ANTIVIRAL AGENTS

1. Introduction

In the 10 years since the last publication of an article on antiviral agents (1), research on this topic has taken on an explosive course, largely because of the growing threat of the epidemic of AIDS (acquired immunodeficiency syndrome) in the western hemisphere, which not only intensified research on HIV (human immunodeficiency virus), but also on other opportunistic viral infections associated with HIV, such as HCV (hepatitis C virus), HBV (hepatitis B virus), and CMV (cytomegalovirus). Thanks to many rapid advances made from chemical, biochemical, as well as molecular biological fronts that led to effective anti-HIV therapies including the most successful combination drug regimen, the threat from HIV infection has been somewhat downplayed in recent years from the edict of a "death sentence" to that of a "manageable illness". On the other hand, a relatively less known virus such as the West Nile virus (WNV), or the less heeded viruses such as HCV and HBV, have suddenly taken up the center stage. In this context, this article will largely focus on viruses of current notoriety and public health concerns, while only brushing up on others that do not evoke alarm at the moment, but are still highly virulent and dangerous when given proper conditions for replication and proliferation. In view of the enormous and ever-expanding literature on antiviral agents in recent years, it will be a mammoth task to provide a comprehensive treatise on this subject, covering all of the known viral maladies and remedies. Therefore, four major viruses have been chosen, for which a relatively more detailed discussion will be given here concerning viral structure and replication as well as recent advances made in antiviral therapies. These four viruses include HIV, HBV, HCV, and WNV. Also, since nucleoside analogues have played a major role as therapeutics in combating these viruses, a special emphasis has been placed on this class of drugs. Before elaborately discussing the mentioned viruses as well as the available antiviral therapies for them individually, this article will briefly delve on the classification of viruses, the general process of viral replication, the potential targets for selective antiviral action, and a few selected natural and synthetic nucleosides as antiviral agents.

2. Classification of Viruses

In the early twentieth century, viruses were classified based on the hosts they infected. Thus, they were grouped into (*a*) plant viruses, (*b*) animal viruses, and (*c*) bacteriophages. The present-day broad classification of viruses is based on the genetic material they contain: DNA or RNA viruses (1). They may contain single-stranded DNA (parvoviruses), double-stranded DNA (herpesviruses), single-stranded RNA (poliovirus), or double-stranded RNA (reoviruses). The RNA viruses are unique in that they are the only living organisms that use RNA to store their genetic information. All other reproducing forms of life employ DNA. The more subtle classification of viruses, however, would include their

hosts, chemical composition (including nucleic acid, protein, presence or absence of lipid envelope), shape, size, and symmetry. The major *animal viruses* can thus be subdivided into 18 categories: (*a*) herpesviruses, (*b*) papovaviruses, (*c*) adenoviruses, (*d*) poxviruses, (*e*) hepadnaviruses, (*f*) retroviruses, (*g*) orthomyxoviruses, (*h*) picornaviruses, (*i*) togaviruses, (*j*) rhabdoviruses, (*k*) paramyxoviruses, (*l*) reoviruses, (*m*) parvoviruses, (*n*) arenaviruses, (*o*) bunyaviruses, (*p*) filoviruses, and (*q*) coronaviruses.

Herpesviruses possess double-stranded, linear DNA that is 120,000–200,000 nucleotides long, icosahedron symmetry, protein coat, and lipid envelope. They include Herpes-simplex virus types 1 and 2 (HSV-1 and HSV-2), which cause recurrent cold sores and lesions (oral: type 1; genital: type 2). They also include Varicella-Zoster virus (VZV) that causes chicken pox and shingles, Epstein-Barr virus (EBV), which causes infectious mononucleosis, and is associated with selected cancers in China and Africa, and cytomegalovirus (CMV), which causes birth defects, and under special circumstances, pneumonia or hepatitis. Human CMV is one of the major opportunistic infections in HIV-infected patients as well as patients of the solid organ and bone marrow transplants.

Papovaviruses contain double-stranded, circular DNA that is 5000–8000 nucleotides long, icosahedral symmetry, and protein coat. They include human papillomaviruses, some of which cause oral or genital carcinomas, while others are responsible for benign genital tumors, polyomavirus that initiates tumors of wide variety in mouse, and simian virus 40 (SV 40), which is monkey virus that initiates tumors in rodents.

Adenoviruses possess double-stranded, linear DNA, 36,000–38,000 nucleotides long, with icosahedral geometry and protein coat. Human adenoviruses cause respiratory or enteric disease and infectious pinkeye. Some types of these viruses are capable of initiating tumors in rodents.

Poxviruses have double-stranded DNA, 130,000–280,000 nucleotides long. They are brick-shaped, and have lipids in the coat. The virion includes an RNA polymerase. Poxviruses that cause infections in man include smallpox, monkeypox, cowpox, tanapox, and *Molluscum contagiosum*.

Hepadnaviruses are part single-stranded and part double-stranded with a circular DNA, 3300–3400 nucleotides long, and possess nucleocapsid, protein coat, and lipid envelope. The virion includes DNA polymerase and reverse transcriptase. An important member of this family is the hepatitis B virus, which is responsible for causing serum hepatitis and liver cancer.

Retroviruses contain two linear, (+) single-strand, RNA molecules per virion, 3500–9000 nucleotides long, and a reverse transcriptase (RNA to DNA). They possess icosahedral shape, protein coat, and a lipid envelope. The retroviral family includes human T-cell leukemia virus-I (HTLV-I) that causes adult T-cell leukemia, human T-cell leukemia virus-II (HTLV-II), which is a possibly linked to hairy-cell leukemia, and human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2), which cause acquired immunodeficiency syndrome (AIDS). In addition, a variety of animal viruses, including Rous sarcoma virus and avian leukosis virus, are classified under retroviruses, and are known to be linked to cancers or immunodeficiencies in animals.

Orthomyxoviruses possess eight linear, (−) single-strand RNA molecules per virion, 13,600 nucleotides long, and a transcriptase (−RNA to +RNA).

They are helical in shape, and have a lipid envelope. *Influenza A Virus*, which causes the respiratory illness, belongs to this family.

Picornaviruses contain a (+) single-strand RNA genome, 7000 nucleotides long. They have icosahedral shape and a protein coat. Members of this family include poliovirus that causes infantile paralysis, rhinovirus that is responsible for common colds, and hepatitis A virus that causes infectious hepatitis.

Togaviruses contain a (+) single-strand RNA genome, 10,000–12,000 nucleotides, and have icosahedral shape, protein coat, and a lipid envelope. The flaviviruses used to be regarded as a genus within the *Togaviridae* family, but in 1984, they were provisionally reclassified as a distinct family (see below).

Rhabdoviruses contain a (−) single-strand RNA genome, 12,000 nucleotides long, and a transcriptase (−RNA to +RNA). They are bullet shaped, with a protein coat and a lipid envelope. Rabies virus, which causes rabies, is a member of this viral family.

Paramyxoviruses possess a (−) single-strand RNA genome, 15,900 nucleotides long. The virion includes a transcriptase (−RNA to +RNA). They have a helical shape, protein coat, and a lipid envelope. Mumps virus and measles virus (MV), which cause mumps and measles, respectively, along with respiratory synctial virus (RSV), which causes common cold-like upper respiratory tract infection in young children, represent this family.

Reoviruses have double-stranded RNA, and 10 chromosomes, 1000–4000 base-pairs. They have icosahedral shape and a protein coat. Rotaviruses, which cause infant enteritis, represent this family.

Parvoviruses are among the smallest, simplest eukaryotic viruses and were only discovered in the 1960s. They are widespread in nature; human parvovirus infections were only recognized in the 1980s. Essentially, they fall into two groups, defective viruses that are dependent on helper virus for replication and autonomous, replication-competent viruses. In all, >50 parvoviruses have been identified. They contain linear, nonsegmented, single-stranded DNA, ~5000 nucleotides long. Most of the strands packaged seem to be (−)sense, but adeno-assiciated viruses (AAVs) package equal amounts of (+) and (−) strands, and all seem to package at least a proportion of (+)sense strands. The virus particles are icosahedral, 18–26 nm diameter, and consist of protein (50%) and DNA (50%). Parvoviruses cause infections in a wide variety of birds and mammals, but 70–90% of most human populations are seropositive. The only known human parvovirus is referred to as B19. The most obvious symptom of B19 infection is a rubella-like rash.

Flaviviruses have single-stranded, positive sense RNA genomes that are 40–50 nm in diameter, 11 kilobase pairs in length. The virions are icosahedral and enveloped. Both hepatitis C virus (HCV) and West Nile virus (WNV), the two dreaded viruses of current notoriety in the western hemisphere, belong to the family of *Flaviviridae*. This family consists of arboviruses (ie, viruses borne by arthropods) that are classified into three genera: the flaviviruses, the pestiviruses, and the hepaciviruses. The flavivirus genus causes many human diseases like dengue fever, yellow fever, encephalitis, and hemorrhagic fevers. Although the natural reservoir of arboviruses is in avians and other animals, they are transmitted by arthropods like mosquitoes. Humans are infected with such viruses after being bitten by an infected arthropod. Pestiviruses only affect

cattle. Hepaciviruses are the hepatitis C viruses, affecting ~3% of the global human population.

Arenaviruses were named after the Latin word arena, meaning sand, because of their granular interior. They are large RNA viruses that contain dense, ribosome-sized particles that give the appearance of sand particles when viewed by an electron microscope. The virus particles are spherical and have an average diameter of 110–130 nm. All are enveloped in a lipid (fat) membrane. The natural hosts of these viruses are generally rats, bats, and mice. The viruses are then shed in the feces and urine to contaminate food and water. When humans consume the infected foods, they contract the infection. Some of them cause meningitis and various hemmorrhagic fevers. Other infections include Lassa fever, lymphocytic choriomeningitis, and Argentinean and Bolivian hemorrhagic fevers.

