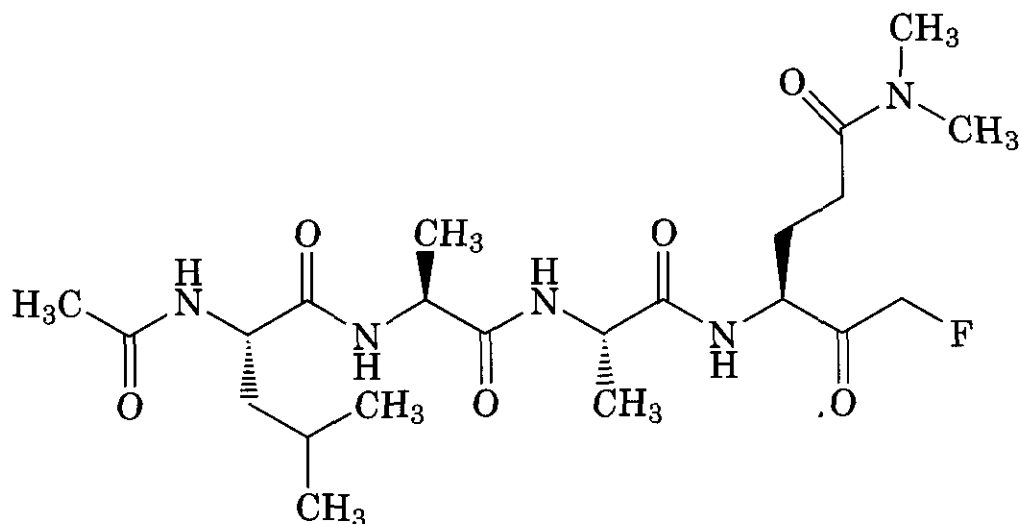
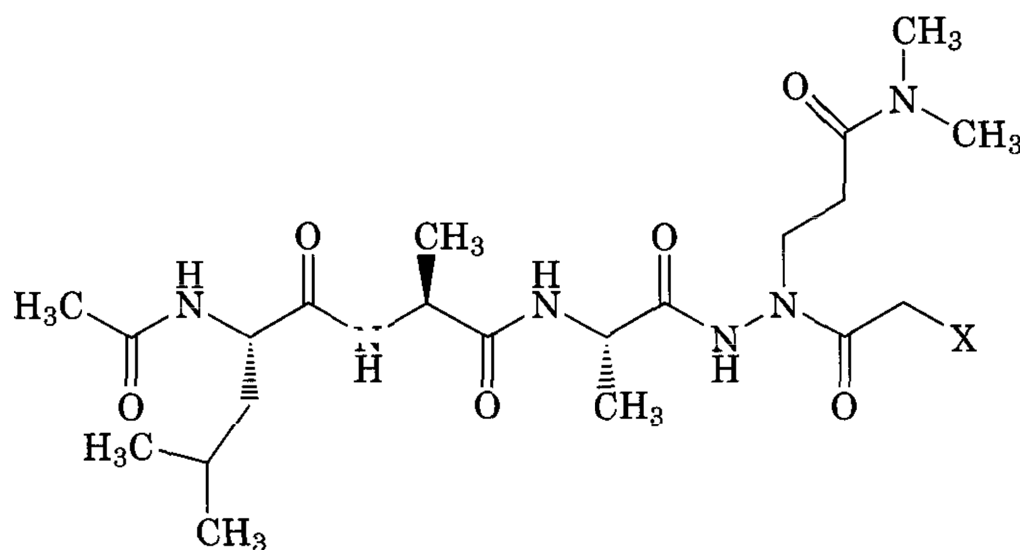


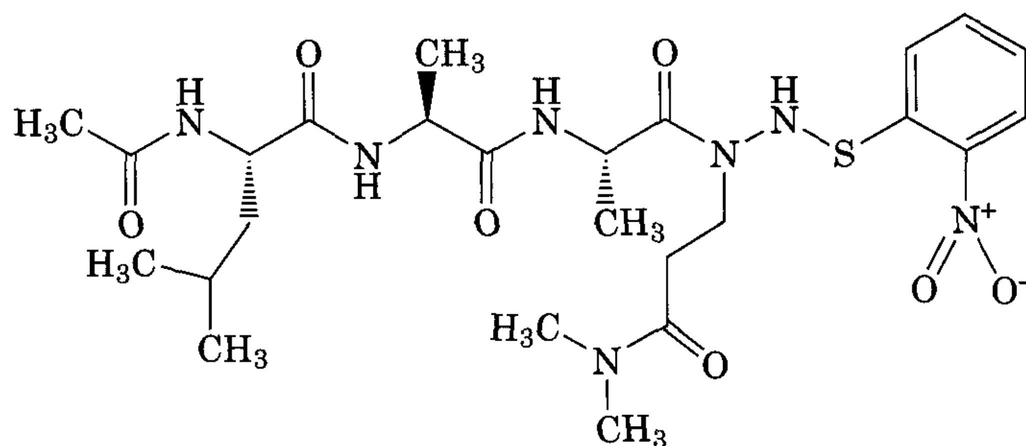
(104)

as monitored by the C^{13} NMR spectrum of enzyme-inhibitor complex and F^{19} NMR, respectively (270).

2.3.2.1.3 Azapeptides. **Azapeptides,** in which peptidic backbones contain hydrazine functionality, have also been known in the design of protease inhibitors (271,272). Vederas and Malcolm, in extension of their previous work on peptidyl haloacetyl ketones, further reported that the haloacetyl azaglutamine peptides (105)and (**106**) as well as the sulfenamide azaglutamine derivative (107) irreversibly reacted with HAV 3C (250). Monitored by electrospray mass spectrometry, it was shown that there was displacement of halogen by the active site thiol of Cys-172. This mechanism of



(105) X = Cl
(106) X = Br



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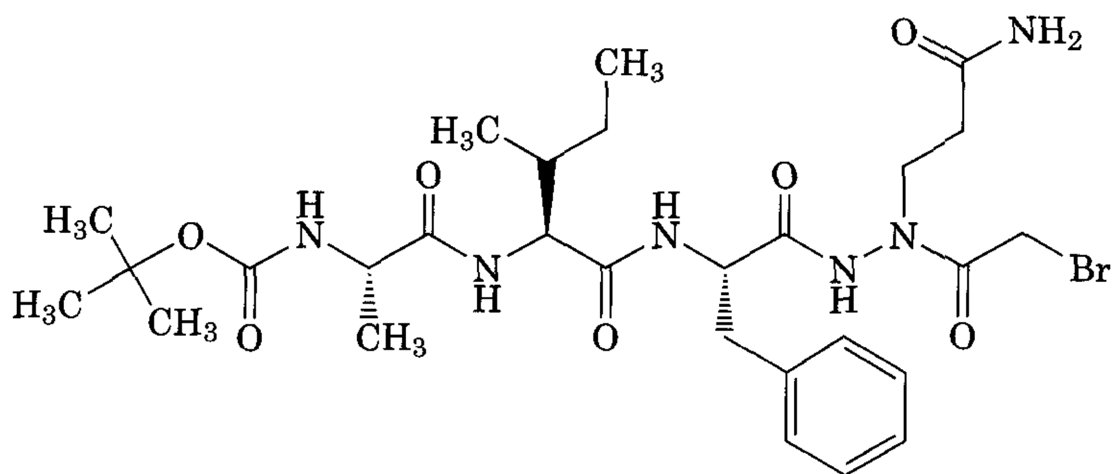
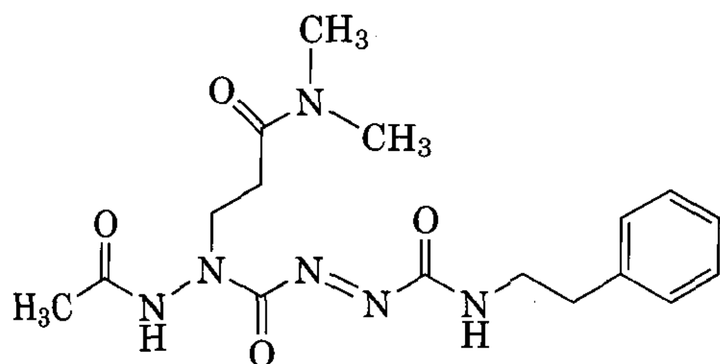


(108)

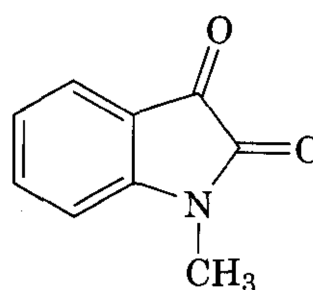
enzyme inactivation (i.e., formation of a covalent adduct between the enzyme and the inhibitor with the loss of a halogen) was further confirmed by the interaction of compound (**108**), prepared independently by Abbott Labs, with HRV-1B 3C protease (249).

Azodicarboxamide derivatives, represented by compound (**109**), were synthesized by Vederas et al. as another series of irreversible 3C protease inhibitors, which formed covalent adducts with the enzyme active site thiol through Michael addition onto the azo moiety (273). Because these compounds react readily with extraneous thiols (e.g., dithiothreitol), they might not be appropriate drug candidates themselves. However, the authors argued that these compounds could be potential tools for probing the enzyme active site because recognition elements could readily be built onto either side of the azo moiety. Most of the current cysteine protease inhibitors recognize on the P region of the active site (273).



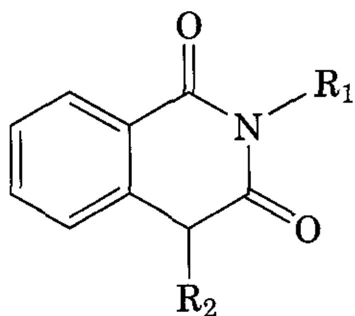
(109)

2.3.2.2 Nonpeptidic Inhibitors. Through a random screening effort, Lilly investigators identified the isatin (110) and the homophthalimides (111) as two interesting lead compounds (274). Subsequent SAR studies resulted in compound LY353349 (112) and LY353352 (113) with much improved enzyme inhibitory activity. Molecular modeling and mass spectrometry studies with LY353349 as the model suggested that this compound is tightly bound to the 3C enzyme in a ratio of 1:1, presumably through the C_3 (non-benzylic) carbonyl under nucleophilic attack by the active site cysteine (274). LY353349 and LY353352 also displayed activity against HRV 2A protease (275). Because the proteolytic cleavage of viral polyprotein is carried out first by the 2A protease followed by the 3C, dual inhibition of both enzymes might result in cooperative inhibition of viral replication (275). This effect could be illustrated by LY353352, which had anti-HRV-14 2A and anti-HRV-14 3C IC_{50} values of 63.3 and 55.4 μM, respectively; however, its anti-HRV-14 activity; exceeded the enzyme inhibitory activity, with an EC_{50} of 15.8 μM. Of interest, a related compound, LY343814 (**114**), showed excellent in *vitro* anti-HRV-14 activity (EC_{50}, 4.2 μM), and was inhibitory to HRV-14 2A (IC_{50}, 20 μM), but not of HRV-14 3C (IC_{50}, >200 μM) (275).

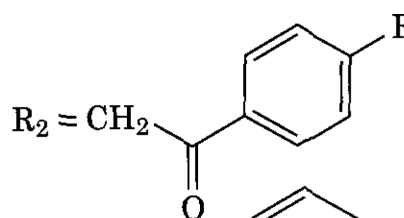


(110) Isatin

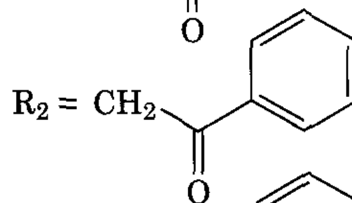
At the same time, Agouron investigators, who, based on the structural determinants



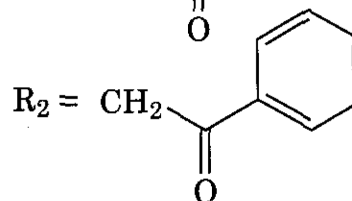
(111) $R_1 = CH_3$



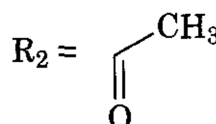
(112) LY 353349 $R_1 = CH_2$ ... S—CH3



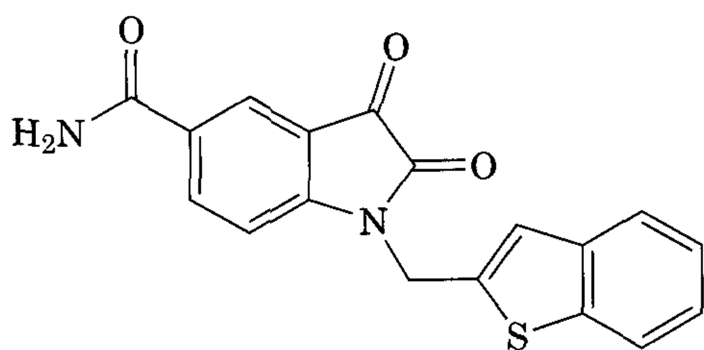
(113) LY 353352 $R_1 = CH_2$ ... O ... CH3



(114) LY 343814 $R_1 = CH_2$ ... CH3



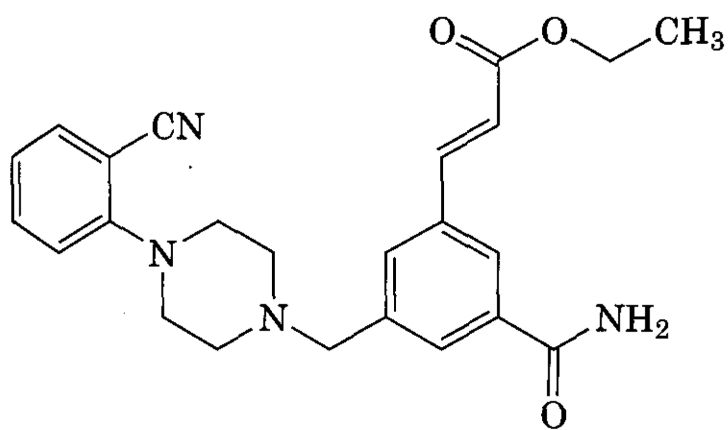
around the scissile cleavage, P_1 recognition, and S_2 sites, envisioned the cyclic a-keto amide isatin structure as a good core for design of small molecule protease inhibitors, reported a series of synthetic isatins (2,3-dioxindoles) (253). Molecular modeling and SAR studies resulted in compound (115) as the



(115)

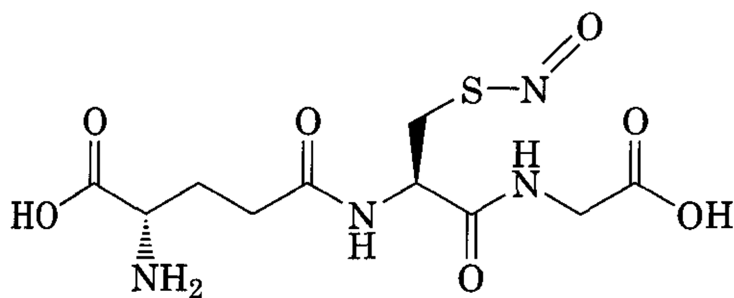
most potent inhibitor of HRV-14 3C protease (K_i, 2 nM) in the series. X-ray co-crystal structure further confirmed the existence of a covalent bond between Cys-147 and the electrophilic C3 of isatin and other important interactions between the enzyme and (115). Disappointingly, because of its apparent cytotoxicity, no *in uitro* anti-HRV-14 activity was demonstrated (253).

In a continuous effort to find orally bioavailable small non-peptide 3C protease inhibitors, Agouron investigators used structure-based design and parallel synthesis on solid support to generate a structurally biased library of Bsubstituted benzamides that contain a C_3 α,β-unsaturated ester moiety (276). The benzamide-Michael acceptor core was designed to mimic the P_1 recognition element of the natural 3C protease substrate and the C_5 substituents were to optimize the binding of the inhibitors to the S_3-S_4 subsites of the enzyme. In such an arrangement, the α,β-unsaturated ester group would be expected to undergo irreversible covalent 1,4-addition by the nucleophilic catalytic cysteine residue on the enzyme (confirmed later by a co-crystal structure). Surprisingly, these compounds generally showed very potent antiviral activity, despite their moderate enzyme inactivation rates. For the most potent compound **(116)**, the antiviral EC„ of 0.6 μM was exceptional, given the modest K_{obs}/I of 139 M/s (276).



(116)

Because nitric oxide (NO) or NO donors could inactivate many cysteine-containing enzymes, HRV **3C protease** might also be susceptible to NO donors. To test this **mechanism-based** hypothesis, Wang et al. demonstrated that S-nitrosothiols [exemplified by GSNO (**117**)] could achieve a time- and **concentra-**

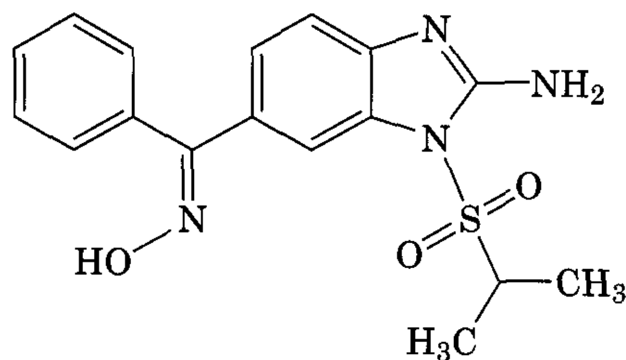


(117) GSNO

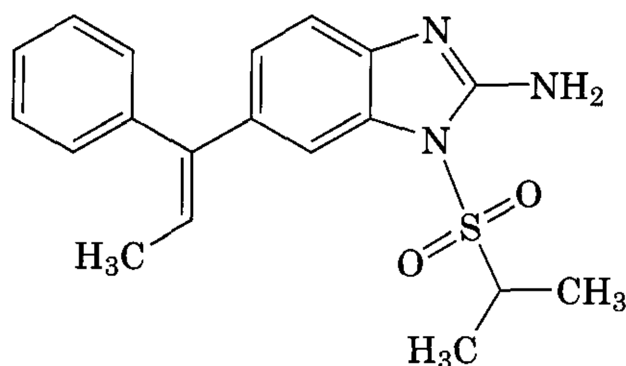
tion-dependent inactivation of HRV-14 **3C protease** with second-order rate constants (277). It was shown that the inhibition by these compounds was caused by the formation of an **S-nitroso adduct** and that the inactivation of the enzyme could be reversed by the addition of nucleophilic thiols, such as **dithio**threitol, to the reaction mixture (277).

2.3.3 Inhibitors of Picornaviral Replication (Enviroxime and Analogs). In the early 1980s, two benzimidazole derivatives, enviroxime (**118**) and enviradene (119) were discovered and evaluated in the clinic by Lily Research Laboratories. However, both compounds failed in clinical studies because of poor oral bioavailability in humans. Enviroxime was also associated with emetic side effects (see references cited in Ref. 278).

Enviroxime possesses significant antiviral activity against both rhinoviruses and enteroviruses. Although a function involving



(118) Enviroxime

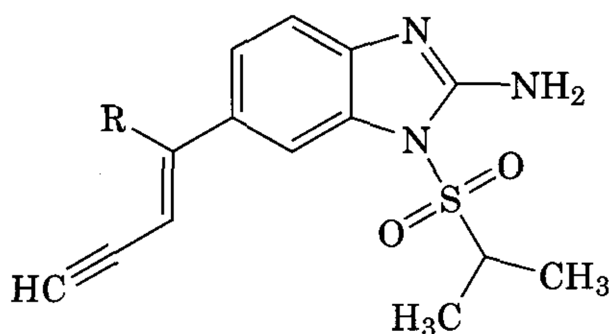


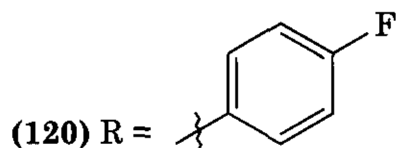
(119) Enviradene

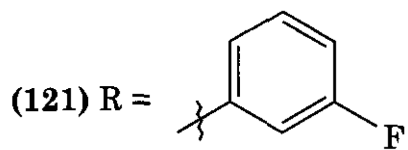
charged residues in the **3A** region of the **3AB** protein of HRV-14 has been suggested as a plausible target for enviroxime, the exact mechanism of action remains unclear. **A** mutant with an increased level of resistance to enviroxime showing mutations in multiple proteins or RNA sequences further suggested that enviroxime might target a complex of viral proteins **and/or** cellular factors (279). Interestingly, efforts to develop drug-resistant mutants mostly resulted in the selection of drug-sensitive mutants that exhibit no increase in their EC_{50} values in standard plaque reduction assays (**280**). Despite extensive efforts, there was no clear evidence to show direct binding between enviroxime and a viral or a host protein. Such a complex mechanism of inhibition might explain the low levels of viral resistance to enviroxime and its related inhibitors (279).