Bunyaviruses are single stranded, (−)sense, RNA viruses in three circular segments of 7, 4, and 2 kilobases, with an envelope and helical symmetry, 90–100 nm in diameter. They have a lipid envelope through which glycoprotein spikes protrude. Within the family *Bunyaviridae* there are two types of viruses that cause disease in humans, the arthropod-borne viruses and the hantaviruses. Arthropod-borne viruses include the bunyaviruses, phleboviruses, and nairoviruses. Hantaviruses, named after the Hantaan river in Korea where it was first discovered in 1978, include six different species. Bunyavirus infection attacks the central nervous system leading to viral encephalitis. Phlebovirus infection leads to two different disease entities that are found at two different geographical locations. One of them, the Sandfly fever, clinically gives rise to fever, rash and arthralgia. The other one, the Rift Valley fever, may lead to complications of retinopathy, meningoencephalitis, haemorrhagic manifestations and hepatic necrosis. Infection with the nairovirus results in an influenza-like illness with fever and haemorrhagic symptoms. The clinical features of hantaviruses include haemorrhagic fever with renal syndrome (HFRS), or its milder form called the nephropathia epidemica, and hantavirus pulmonary syndrome (HPS), characterized by sudden onset of coughing, dyspnoea, pulmonary oedema, pleural effusion and shock.

Filoviruses contain single, unsegmented, (−) sense RNA, ~19 kilobases long. They appear in several shapes, a biological feature called pleomorphism. These shapes include long, sometimes branched filaments, as well as shorter filaments shaped like a “6”, a “U”, or a circle. Viral filaments may measure up to 14,000 nm in length, have a uniform diameter of 80 nm, and are enveloped in a lipid (fatty) membrane. They have strong structural and genetic similarities to both the rhabdoviruses and paramyxoviruses. They cause severe hemorrhagic fever in humans and nonhuman primates. So far, only two members of this virus family have been identified, including the Marburg virus and the Ebola virus.

Coronaviruses are irregularly shaped particles, ~60–220 nm in diameter, with an outer envelope bearing distinctive, ‘club-shaped’ peplomers that give its ‘crown-like’ appearance, and hence, its family name. Their genomes contain nonsegmented, single-stranded, (+) sense RNA, 27–31 kilobases long, the longest of any RNA virus. They infect a variety of mammals and birds. The exact number of human isolates are not known as many cannot be grown in culture. They cause common respiratory and occasional enteric infections in infants older than 12 months.

3. The General Process of Viral Infection and the Available Remedies

In order to discover site- or process-specific antiviral agents, it is important to understand the specific biochemical processes that occur during viral infection. In all, there are seven stages (2) in a typical viral infection process: (*a*) *adsorption*: The attachment of the virus to specific receptors on the cell surface; (*b*) *penetration*: The viral entrance into the cell by penetration through plasma membrane; (*c*) *uncoating*: The release of viral nucleic acid from the covering proteins; (*d*) *transcription*: The production of viral mRNA from the viral genome; (*e*) *translation*: The synthesis of viral proteins, including coat proteins and enzymes necessary for viral replication, as well as replication of viral nucleic acid (ie, the parental genome or complimentary strand); (*f*) *virion assembly*: The assembly of individual components of the viron (nucleic acid and structural proteins synthesized in stage *e*), and transportation to the site of nucleocapsid assembly, followed by autocatalytic assembly; (*g*) *release*: For viruses with icosahedral symmetry that do not have an envelope, this stage comes after disintegration of host cell as a result of the killing action of the infecting virus; for enveloped viruses, the assembled nucleocapsids move toward the modified membrane areas where the synthesized viral matrix protein replaces the cellular membrane proteins, and then nucleocapsids bud through the modified membrane, wrapping themselves into a portion of membrane in the process.

The preferred approach to combat viral diseases is the prevention of infection by active immunization. There are a number of successful vaccines for prophylaxis of some viruses such as polio, mumps, measles, influenza, encephalitis, hepatitis, and smallpox. On the other hand, there has been less success in the prevention of viruses such as the HIV, HSV, and RSV. Therefore the need for effective medicines to treat these viruses is urgent. Furthermore, millions of people around the globe are still suffering from a variety of viral diseases for which the vaccines already exist. For example, with >1 million child deaths per year, the measles virus (MV) ranks eight as the cause of death worldwide, especially in the developing countries (3). Despite large vaccination campaigns, MV is still resisting eradication, and there is no available therapeutic treatment (4). The MV infection causes a respiratory disease which is, more often than not, controlled solely by the immune response. The uncontrolled MV infection can lead to a severe immunosuppression that is responsible for additional opportunistic infections (5,6). Furthermore, in certain cases, MV establishes persistent infection of the brain leading to neurological complications (7). Also, some viruses are known to have very long latency period (8). Papova viruses may remain latent for years following childhood activation. These viruses reactivate and lead to viral diseases once T-lymphocyte hyporesponsiveness develops, either as a result of exogenous therapy, as in transplant recipients, or because of endogenous factors such as cancer or AIDS.

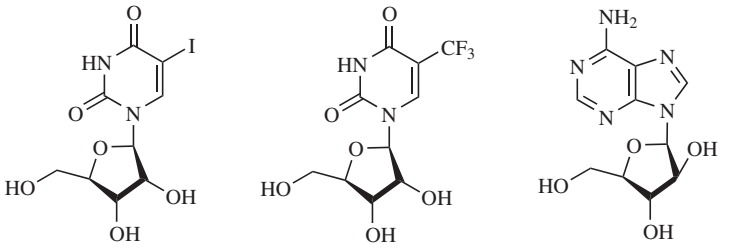
Two major virus-specific processes are normally targeted in order to develop selective antiviral agents: (*a*) early events, including adsorption, penetration and uncoating, and (*b*) later synthetic events that concern intracellular replication of the virus. In the fist stage of virus activity, heparin, an anionic polyelectrolyte of relatively high molecular weight (>5000 D) has shown to favor the formation of a complex with HSV that prevents virus from establishing an effective interaction

with cell membrane (9,10). This phenomenon is ascribed to the unique anionic structure, an acid mucopolysaccaride (MW = 13,000 D), built by sulfated D-glucosamine and D-glucuronic acid units. Although there is no evidence heparin is toxic for the host cells, its antiherpes virus action is essentially nonspecific because of the ionic nature of the chemical-virus interaction. The electrostatic interaction can also be established between heparin and the positively charged groups projected out of cell membrane. Once virus successfully makes contacts with the host membrane, heparin is no longer effective. On the other hand, oligopeptides have comparably more potential as candidates in inhibiting the early activities of certain types of viruses. Sequence-specific oligopeptides that mimic the N-terminal region of the paramyxovirus F1 polypeptide (11) (16–19) are specific inhibitors of paramyxoviruses (12). Oligopeptides that mimic the N-terminal region of the orthomyxovirus polypeptide specifically inhibit influenza viruses (11). Recently, two synthetic proteins (DP-107 and DP-178) (13), which mimic the separate domains within the HIV-1 transmembrane (TM) protein-gp4l, have been found to be stable and potent inhibitors of HIV-1 infection and fusion. This inhibitory effect can be intensified by increasing the length of the oligopeptide or by the presence of a carbobenzyloxy group on the N-terminal amino acid, whereas the esterification of the C-terminal amino acid decreases the activity. It was proposed that the antiviral effects of these oligopeptides are due to interference with binding of the N-termini of the viral envelope glycoproteins to specific receptors on the mammalian host cell membrane. Adamantane derivatives, amantadine hydrochloride (Symmetrel or Symadine) and rimantadine hydrochloride (α-methyl-1-adamantane methylamine hydrochloride or Flumadine) salts, are commercially available drugs for prevention and treatment of type A influenza viruses. Although Symmetrel is the first antiviral drug licensed in United States nearly 50 years ago, its mechanism of antiviral action remained unclear until recently. Early research using electron microscopy and pulse-labeling techniques revealed that amantadine acted at some point after late uncoating, but before initiation of viral RNA transcription (14). Rimantadine and amantadine have no inhibitory effect on the activity of viral polymerase; instead, the synthesis of the latter enzyme is prevented. Recently, the structure and function of the small protein-M2 in influenza A were elucidated (15). This protein has a single transmembrane helix that associates to form a tetramer *in vivo*, which forms proton-selective ion channels. This association is a pH dependent monomer–tetramer equilibrium. Upon binding of amantadine, the equilibrium shifts to tetrameric species. At higher pH, close to the pK_a of a histidine side chain where the protonation occurs within the transmembrane helices, the binding of amantadine is favored, which pushes the equilibrium toward the tetramer. It is suggested that amantadine competes with protons for binding to the deprotonated tetramer, thereby stabilizing the tetramer in a slightly altered conformation. It leads to the blockage of proton flux.

Other examples of antiviral agents targeted at the early events of viral activity include DIQA (3,4-dihydro-1-isoquinolineacetamide HCl), which has a broad-spectrum antiviral activity against lethal influenza A virus, echovirus, Columbia SK virus, herpes simplex virus, and rhino virus (16,17). The mechanism of its action involves the inhibition of virus penetration into the host cell membrane (16). Arildone (4-[6-(2-chloro-4-methoxyphenoxy)hexyl]-3,5-heptanedione),

also a broad-spectrum antiviral agent against a number of RNA-containing and DNA-containing viruses, has been reported to interact directly with the polio virus capsid proteins in a way as to inhibit the uncoating of the virus and to prevent the subsequent virus-induced inhibition of cellular protein synthesis (18). It also showed inhibition of herpes virus DNA and virus-specific protein synthesis by acting on an early event in the virus replication cycle (19).