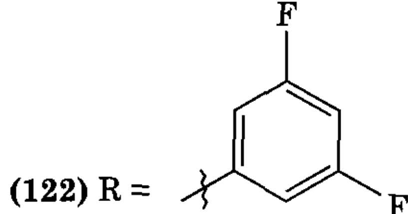
Intrigued by the potential of enviroxime and its analogs, efforts to synthesize new analogs have recently been renewed with an emphasis on optimizing antiviral activity while maximizing oral bioavailability. It has been known that the vinyl oxime moiety of **enviroxime** is metabolically labile (**281**). In the case of enviradene, the vinyl methyl group also undergoes rapid allylic oxidation to a **hydroxy-**

methyl metabolite resulting in very low blood levels of enviradene in man and **monkeys.** The bioavailability improved when methyl was replaced with by acetylene, the resulting **vinyl-**acetylene derivative showed increased levels in blood in monkeys (282). Moreover, fluorine substitution on the left-hand aromatic ring further enhanced the oral blood level (280, 282). These compounds remained as potent and potentially broad-spectrum **anti-picorna-**viral inhibitors as illustrated by compound **(120)**, which inhibited poliovirus-1 (Mahoney)





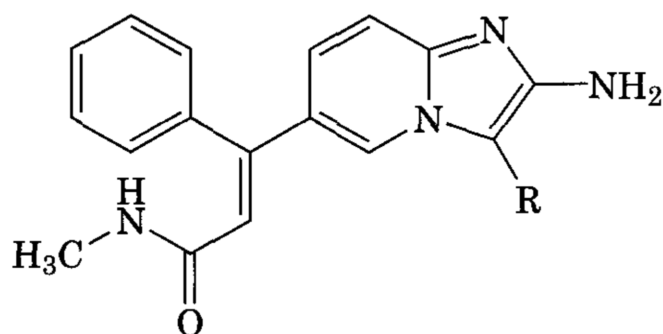


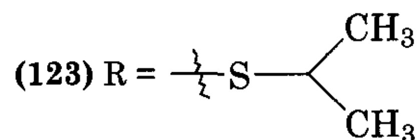


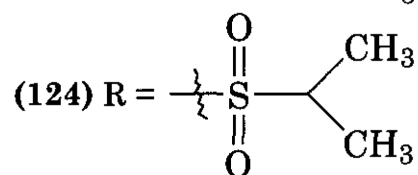
and HRV-14 with EC_{50} of 0.04 and 0.13 μg/mL, respectively, in a plaque reduction assay. It was also efficacious by oral administration in treating a coxsackie A21 infection in CD-1 mice (278,282).

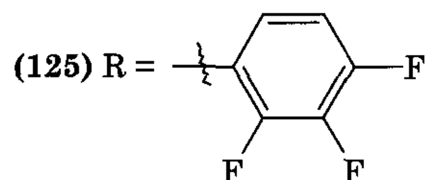
In a study on cytochrome P450 function and hepatic porphyrin levels in mice, two fluoro-substituted vinylacetylene **benzimid-**azoles, (121) and **(122)**, which were shown to attain significant levels of plasma concentrations after oral dosing, caused a marked effect on liver enzymes and hepatic porphyrin levels. The multiple dose studies showed significant increases in liver weights as well as increases in serum levels of enzymes suggestive of **hepa-**totoxicity (278). These hepatotoxic effects are most likely related to the acetylene moiety because structurally similar enviroxime and enviradene were free of these adverse effects during their respective preclinical and clinical evaluations. Nevertheless, there are marketed alkyne-containing drugs that do not have obvious detrimental effects on **P450,** its function, or hepatic porphyrin levels (278).

In studies with a series of C2 analogs of enviroxime, it was found that primary amino substitution, as in enviroxime, was the most **antivirally** active. The activity was reduced with those substituted with a larger group at C_2, which provided a repulsive steric interaction at N_3, resulting in less flexible conformation. In the case of enviroxime, a hydrogen atom of C_2 amino was shown to form an intramolecular hydrogen bond with the N_1 sulfonyl oxygen. This interaction might act to enhance the activity by holding the second hydrogen in a desirable orientation toward the enzyme active site (283). However, such internal hydrogen bonding might not be important in a series of 2-amino-3-substituted-6-[(E)-**1-phenyl-2-(*N*-methylcarbamoyl)vinyl]imid-azo[1,2-α]pyridines,** as evidenced by the reduction of anti-HRV-14 activity when the **sul-**fite in compound **(123)** (EC_{50}, 0.17 μg/mL; by a plaque reduction assay) was replaced with corresponding sulfone as shown in (124) (EC_{50}, 0.64 μg/mL). The potency remained the same even the sulfite in (123) was replaced



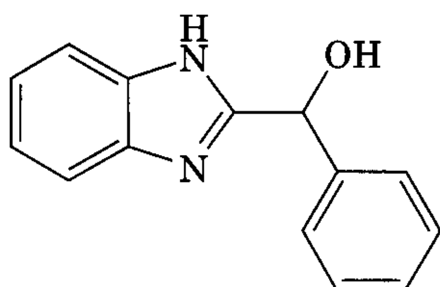




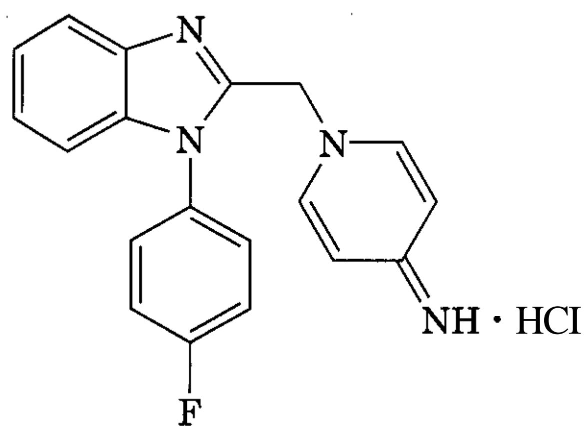


with a fluorine substituted aromatic ring, as demonstrated by compound (**125**) (284). When enviroxime (118) was combined with disoxaril (WIN 51711) (**82**), the combination exerted a significant synergistic inhibitory effect on poliovirus-1 (Mahoney) replication in FL cells, without concomitant synergic cytotoxic effect (285, 286). The combination also demonstrated synergistic in vitro antiviral effect in FL cells and in vivo protective effect in newborn mice infected with coxsackievirus B1 (287).

2.3.4 Inhibitors of Picornaviral Protein 2C. The 2C protein of picornaviruses is a multi-Functional and highly conserved non-structural protein involved in viral RNA replication, viral encapsidation, and other functions, such as membrane binding, RNA binding, protein–protein interactions, and NTPase activity (288, 289). Recently, mutational analyses with drug-resistant and drug-dependent variants revealed that two previously known benzimidazoles, HBB (126) (288, 290) and MRL-1237 (127) (291), exert their antiviral action



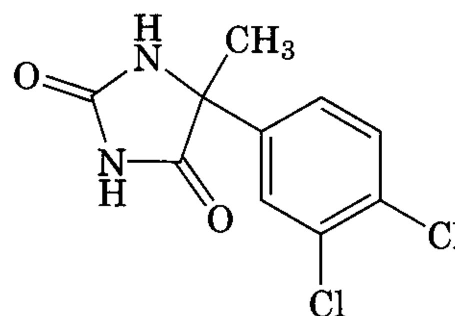
(126) HBB



(127) MRL-1237

on the 2C protein. However, the exact mode of action of these compounds remains to be elucidated.

Similar studies with 5-(3,4-dichlorophenyl) methylhydantoin (128) suggested that the hydantoin derivative inhibits the encapsidation

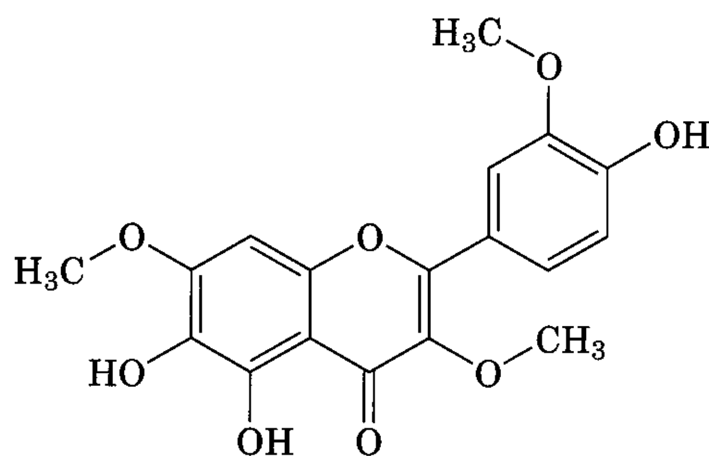


(128) 5-(3,4-Dichlorophenyl)methylhydantoin

function of the 2C protein (292). This finding was further confirmed with experiments in a cell-free system showing that this compound is an inhibitor of poliovirus assembly. It also inhibited the post-synthetic cleavage of poliovirus (293).

2.3.5 Other Anti-Picornaviral Inhibitors

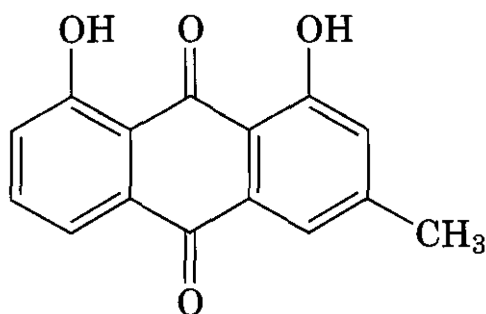
2.3.5.1 Natural Products and Synthetic Analogs. By employing antiviral assay-guided fractionation of the ethanolic extract of *Pterocaulon* sphacelatum, a traditional medicinal plant used by the Australian aboriginal people, Semple et al. reported the isolation of the flavonoid chrysosplenol C (**129**), which.



(129) Chrysosplenol C
(3,7,3′-Trimethoxy-5,6,4′-trihydroxyflavone)

showed in vitro activity against poliovirus with an EC_{50} of 0.27 μg/mL (0.75 *μM*), and a maximum non-toxic concentration to proliferating Buffalo green monkey (BGM) kidney cells of 4 μg/mL (294). This was the first report of isolation of chrysosplenol C from the genus Pterocaulon.

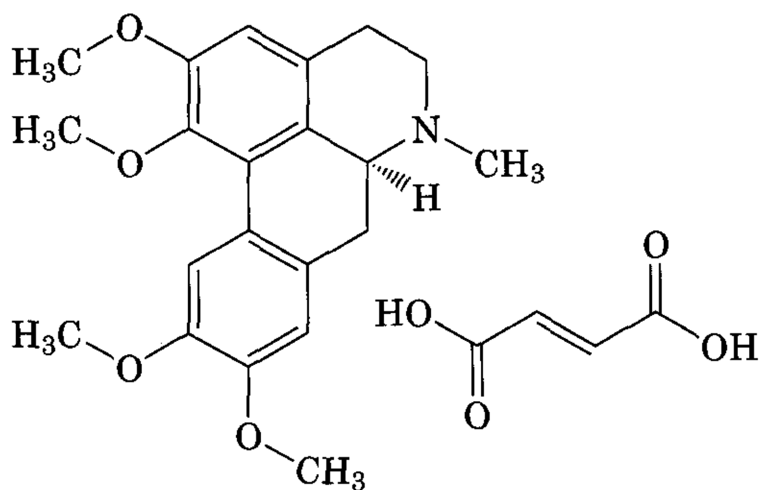
The same group of investigators also discovered chrysophanic acid (**130**), isolated from another Australian aboriginal medicinal plant,



(**130**) Chrysophanic acid
(1,8-Dihydroxy-3-methylanthraquinone)

Dianella longifolia, to be inhibitory to the replication of poliovirus types 2 and 3 in BGM kidney cells, with EC_{50} values of 0.21 and 0.02 $\mu g/mL$, respectively (295). The maximum non-toxic concentration for proliferating BGM cells was 12.5 $\mu g/mL$. This compound did not have an irreversible virucidal effect on poliovirus particle. Rather it might inhibit an early stage in the viral replication cycle as suggested by a time-of-addition study. Disappointingly, this compound did not show in *vitro* activity against coxsackievirus types A21 and B4 and human rhinovirus-2. Nevertheless, this was the first report of activity of an anthraquinone derivative against a non-enveloped virus (295). Structurally related anthraquinones have been shown to inhibit enveloped viruses by both virucidal and non-virucidal mechanisms (see references cited in Ref. 295).

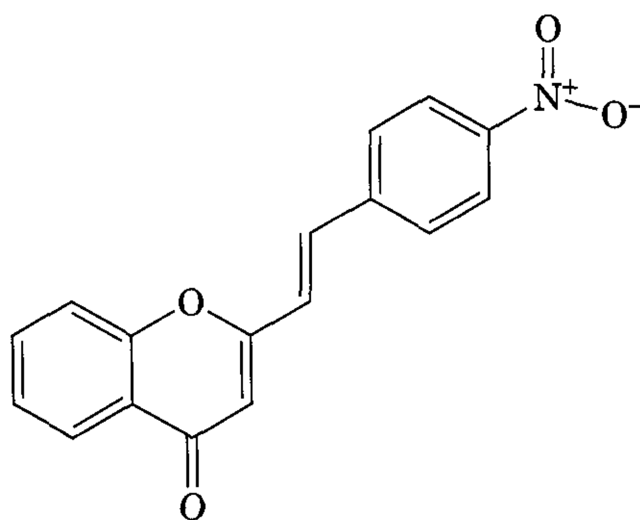
A series of aporphinoid alkaloids were also shown active against poliovirus type 2 (296). Glaucine fumarate (**131**) was among the most active ones. It inhibited poliovirus replication



(**131**) Glaucine fumarate

in Vero cells with EC_{50} and CC_{50} of 9 and 142 μM (in a CPE assay), respectively. It was active even when added 1 h post-infection. This activity was further confirmed by reduction of virus yields after a single cycle of replication. The nature of the 1,2-substituents of the isoquinoline moiety was critical for activity and cytotoxicity; a methoxyl group at C_2 position seemed to be of most importance for anti-polioviral activity (296).

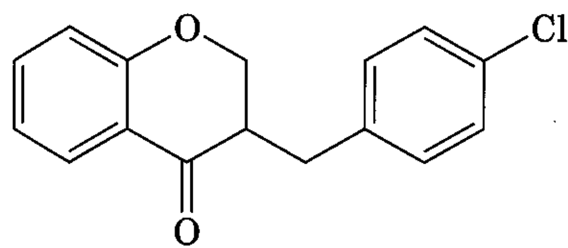
Desideri et al. have reported the anti-picornavirus activity of two series of synthetic flavonoids. First, 2-styrylchromones were evaluated against two selected human rhinoviruses, HRV-1B and HRV-14, by a plaque reduction assay in HeLa cells; it was shown that the majority of the compounds interfered with replication of both viruses. The most active compound was 4-nitro-2-styrylchromone (**132**),



(**132**)

which had EC_{50} values of 3.9 and 1.3 μM for HRV-1B and HRV-14, respectively. Its maximum non-toxic concentration for HeLa cells was 12.5 μM (297). In a second series of compounds, all synthetic homo-isoflavonoids were weakly effective against poliovirus-2, whereas they exhibited a variable degree of activity against HRVs 1B and 14 (298). Interestingly, the configuration of the chiral center in position 3, as illustrated by isoflavanone (133) did not seem to influence the activity against both rhinovirus serotypes, because the two enantiomers and the corresponding racemate were equipotent (299).

2.3.5.2 ***Other Compounds.*** Although combinatorial chemistry has been recognized as a powerful tool for drug discovery, application of



(133)

this technology has not been found widely used in antiviral research. One interesting work was recently reported in a communication by Lilly investigators who used a solution phase synthesis, coupled with a solid-supported aminomethylpolystyrene as "covalent scavenger" for removing isocyanate impurities, to yield equimolar mixtures of ureas for antirhinoviral testing in a whole cell assay (300). Subsequent deconvolution of hit mixtures led to two low cytotoxic leads, (134) and (135), with micromolar inhibitory activity against HRV-14.

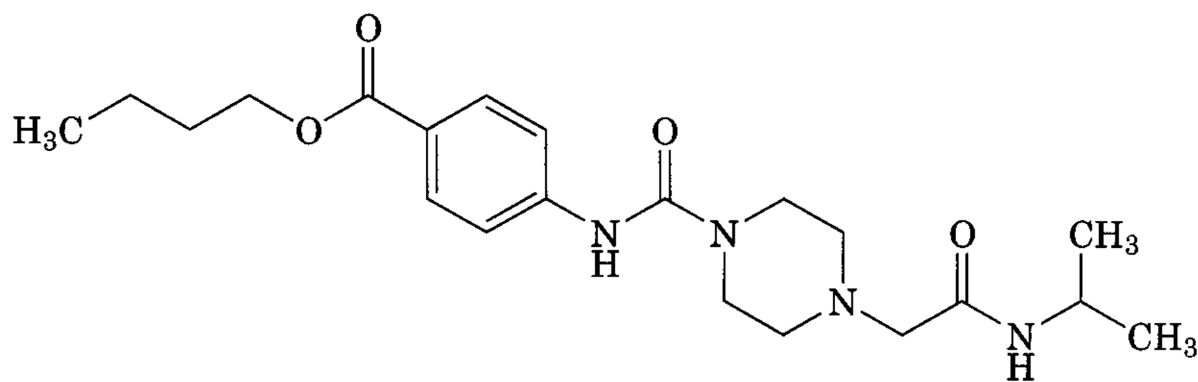
2.4 Hepatitis C Virus

The HCV genome is a 9.5-kilobase, single-stranded, positive-sense RNA molecule, containing a single open reading frame that encodes for a polyprotein of 3010–3033 amino acids. This polyprotein undergoes maturational processing in the cytoplasm or in the endoplasmic reticulum (ER) of the infected cell to produce at least 10 mature proteins (C, E1, E2, P7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B). In addition, an unusual feature of the HCV viral genome is the presence of two long and highly ordered untranslated regions at both 5' and 3' ends. All of these viral functions are potential target for therapeutic intervention; nevertheless, recent advances in anti-HCV drug development have largely focused on the 5' untranslated region, the core, and the NS3 protein.

2.4.1 Inhibitors of HCV 5' Untranslated Region and Core Gene

2.4.1.1 Ribozymes and Antisense Oligonucleotides. The 5' UTR, which encodes the HCV internal ribosome entry site (IRES), and the core gene encoding the nucleocapsid protein of HCV are highly conserved among HCV isolates, making them attractive targets for ribozyme- and antisense oligonucleotide–based antiviral strategies (301).

Ribozyme Pharmaceuticals (RPI) reported their design and synthesis of hammerhead ribozymes targeting various conserved sites in the 5' untranslated region (UTR). These ribozymes significantly reduced HCV 5' UTR-mediated expression in a 5' UTR-luceferase reporter system, as well as inhibited replication of an HCV-poliovirus chimera (302). Moreover, a nuclease resistant ribozyme, tar-



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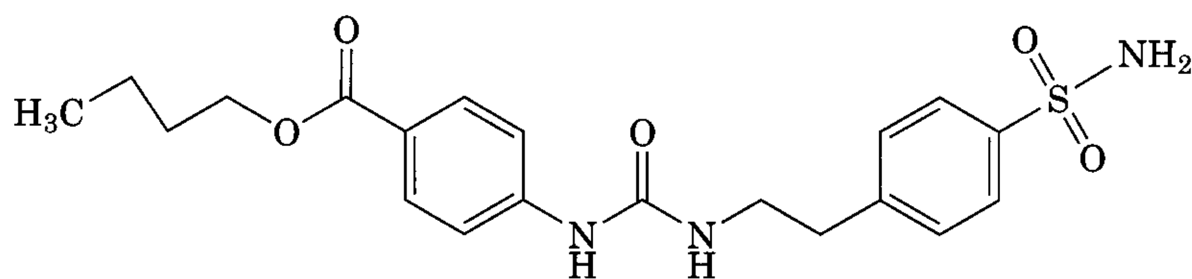


(135)

geting site 195, was selected for pharmacokinetics and tissue distribution studies after intravenous and subcutaneous administration in mice. The results showed that the ribozyme can be taken up and retained in the liver cells (303). RPI has recently completed their clinical trials of a 28-day safety and pharmacokinetic study of Heptazyme (LY 466700) following daily subcutaneous injections. Phase II clinical trials to study the drug's dose-ranging and efficacy makers in chronic HCV patients have been planned (RPI Press Release, September 28,2000).

Wu et al. reported that two hammerhead ribozymes, which were designed to target just upstream of the start codon of the viral transcript and the core, respectively, were capable of suppressing HCV-luciferase reporter gene expression in a cell-free system and in transfected Huh7 cells (304). The same two regions were also targeted by DNA analogs of ribozymes (305). The third approach used by the group was to apply antisense oligonucleotides directed against a sequence in the 5'UTR IRES region and a region of the UTR overlapping the core protein translational start site of HCV. They reported that the antisense oligonucleotides, in the form of asialoglycoprotein-polylysine complexes, could be delivered by receptor-mediated endocytosis and caused specific inhibition of HCV-directed protein synthesis, as monitored by the expression of luciferase activity, in cells (306).

Patients infected with HCV genotype 1b have shown the poorest rate in response to interferon therapy. Kay et al. incubated total RNA from HCV 1 b positive human livers, containing plus and minus strands, with a library of hammerhead ribozymes, and thereby isolated several effective ribozymes directed against a conserved region of the plus and minus strand of the HCV genome (307). The ribozymes were found to reduce or eliminate the respective plus or minus strand HCV RNAs in cultured cells and from primary human hepatocytes obtained from infected patients (307). Other studies by Hayashi et al. were targeting the core region of HCV 1b for the design of hammerhead ribozymes. They found that the ribozyme, whose cleavage site is located nearest to the initiation codon of the HCV ORF, showed the most efficient cleavage of the target RNA. On the other hand, the ribozyme with the cleavage site located farthest from the initiation codon blocked viral translation in a rabbit reticulocyte lysate most efficiently (308).

Hairpin ribozymes targeting HCV 5' UTR and capsid gene regions were reported by Barber et al. (309, 310). Because the 5' UTR contains considerable secondary structures, which could interfere with the cleavage activity of a ribozyme, the authors also prepared facilitator RNAs, trying to help to relax the secondary structure, and therefore, enhance the binding and activity of the ribozyme.

Different domains within the 5' UTR and core region have also been exploited as potential targets for inhibition of HCV translation by antisense oligonucleotides and oligodeoxynucleotides (ODNs). Isis Pharmaceuticals reported two phosphorothioate ODNs, ISIS 6095, which targeted a stem-loop structure within the 5' UTR known to be important for IRES function (nt 260–2791, and ISIS 6547, which targeted sequences spanning the AUG used for initiation of HCV polyprotein translation (nt 330–3491, effectively inhibited HCV gene expression as monitored in transformed hepatocytes (311). Reduction of RNA levels and the subsequent protein levels by these phosphorothioate ODNs was associated with RNase H cleavage of the RNA strand of the oligonucleotide–RNA duplex. On the other hand, 2'-modified (e.g., 2'-*O*-methoxyethyl) phosphodiesters oligonucleotides inhibited HCV core protein synthesis with comparable potency to phosphorothioate ODNs by an RNase H-independent mechanism (311,312). In mice infected with an HCV-vaccinia virus recombinant, subcutaneous administration of ISIS 6547 and ISIS 14803 showed specific and dose-dependent inhibition of an HCV-luciferase reporter gene expression in the livers (313). (The two 20-base oligomers have the same sequence, but ISIS 14803 has 5-methylcytidine residues at all respective cytidine positions in ISIS 6547.) In March 2000, the company initiated clinical trials with ISIS 14803 in patients who failed interferon or interferon-plus-ribavirin therapy (314). In addition, investigators of Isis Pharmaceuticals reported a new probing strategy by using hybridization affinity screening and RNase H cleavage anal-

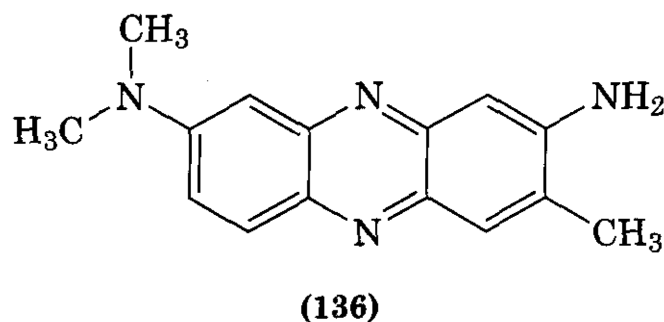
ysis on a fully randomized sequence DNA oligonucleotides (10-mer) library to identify energetically preferred hybridization sites on folded target RNA (315). The hypothesis is that binding-optimized shorter oligomers (10–15-ers) may have equivalent or greater affinity and specificity for hybridization site than longer ones (e.g., 20-mers).

Caselmann et al. reported that a phosphorothioate ODNs complementary to nucleotides 326–348 spanning the 3' end of the UTR and the start codon of the polyprotein precursor was very efficient in inhibiting HCV gene expression (316, 317). Moreover, strong inhibition of HCV gene expression was still remained with modified oligomers having methylphosphonate or benzylphosphonate modifications located at the termini (316, 318). Inhibition correlated with induction of RNase H activity. Furthermore, these oligomers could be coupled with cholesterol or bile acid to enhance their lipophilicity for improved liver specific delivery (319).

An independent investigation conducted by Vidalin et al. identified a domain within 5' UTR, which contains the conserved pyrimidine-rich tract (nt 103–138), as a new region susceptible to ODN inhibition (320). They also evaluated a-anomer phosphodiesters ODNs. It was found that α-ODNs inhibited HCV translation as efficiently as their β-ODNs counterparts. Wands et al. demonstrated that translation of HCV RNAs was efficiently inhibited by antisense RNA when the HCV core-luciferase cDNA was co-transfected with antisense RNA-producing contracts in Huh7 cells (321).

2.4.1.2 inhibitors of HCV internal Ribosome Entry Site. As a crucial RNA genomic structure required by HCV for initiation of translation, HCV internal ribosome entry site (IRES) has become an attractive target for therapeutic intervention. A small yeast RNA (a 60-nt-long RNA called inhibitor RNA or IRNA), which has previously shown to selectively block internal initiation of translation programmed by poliovirus RNA, has also shown to block HCV IRES-mediated translation in transient transfection of hepatoma cells (Huh-7) and a hepatoma cell line constitutively expressing IRNA. In these cells, it was further shown that replication of chimeric poliovirus containing the HCV IRES element was blocked (322). Site-directed mutagenesis studies suggested that the secondary structure of IRNA might be important in its competing with viral IRES structural elements for the binding of cellular proteins required for IRES-mediated translation (323).

Using an in uitro assay in which IRES-dependent translation of luciferase function could be selectively suppressed by the presence of inhibitor, BioChem Pharma and OSI Pharmaceuticals reported the findings of HCV IRES inhibition by phenazine and phenazine-like molecules by screening a compound library and fungal extracts (324). The hit compound (136) is also known as neutral red,



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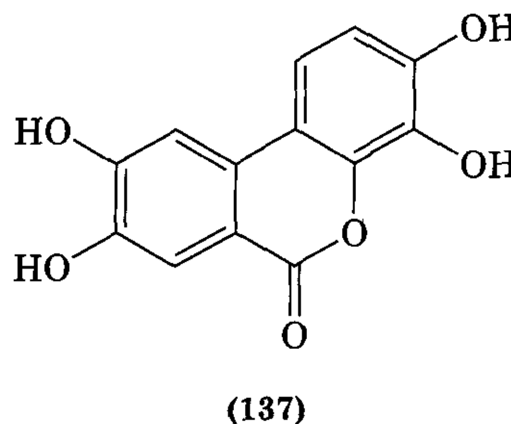
which is a dye used commonly in antiviral assays. The central ring seemed crucial for activity because the open ring analogs exhibited much lower or no activity. Polar substituents at positions 2 and 8 were also important for the inhibitory activity (324). Finally, Eisai Co. has patented several low molecular weight inhibitors, such as compounds (137) and (138) (see references in Refs. 324–326).



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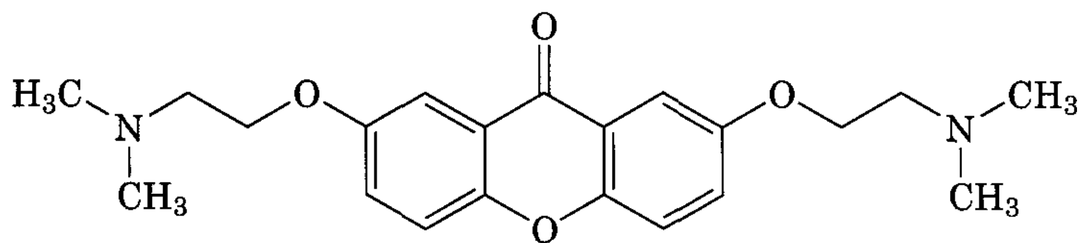
2.4.2 HCV NS3 Protein. NS3 is a multifunctional protein in which the N-terminal (ca. 180 amino acids) encodes a serine protease responsible for a distinct temporal hierarchy event of cleavage of NS3/4A, NS4A/4B, NS4B/5A, and NS5A/5B junctions, generating four



(138)

mature viral non-structural proteins, including NS4A, NS4B, NS5A, and NS5B. The remaining C-terminal two-thirds (ca. 450 amino acids) of the NS3 protein encode a nucleic acid–stimulated nucleoside triphosphatase (NTPase) and a helicase activities (327).

The interaction between the NS3 and its cofactor NS4A is required for the proteolytic activity of NS3. NS4A is a relatively small protein (54 amino acids). Binding of NS4A brings about several conformational changes, resulting in the stabilization of the conformation of the N-terminal domain of the protease and optimization of the alignment of the catalytic triad of His-57, Asp-81, and Ser-139 (327, 328). The structure of the isolated NS3 protease domain either in the presence or in the absence of the cofactor peptide was resolved by X-ray crystallography and by NMR spectroscopy.

A zinc ion is coordinated tetrahedrally by Cys-97, Cys-99, Cys-145, and His-149 at a site located remote from the active site. Interestingly, these residues are conserved in all known HCV genotypes. The zinc ion is believed to be required for structural integrity and activity of the enzyme (329,330).

The NS3 serine protease has been regarded as one of the preferred targets for the development of anti-HCV agents because it presents three potential targets for antiviral design: (1) the enzyme active site, (2)the structural zinc-binding site, and (3) the NS4A binding site (327). However, the interaction between NS3 and NS4A is considered an unlikely target for the development of inhibitors because this region involves a very large surface area and the two components are tightly intercalated (328, 331).

2.4.2.1 Inhibitors of HCV NS3 Protease

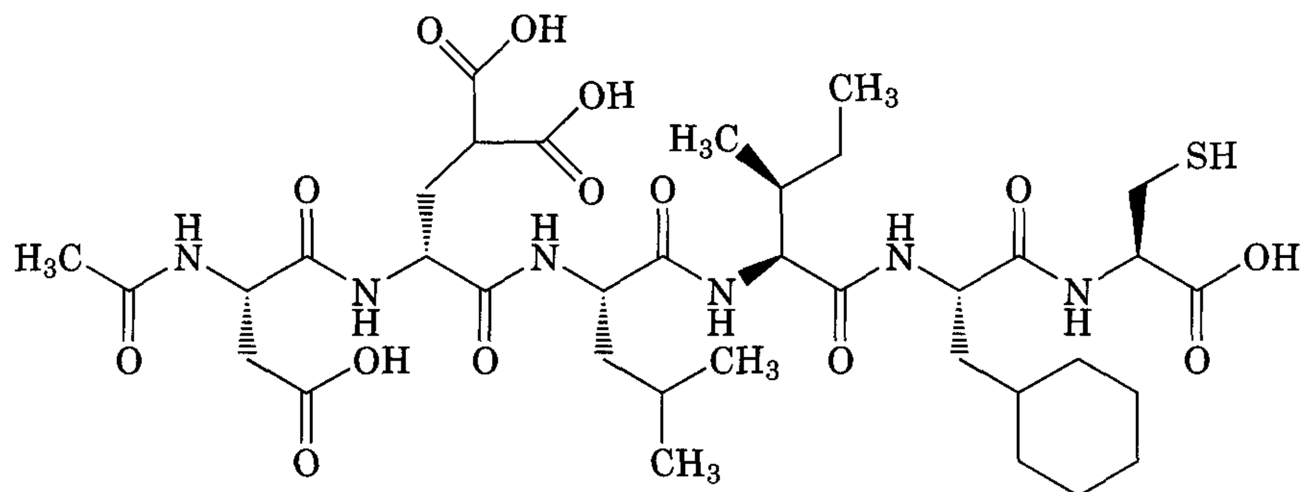
2.4.2.1.1 Peptide-Based Inhibitors. The NS3-dependent cleavage sites of the polyprotein have a consensus feature with cleavage occurring after cysteine (at the three intermolecular cleavage sites) or threonine (at NS3-4A intramolecular cleavage site) (Table 10.3). Other conserved features are an acid residue in the P, position (aspartic acid or glutamic acid), a small residue (serine or alanine) in $P_{1'}$, and a hydrophobic residue in the $P_{4'}$ position (332). Crystal structural studies have shown that the active site of the HCV enzyme is extended, shallow, with very little surface feature, and solvent exposed, requiring multiple weak interactions for binding of substrates and inhibitors. Moreover, NS3/4A is an induced-fit enzyme, requiring both the cofactor and the substrate to acquire its bioactive conformation (333). These characteristics pose significant challenge for the design of inhibitors. This challenge is demonstrated by the fact that common protease inhibitors, including serine protease inhibitors, are not effective against the HCV NS3 protease or are only active at high concentrations (334, 335).

The minimum length required for a peptide substrate is a decamer spanning from P, to $P_{4'}$ and incorporating preferentially all of these

Table 10.3 Amino Acid Sequences of HCV Protease Cleavage Sites (331)

	$P_1 - P_{1'}$
NS3–4A	H_2N . . . **Asp** Leu Glu Val Val Thr – Ser Thr **Trp** Val . . . OH
NS4A-4B	H_2N . . . **Asp** Glu Met Glu Glu Cys – Ala Ser His Leu . . . OH
NS4B-5A	H_2N . . . **Asp** Cys Ser **Thr** Pro Cys – Ser Gly Ser Trp . . . OH
NS5A-5B	H_2N . . . Glu **Asp** Val Val Cys Cys ~ Ser Met Ser **Tyr** . . . OH

–, scissile bond.



(139)

conserved features (336). Because it will be difficult to develop a large **peptide** substrate into therapeutic agent, recent research effects have been looking into smaller peptide-based inhibitors guided by SAR analyses of substrate specificities.

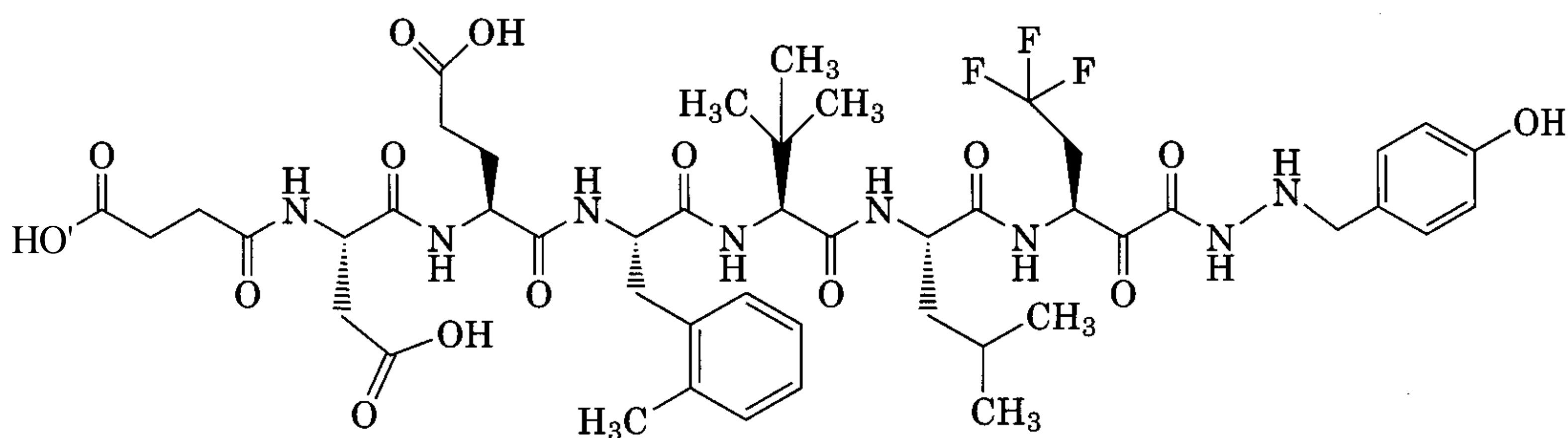
Investigations reported independently by **Llinàs-Brunet** et al. of Boehringer Ingelheim (Canada)(337) and Steinkiihler et al. of IRBM in Italy (**338**) have shown that the NS3 protease undergoes inhibition by the N-terminal cleavage products of substrate **peptides** derived from the **NS4A-4B** [**e.g.**, Asp-Glu-Met-**Glu-Glu-Cys (338)], NS4B-5A, and NS5A-5B** [**e.g.**, Asp-Asp-Ile-Val-Pro-Cys (**337**)] cleavage sites.

Based on these hexapeptides, attempts have been tried to maximize the enzyme-inhibitor interaction through optimization of each amino acid residue by using single amino acid substitution and combinatory chemistry. The following are some substrate specificities observed in the SAR studies. (1)Efficient inhibitor binding requires two electrostatic interactions that involve both the P_1 carboxylic acid and a P_6 acidic residue. Moreover, the $\mathbf{P_1}$ carboxylic acid functionality contributes most to the potency and specificity to these inhibitors (337,339,340). **(2)**$\mathbf{P_2}$–$\mathbf{P_4}$ sites prefer hydrophobic residues (332, 339, 341). **(3)** At the $\mathbf{P_5}$ site, a negative charge is not an absolute requirement (339). Substitutions with D-amino acids generally result in enhanced potency (337, 339,342).

After sequential optimization of the initial **NS4A-4B** inhibitor sequence (Ac-Asp-Glu-Met-Glu-Glu-Cys-OH; IC_{50}, 1 μM), **Ingallinella** et al. at IRBM reported a more than 600-fold increase in potency observed with Ac-Asp-D-Gla-Leu-Ile-Cha-Cys-OH (139) (IC_{50}, 1.5 nM) (339). It should be noted that the enzyme assay was based on the **protease** domain of NS3 protein. Shortening the length of the peptidic inhibitors has shown to have significant detrimental effect on the binding of inhibitors to the enzyme. However, **it** was later shown that the decrease in potency was much less when determined in the assay system that uses the full-length NS3 protein (protease-helicase/NTPase), suggesting that the helicase domain might have a significant influence on binding of **protease** inhibitor to the enzyme (343). It should also be noted that the IC_{50} values vary according to different assay systems used by different groups of investigators.

A common strategy for the design of protease inhibitors is to incorporate an electrophilic carbonyl group like aldehyde (332,340, **344**), boronic acid (332, **345**), trifluoromethyl ketone (**340**), ketoamide (**340, 346, 347**), ketoacid (344,348,3491, and **diketone** (**346**) to the C-terminal (in place of the scissile amide bond) of a synthetic peptidic molecule as serine trap. The electrophilic carbonyl groups are anticipated to form a transition-state analog of the tetrahedral intermediate with the y-OH nucleophile of the active site serine residue (340, **349**).

However, because that there is a strong preference for cysteine at the $\mathbf{P_1}$ position (337) and the **cysteine's** nucleophilic **sulfhydryl** side-chain is incompatible with the presence of an electrophile within the same molecule, this cysteine needs to be replaced with other small, hydrophobic amino acids to avoid this potential intramolecular interaction, which gener-



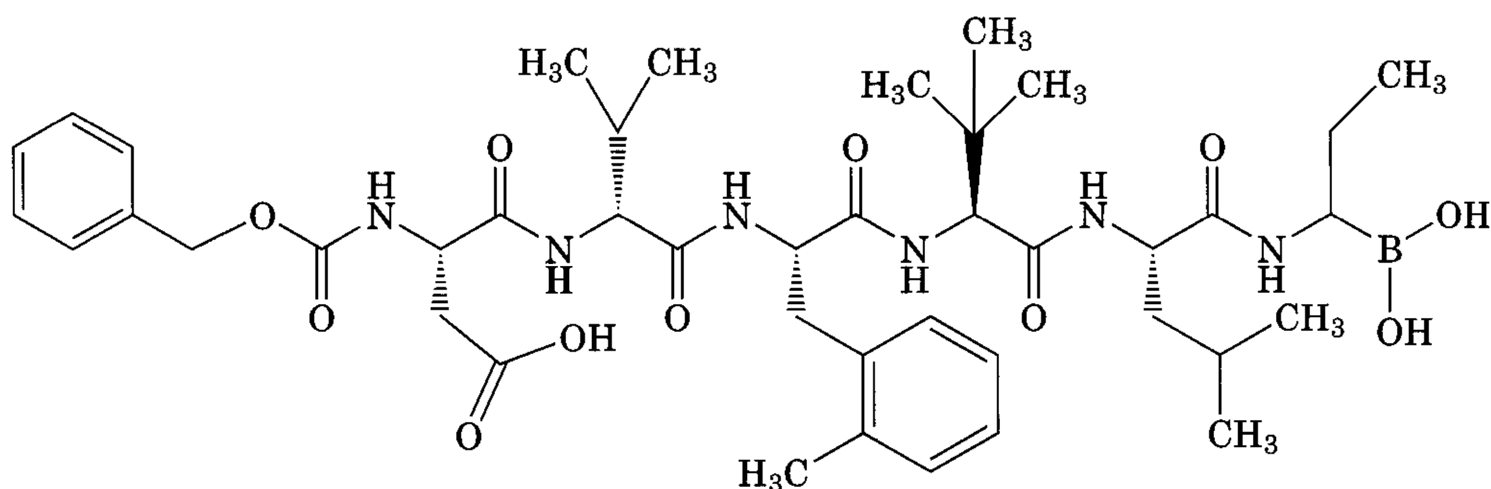
(140)

ally leads to inactivation of the inhibitor. a-Amino acids such as allylglycine, propargylglycine, aminobutyric acid, trifluroaminobutyric acid (**332**), difluoroaminobutyric acid (344, 348, **349**), 1-amino-cyclopropylcarboxylic acid (**342**), and **norvaline** (340, 342) have been reported as the P_1 cysteine mimics.