There exists a much larger pool of synthetic drugs that target the later events in a virus life cycle as compared to only a few synthetic or natural products that target the early events. These events are known to be virus specific. The viruses synthesize and utilize specific enzymes and proteins, and more importantly, the replication of viral genetic codes is also virus specific. The specificity in the synthesis of viral DNA or RNA is conferred by the virus-specific enzymes such as kinases, helicases, polymerases, transcriptases, reductases, etc. Viruses are more prone to mutations as compared with other microorganisms. The mutation rate of a virus is much higher than that of its host cell. Its mutants possess an excellent chance to be accommodated in the new host cell and escape from the host immune responses. On the other hand, high mutation rate means less selectivity toward substrates for the enzymes involved in DNA/RNA replication process. Once the potential drug candidate (an unnatural nucleotide analogue, for example) enters the catalytic site, it may disrupt or terminate the activity of enzymes. The unnatural nucleotides can be incorporated into DNA double helix, distort the DNA structure, and utimately stop the virus replication. For example, the nucleoside analogues idoxuridine (5-iodo-2′-deoxyuridine) (20,21), trifluridine (5-trifluoromethyl-2′-deoxyuridine) (22,23), and vidarabine



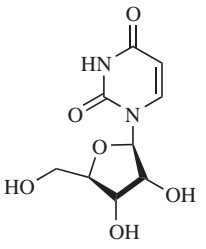
(1-β-D-arabinofuranosyladenine or Ara-A) (20,21,24) appear to block replication in herpesviruses by three general mechanisms: first, as the monophosphates, they inhibit the formation of precursor nucleotides required for DNA synthesis; second, as triphosphates, they inhibit DNA polymerase; and third, the triphosphates are incorporated into DNA, which then does not function normally. For example, the DNA containing idoxuridine is more susceptible to strand breakage as well as to miscoded errors in RNA and protein synthesis.

There are also many compounds that fall outside the nucleoside family. A distinct example comes from the ever-growing fight against AIDS. In the late stage of a virus life cycle, the virus-specific processing of certain viral proteins by viral or cellular proteases is crucial. It was revealed that HIV expresses three genes as precursor polypepteins. Two of these gene products (designated as P55gag and p160 gag-pol proteins) undergo cleavage at several sites by a

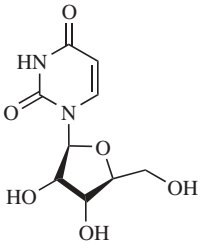
virally encoded protease to form structural proteins and enzymes required for replication (25). This fact has stimulated the research efforts to find safe and effective inhibitors for the viral protease. It is believed that the inhibitors should resemble a small portion of the substrate polyprotein structure but contain an isosteric replacement for the scissible (hydrolyzable) peptide bond that mimics the transition state for the hydrolysis of that bond, which is stable against cleavage. This has led to the discovery of several successful clinical candidates for HIV infection.

4. Nucleoside Analogues as Antiviral Agents

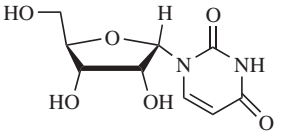
Initially, the term "nucleoside" was referred to the purine and pyrimidine *N*-glycosides derived from nucleic acid. However, after the discovery of pseudouridine (5-β-D-ribofuranosyluracil), a natural constituent of tRNA, it became a common practice to consider even those molecules whose heterocyclic rings are connected to the sugar moieties at the anomeric junctions through carbon–carbon single bonds. Such compounds are classified as *C-nucleosides* (26,27). Another interesting class of nucleosides, called *L-nucleosides* (28,29), are lately emerging as powerful antiviral compounds. The sugar parts of these nucleoside analogues possess the L- instead of the natural D-configuration. Furthermore, considering the possibility of existence of the carbon linking the base to the heterocycle into α- or β- anomeric form (β being the natural form), there exists an additional category of α-*nucleosides*. These different classes of nucleosides are contrasted below, using uridine as an example.



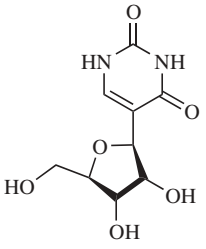
D-Uridine



L-Uridine

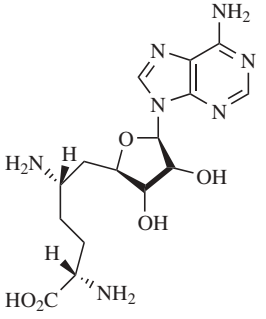


α-Uridine

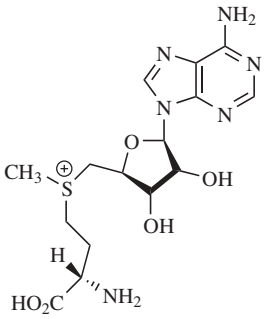


C-Uridine
(pseudouridine)

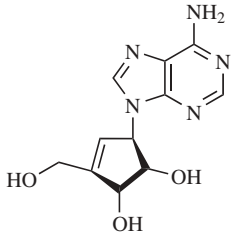
Among the naturally occurring nucleosides, sinefungin, an antifungal antibiotic isolated from *Streptomyces griseolus* (30), and its related metabolite A9145C (31), were found to be potent inhibitors of Newcastle disease virion, vaccinia virion mRNA (guanine-7-)-methyltransferase and vaccinia virion mRNA (nucleoside-2′)-methyltransferase. The structure of sinefungin is close to that of *S*-adenosylmethionine (AdoMet) with the methylthio group replaced by an aminomethylene group. They were found to be competitive inhibitors of the *S*-adenosyl-L-methionine-dependent enzymes. Neplanocin A, a carbocyclic analogue of adenosine with a unique cyclopentene structure, was isolated from the culture filtrate of *Ampullariella regularis* A11079 in 1980 (32). It has potent antitumor as well as antiviral activity. It acts primarily as an *S*-adenosylhomocysteine (AdoHcy) hydrolase inhibitor (33), which accounts for its broad-spectrum antiviral activities. AdoHcy hydrolase plays a key role in methylation reactions that depend on *S*-adenosylmethionine (AdoMet) as a methyl donor.



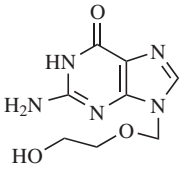
Sinefungin



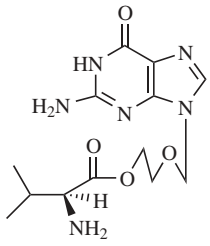
AdoMet



Neplanocin



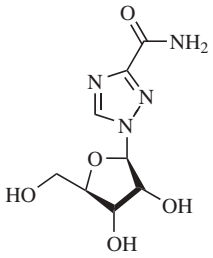
Acyclovir



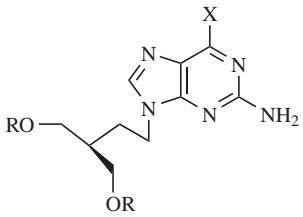
Valaciclovir

In contrast to the limited number of natural nucleosides, numerous synthetic nucleosides are now available for treating viral infections. This is because of the unlimited possibilities for modifications both at the carbohydrate and the base sites. Acyclovir or ACV [9-(2-hydroxyethoxymethyl)guanine] (34–43) and its oral prodrug–Valaciclovir (Val–ACV) (35,38,44–53) are the two most commonly prescribed drugs for the treatment of HSV infections. These compounds contain a unique structure as compared with the naturally occurring guanosine in that an acyclic side chain is designed to replace the cyclic ribose moiety. When tested in cell culture, the majority of isolates of HSV are sensitive to ACV. The ACV-resistant strains are rarely found in clinical practice among immunocompetent

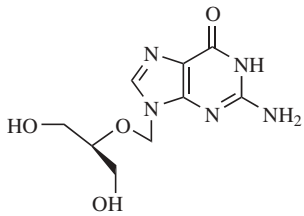
patients (<1% isolates). Resistant HSV occurs much more frequently, however, among immunocompromised patients during treatment (~5% isolates). Acyclovir and valaciclovir also show activity against human cytomegalovirus (HCMV) and varicella-zoster virus (VZV) (44,50,51,54,55). To date, acyclovir is the standard for the treatment of mucosal, cutaneous, and systemic HSV-1 and HSV-2 infections (including herpes encephalitis and genital herpes) and VZV infections. Valaciclovir was discovered to achieve substantially higher plasma levels of acyclovir than oral acyclovir itself. It has proven to be particularly useful in the treatment of herpes zoster and in the prevention of HCMV disease after renal transplantation (56). Other acyclic nucleoside analogues, besides ACV and Val–ACV, that are available for treatment of diseases caused by viruses belonging to the herpes family (comprising HSV-1, HSV-2, HCMV, and VZV), include penciclovir, famciclovir, ganciclovir, and cidofovir (35,36,45–47,49,53,57–74).



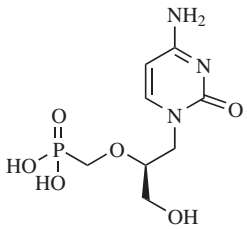
Ribavirin



R = H, X = OH, Penciclovir
R = Ac, X = H, Famciclovir



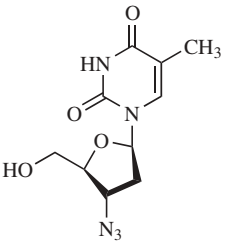
Ganciclovir



Cidofovir

Ribavirin, which was synthesized nearly 30 years ago (75) by the research group at ICN Pharmaceuticals, is a broad-spectrum antiviral agent (76–79). Instead of a usual purine or pyrimidine base, it has a five-membered triazole ring with a carboxamide substitution at position-3 of the heterocycle. A large number of RNA and DNA viruses are sensitive to ribavirin, including the respiratory syncytial virus, influenza, parainfluenza, herpes viruses, and RNA tumor viruses. It was found that 5′-monophosphate of ribavirin accounts for the antiviral action in mammalian cell culture (80). The 5′-monophosphate derivative of ribavirin was a potent inhibitor of the enzyme IMP dehydrogenase, thereby preventing the conversion of IMP to xanthine monophosphate (XMP) (81,82). XMP is required for guanosine triphosphate (GTP) synthesis. Thus, the antiviral activity of ribavirin might be due to the inhibition of GTP biosynthesis in virus-infected cells, which in turn results in the inhibition of viral nucleic acid synthesis. Several other

possible mechanisms have been proposed (78,79), which include the recently described activity as an RNA mutagen (77,83–85,87,88,90). Ribavirin triphosphate (RTP) inhibits viral RNA polymerases. It also prevents the capping of viral mRNA by inhibiting guanyl N^7-methyltransferase. It was suggested that the phosphorylation of ribavirin was most likely accomplished by deoxyadenosine kinase (86). The kinetic studies from rat liver preparations showed that ribavirin and deoxyadenosine competitively inhibited the phosphorylation of each other. To date, success has been achieved with the aerosol use of ribavirin in treating respiratory syncytial virus infection in infants and young children (87–89). It also showed clinical benefit in treating severe and life-threatening infections caused by the Lassa fever virus (90). It is the first antiviral drug that is able to reduce mortality in a highly lethal systemic disease by more than 90%. Furthermore, ribavirin is the only approved nucleoside analogue for the treatment of hepatitis C virus (HCV) infections, but the approval is limited to combination therapy with interferon, another drug used against HCV. HCV currently threatens the global public health with more than 200 million people having been infected worldwide (85,91,92). However, there have been a few documented side effects associated with the use of ribavirin. The treatment results in a fall in transaminase levels and some decrease in hepatic inflammation.



AZT

AZT (azidothymidine) and other 2′,3′-dideoxynucleoside analogues that are currently employed for treating HIV infections, are inhibitors of the HIV reverse transcriptase (HIV RT) (93,94). Their mechanism of action is believed to be the chain termination of nucleic acid synthesis during the RT-catalyzed reverse transcription of HIV RNA genome into its complementary DNA strand (95). The nucleoside analogue is first converted into its 5′-triphosphate derivative by the host kinases, which then is incorporated into the developing DNA strand opposite to an adenosine residue in the viral RNA template. The lack of the 3′-hydroxy group in AZT, which is crucial for the chain extension, prevents further incorporation of nucleotide building blocks beyond the point of insertion, thus leading to chain termination.

5. The Viruses of Current Health Concern and the Related Antiviral Therapy

As mentioned under Introduction, the following four viruses are currently of prime health concern worldwide: HIV, HBV, HCV and WNV, and so, this article

will focus on these viruses and the progress being made on antiviral therapy to treat each of these viral infections.

5.1. Human Immunodeficiency Virus (HIV). Perhaps no other virus in recent history has stirred more global panic and paranoia than HIV, an etiological agent causing the acquired immunodeficiency syndrome (AIDS) (96–98). The AIDS epidemic has made more impact on public health than even the black plague of the late Middle Ages. With >25 million people vanishing worldwide due to its infection since the early 1980s, and >40 million individuals currently infected with, HIV is one of the deadliest viruses ever to hit the mankind (99). Despite intense efforts from several research fronts including chemistry, biochemistry, biology, and biotechnology, and not to mention epidemiology and prevention measures, the fight to conquer HIV altogether still remains largely elusive. As proven techniques of viral attack seem inadequate against HIV, and chances for a suitable vaccine continue to be disappointing, the current research trend is to focus on the complete viral life cycle and the replication process for new targets. The ultimate success may lie in the power of modern molecular biology to explore every aspect of the HIV life cycle and every response of the human body toward viral invasion (97). The tools of biotechnology have greatly aided in sequencing the viral genome as well as the proteins that are associated with it. So, it is important to review the current status of knowledge on the viral structure and its life cycle (100–106) before delving into what is being targeted for antiviral therapy (107,108).

As classified earlier, HIV is a retrovirus consisting of two copies of a single-stranded RNA genome and a few replicative and accessory proteins within the boundaries of a lipoprotein shell (see Figure 1), known as the viral envelope. Embedded in the viral envelope is a complex protein known as *env*, which consists of an outer protruding cap glycoprotein (gp) 120, and a stem gp41. Within the viral envelope is an HIV protein called p17 (matrix), and within this is the viral core or capsid, which is made of another viral protein p24 (core antigen). The major elements contained within the viral core are two single strands of HIV–RNA, a protein p7 (nucleocapsid), and three enzyme proteins, p66 (reverse transcriptase), p11 (protease), and p31 (integrase) (100–106).

Infection begins when an HIV particle encounters a target T-Helper cell of the host containing a surface receptor molecule called CD4 (109). The virus particle uses gp120 to attach itself to the host cell membrane and then enters. Within the cell, the virus particle releases its RNA as well as the crucial enzyme *reverse transcriptase* (HIV RT), which converts the viral RNA into a cDNA copy. This new HIV–DNA then moves into the cell's nucleus where, with the help of the enzyme *integrase*, it is then inserted into the host cell's DNA. Once into the host cell's genes, HIV DNA is called a *provirus*. The HIV provirus is then replicated by the host cell, which then cranks out multiple copies of its own genome, and produces the mRNA necessary for creation of viral proteins. The fully replicated and packaged viral particles then bud out of the cell to infect fresh new cells.

In addition to HIV *reverse transcriptase* and *integrase*, the two key enzymes involved in the viral replication process as described above, a third enzyme called HIV *protease* is also a viable target for antiviral therapy. After replication within a host cell, when new viral particles are ready to break off to infect other cells,

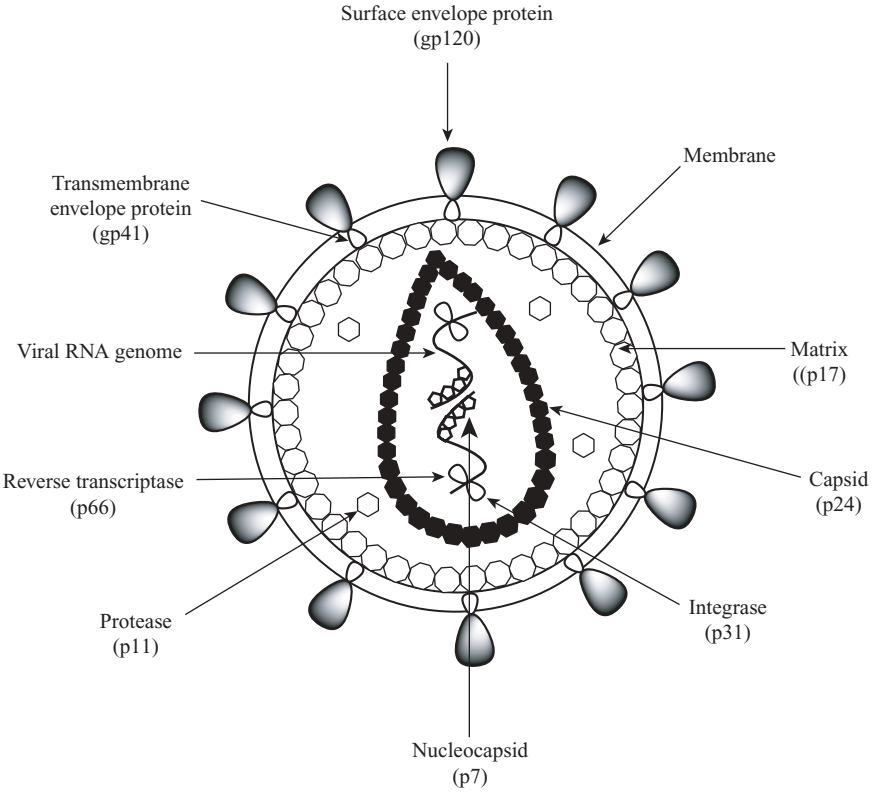


Fig. 1. The molecular structure of HIV.

protease plays a vital role in cutting longer protein strands into smaller parts needed to assemble a mature virus. These shorter polypeptides include three structural proteins, capsid (p24), matrix (p17), and nucleocapsid (p7), encoded by the viral *gag* gene, as well as three enzymes that are crucial for replication, including a reverse transcriptase (p51), an integrase (p31), and an RNAse H (p15), in addition to a new protease (p11), encoded by the viral *pol* gene. Thus, when protease is blocked, the new viral particles fail to mature.

Infection of the host T-cells by HIV involves a process of receptor interaction and membrane fusion. The viral *env* gene codes for the envelope protein (ENV) gp160. The latter is cleaved by HIV protease into two protein fragments called gp120 and gp41. While gp120 binds to the CD4 receptor on the surface of the host immune cells, gp41 mediates the fusion between viral and cellular membranes.

HIV protease thus plays a critical function in the HIV life cycle. Consequently, the detailed structural analysis of HIV protease has led to the discovery of protease inhibitors (110–115), one of the important components in the *highly active antiretroviral therapy*, commonly referred to as HAART Therapy (116–118) that consists of multiple drug regimen aimed at different targets in the viral life cycle. The "cocktail" regimen normally includes a protease inhibitor along with two HIV RT inhibitors, one a nucleoside and the other a nonnucleoside.

The HAART therapy has been highly successful in preventing AIDS-related deaths in the industrialized world, although it had relatively low impact in the developing world because of the high cost of medication.

5.2. Antiviral Therapy for HIV Infections. *Vaccines.* To date, over 60 phase I/II trials of 30 candidate vaccines against HIV have been conducted worldwide (98,119–133). Most initial approaches focused on the HIV envelope protein, produced in insect, bacteria, yeast, or mammalian cells, which was logical given that envelope is the primary target for neutralizing antibodies in HIV-infected persons. At least 13 different gp120 and gp160 envelope candidates have been evaluated in phase I/II trials, predominantly through NIAID-supported AIDS vaccine evaluation group. Most research focused on gp120 rather than gp140/gp160, as the latter are generally more difficult to produce and did not initially offer any clear advantage over gp120 forms. Overall, they have been safe and immunogenic in diverse populations, and have induced neutralizing antibody in nearly 100% recipients, but rarely induced CD8+ cytotoxic T lymphocytes (CTL) even when formulated in novel adjuvants that effectively induced CTL in mice, although mammalian-derived envelope preparations have been better inducers of neutralizing antibody than candidates produced in yeast and bacteria. CTLs recognize surface markers on other cells that have been labeled for destruction. In this way, CTLs help to keep virus-infected (or malignant) cells in check. The antibodies induced by these early envelope preparations rarely neutralized primary isolates of HIV.