Combining the optimal substitutions, **Roche (Welwyn,** UK) reported a series of nanomolar peptide-based inhibitors as represented by compound (140) (IC_{50}, 4 n*M*) (347) and compound (141) (IC_{50}, 80 n*M*) (345). Boehringer Ingelheim (Canada) reported compound (142) as a HCV serine protease-specific inhibitor (IC_{50}, 27 n*M*) (342). It is of interest to note that large aromatic group in P_2 resulted in stronger binding. N-terminal truncation yielded tetrapeptide (**143**), which is smaller but still remains potent (IC_{50}, 3.5 *μM*) (**342**).

IRBM showed that hexapeptide a-ketoacid (**144**), incorporating difluoroaminobutyric acid in the P_1 position, acted as a potent, slow-binding inhibitor of the NS3 **protease** (IC_{50}, **1** n*M*) (349). The mechanism of action was thought to involve the rapid formation of a reaction intermediate followed by a slow conversion into a tight 1:1 covalent complex (349). Similar mechanism of inactivation was also suggested for tripeptide (145) (IC_{50}, **1.4** *μM*) (**344, 349**), and this was further supported by the crystal structure of the inhibitor bound to the HCV **NS3-4A** complex (344, 348). The same investigators have also incorporated the crucial a-ketoacid moiety into the preparation of peptidomimetics (**e.g.,** compound (146); IC_{50}, 0.18 *μM*) by de *novo* design (**344**).

Peptidic inhibitors described so far are mainly based on the determinants of ground-state substrate binding to the enzyme, which reside in the P region. The P' region of the substrate is important for catalysis, whereas it contributes little **to binding (351). Nevertheless**, there are binding **pockets** in the S′ region that can be exploited for inhibitor binding. Ingallinella et al. of IRBM took non-cleavable decapeptides spanning P_6–$P_{4'}$, sequentially optimized the P' residues, and generated subnanomolar inhibitors (**351**). For instance, the IC_{50} **for the decapeptide** Ac-Asp-D-Glu-Leu-Ile-Cha-Cys-Pro-Cha-Asp-Leu-NH2 was <0.2



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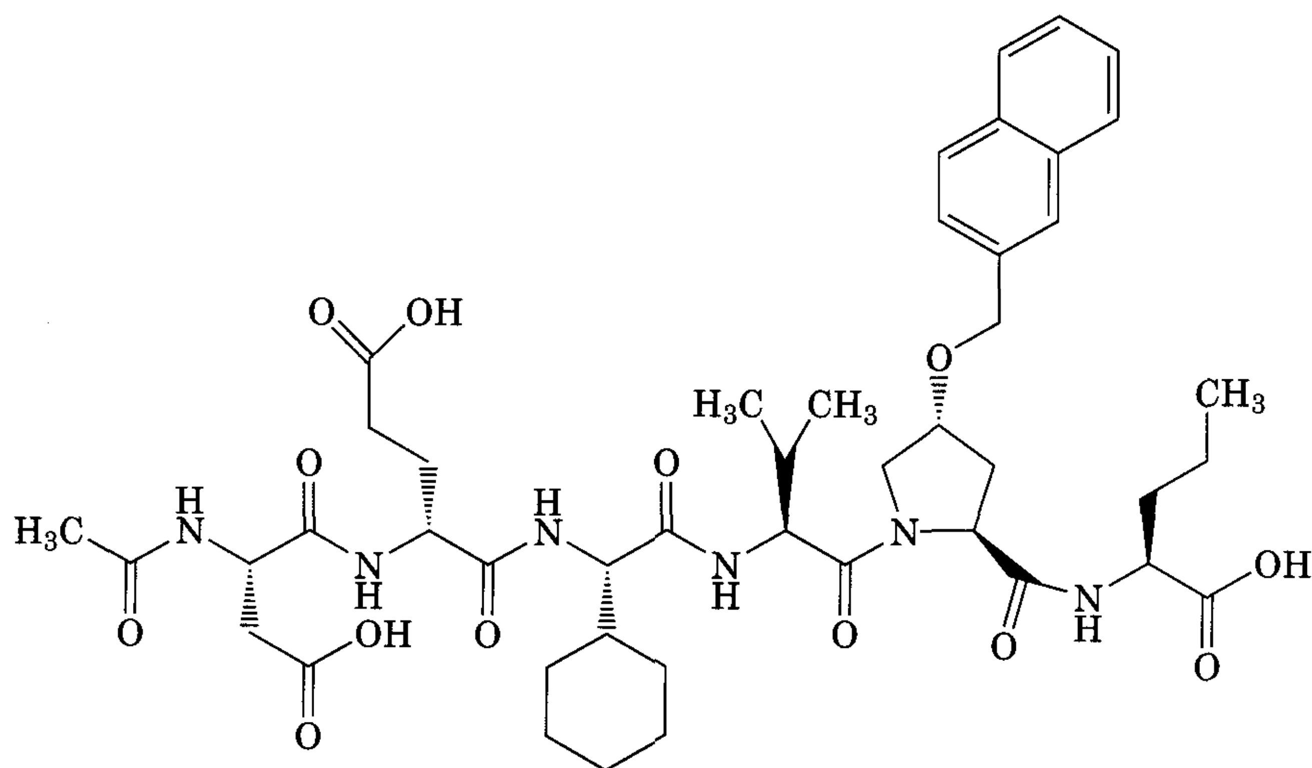
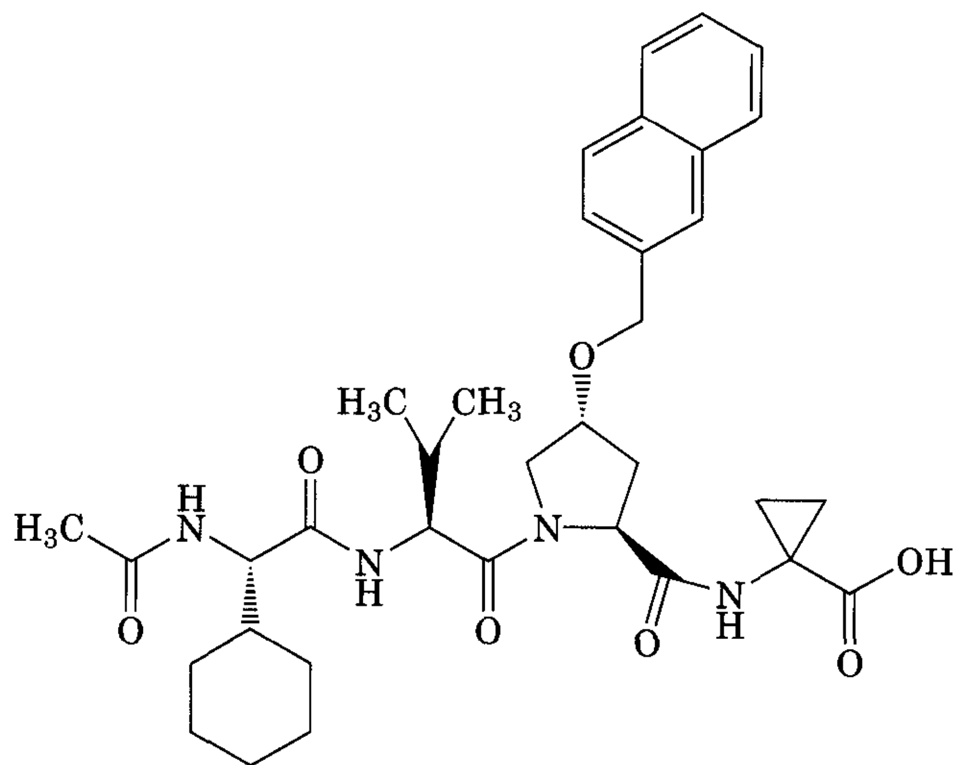


(142)

n*M*, whereas the IC_{50} of hexapeptide Ac-Asp-D-Glu-Leu-Ile-Cha-Cys-OH, which is corresponding to P_6–P_1, was 15 n*M* (351).

2.4.2.1.2 Low Molecular Weight Inhibitors. Through a screening effort, Sudo et al. showed a number of benzamide derivatives as having activity against NS3-4A protease (352). The most active one in the series was RD3-4082 (**147**) (IC_{50}, 5.8 μ*M*), which contains a long N-alkyl chain. However, this compound was also inhibitory to other seine proteases. The most selective compound in the series against HCV serine protease was RD2-4039 (**148**), which has a N-phenyl moiety. Interestingly, RD2–4039 seemed to share a common structure with other HCV serine protease selective inhibitors, such as (**149**), reported independently by Kakiuchi (353). Compound (**149**) was found to be a non-competitive inhibitor, suggesting that these compounds do not bind to the substrate-binding pocket.



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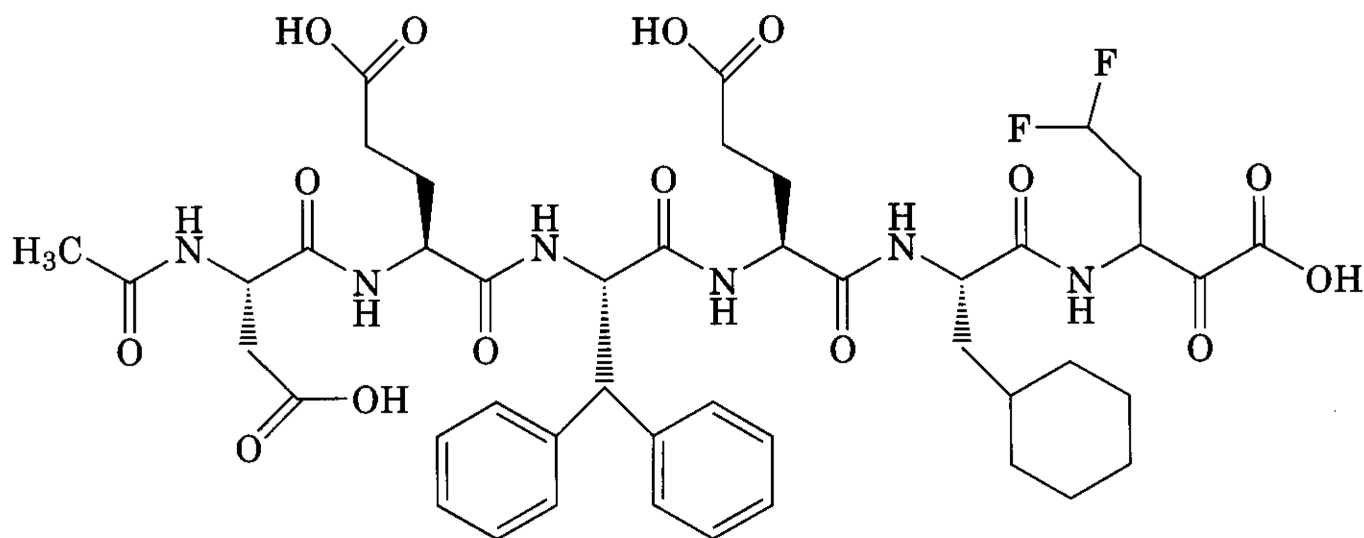

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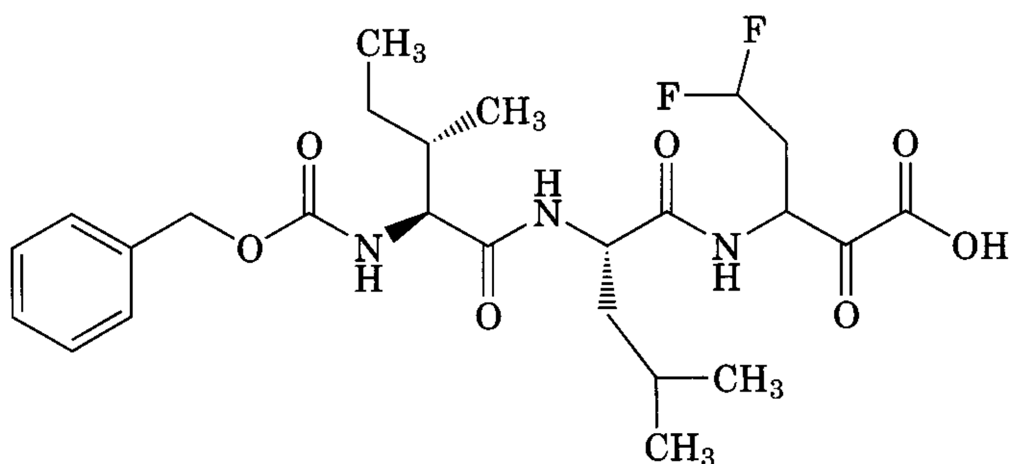

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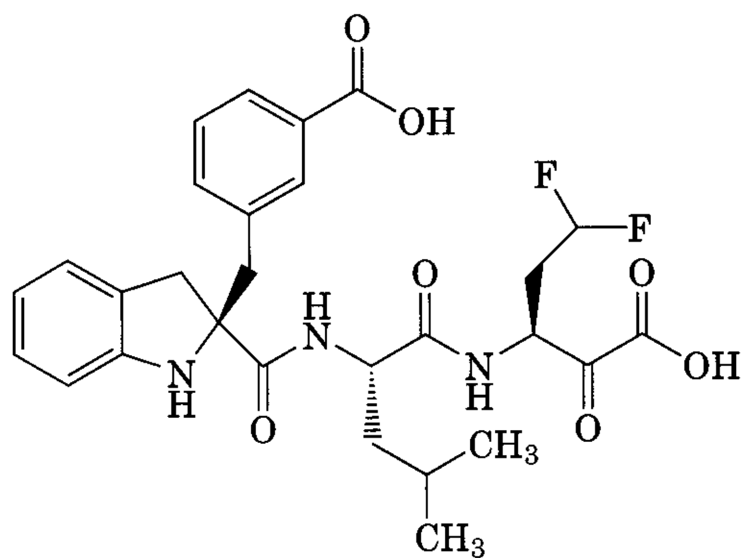


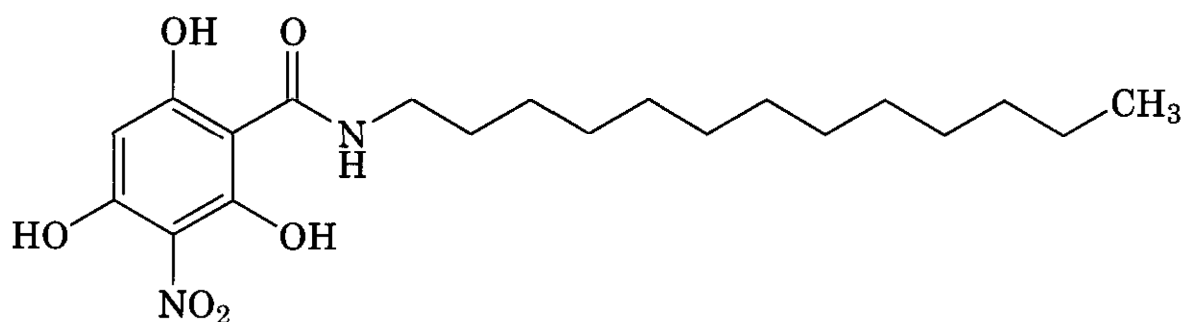
(146)

Derivatives of rhodanine have also been reported through screening, e.g., compound (150) (353) and RD4-6205 (151)(354), as well as through synthesis, e.g., compound (**152**) (355). Unfortunately, these compounds also showed to be non-selectively inhibitory to at least one other serine protease (chymotrypsin, trypsin, plasmin, and elastase) in addition to their activity against HCV serine protease. Kinetic analysis revealed that RD4-6205 inhibits the HCV protease in a non-competitive manner.

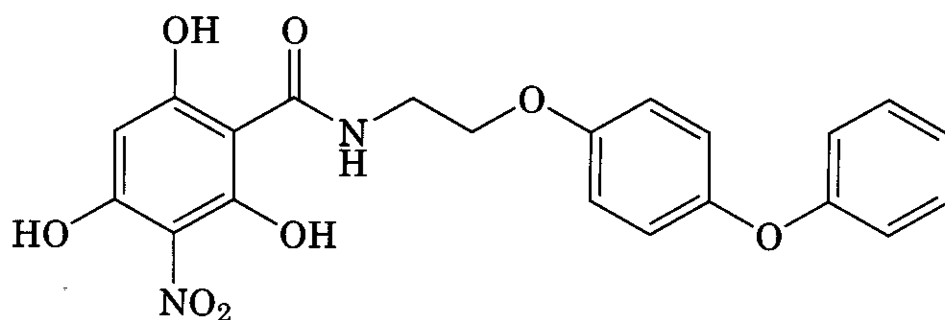
HCV protease-guided screening of natural products yielded Sch 68631 (153) isolated from the fermentation culture broth of ***Streptomyces*** sp. (356), Sch 351633 (**154**), isolated from the fungus ***Penicillium griseofulvum*** (357), and mellein (155) from ***Aspergillus ochraceus*** (358).

Because HCV NS3 protease is a zinc-containing protein, Zn-ejecting compounds, such as **2,2′-dithiobis[(*N*-phenyl)benzamide] (156)**, which might interfere metal ligation could show enzyme inhibitory activity (328). However, zinc is essential for several cellular enzymes; ejecting zinc might cause non-selective interference with normal cell metabolism (331).