In an effort to induce both CTL and antibody responses, the attention was turned to evaluating a combination vaccine approach in which two types of vaccines are used (124,125,129,131). Most commonly referred to as "prime-boost", this has involved an immunization (priming) with a recombinant viral vector followed by or combined with boosting doses of recombinant protein. Three recombinant attenuated vaccinia vectors and five recombinant canarypox vectors have been evaluated in phase I trials alone and in combination with a recombinant protein envelope boost. In general, vaccinia-immune individuals have not responded as well as vaccinia-naïve individuals to vaccinia vectors, although there has been no difference in the response of these groups to recombinant canarypox vectors. All recombinant viral vectors have been safe and immunogenic to date, and have been shown to prime the immune response to an envelope boost, thereby necessitating fewer doses of recombinant protein to reach maximum antibodies titers. However, the antibodies elicited in prime-boost protocols so far have a limited breadth of reactivity.

The availability of several recombinant canarypox vectors has provided interesting results that may prove to be generalizable to other viral vectors. Canarypox is the first candidate HIV vaccine that has induced cross-clade functional CTL responses. Increasing the complexity of the canarypox vectors by inclusion of more genes/epitopes has increased the percent of volunteers that have detectable CTL to a greater extent than did increasing the dose of the viral vector. Importantly, CTLs from volunteers were able to kill peripheral blood mononuclear cells infected with primary isolates of HIV, suggesting that induced CTLs could have biological significance.

Other strategies that have progressed to phase I trials in uninfected persons include peptides, lipopeptides, DNA, an attenuated Salmonella vector,

lipopeptides, p24, etc. To date, none has proven as effective in eliciting human CTL and/or antibody as the recombinant canarypox-envelope combination. Merck has advanced a candidate DNA vaccine (125,129,134–136) containing a codon-optimized gag gene to phase I trials. In 2001, NIAID began phase I trials of a vaccine that contained DNA for the *gag* and *pol* genes. Gag and Pol are considered good candidates for developing an AIDS vaccine as they are relatively constant across different virus strains and account for a large percentage of total virus protein. Other approaches to improve the immunogenicity of DNA vaccines are being pursued and may enter phase I trials over the next few years.

In summary, clinical trials of candidate HIV vaccines have so far been only informative. In the absence of validated correlates of immune protection, larger trials of the most promising candidates will be needed. Furthermore, as promising candidates advance to efficacy trials, there does appear to be room for improvement. There is at least as much if not more known about the HIV genome than other pathogens for which vaccines have successfully been made. Advances in genomics and micro-array technologies will likely have multiple applications in the field of HIV vaccine development. For one, new DNA approaches in which combinations of DNA containing genes of different clades are currently in preclinical research. Methods that help identify optimal DNA sequences for inducing CTL in proposed trial populations with defined HLA backgrounds could help increase the immunogenicity of these and other approaches. In addition, there will be a need to apply new, highly sensitive techniques for HIV detection to determine true infection and to detect infection in small volume samples in high through put assays. Finally, the advent of micro-array technologies could prove to be useful in exploring and cataloguing immune response genes that are up or down regulated and that correlate with protection.

Chemotherapy. Currently, there are a total of 16 drugs that have been approved by the U.S. Food and Drug Administration (FDA) for the treatment of AIDS (93,94). Seven out of the 16 are nucleoside-based reverse transcriptase inhibitors (NRTI), three are nonnucleoside reverse transcriptase inhibitors (NNRTI), and 6 are protease inhibitors. NRTIs (Figure 2) include AZT (zidovudine) (93,137,138), ddC (93,139) (zalcitabine), ddI (didanosine) (93,139), d4T (stavudine) (93,140,141), 3TC (lamivudine) (93,137,142,143), abacavir (93,94,144–148), and Bis-POC-PMPA (tenofovir disoproxil) (93,94,147,149–152), while the approved NNRTIs (Figure 3) are nevirapine (viramune) (153), delavirdine (93,154,155), and efavirenz (sustiva; stocrin) (93,144,155–157). The six protease inhibitors that have been FDA-approved include saquinavir (93,94,112,113, 115,144,147,158,159), indinavir (93,94,112,115,147,160,161), ritonavir (93,94, 111–115,144,147,162,163), nelfinavir (93,94,112,113,115,147,161), amprenavir (93,94,112,113,147,162,164–170), and lopinavir (93,94,111–114,144,171,172) (Figure 4). The HIV reverse transcriptase (RT) (173–178), coded by the *pol* gene, is both RNA- and DNA-dependent polymerase. While HIV–RT makes a DNA copy of the viral RNA template, RNAse H of the virus chews away the RNA strand from the initially formed RNA–DNA hybrid. This will allow further synthesis of viral DNA duplex, which can then integrate into the host genome assisted by viral integrase. HIV–RT has been the key target of anti-AIDS drugs for a number of years, and still continues to be the major focus in the HAART therapy (179) described earlier. All of the nucleoside RT inhibitors

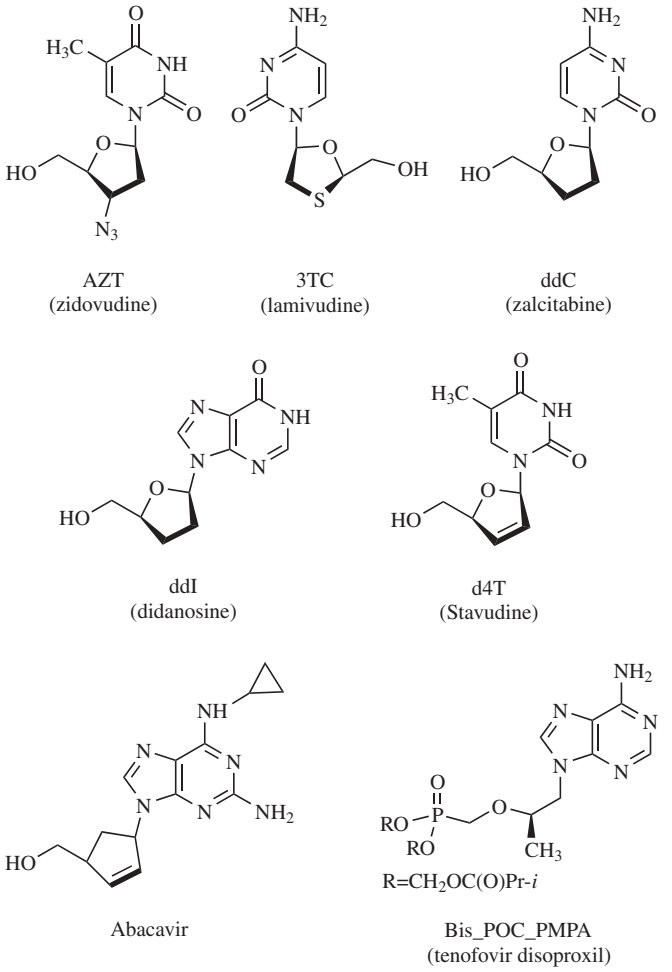


Fig. 2. FDA-approved nucleoside reverse transcriptase inhibitors (NRTIs) against HIV.

(NRTIs) (93,94,179,180) share a common mechanistic principle in that they are phosphorylated *in vivo* in the host cells, and subsequently are incorporated into the developing viral nucleic acids. This in turn results in nucleic acid chain termination since NRTIs lack the crucial 3′-hydroxy group that is necessary for chain extension.

Like NRTIs, NNRTIs (93,147,155,161,179–184) also target HIV–RT. However, unlike NRTIs, they bind RT at a secondary or allosteric site instead of at the active sites utilized by the NRTI. This causes conformational change in RT, which leads to alteration of the active site pocket. The change results in reduced binding of naturally occurring nucleosides and thus reduced viral cDNA elongation. NNRTIs are direct inhibitors of HIV reverse transcriptase that, unlike RTIs, are not incorporated into the viral DNA molecule. A major advantage of NNRTIs, therefore, is that these compounds require no phosphorylation by cellular enzymes in order to be active. NNRTIs work synergistically with

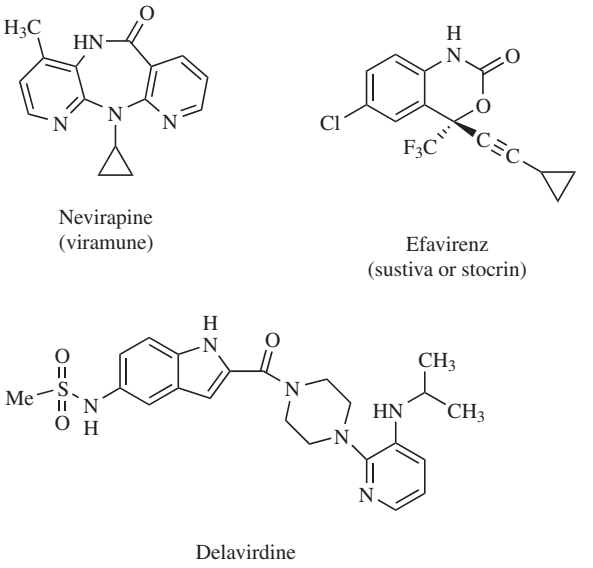


Fig. 3. FDA-approved nonnucleoside reverse transcriptase inhibitors (NNRTIs) against HIV.

most NRTIs, and are demonstrating impressive efficacy in increasing immunologic markers and decreasing viral load markers in HIV-infected patients. While they are very potent antiretrovirals, they also suffer from a major drawback in that the resistance against them can develop quickly if the drugs are not taken exactly as prescribed, and once the resistance develops to one drug in the class, there will probably be a resistance to all the drugs in that class. Thus, NNRTIs appear to be highly cross-resistant. A mutation at position 103 on the HIV reverse transcriptase gene is known to confer resistance to all of the agents in the class. However, this mutation does not confer resistance to drugs in other classes.