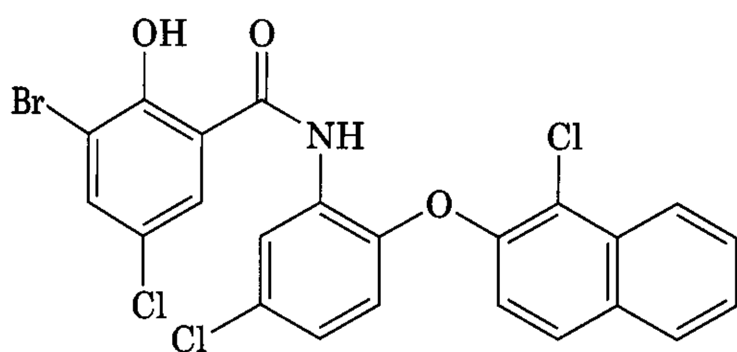
Other series of compounds, such as APC-6336 (**157**), might take advantage of forminga



(147) RD3-4082



(148) RD2-4039



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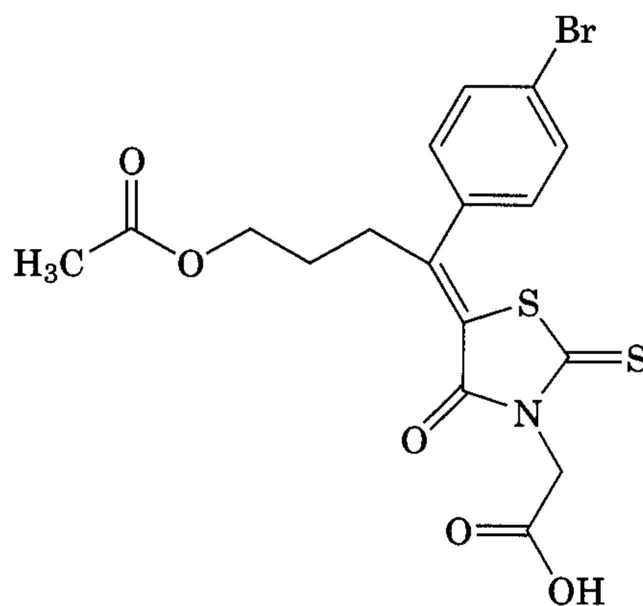
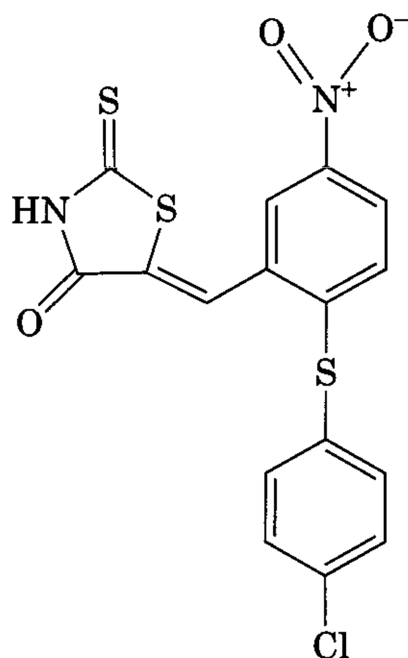


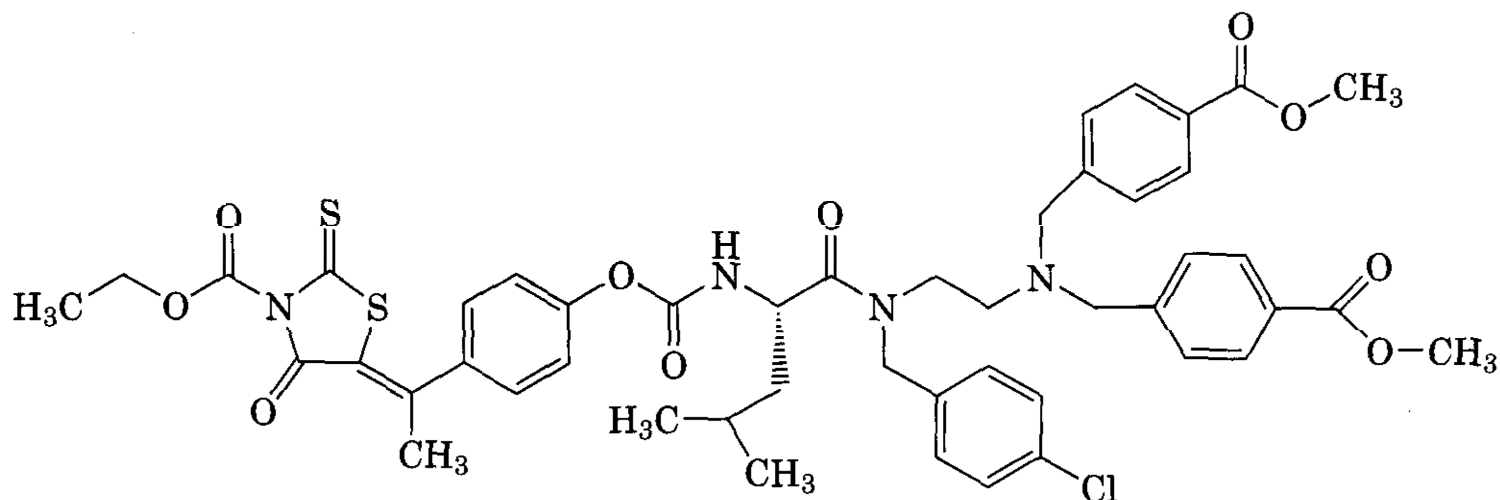
(151) RD4-6205



(150)

tetrahedral coordination with the zinc atom to help anchor their binding to the active site and thereby bring about enzyme inhibition. APC-6336 showed sub-micromolar inhibition against HCV NS3 in the presence of Zn^{2+}. The activity dropped more than 800-fold in the absence of Zn^{2+} *(359).*

2.4.2.1.3 Macromolecules. Nishikawa et al. have used a genetic selection strategy, which involves repeated rounds of selection and amplification, to isolate NS3-binding RNA aptamers from pools of random RNA.



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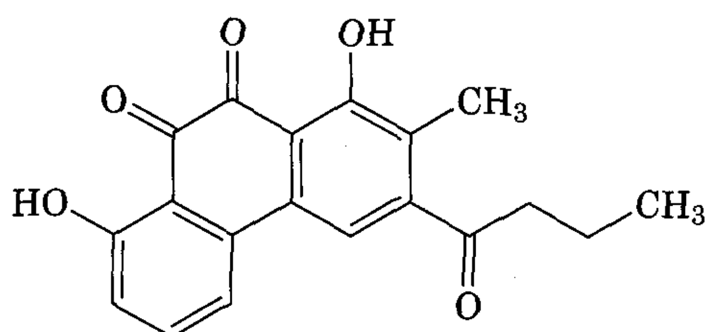
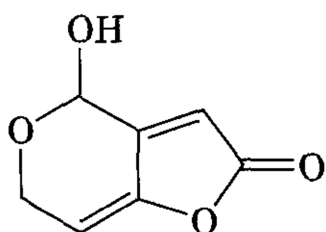
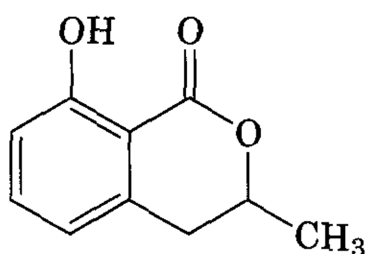


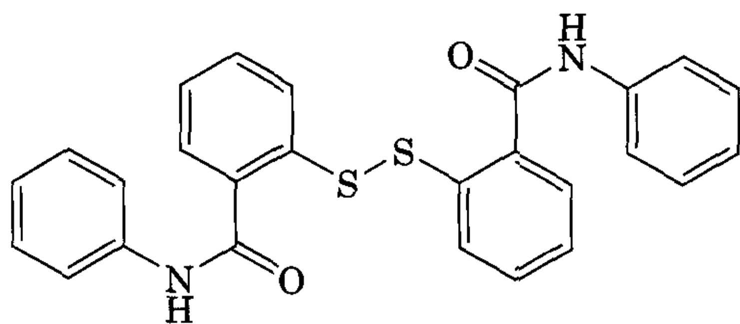
(153) SCH 68631



(154) SCH 351633



(155) Mellein

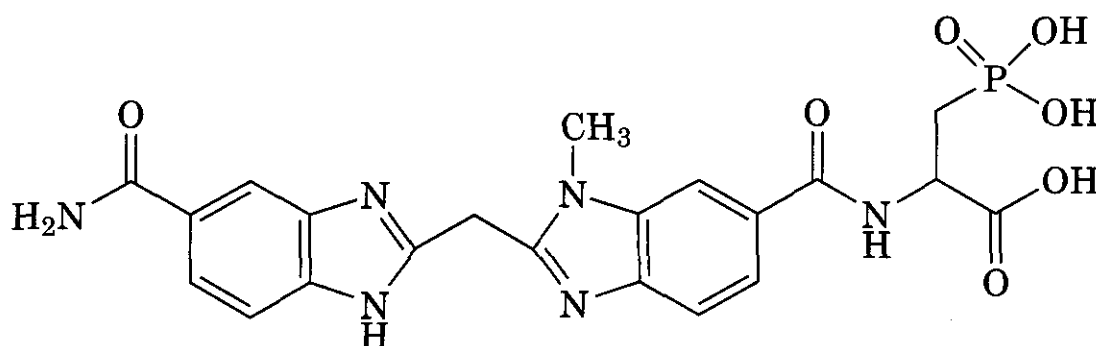


(156)

The aptamers so selected inhibited both the proteolytic and helicase activities of NS3 (360, 361). High affinity RNA aptamers selected by using truncated NS3 containing only the protease domain (ΔNS3) could bind to ANS3 with a binding constant of about 10 n*M* and inhibit approximately 90% of the protease activity of ANS3 and a full-length NS3 protein fused with maltose-binding protein (362). Amino acid residues essential for aptamers binding have been revealed by surface plasmon resonance measurements (363).

Sollazzo et al. reported a similar affinity strategy for selection of macromolecular inhibitors, called minibodies ("minimized" antibody-like proteins), by phage display techniques (364). Based on known determinants for NS3 protease substrate recognition, affinity selection from a biased repertoire of minibody variants identified a competitive inhibitor of this enzyme (365). Moreover, characterization of the minibody inhibitor led to the synthesis of a cyclic hexapeptide mimicking the bioactive loop of the parent macromolecule. The same authors also reported the design of nanomolar NS3 protease inhibitors generated by reshaping the active site-binding loop of eglin c, which is a known potent inhibitor of several serine proteases isolated from *Hirudo medicinalis* (366).

More recently, Ueno et al. reported the development of a monoclonal antibody (Mab), 8D4, which recognized the active site of HCV NS3 protease (367). Interestingly, the variable fragment (Fv) of 8D4 had an inhibition profile almost identical to that of the parent IgG (368).



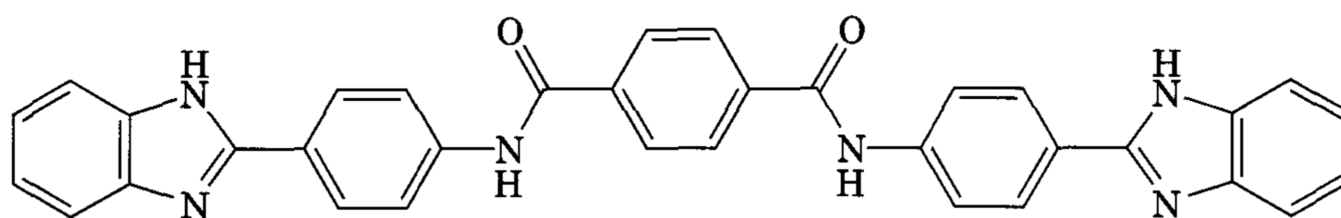
(157) APC-6336

2.4.2.2 Inhibitors of HCV NS3 Helicase. The helicase activity associated with HCV NS3 protein has also been targeted for potential therapeutic intervention (369). The crystal structure of this enzyme has been determined to provide insights into the mechanism of unwinding (369–372). In addition to unwinding dsRNA, this enzyme also unwinds dsDNA (373). Using a DNA duplex substrate and recombinant HCV NS3 produced in E. *coli*, Biochem Pharma recently reported an assay system for HCV NS3 helicase activity that might be suitable for high-throughput screening of potential inhibitors (374). Other reported assay systems include an ELISA using a non-radioactive dsRNA substrate (375) and a scintillation proximity assay using radio labeled RNA/DNA hetero-duplex as the substrate (376). These assays can be amenable to high-throughput mode.

Notwithstanding, there have been only a few HCV helicase inhibitors reported. ViroPharma has patented two series of long-chain compounds as low micromolar inhibitors (158 and 159) (see references in Refs. 326 and 377). A preliminary SAR studied by Sim et al. showed that the essential elements for inhibitory activity were the NH group within the benzimidazole ring, the benzene group at the C_2 position of benzimidazole, and the nature of the linker (378).

By studying with ribavirin 5'-triphosphate, the ATP-binding domain of NTPase/helicase has been suggested as a potential antiviral target (379). Ribavirin 5'-triphosphate showed a competitive inhibitory mechanism with respect to ATP.

2.4.3 Inhibitors of NS5B RNA-Dependent RNA Polymerase. The HCV NS5B protein, which encodes RNA-dependent RNA polymer-



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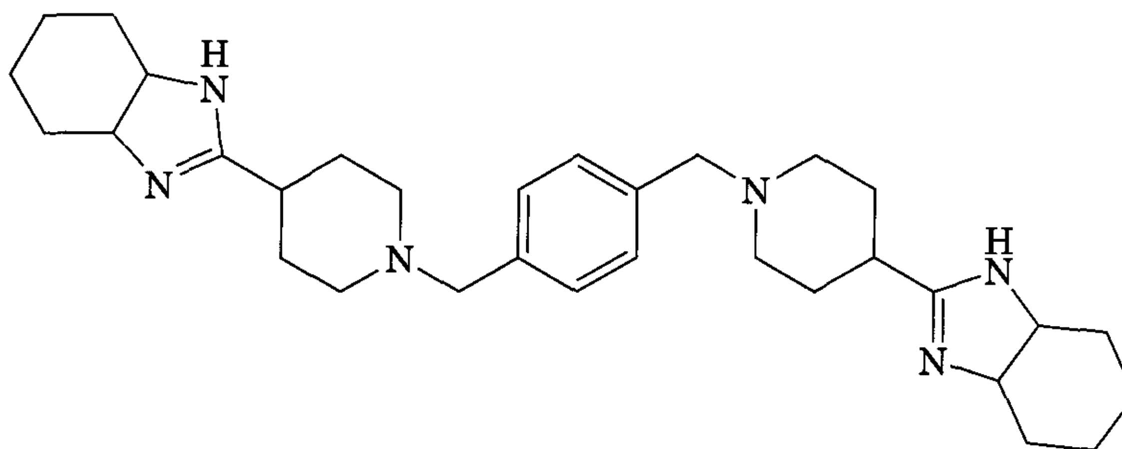

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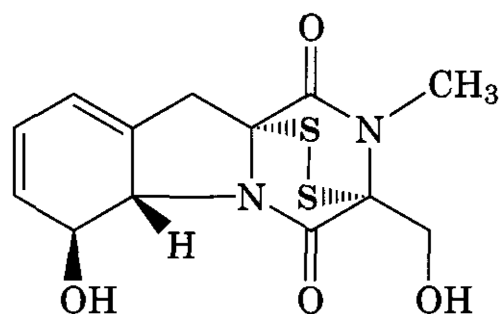
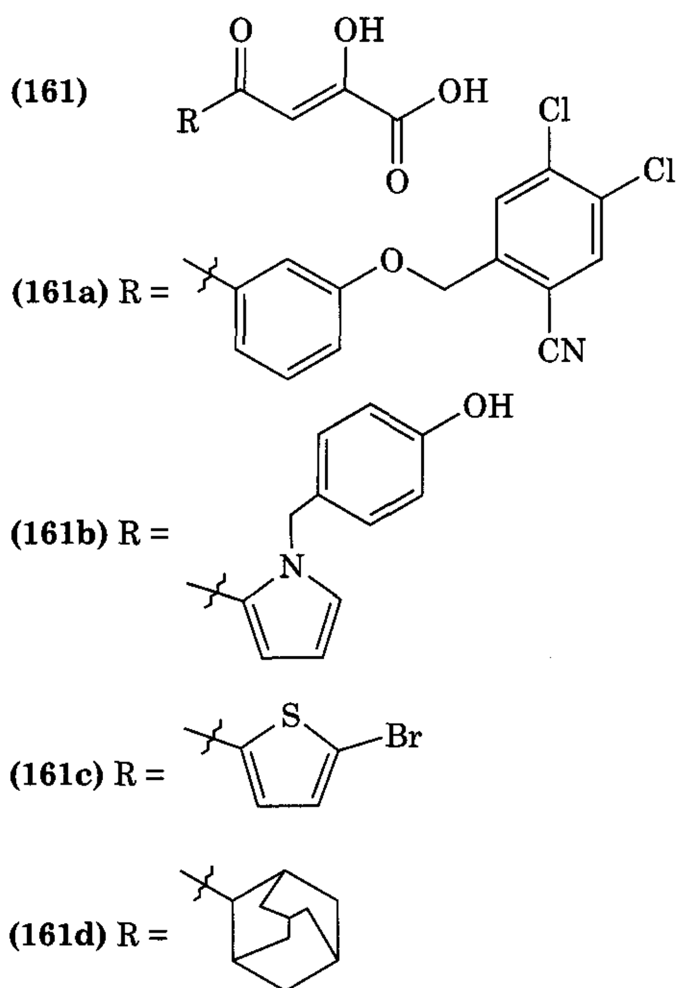
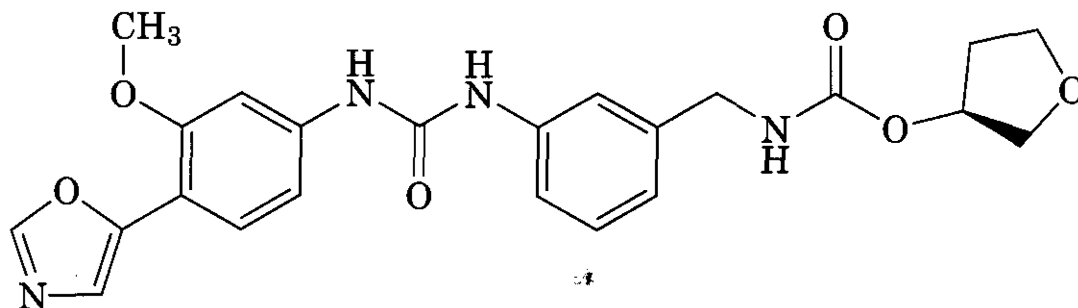


(160) Gliotoxin



ase (RdRp) activity, involves in the synthesis of complementary (−)-**RNA** using the genome as the template and the subsequent synthesis of genomic RNA using this (−)-**RNA.** In addition to its important role in the viral RNA replication, the **NS5B** amino acid sequence is highly conserved among different HCV strains, this protein has been considered as an attractive target for antiviral therapy (377, 380). Biochemical and kinetic characterizations and the crystal structure of this enzyme have been reported (380–383). Gliotoxin (**160**), a known poliovirus 3D RdRp inhibitor, inhibited HCV **NS5B** RdRp in a **dose-dependent** manner (**381**); however, broad-spectrum antiviral agent ribavirin (in triphosphate form) did not show any effect (380). In a recent review article, there were several patented diketoacids (**161a-d**) cited as low nanomolar inhibitors (377).

2.4.4 **VX-497.** Although ribavirin may inhibit viral replication through multiple mechanisms of action, the major event is thought to be a depletion of the intracellular GTP and **dGTP** pools as a result of the inhibition of inosine 5'-monophosphate dehydrogenase (IMPDH) (384) (for a review of IMPDH and its inhibitors, see Refs. 385–388). Because (1) HCV infection involves both viral proliferation and liver inflammation and (2) blocking IMPDH could block the proliferation of certain cell types, such as lymphocytes (**389**), and the growth of viruses, including viruses closely related to HCV, such as BVDV (**384**), Vertex Pharmaceuticals investigators rationalized that inhibition of IMPDH might have potential to treat HCV infection (390). However, studies by Schering-Plough investigators pointed to the opposite that inhibition of IMPDH is unlikely to be effective approach, because the major mechanism of action of ribavirin is not related to its inhibition of host IMPDH (391). The hypothetical role of IMPDH in HCV life cycle is under scrutiny with the studies of VX-497 (162). VX-497 is a potent, reversible uncompetitive IMPDH inhibitor currently in clinical trials conducted by Vertex to treat **HCV** patients unresponsive to IFN (392) as well as in combination with IFN to treat patients who have not previously



(**162**) VX-497

received HCV antiviral therapy (Vertex Pharmaceuticals Press Release, July 3, 2000). The company has also conducted clinical trials of VX-497 as a treatment for psoriasis, an autoimmune disease of skin.

The process leading to the discovery of VX-497 began with a molecular shape cluster-based screening of chemical library, followed by molecular modeling using DOCK program to model screening leads into the enzyme active site generated from the X-ray crystal structure of mycophenolic acid (MPA) bound to IMPDH (390). The crystal structure of VP-497 enzyme complex revealed several new interactions that are not observed in the binding of MPA (390).

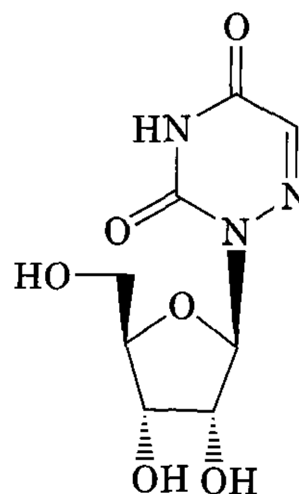
VX-497 is a nanomolar inhibitor of both isoforms of human IMPDH ($K_i = 7$ and 10 nM against type I and II, respectively) (390). Its K_i values are approximately 30-fold lower than that of ribavirin. In cultured cells, VX-497 showed broad-spectrum activity against several DNA and RNA viruses. Particularly, it was 17- to 186-fold more potent than ribavirin against HBV, HCMV, RSV, HSV-1, PIV-3, EMCV, and VEEV (384, 393). Moreover, the finding that both compounds' antiviral effects could be reversed by the addition of guanosine further strengthened the association of inhibition of IMPDH with the antiviral activities of both compounds (384).