Two other classes of drugs against HIV that are currently under clinical trials are *Integrase* (93,94,185–191) and *Fusion* Inhibitors (192–195). HIV integrase is the third key enzyme in HIV replication besides protease and reverse transcriptase. As described earlier, the integration of provirus into the host genome is catalyzed by virally encoded integrase which has multiple functions. First, it acts as an exonuclease to cut the complementary viral DNA produced by HIV–RT to the appropriate size. Second, it serves as an endonuclease to cut the host DNA so as to facilitate insertion of the provirus. Finally, it acts as a ligase to fuse the host and viral DNAs into a seamless whole. Currently, a few drugs are being developed for inhibition of this integration step. At the most recent fourteenth International AIDS conference held in Barcelona, Spain in July 2002, Merck and Co. presented the results of clinical and animal trials of its two HIV integrase inhibitors, called L870810 and L870812. Initial data indicate that they are safe and well-tolerated in healthy volunteers.

HIV Fusion Inhibitors are a new class of drugs that bind to the viral protein gp120 and prevent HIV from infecting host cells. The virus is basically frozen

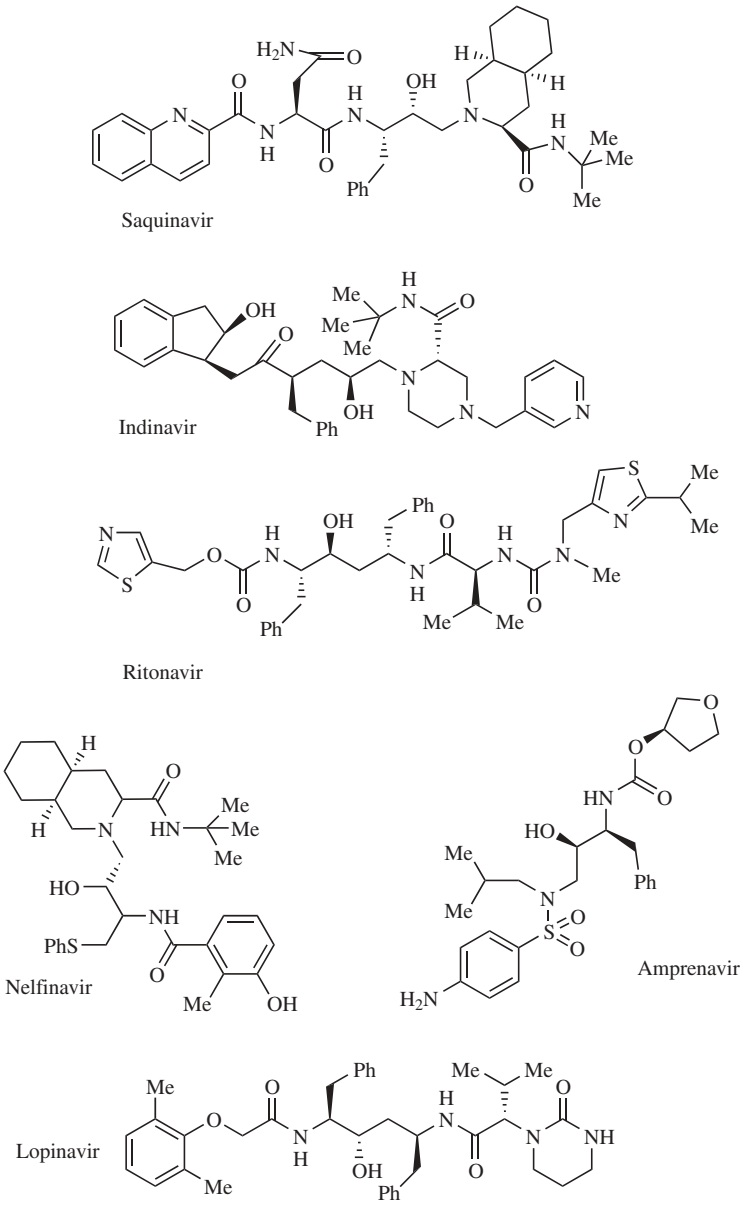


Fig. 4. FDA-approved protease inhibitors against HIV.

on the surface of the cell preventing it from entering the cell, and therefore cannot propagate in an HIV-infected person. The drug called T-20, which is being developed by Trimeris Research, belongs to this category. T-20 is a polypeptide (L-phenylalaninamide, *N*-acetyl-L-tyrosyl-L-threonyl-L-seryl-L-leucyl-L-isoleucyl-L-histidyl-L-seryl-L-leucyl-L-isoleucyl-L-.alpha.-glutamyl-L-.alpha.-glutamyl-L-seryl-L-glutaminyl-L-asparaginyl-L-glutaminyl-L-glutaminyl-L-.alpha.-glutamyl-L-lysyl-L-asparaginyl-L-.alpha.-glutamyl-L-glutaminyl-L-.alpha.-glutamyl-L-leu-

cyl-L-leucyl-L-.alpha.-glutamyl-L-leucyl-L-.alpha.-aspartyl-L-lysyl-L-tryptophyl-L-alanyl-L-seryl-L-leucyl-L-tryptophyl-L-asparaginyl-L-tryptophyl-). Data from initial clinical trials show that it works effectively in patients for whom other types of drugs no longer work well. All patients in the clinical studies had already developed resistance to all three types of anti-HIV drugs now on the market, and many were developing full-blown AIDS. At the fourteenth International AIDS conference held in Barcelona, Spain in July 2002, T-20 was hailed as one of the most exciting things to happen since the discovery of protease inhibitors.

Another interesting antiviral therapy that is currently being actively pursued by a number of researchers is based on the enzyme ribonuclease H (RNAse H) of HIV (196). The latter is responsible for digesting the RNA strand of the initially formed RNA–DNA hybrid from the viral RNA template. This important property of RNAse H is exploited in promoting the enzyme-catalyzed destruction of the viral mRNA target via formation of RNA–DNA duplexes employing appropriately designed complementary antisense oligonucleotides (AON). While the success has so far been limited, many important criteria are emerging to enable to draw correlation between the structure of the hybrid and its property as a suitable substrate for RNAase H, as well as to reveal the crucial structural requirements for AONs to preserve their RNAse H potency (197).

In summary, despite enormous progress made in understanding its life cycle as well as its potentially viable targets for the development of antiviral therapies, HIV remains an elusive virus even in the face of the successful HAART therapy. The major obstacle in conquering HIV through therapy concerns the high level of viral mutagenicity and the consequent drug resistance. Its eerie ability to integrate into the host to kill the very cells that are normally mobilized to confront the invader makes HIV a formidable virus. Neither the efficacious long-term therapies nor the uniformly effective vaccines against HIV are yet close in sight, but the international research to fight the virus continues unabated.

5.3. Hepatitis B Virus (HBV). According to the estimates of World Health Organization (WHO), HBV has infected over two billion people worldwide, making it one of the most ubiquitous human pathogens on earth, and ranking third in the global illnesses behind venereal disease and chickenpox (198–206). Approximately 500 million of the infected people are chronic carriers, and about 1 million die each year from HBV-related chronic liver disease. Most people are from Asia, Africa, and the western Pacific, although >1.5 million are infected in the United States, and $\sim$15,000 new cases are detected each year. Mother to infant transmission accounts for most cases in the undeveloped countries, whereas unsafe sex and body fluid contacts are the major forms of transmission in the developed countries. The viral transmission occurs primarily through blood and/or sexual contact, though other methods of transmission have also been suggested. Transmission is most efficient via percutaneous mode, whereas sexual transmission is somewhat inefficient. The virus is primarily found in the blood of infected individuals, and virus titres as high as 10 billion virions per milliliter of blood have been reported. HBV has also been detected in other body fluids including urine, saliva, nasopharyngeal fluids, semen, and menstrual fluids. However, HBV has so far not been detected in feces, perhaps

due to inactivation and degradation within the intestinal mucosa or by the bacterial flora.

HBV is responsible for both acute and chronic hepatitis (198,207–210). Individuals infected with acute HBV show no apparent clinical signs of the disease, but at the end of the incubation period, a flu-like symptoms, such as fever, fatigue, and general discomfort, and in some cases jaundice, will occur. About 2–10% of the adult acute HBV carriers will become chronic carriers of the disease, but in infants this percentage is >90% via neonatal exposure. An average of 25–40% of the chronic carriers will develop liver cirrhosis and primary hepatocellular carcinoma (HCC) (211–220), which are the major causes of morbidity and mortality. In the last few decades, the correlation between HBV and the development of HCC has been well established, although the mechanism by which HBV transforms hepatocytes still remains elusive. Before HBV can transform a cell, the virus must first infect it. However, the mechanism through which HBV enters hepatocytes has not been resolved despite further understanding of the viral proteins involved. Vaccines are available against HBV, but they may not be 100% effective against all variants of HBV. Furthermore, there is no cure for individuals already infected. Much more research is needed before we fully understand and control the spread of this infectious agent. The HBV life cycle is depicted in Figure 5 (210,221–230). As mentioned above, the virus attachment and entry into the host cell, as well as the cellular receptor for the virus are as yet poorly understood. After the initial entry, the viral core particle is translocated into the host nucleus. The viral DNA then becomes matured, forming a covalently closed circular DNA (called *ccc*DNA or supercoiled DNA). The *ccc*DNA remains episomal and serves as a template for cellular RNA polymerase II, giving rise to many viral RNA transcripts. The largest of these RNA transcripts serves as both mRNA for the viral polymerase (HBV DNA polymerase) and pregenomic RNA, which is slightly larger than genomic size, and is translated and packaged into viral particles. Concurrently, the smaller RNA transcripts-PreS- and PreC- are translated into surface and core proteins of the virus. The viral DNA is synthesized using reverse transcription of the pregenomic RNA by HBV DNA polymerase. The initial synthesis of (−) strand DNA is followed by synthesis of a short (+) strand DNA in a remarkable process, unique to HBV, called *priming*. Priming involves a specific tyrosine residue located at the N-terminus of HBV polymerase, which forms a covalent bond with the initiating deoxynucleotide residue, normally a dGTP. Priming is templated by a bulge sequence in the stem-loop structure (epsilon or,) on the pregenomic RNA, and results in a short DNA oligomer, covalently linked to the polymerase. This covalent enzyme–DNA adduct is then translocated to the appropriate complementary sequence at the 3′-end of pregenomic RNA. The (−) strand is then elongated via reverse transcriptase activity of the polymerase. The newly synthesized partially double-stranded viral DNA is either recycled as a resource for *ccc*DNA or functions as the viral nucleic acids in the matured virions budding out of the host cells.