2.4.5 Model Systems to Study HCV Replication. Robust and reliable cell-based in vitro screening systems are critical for the development of antiviral drugs. Unfortunately, no natural human liver cells are available for this purpose. Many cell lines have been described as methods for in vitro HCV replication; however, these systems are generally limited by the poor reproducibility and support low level of HCV replication (for a review of HCV cell culture systems, see Refs. 394–397). More recently, two independent research groups led by Bartenschlager (398) and by Rice (399), respectively, reported their efforts in the development of highly efficient cell culture systems based on the self-replication of engineered HCV sub-genomic RNAs (replicons) in transfected human hepatoma cell (Huh7) line. In these replicons, the neomycin phosphotransferase gene replaced the HCV structural genes. Using these systems high-level natural HCV RNA replication could be maintained stably for considerable periods of time and the replication was totally dependent on the activity of HCV NS5B RNA polymerase. Although these replicons do not represent a full natural viral replication and do not produce infectious virus particles, the systems seem to be suitable for the evaluation of HCV replication inhibitors because the replicons encode all the viral functions required for RNA replication (397, 400). Ultimately, an ideal system, which hopefully can happen in the future, is one that efficiently produces infectious HCV as well as a permissive cell line (395). Various animal models such as HCV SCID mouse model and HCV-trimera mouse model (401, 402) have been reported; however, the only reliable animal model for HCV infection is the chimpanzee (for a review, see Refs. 394,396).

2.5 Flaviviruses

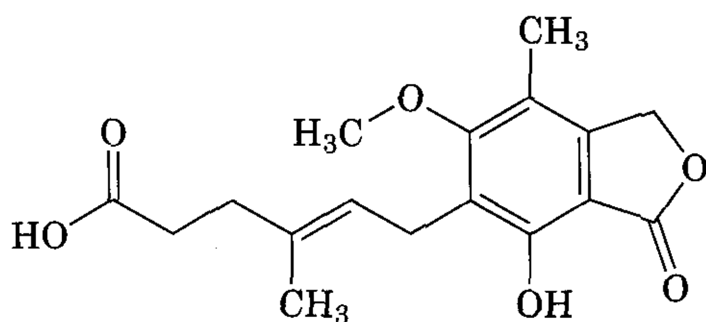
The family of flaviviridae contains three genera: hepacivirus [hepatitis C virus (HCV)], flavivirus [e.g., yellow fever virus (YFV), dengue virus (DENV), Japanese encephalitis virus (JEV), West Nile virus (WNV)], and pestivirus [e.g., bovine viral diarrhea virus (BVDV)]. This section will discuss the discovery and development of inhibitors of flaviviruses (for a review, see Ref. 403).

By screening compounds in cultured Vero cells, several known antiviral agents, such as interferon-a, ribavirin, and 6-azauridine (**163**) (an OMP decarboxylase inhibitor), proved effective in reducing viral cytopathic effect (CPE) induced by flaviviruses (404). In a

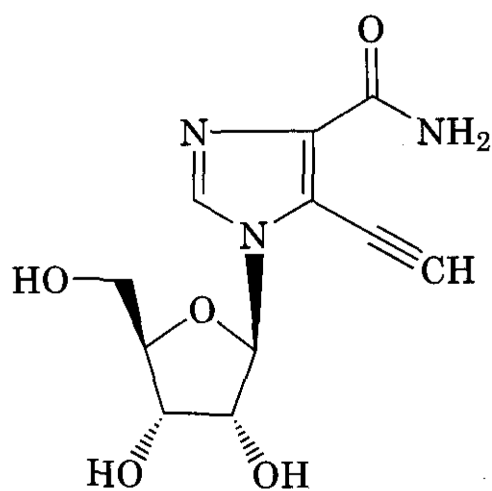


(163) 6-Azauridine

separate report, YFV (vaccine strain 17D) was shown to be highly sensitive to two IMPDH inhibitors, mycophenolic acid (164) and EICAR (**165**) as well as to a dihydrofolate reductase inhibitor, methotrexate (**166**) (405).

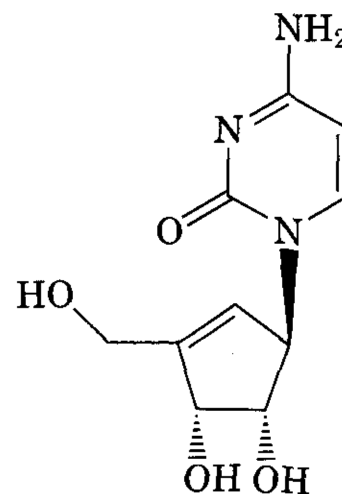


(164) Mycophenolic acid



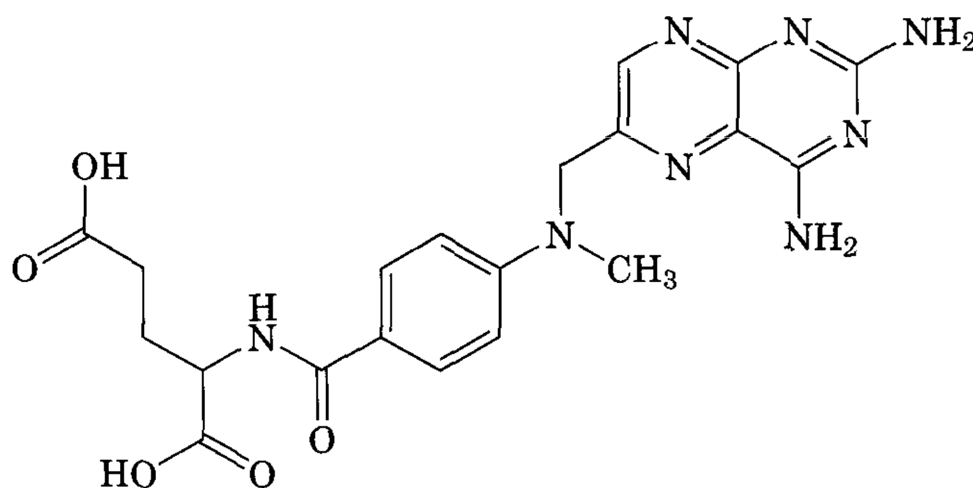
(165) EICAR

The recent outbreak of West Nile virus (WNV) in the United States has motivated a search of potential antiviral agents. Recent reports confirmed that WNV was susceptible to 6-azauridine (**163**) (406), cyclopentenyl cytosine (CPE-C) (167) (a CTP synthetase inhibi-



(167) CPE-C

tor) (406, 407), mycophenolic acid (**164**), and ribavirin (406, 408) in cell cultures. [Note: 6-azauridine was also inhibitory to sandfly fever Sicilian virus (a phlebovirus in the family of bunyaviridae) replication in *vitro* (409, 410).] Using cytopathic effect assay, ribavirin was active in MA-104 cells (EC_{50} = 5 μg/mL), but it was not very active in Vero cells (EC_{50} = 178 μg/mL) (411). This difference in activity might be because of differences in phosphorylation of ribavirin in different cell lines. Using virus yield reduction assay, the antiviral effect of 6-azauridine was seen at day **2,** but not at day 6 post-infection (406). It seemed that the inhibition of virus replication by 6-azauridine was transient. On the other hand, the virus yield reduction result of ribavirin was essentially the same at day 2 and day 6 in MA-104 cells, suggesting the antiviral effect of ribavirin was sustained over a 6-day period (411). The modes of ribavirin action against WNV might involve both cellular metabolism and lethal mutagenesis of the viral genome. Riba-



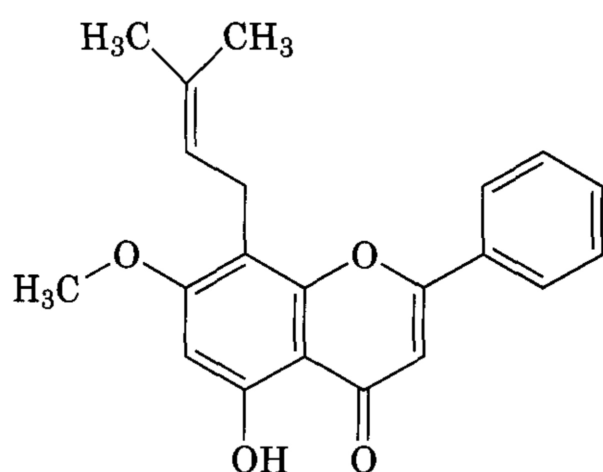
(166) Methotrexate

virin has been shown as an RNA virus mutagen that would cause RNA virus error catastrophe (22,412).

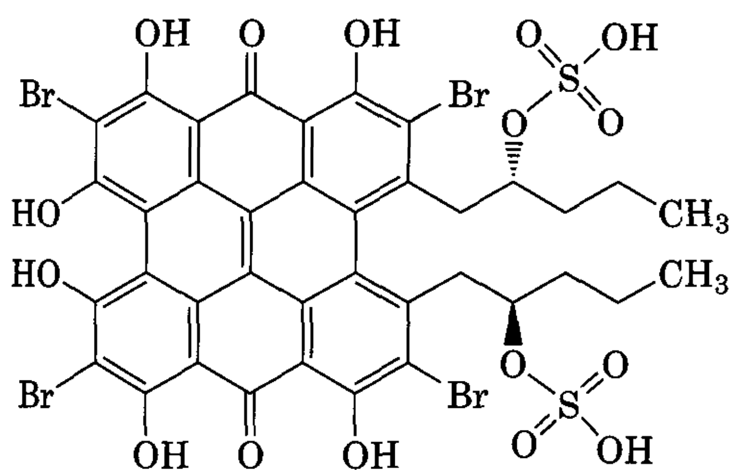
The viral genome of mosquito-borne dengue virus encodes a single polyprotein, which undergoes subsequent proteolytic processing to form mature proteins by a combined action of host proteases and a virus-encoded, two-component protease, NS2B-NS3 serine protease (413). The crystal structure of the catalytic component of the NS3 at 2.1 Å resolution was recently reported (414–416). It is anticipated that specific NS3 inhibitors could be designed based on this structure. Nevertheless, several natural products with in vitro activity against dengue virus have been reported recently; these included glabranine (168) isolated from Tephrosia sp (417), and gymnochrome D (169)isolated from the living fossil crinoid Gymnocrinus richeri (418).

Bafilomycin A1 (**170**), a macrolide antibiotic isolated from the fermentation of ***Streptomyces*** griseus, was shown to inhibit the growth of Japanese encephalitis virus (JEV) in Vero cells (419). Viral inhibition correlated with the disappearance of acidified cellular compartments such as endosomes and lysosomes (referred to as ELS), suggesting that bafilomycin Al-sensitive vacuolar-type proton pumps are responsible for the acidification of ELS and that the acidified compartments are essential for the early phase of JEV infection (419).

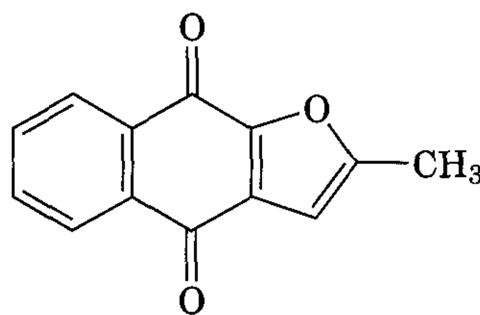
The effect of synthetic derivatives of natural furanonaphthoquinone on the replication of JEV was also demonstrated in Vero cells (420). The most active compound in the series, FNQ3 (**171**), effectively inhibited the expression of viral proteins and also genomic RNA.



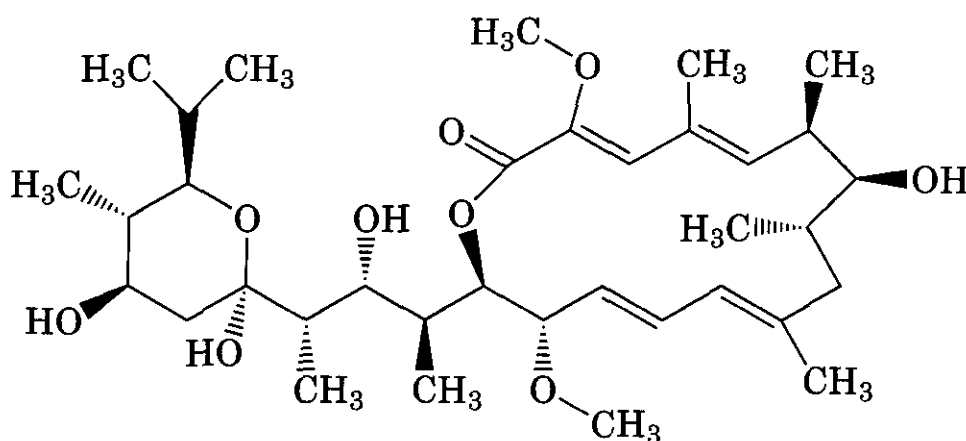
(168) Gabrauine



(169) Gymnochrome D



(171) FNQ3

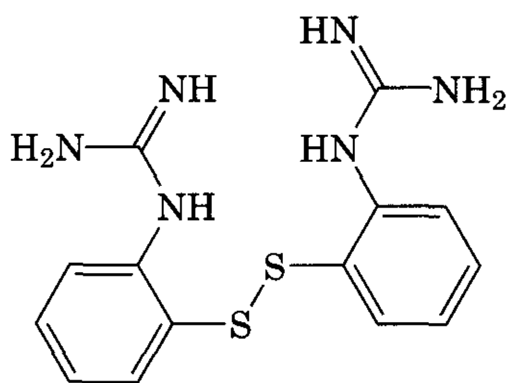


(170) Bafilomycin **A 1**

In addition, envelop protein E was much more inhibited than viral non-structural protein NS3 (420).

2.6 Arenaviruses

A number of anti-retroviral Zn-finger active compounds were tested *in vitro* against arenaviruses and the results demonstrated that (**172**), (**173**), and (**174**) have activities against



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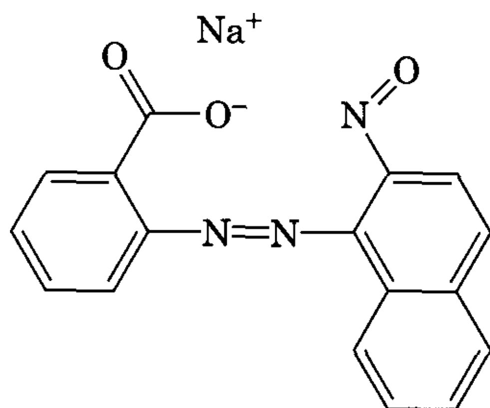

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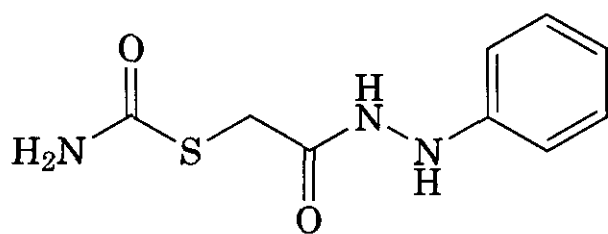
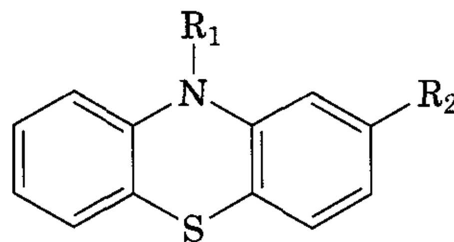
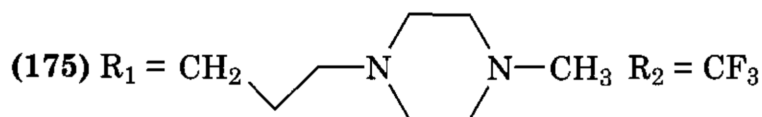
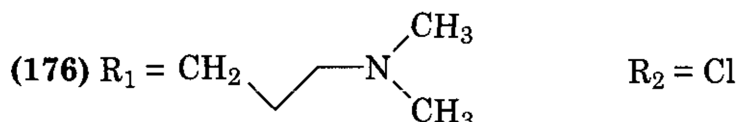


(174)

attenuated and pathogenic strains of Junin virus, the etiological agent of Argentine hemorrhagic fever, as well as antigenically related Tacaribe virus and Pichinde virus (421). Other pharmacologically active compounds reportedly showed *in vitro* activity against Junin, Tacaribe, and/or Pichinde virus included two phenotiazines [trifluoperazine (**175**) and chlorpromazine (**176**) (422)], two





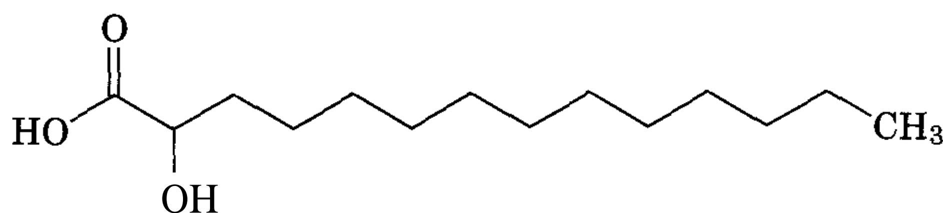


myristic acid analogs [2-hydroxymyristic acid (177) and 13-oxamyristic acid (178) (423)], and a brassinosteroid (**179**) (424). [Note: chlorpromazine also displayed strong inhibition of hepatitis A virus replication in BS-C-1 cells, possibly by preventing virus uncoating (425). Moreover, Prusiner et al. recently reported that chlorpromazine exhibited micromolar inhibition of formation of disease-causing isoform of the normal host prion protein in cultured cells chronically infected with prions (426).]

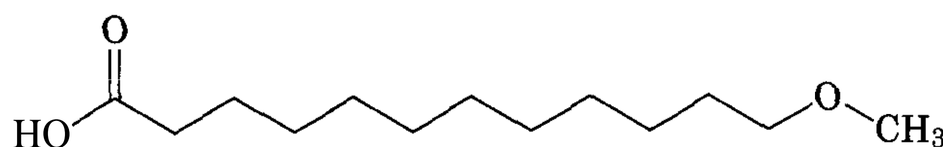
2.7 Rotavirus

Brefeldin A (BFA) (**180**) has been known as having specific activity in blocking protein transport from the endoplasmic reticulum (ER) to the Golgi complex. Because rotavirus uses ER for maturation, treatment with BFA was found to reduce progeny virus yield by 99% at 0.5 μg/mL (427). Electron microscopy analysis revealed that BFA interfered with the transition from the enveloped particle to the mature double-shelled rotavirus (427).