5.4. Antiviral Therapy for HBV Infections. *Vaccine.* HBV vaccine is the first successful *recombinant vaccine* against a human infectious disease, in particular, against a mucosal virus (127,213,216, 231–249). The original vaccine, prepared in 1978 and licensed in the United States in 1981, was based on the

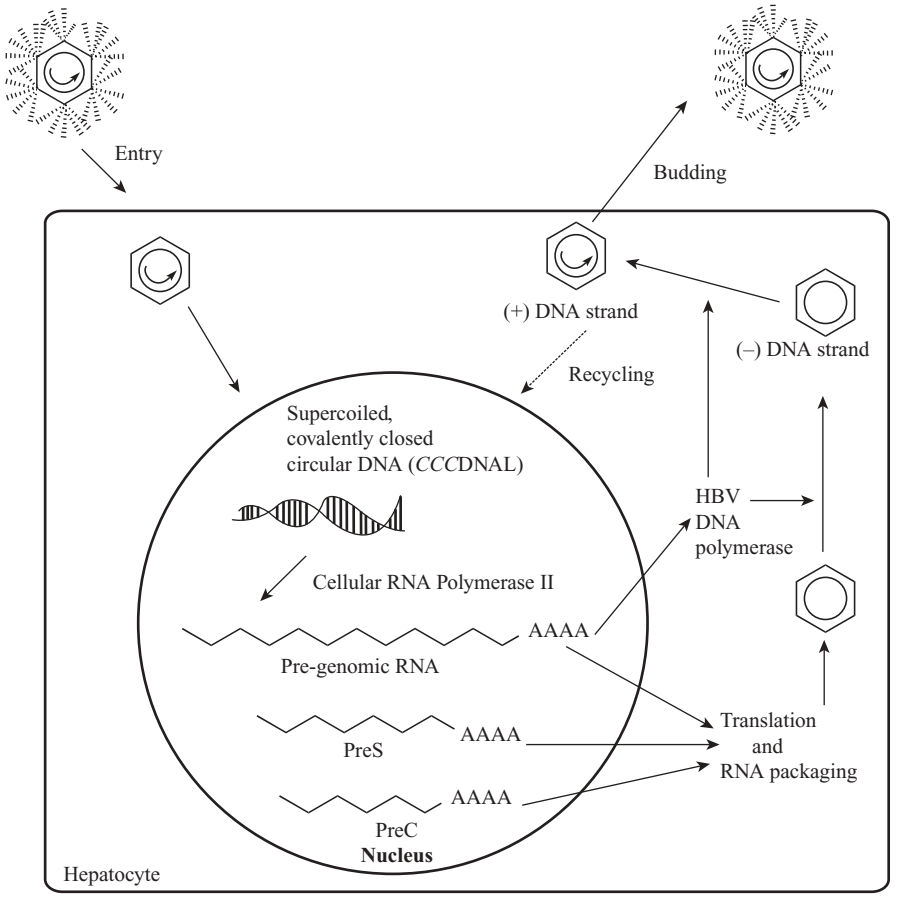


Fig. 5. The life cycle of the hepatitis B virus.

viral envelope protein (HBsAg). The latter was isolated and purified from the plasma of individuals infected with chronic HBV. The plasma vaccine has been replaced with the recombinant vaccine, prepared from yeast, mainly because the principal source of HbsAg-positive plasma was the same population that was also at the highest risk of contracting AIDS. Although the vaccine can help prevent the spread of HBV, it is not useful for those 350 million people who have already been chronically infected with the virus.

Anti-HBV Therapy. (*a*) *Interferons:* Interferons are a family of proteins—α, β, ω, and γ—that are induced in response to viral infections or double-stranded RNA. Interferon α is the most effective against HBV, and has been approved by FDA in 1991 for treatment of chronic HBV infections (212,213,215,250–255). Relapse of the disease after discontinuation of treatment, side effects, high expense of the drug, and the necessity to administer the drug only through injection are some of the limitations of interferon therapy. (*b*) *Nucleoside Analogues*: Nucleoside analogues are currently the most intensely studied anti-HBV agents (28,213,224,227,236,250–252,256–272). Some of the leading candidates include

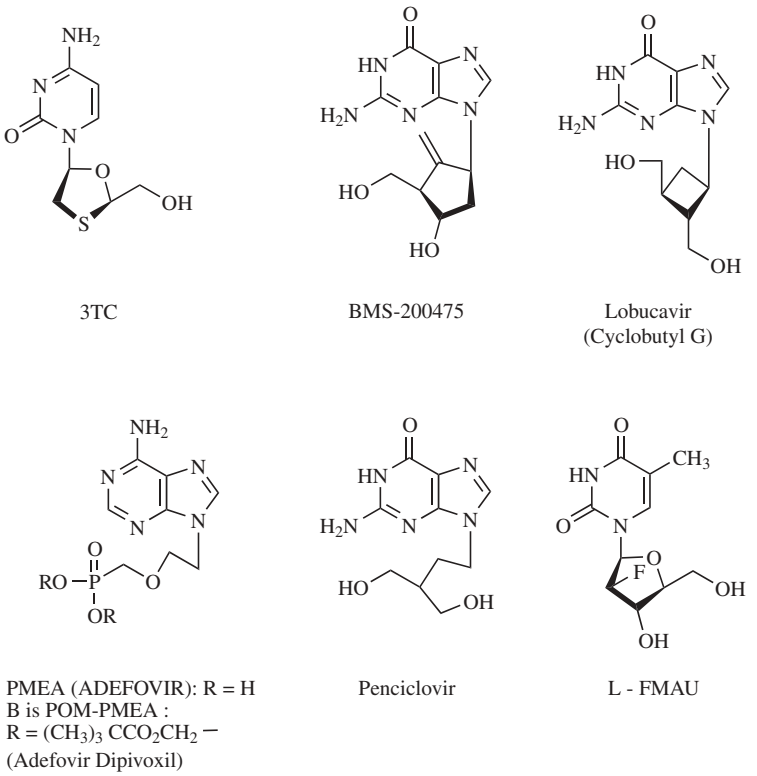


Fig. 6. Nucleoside inhibitors of replication of the hepatitis B virus (HBV).

3TC (lamivudine) (28,250,252,256,257,260–262,273–275), BMS-200475 (276–281), lobucavir (230,282–284), PMEA (adefovir) (285–289), adefovir dipivoxil (259,265,282,290–299), penciclovir (famciclovir) (257,300–306), and L-FMAU (227,262,282,307–317) as outlined in Figure 6. The clinical trials with a number of nucleoside analogues, however, were either unsuccessful or accompanied by severe toxicity. Lamivudine (3TC) is the first, effective, and reasonably well tolerated, oral treatment for chronic HBV infection, approved by FDA. The other approved drug for chronic HBV infection is adefovir dipivoxil. Lamivudine is an inhibitor of RT, and is in clinical use in HIV-infected individuals. The use of lamivudine on patients with HBV infection clearly shows the development of resistance arising from base-pair substitutions at a specific locus called YMDD of the viral DNA polymerase, resulting in significant clinical problem (275). So, the future of HBV chemotherapy may reside in combination drug therapy with newer, less toxic nucleoside analogues, along with other classes of agents including immunomodulators. With the discovery of 3TC, a nucleoside with a sugar moiety in the unnatural L-configuration, and its potent dual activity against both HIV and HBV, the interest in L-nucleoside analogues has taken on an explosive course. A number of L-nucleosides are currently undergoing preclinical and clinical trials against both HIV and HBV as well as other viral

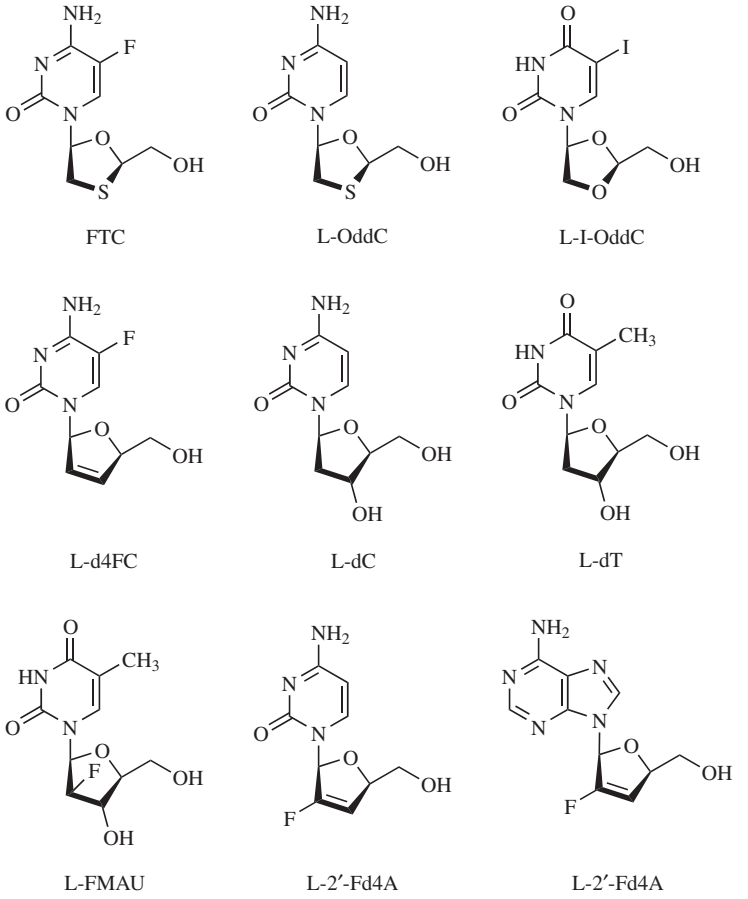


Fig. 7. L-Nucleoside analogues that are currently undergoing preclinical and clinical trials against HIV, HBV, and other viral infections.

infections, and are listed in Figure 7. Beneficial features of L-nucleosides include an antiviral activity comparable or sometimes greater than their natural D-counterparts, a more favorable toxicological profile, and more importantly, a greater metabolic stability due to their lower susceptibility to catabolic and hydrolytic enzymes. The synthesis and biology of L-nucleosides have been the object of many recent reviews (28,29,318).