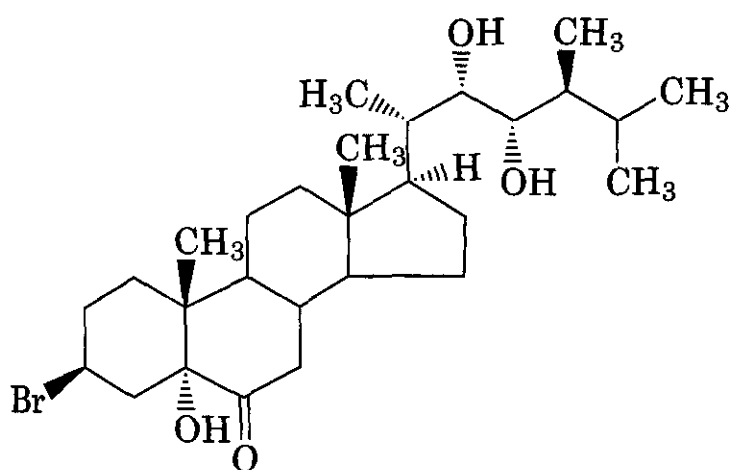
Sialic acid–containing surface glycoproteins of mature small intestine epithelial cells have been proposed as binding sites for animal rotaviruses. Therefore, as in the case of influenza virus, anti-rotaviral compounds might be derived from sialic acid-based compounds, particularly from multivalent sialic acid derivatives. Synthetic sialylphospholipid (181) was shown to exhibit dose-dependent inhibition against simian (SA-11 strain) and human (MO strain) rotaviruses in Rhesus monkey kidney cells (MA-104). The EC„ values against SA-11 and MO were 4.4 and 16.1 *μM*, respectively



(177) 2-Hydroxytetradecanoic acid



(178) 12-Methoxydodecanoic acid



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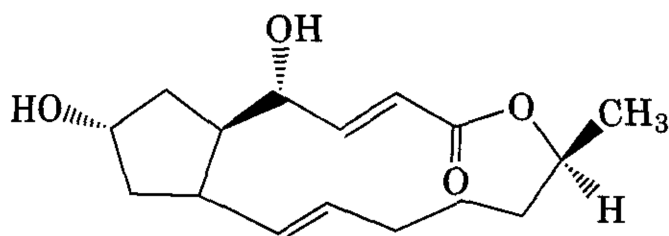
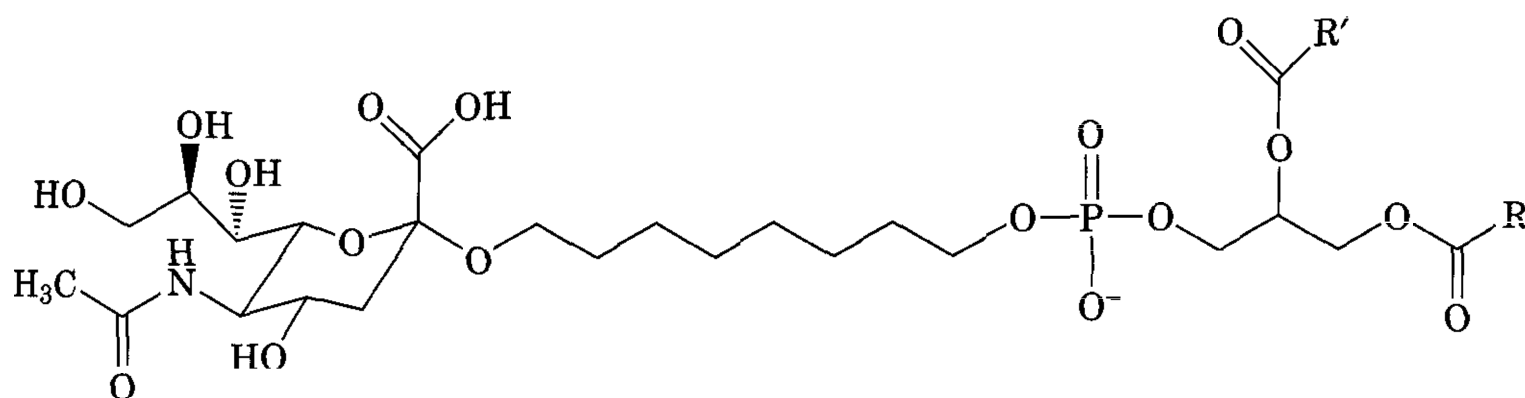


(180)

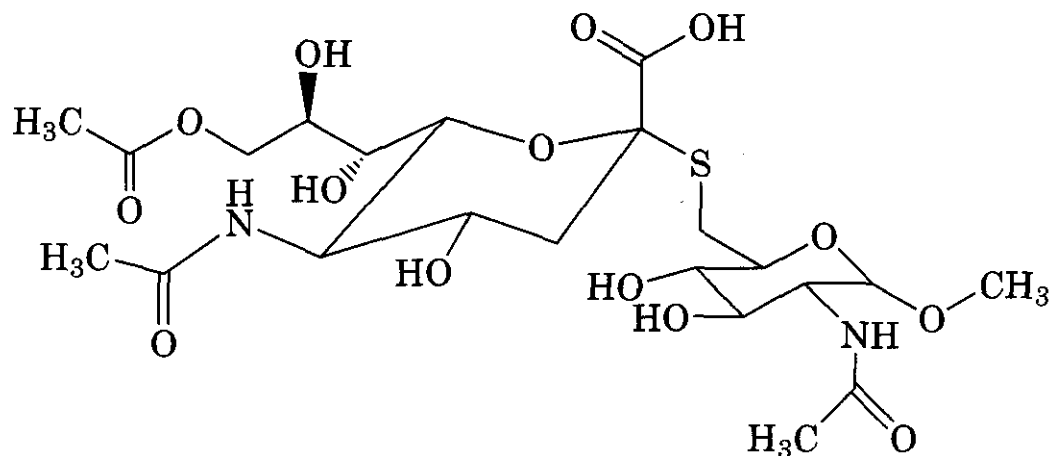
(428). A number of thioglycosides of sialic acid, exemplified by compound **(182)**, have also been evaluated. Bovine **(NCDV) rotavirus** seemed to be the most sensitive serotype to these compounds, whereas human rotavirus (**Wa** strain) was not (429). More recently, sulfated colominic acid **(183)** was shown to exhibit suppressive effect on simian **rotavirus SA-11** and human rotavirus MO infections, but not on Wa, which is a sialic **acid–independent rotavirus** (430). However, Wa rotavirus remained sensitive to other **carbohydrate-containing** compounds, such as neohesperidin (184) and hesperidin **(185)** isolated from the fruit of *Citrus aurantium* (431, 432). On the other hand, the aglycone, hesperetin, was not active.

2.8 Rubella Virus

To explore the chemical and biological relationships of 6-substituted uracil derivatives, a family of 2-methoxy- and **2-methylthio-6-[(2′-alkylamino)ethyl]-4(*3H*)-pyrimidinones**, represented by compound **(186)**, have shown various effects on virus yields in a plaque assay in Vero cells against vesicular stomatitis virus, sindbis virus, and rubella virus (433). The anti-rubella activity exhibited by (186) was



(181) Sialylphospholipid



(182)

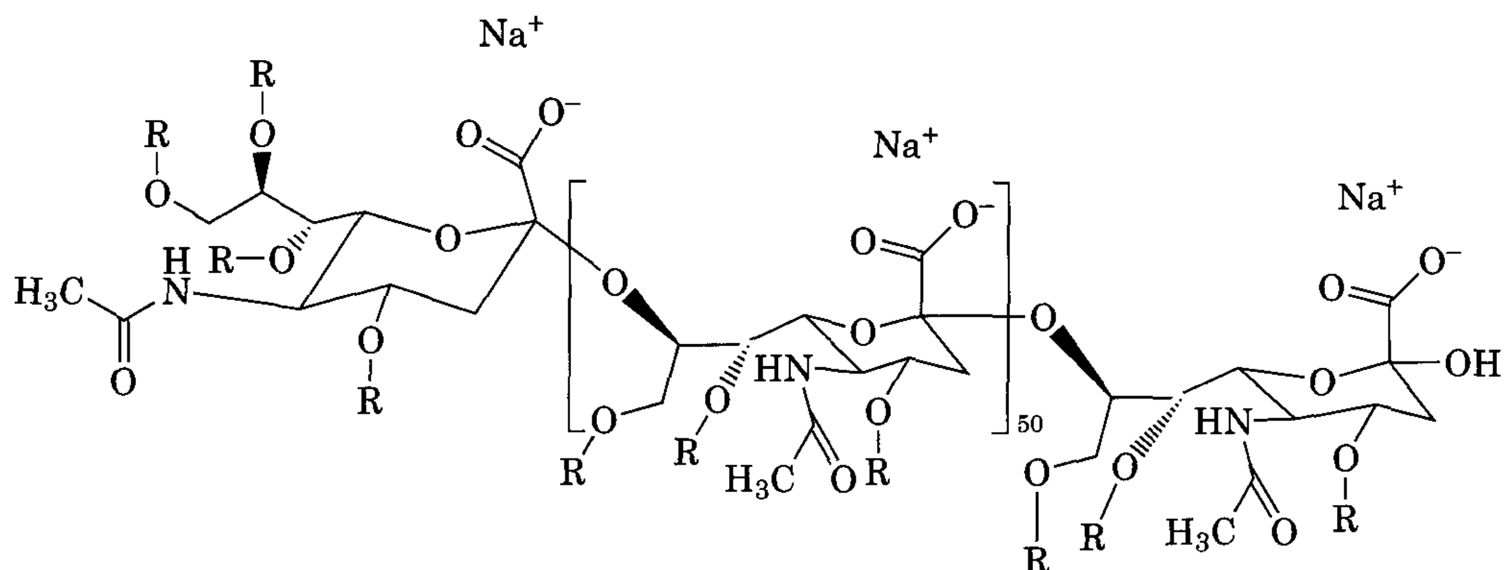
the most notable, with EC_{50} and CC_{50} of 4 and 250 μg/mL, respectively.

2.9 Broad-spectrum Antiviral Compounds

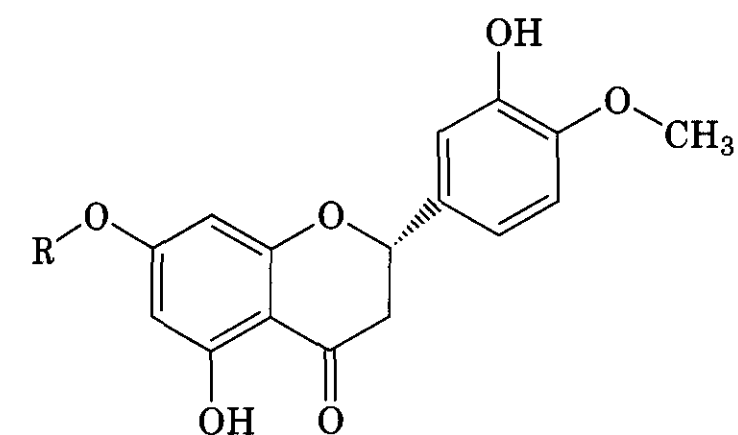
2.9.1 *S*-Adenosyl-L-Homocysteine Hydrolase Inhibitors. S-adenosyl-L-homocysteine (SAH) hydrolase is an intracellular enzyme that regulates biological transmethylation in general. Because many animal viruses require SAH hydrolase in the methylation of the 5'-terminal residue of viral mRNA for forming the 5'-methylated cap structure necessary for viral protein translation and replication, this enzyme has been recognized as a suitable target for antiviral chemotherapy (434). Its inhibitors have generally shown broad-spectrum-activity against orthopox-, paramyxo-, rhabdo-, filo-, bunya-, arena-, and reoviruses (for a review, see Ref. 142). This enzyme has also been considered as an attractive target for parasite chemotherapy (435, 436) and other medical indications (437). More recent studies with SAH hydrolase inhibitors on the replication of measles virus and Ebola virus are discussed in the respective sections.

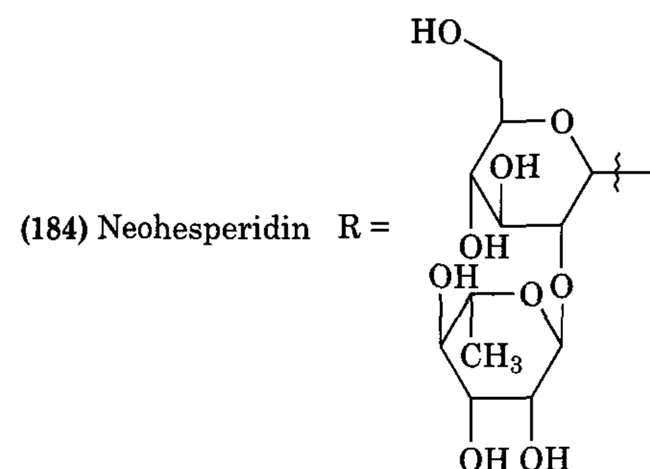
Barnard et al. reported that both D-5'-noraristeromycin (187) and its L-isomer (188) (synthesized by Schneller et al.) were having potent *in vitro* anti-measles virus (MV) activity as determined by cytopathic effect reduction assay and by virus yield reduction assay (438). The D-like analog (189) was also potent MV inhibitors even when added to infected cells 24-h post-virus exposure, implying that an event occurring late in infection such as assembly or egress could be affected. When combined with ribavirin, these compounds demonstrated synergistic (additive) inhibition of MV replication at several concentrations.

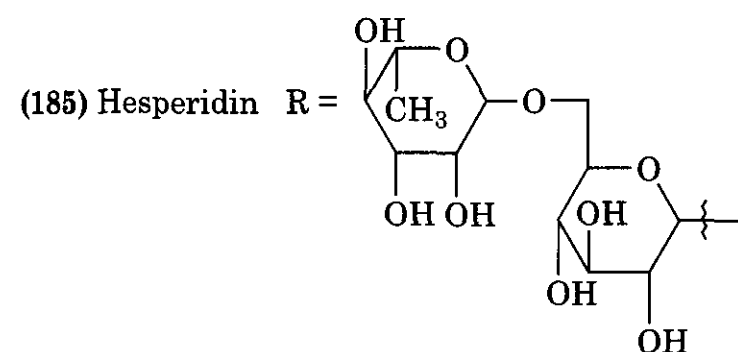
Huggins et al. have recently established a lethal mouse model suitable for evaluation of prophylaxis and therapy of Ebola virus (a filovirus) (439). Intraperitoneal administration, thrice daily, of carbocyclic 3-deazaadenosine

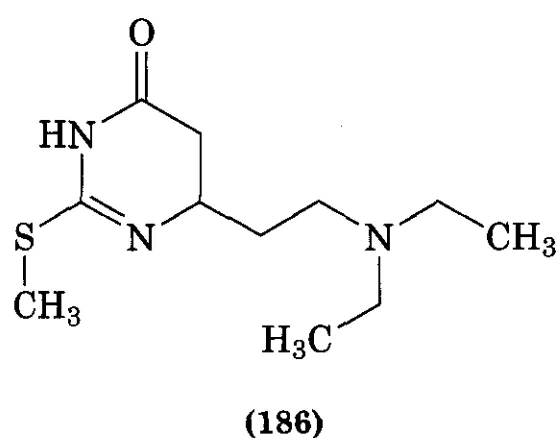


(183) R = H or SO_3Na

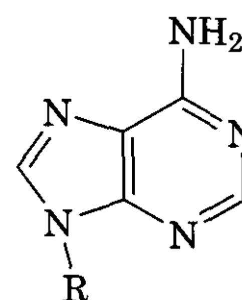


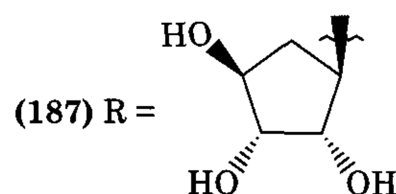


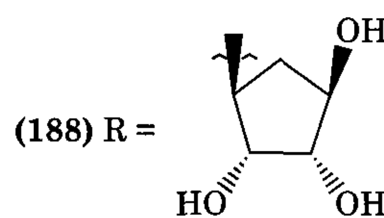


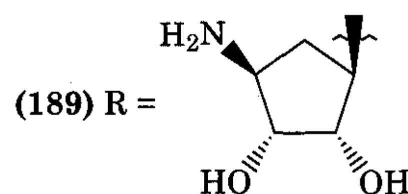


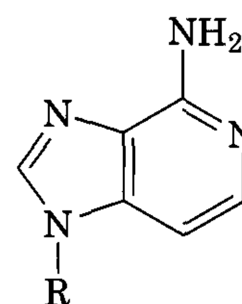
(186)

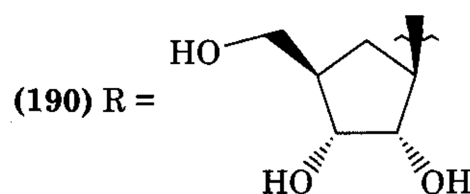


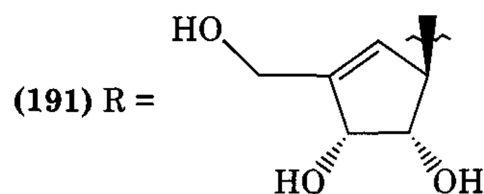








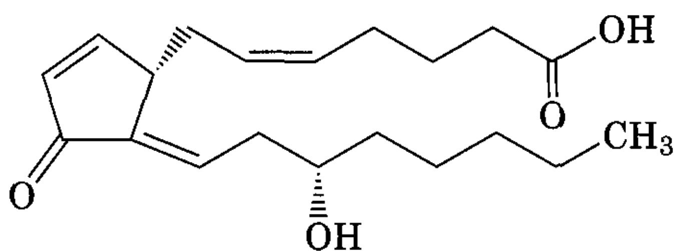




(190) significantly protected **BALB/c** mice from lethal infection with mouse-adapted Ebola Zaire virus, providing treatment was initiated on day – 1, 0, or 1 day relative to time of virus challenge (**440**). Treatment with 2.2 **mg/kg** initiated on day 3 post-infection still resulted in 40% survival. In another study, a single subcutaneous dosing of 80 **mg/kg** or less of **carbocyclic** 3-deazaadenosine, or of 1 **mg/kg** or less of 3-deazaneplanocin **A** (**191**), provided equal or better protection, without causing toxicity (441). One dose of drug given on day 1 or 2 significantly reduced serum virus titers and resulted in survival of most or all animals. However, drug treatment given within 1 h after infection (day 0) was less effective. In SCID mice, single or multiple drug treatment suppressed Ebola replication, but did not prevent death (441). The prolonged efficacy of these two SAH hydrolase inhibitors demonstrated a

potential useful antiviral strategy in that drug treatment begins early in infection with high but non-toxic doses, to hold viral burden below the lethal threshold until the host immune system eliminates the infection (441).

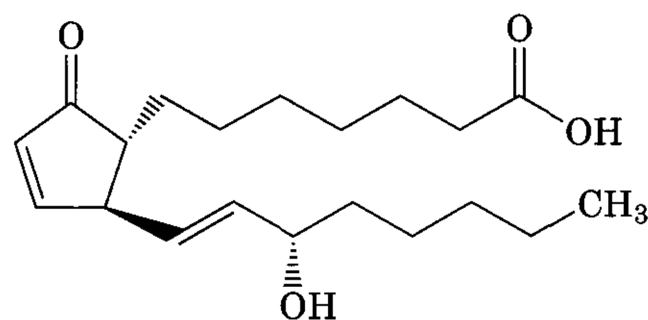
2.9.2 Prostaglandins. Cyclopentenone prostaglandins (PGs) have reportedly shown inhibitory activities against a variety of RNA and DNA viruses, including influenza A PR8, Sendai, poliovirus, VSV, Sindbis, rotavirus, HIV-1, vaccinia, HSV-1, and HSV-2 in cultured cells (reviewed by Santoro in Ref. 442). Only the cyclopentenone PGs were effective in inhibiting viral replication. It might be that the presence of an α,β-unsaturated carbonyl group allows to form Michael adducts with proteinic nucleophiles and to bind covalently to the target proteins (443). The plausible mechanism of action of PGs includes the induction of cytoprotective heat shock protein (HSP) synthesis, notably of the 70K heat shock protein (HSP 70) in human cells, through activation of heat shock transcription factor 1 (HSF 1) (443). A recent study by Santoro et al. showed that Δ^{12}-PGJ_2 (192) effectively inhibited viral protein synthesis of influenza virus A/PR8/34 (H1N1) as long as the host MDCK cells were synthesizing HSP 70, whose synthesis started 3 h after Δ^{12}-PGJ_2 treatment and continued for at least 12 h in both uninfected and virus-infected cells (444). Nevertheless, the mechanism by which HSP can interfere with viral protein synthesis remains to be elucidated.



(192) Δ^{12}-PGJ_2

Δ^{12}-PGJ_2 caused a dose-dependent reduction of influenza A/PR8/34 (H1N1) virus production in infected MDCK cells (greater than 95% at 6 μg/mL), and this antiviral effect could be sustained for at least 72 h post-viral infection. Drug treatment did not affect cell viability. In fact, the treatment actually prevented the virus-induced inhibition of cellular RNA synthesis. Intraperitoneal administration of Δ^{12}-PGJ_2 to PR8 virus-infected mice significantly reduced the virus titers in the lungs and increased the survival rates (50–60% of the animals that received a daily dosing of 5 μg/mouse for 7 days survived the infection). This compound was well tolerated by the animals (444).

At the same concentration of 6 μg/mL, PGA, (193)only modestly and transiently inhibited influenza PR8 replication in cultured cells. On the other hand, non-cyclopentenone PG of the E and D types, which are not able to induce HSP synthesis, did not affect PR8 virus replication (444). In an independent study, PGA, showed in *vitro* effect on the replication of avian influenza A, Ulster 73 (H7H1) (445).



(193) PGA_1

Intriguingly, despite the fact that both PGA1 and Δ^{12}-PGJ_2 inhibit poliovirus replication in a dose-dependent manner, infection with poliovirus seemed to inhibit, rather than induce, HSP 70 synthesis in PG-treated HeLa cells (446). Because cyclopentenone PGs lack of ability to induce heat shock response in polioviral-infected cells, poliovirus protein synthesis was not inhibited by PGs, suggesting that cyclopentenone PGs could interfere with a late event in the virus replication cycle, such as protein assembly and maturation of poliovirus virions (446).

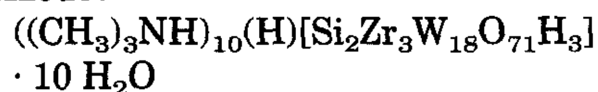
Infection of monkey kidney MA104 cells with SA-11 simian rotavirus, a nonenveloped double-stranded RNA virus, was also shown to inhibit PGA_1-induced HSP 70 synthesis (447). Electron microscopic analysis revealed that, in the present of PGA,, most of the virus particles remain in the membrane-enveloped intermediate form, and virus maturation is impaired. This effect might be caused by inhibition of glucosamine incorporation into

the NSP4 glycoprotein as well as partial inhibition of VP7 and VP4 synthesis in PGA_1-treated cells.

Because PGs inhibit viral replication by acting on multiple cellular and viral targets, their potential use in the treatment of viral diseases remains as an open question.

2.9.3 Polyoxometalates. Polyoxometalates (POMs) are oligomeric aggregates of metal cations bridges by oxide anions that form by self-assembly processes (for a review on POMs in medicine, see Ref. 448). Previously, POMs have demonstrated broad-spectrum antiviral activity against many enveloped viruses (e.g., HIV, influenza, paramyxoviruses, herpesviruses, etc.) (448). More recent studies by Schinazi and Sidwell showed that a series of germanium- or silicon-centered POMs with the Barrel (e.g., JM2919), Keggin (e.g., JM2921), or double Keggin (e.g., JM2927) structure were highly inhibitory to influenza (both types of A and B) viruses (449) and respiratory syncytial virus (450). The *in vitro* anti-RSV results strongly suggested inhibition of virus attachment as the primary mode of action (450). In the case of influenza, greatest *in vitro* efficacy was also seen during the period of viral adsorption and penetration (449). A more precise mechanism of action was revealed by a study with a Keggin-type PM-523 by Shigeta and Schinazi, showing that inhibition of influenza replication in MDCK cells by POMs was not because of inhibition of virus binding to cells, but was associated with inhibition of fusion of the viral envelop to the cellular membrane (451).

Generally, recent clinical isolates of influenza A were more susceptible to these compounds than older, laboratory-adapted strains; H1N1 viruses were more sensitive to these effects than the H3N2 viruses (449). When PM-523 was combined with ribavirin, synergistic anti-influenza (H1N1) effects were demonstrated both *in vitro* and in mice (by intranasal administration) (452). In a separate investigation in mice, Liu et al. showed HPB-2 (given either orally or intraperitoneally) to be more effective than ribavirin in preventing deaths and lowering lung consolidation (453).

JM2919:

$((CH_3)_3NH)_{10}(H)[Si_2Zr_3W_{18}O_{71}H_3]$
$\cdot 10\,H_2O$

JM2921: $K_7[A\text{-}\alpha\text{-}GeNb_3W_9O_{40}] \cdot 18H_2O$

JM2927: $K8[A\text{-}\beta\text{-}Si_2Nb_6W_{18}O_{77}]$

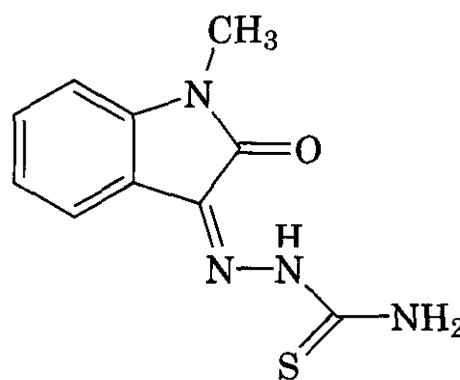
PM-523: $(iPrNH3)_6(H)[PTi_2W_{10}O_{38}(O_2)_2]$
$\cdot H_2O$

HPB-2: $Ce_2H_3[BW_9{}^{VI}W_2{}^{V}Mn(H_2O)O_{39}]$
$\cdot 12\,H_2O$

3 ORTHOPOXVIRUSES

Smallpox is presumably one of the most attractive pathogens to a potential bioterrorist because it meets the twin criteria of high transmissibility and high mortality. In addition, survivors are left with disfiguring sequelae. Historically drugs were tried both for treatment of smallpox and for prophylaxis of contacts but rarely in well-controlled clinical trials. Post-exposure prophylaxis with vaccinia immune globulin (VIG) demonstrated a modest anecdotal benefit when given to close contacts of smallpox patients along with revaccination, yet this scenario is not altogether relevant when an ever-increasing portion of the population has not received even a primary vaccination, and supplies of VIG are limited (454–456).

In 1963, post-exposure prophylaxis with marboran (N-methylisatin β-thiosemicarbazone) (**194**) was hailed as "the most significant



(194) Methisazone (Marboran)

advance in smallpox control since the days of JENNER." (457). However, this influential study was seriously flawed by current standards because most subjects were successfully vaccinated in infancy and revaccinated before receiving therapy. In addition, the study

groups were not randomized and subject compliance with the dosing schedule was not adequately ascertained (458). This last point is especially relevant because marboran caused severe nausea and vomiting in approximately one-half of treated subjects. Other investigators conducted a well-designed post-exposure prophylaxis study of a similar thiosemicarbazone (4-bromo-3-methylisothiazole-5-carboxaldehyde thiosemicarbazone, M&B 7714) in India (459). Enrollees were limited to those without previous vaccination before their contact with an index case of smallpox. Although there was a small decrease seen in the incidence of smallpox in the treated group, there was no decrease in mortality of those who acquired smallpox. The authors concluded that post-exposure treatment was not appropriate for routine use as the benefit was small and the drug poorly tolerated—52% of those in the treatment group refused to complete their treatment course.

On the basis of encouraging data in animal models of poxvirus infections, a controlled clinical trial was undertaken in India to evaluate the treatment of smallpox with M&B 7714, the thiosemicarbazone described above that showed a small amount of benefit as a prophylactic agent. Unfortunately, this compound showed no therapeutic benefit in patients with or without prior vaccination (460).

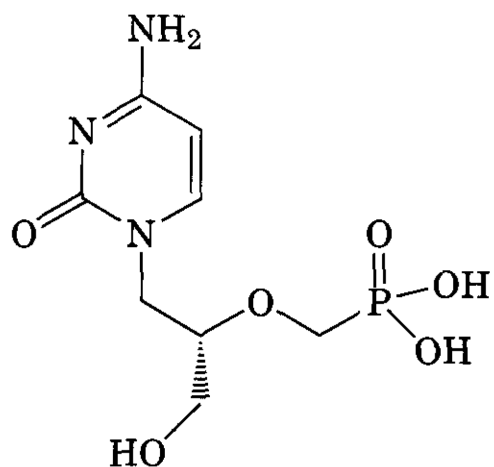
Other antiviral agents were studied in small trials. An initial uncontrolled treatment study in Bangladesh of nine patients with cytosine arabinoside reported that eight survived compared with an expected 45% mortality in this area (461). This report was quickly followed by two randomized hospital-based controlled studies in Ethiopia and Bangladesh that showed no benefit for treatment (462, 463). The observation in one study that three treated patients who seemed to be improving died late in the course of infection is worrisome in view of cytosine arabinoside's immunosuppressive activity (463). Adenosine arabinoside was also studied in Bangladesh in a small double-blind placebo-controlled trial, and no differences were found between placebo and Ara-A treated groups in mortality, fever days, or duration of days of virus isolation.

Another historically important medical need was for prophylaxis and treatment of complications for vaccination for smallpox. Although vaccination is undeniably effective, it is a live vaccine and not sufficiently attenuated to prevent its unwanted replication in people with impaired immune systems. There are four complications of vaccination that are considered serious. Three of these involve viral replication and should potentially be responsive to antiviral therapy. These are as follows. (1) progressive vaccinia in which the original vaccination lesion gradually extends rather than resolves and new lesions appear at noncontiguous sites. This is almost always fatal. (2) Eczema vaccinatum in which lesions appear on previously normal as well as eczematous areas of skin. The prognosis is correlated with the extent of skin involved. (3) Generalized vaccinia, a generalized skin eruption, has a good prognosis. The fourth serious complication is post-vaccinial encephalitis and is thought to result from immunpathology rather than viral replication. In the absence of treatment progressive vaccinia is usually fatal (458), and the U.S. mortality rate for eczema vaccinatum in very young children was approximated as 33%. Fortunately treatment with VIG seems to significantly improve survival to about 93%, although complication rates are too low to conduct controlled clinical trials (455.458).

Historical data on complication rates from the past will probably not be reliable predictors of future rates, should any government undertake the vaccination of large segments of the population to deter or ameliorate the consequences of a potential terrorist use of smallpox. The world's population has changed dramatically since the middle of the 20th century. Immunocompromised individuals comprise a much larger proportion of the overall population as a result of advances in transplantation and cancer treatment as well as the global devastation caused by HIV. In addition, the incidence of atopic dermatitis has dramatically increased in recent decades. As supplies of VIG are very limited, it may be as or even more important to identify an effective chemotherapeutic agent for the treatment of vaccinia complications as for the treatment of smallpox. Fortunately, because the viruses are

closely related, most antiviral agents with activity against one of these viruses is likely to also inhibit the other.

New preclinical data support use of cidofovir (195)for both treatment of smallpox and of treatment of complications of vaccination. Accordingly, the Department of Health and Human Services has prepared and sponsored Investigational New Drugs (INDs) for both potential indications.



(195) Cidofovir

3.1 Inhibitors of Orthopoxviruses

Concerns about possible unnatural outbreak of smallpox (such as in a bioterrorist attack), prompted a renewed interest in the search for antiviral agents that might be useful to treat smallpox (variola). Because evaluation of antivariola compounds cannot be done in a laboratory without a BSL-4 facility, and variola (as well as monkeypox) does not cause disease in adult mice (464), routine preclinical assessment of potential anti-variola compounds can only be studied in systems using surrogate viruses, such as vaccinia and cowpox viruses.

Very recently De Clercq published an influential review article to summarize the research on vaccinia virus inhibitors in his and others' laboratories (465). The inhibitors might serve as a paradigm for the chemotherapy of poxvirus infections. According to this review, the inhibitors could generally divide into two major categories — nucleoside derivatives and non-nucleoside organic molecules.

3.1.1 Methisazone (Marboran). Of the non-nucleoside compounds, methisazone (194), a thiosemicarbazone derivative of isatine (110), is worthy of note. In the early 1960s, Bauer et al. first showed that methisazone protected infant mice from fatal encephalitis caused by intracerebral injection of variola virus (464). Around that time methisazone was used in a case of eczema vaccinatum and this appeared to be the first clinical use of antiviral drug in man (464). As discussed above, clinical experience with methisazone has also included the treatment of vaccinia gangrenosa, prophylaxis of vaccinia infection, prophylaxis of smallpox (the main indication for the use of the drug), and treatment of smallpox (466). In any event, methisazone could serve as the lead for the design of a next generation drug with much improved pharmacological properties and safety profile.

3.1.2 Nucleoside Derivatives

3.1.2.1 Cidofovir (HPMPC). To promptly identify an anti-poxvirus drug that could be immediately available in the event of a bioterrorism attack, initial attention has focused on currently approved antiviral agents. Recent preclinical studies against vaccinia and cowpox viruses have identified cidofovir (CDV) (195) as a promising candidate. Cidofovir was first described in the literature in 1987 by De Clercq and Holý (467) and was approved in 1996 by the U.S. FDA as an intravenous treatment for human cytomegalovirus (HCMV) retinitis in AIDS patients under the licensed name Vistide (468–470). Once inside the cells, cidofovir follows two-step phosphorylation by cellular enzymes first to cidofovir monophosphate, CDVp, (e.g., by pyrimidine nucleoside monophosphate kinase) then to cidofovir diphosphate, CDVpp (e.g., by pyruvate kinase) (471). The latter, structurally analogous to a nucleoside triphosphate, serves as a competitive inhibitor of dCTP and an alternative substrate for HCMV DNA polymerase (472,473). Incorporation of a single cidofovir molecule causes a 31% decrease of in the rate of DNA elongation by HCMV DNA polymerase; incorporation of two consecutive molecules prohibits the DNA from further elongation (474). Furthermore, the intracellular cidofovir metabolites, namely CDVp, CDVpp, and CDVp choline, have very long half-life and these molecules confer a long-lasting antiviral response

of cidofovir and infrequent dosing for antiviral therapy (469,475,476).

Cidofovir has broad-spectrum activity against various DNA virus, including polyomaviruses, papillomaviruses, adenoviruses, herpesviruses, and poxviruses (468, 469, 475, 476). Huggins et al. reported that, in Vero and BSC-40 cells, cidofovir inhibited vaccinia, cowpox, camelpox, and monkeypox viruses with EC_{50} values in the range of 30–90 μM and variola virus in the range of 10 μM (Table 10.4) (477). It seemed that variola virus was most sensitive to cidofovir than the other orthopoxviruses in this particular study.

Several different animal models have been used to assess the therapeutic potential of cidofovir for the treatment of poxvirus infections. In earlier studies, De Clercq et al. had used intravenous injection of vaccinia virus to infect the mice and measured the suppression of tail lesion formation to assess a compound's antiviral effect (465). Similarly infected SCID mice also die from the disseminate vaccinia infection in addition to the development of tail lesions. In such infected SCID mice, cidofovir was shown to significantly delay the mean day of death using either treatment or prophylactic regimen (478). However, inoculation of virus by injection does not simulate the respiratory exposure that occurs in natural smallpox infection nor in a bioterrorist scenario, namely infection acquired by aerosol route. To mimic the natural infection, Bray et al. (479) and Smee et al. (480–482) demonstrated that aerosol or intranasal infection of BALB/c mice with vaccinia virus or cowpox virus caused the infected animal to develop pneumonia, lose weight, and eventually die from the disease. The efficacy of cidofovir observed in these new models can be summarized by the following.

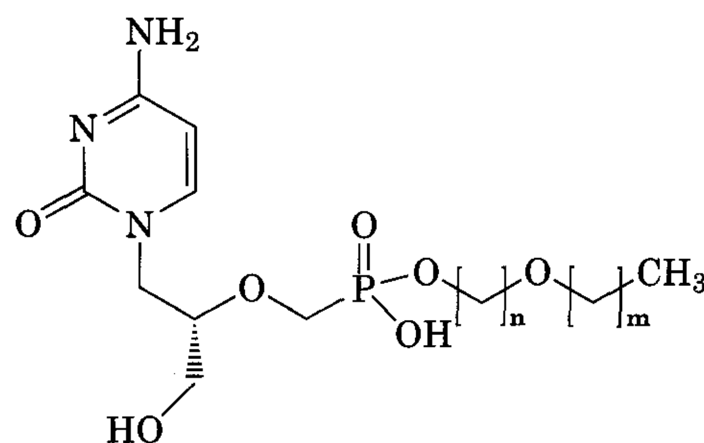
Cidofovir was active by intraperitoneal and intranasal routes against wild-type virus infections at non-toxic doses. It was not effective against infections caused by the cidofovir-resistant cowpox virus, and is not active orally.

- The efficacy of cidofovir against wild-type cowpox virus infections was similar to its activity against vaccinia virus infections.
- Mice could benefit from as little as a single treatment given a few days before or up to 4 days after virus exposure.
- Daily dosing with cidofovir was more beneficial than the single treatment regimen.

In a meeting presentation, Huggins reported that cynomolgus monkeys infected with monkeypox by small particle aerosol inoculation developed classical poxvirus lesions and pulmonary distress and that treatment of cidofovir, initiated on the day of infection, completely protected the animals from clinical and laboratory signs of disease (483). Topical cidofovir has been used to treat molluscum contagiosum (poxvirus) infections in AIDS patients (484).

In a bioterrorist attack, the number of exposed individuals is expected to be large; therefore, it may be technically difficult to administer cidofovir by injection. Because oral bioavailability of cidofovir is less than 5%, administration with an orally active prodrug form of cidofovir would be an ideal alternative under such circumstance.

Several reports by Hostetler et al. have shown that the oral bioavailability of nucleosides could be improved by conjugation with certain ether lipid groups, presumably by increasing oral absorption and cell membrane penetration (see references in Ref. 485). Cidofovir derivatives, HDP-CDV (**196**) and ODE-CDV (**197**), obtained by esterification of cido-



(196) HDP-CDV n = 3, m = 15
(197) ODE-CDV n = 2, m = 17

fovir with two long-chain alkoxyalkanols (3-hexadecyloxy-1-propanol and 3-octadecyloxy-1-ethanol, respectively) significantly enhanced both antiviral potency and selective indexes

Table 10.4 ***In*** *Vitro* **Activity of Cidofovir, Ribavirin, Methisazone, Carbocyclic 3-Deazaadenosine, and 3-Deazaneplanocin A Against Vaccinia, Cowpox, Camelpox, Monkeypox, and Variola Viruses (477)**

	EC_{50} (*μM*) in Indicated Cell Line											
	Vaccinia (Copenhagen)		Cowpox (Brighton)		Camelpox (Somalia)		Monkeypox (Copenhagen)		Variola (Bangladesh)		Variola (Congo)	Variola (Garcia)
	Vero	BSC	Vero	BSC	Vero	BSC	Vero	BSC	Vero	BSC	BSC	BSC
Cidofovir (195)	91	26	77	36	27	34	94	57	5	8	10	11
Ribavirin (3)	398	107	615	98	340	102	238	107	9	6	3	7
Methisazone (194)										258		
Carbocyclic 3-deazaadenosine (190)	2		>250		30		44		1	1	2	4
3-Deazaneplanocin A (191)	0.07		>250		2		2		0.01	0.06	0.02	0.08

EC_{50}, concentration of the compound required to inhibit viral-induced effect (cytopathic effect or plaque formation) by 50% in cell culture.

Table 10.5 *In Vitro* Activity of CDV, HDP-CDV, and ODE-CDV Against Vaccinia and Cowpox Viruses in HFF Cells (485)

	Vaccinia (6 Strains)			Cowpox (Brighton)		
	EC_{50} (μM)	CC_{50} (μM)	SI	EC_{50} (μM)	CC_{50} (μM)	SI
Cidofovir (CDV) (**195**)	10.1–46.2	278	6.0–27.5	44.7	278	6.2
HDP-CDV (**196**)	0.2–1.2	31	25.8–155	0.6	31	50
ODE-CDV (197)	0.1–0.4	14.3	35.8–143	0.3	14.3	49

EC_{50}, concentration of the compound required to inhibit viral-induced effect (cytopathic effect or plaque formation) by 50% in cell culture; CC_{50}, concentration of the compound required to induce 50% normal cell morphological change or inhibit 50% normal cell growth in cell culture; SI, CC_{50}/EC_{50}.

over the parent compound in human foreskin fibroblast (HFF) cells using a plaque reduction assay (Table 10.5) (485). Importantly, the potency against variola was also increased by more than 100-fold with the alkoxyalkyl prodrugs [data from J. Huggins, (485)]. These long-chain alkoxyalkyl esters of cidofovir resemble readily absorbed dietary phospholipids as evident by the observation that cellular uptake of ^{14}C-labeled HDP-CDV was severalfold greater than that observed with ^{14}C-labeled CDV in human lung fibroblast cells (485).

3.1.2.2 Ribavirin and Other Nucleoside Derivatives. The other nucleoside derivatives discussed in De Clercq's review could attribute their mechanisms of action to the inhibition of viral DNA synthesis (e.g., adenine arabinoside) or one of the following enzymes: IMP dehydrogenase [e.g., ribavirin (3) and EICAR (**165**)] SAH hydrolase [e.g., 5'-noraristeromycin (**187**), carbocyclic 3-deazaadenosine (**190**), and 3-deazaneplanocin A (**191**)], OMP decarboxylase [e.g., 6-azauridine (**163**)], CTP synthetase [e.g., CPE-C (**167**)], and thymidylate synthase (e.g., 5-nitro-2'-deoxyuridine).

Ribavirin is another approved drug that may have the potential to treat poxvirus infections because its broad-spectrum antiviral activity also encompasses vaccinia virus (465). The anti-vaccinia activity could be attributable to the inhibition of IMP dehydrogenase by ribavirin 5'-monophosphate (486), as well as inhibition of the capping of vaccinia mRNA by ribavirin 5'-triphosphate (487). As shown in Table 10.4, although ribavirin was a weaker inhibitor than cidofovir against vaccinia, cowpox, camelpox, and monkeypox, it was comparable with cidofovir against variola virus. In a separate study, Smee and Huggins reported that ribavirin inhibited plaque formation caused by camelpox, cowpox, monkeypox, or vaccinia viruses by 50% at approximately 2–12 μM in mouse 3T3 cells and at 30–250 μM in African green monkey kidney (Vero 76) cells (488,489). The greater potency and increased toxicity of ribavirin in 3T3 cells was due, at least in part, to the higher amount of ribavirin taken into 3T3 and greater accumulation of mono-, di-, and triphosphate forms of the drug in 3T3 cells than in Vero cells (489). Ribavirin was marginally active (EC_{50}, 281 μM) against vaccinia and not active against cowpox virus when tested in HFF cells (485).

In animal models, ribavirin protected vaccinia tail lesion formation in mice (465). More recently Smee et al. showed that mice could not survive a high intranasal cowpox virus challenge, but ribavirin-treated animals lived several days longer than placebos (488, 490). However, under less severe cowpox virus infection, high dose of subcutaneous ribavirin (100 mg/kg/day) could completely protect the infected mice from death, and lower doses could also improve the survival rate. Ribavirin treatment followed sequentially by cidofovir significantly increased the mean time to death beyond that achieved with ribavirin alone (490).

Carbocyclic 3-deazaadenosine (190) and 3-deazaneplanocin A (**191**), two potent inhibitors of S-adenosylhomocysteine hydrolase, seemed to have in *vitro* activity against various poxviruses, except cowpox in Vero cells (Table 10.4). The sensitivity of poxviruses to this series of compounds in various cell lines is a subject of further investigations.

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