Recently, we reported that the ring-expanded nucleosides **REN-1** (319) and **REN-2** (320) containing the imidazo[4,5-*e*][1,3]diazepine ring system, along with nucleoside **REN-3** (321), containing the imidazo[4,5-*e*][1,2,4]triazepine ring system (Figure 8), exhibit potent and selective *in vitro* anti-hepatitis B virus (anti-HBV) activity in cultured human hepatoblastoma 2.2.15 cells (322). The 50% effective concentration (EC_{50}) values for inhibition of extracellular virion release are 0.39, 0.13, and 4.2 μM, respectively. The compounds were also able to inhibit intracellular HBV DNA replication intermediates (RI) in 2.2.15 cells following 9 days of treatment. In addition, they exhibited very low cellular toxicities

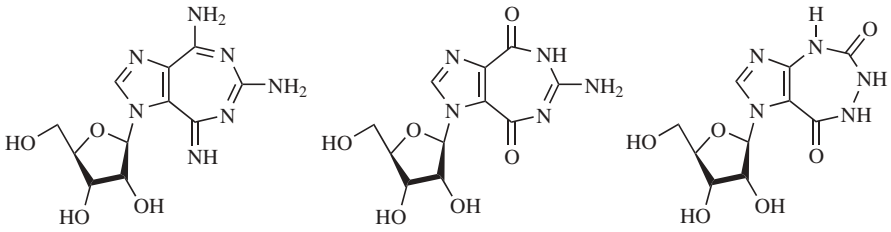


Fig. 8. Ring-expanded nucleosides with potent *in vitro* anti-hepatitis B virus (anti-HBV) activity with little, if any, toxicity.

with the respective selective indices (SI) of 1262, 18,526, and 439. The EC_{50} value for inhibition of virion DNA synthesis by **REN-2** suggested that it was two- to threefold less potent than 3TC. Comparison of the antiviral activity of **REN-1** and **REN-2** reveals that the replacement of the amino groups on the seven-membered heterocycle with oxygen increased the *in vitro* anti-HBV activity by threefolds. More importantly, this change in the structure resulted in a decrease in the *in vitro* cellular toxicity of **REN-2** ($CC_{50} = 2427$ μM) by fivefolds compared with toxicity of **REN-1** ($CC_{50} = 501$ μM) in confluent 2.2.15 cells. It was interesting to note that all three compounds showing antiviral activity were riboside analogues since 2′-deoxy analogues of both **REN-1** and **REN-2** were found to be inactive against HBV.

We then evaluated nucleosides **REN-1**, **REN-2**, and **REN-3** for their ability to inhibit viral RNA synthesis in 2.2.15 cells (322). Since HBV uses host cellular RNA polymerase II for the transcription of viral RNA from the covalently closed circular HBV–DNA during its replication, any effect on the synthesis of viral RNA by these compounds would mean interference with the cellular RNA polymerase which could lead to unacceptable cellular toxicity. Like 3TC, all three compounds showed no inhibition of the synthesis of viral 3.6 and 2.1 kb RNA by HBV in 2.2.15 cells. In spite of no suppression of viral RNA synthesis in the presence of these compounds, it was interesting to see that treatment of 2.2.15 cells with these compounds, unlike 3TC, did result in the reduction of viral protein synthesis especially that of the core antigen. The significance of this observation is not clear at the present time.

Another interesting and useful observation was that the antiviral activity exhibited by **REN-1**, **REN-2**, and **REN-3** was specific against HBV (322). These compounds were also tested against HIV, herpes simplex virus (HSV-1 and HSV-2), cytomegalovirus (CMV), VZV and EBV. They showed no antiviral activity against any of these viruses.

In vitro cellular toxicity of **REN-1**, **REN-2**, and **REN-3** was evaluated in several stationary and rapidly growing cell systems. Toxicity of **REN-2** was also studied in bone marrow precursor cells (by erythroid burst forming units and granulocyte macrophage CFU). In bone marrow precursor cells, **REN-2** had CC_{50} values that are comparable to those exhibited by 3TC. In rapidly growing human HFF cells and Daudi cells, all three compounds were found to be nontoxic up to 100 and 50 μM concentrations, respectively. In summary,

ring-expanded nucleosides represented by compounds **REN-1**, **REN-2**, and **REN-3** carry excellent promise as therapeutic agents against chronic HBV infections, and to that end, the efforts are currently underway in our laboratory.

5.5. Hepatitis C Virus (HCV). The hepatitis C virus (HCV) is one of the most dreadful infectious diseases of modern times, which has currently infected >175 million people worldwide and >5 million in the United States (323–332). What makes it so dreadful is that most people do not even know that they have been infected with the virus as it can remain dormant for scores of years in the infected individual without revealing any signs or symptoms of the disease. Some estimates say the number of HCV-infected individuals may be four times the number of those infected with the AIDS virus, the main differences being that hepatitis C does not kill as quickly as AIDS. Until 1989, HCV was known by the name non-A, non-B hepatitis, when scientists at Chiron, Inc. succeeded in isolating portions of the HCV genome and conclusively demonstrated that the virus was indeed responsible for the noted pathogenicity that did not fit the category of either the A or B type hepatitis, and so classified it as type C. Subsequently, the complete genomes of various HCV isolates were cloned and sequenced by several research groups.

HCV is one of the major causes of chronic liver disease in the United States (323,324). It accounts for ~15% of acute viral hepatitis, 60–70% of chronic hepatitis, and up to 50% of cirrhosis, end-stage liver disease, and liver cancer. Hepatitis C causes an estimated 8,000–10,000 deaths annually in the United States. Hepatitis C is the major reason for liver transplants in the United States, accounting for 1000 of the procedures annually. A conspicuous characteristic of hepatitis C is its tendency to cause chronic liver disease. At least 75% of patients with acute hepatitis C ultimately develop chronic infection, and most of these patients have accompanying chronic liver disease. But chronic hepatitis C varies greatly in its course and outcome. At one end of the spectrum are patients who have no signs or symptoms of liver disease and completely normal levels of serum liver enzymes. Liver biopsy usually shows some degree of chronic hepatitis, but the degree of injury is usually mild, and the overall prognosis may be good. At the other end of the spectrum are patients with severe hepatitis C who have symptoms, HCV–RNA in serum, and elevated serum liver enzymes, and who ultimately develop cirrhosis and end-stage liver disease. In the middle of the spectrum are many patients who have few or no symptoms, mild-to-moderate elevations in liver enzymes, and an uncertain prognosis. Some patients learn they have hepatitis C only after a routine physical or when they donate blood and a blood test shows elevated liver enzymes. Further testing for HCV antibodies using the enzyme immunoassay (EIA) test and a supplemental test such as the "Western blot" or HCV–RNA detection can positively identify the infection. A liver biopsy shows disease manifested by damage already done to the liver. It is estimated that at least 20% of patients with chronic hepatitis C develop cirrhosis, a process that takes 10–20 years. After 20–40 years, a smaller percentage of patients with chronic disease develop liver cancer.

The virus is transmitted primarily by blood and blood products (323,324). The majority of infected individuals have either received blood transfusions prior to 1990 (when screening of the blood supply for HCV was implemented) or have used intravenous drugs. Sexual transmission between monogamous

couples is rare but HCV infection is more common in sexually promiscuous individuals. Perinatal transmission from mother to fetus or infant is also relatively low but possible. Many individuals infected with HCV have no obvious risk factors. Most of these persons have probably been inadvertently exposed to contaminated blood or blood products.

HCV is also considered an opportunistic infection in HIV-infected individuals, and about one quarter of them are also infected with HCV (333–347). Since HCV is transmitted primarily by large or repeated direct percutaneous (ie, passage through the skin by puncture) exposures to contaminated blood, coinfection with HCV is common (50–90%) especially among HIV-infected injection drug users. Also, HCV infection progresses more rapidly to liver damage in HIV-infected persons. HCV infection may also impact the course and management of HIV infection. Prevention of HCV infection for those not already infected and reducing chronic liver disease in those who are infected are important concerns for HIV-infected individuals and their health care providers.

HCV is a member of the family of RNA viruses called *Flaviviridae* (348–351) to which also belongs the West Nile virus, another frightful virus of current notoriety in the United States and the western hemisphere as stated in the introduction. The viruses of the *flaviviridae* family are small, enveloped, spherical particles of 40–50 nm in diameter with single-stranded, positive sense RNA genomes (352–354). They are known to be the cause of severe encephalitic, hemorrhagic, hepatic, and febrile illnesses in humans. The viral genome encodes a polyprotein of 3000–4000 amino acids that is processed by host-cell and viral proteases into three structural (C, prM, and E) and seven non-structural (NS) proteins (see Figure 9) (352,354,355). Among these proteins the NS3 appears to be the most promising target for antiviral agents because of the multiple enzymatic activities associated with this protein. NS3 exhibits serine protease-, RNA-stimulated nucleoside triphosphatase (NTPase)-, and RNA helicase activities (356–358). The catalytic domain of the chymotrypsin-like NS3 protease has been mapped to the NH2-terminus region of the NS3, whereas the NTPase and the helicase activities are associated with the COOH-terminus of NS3

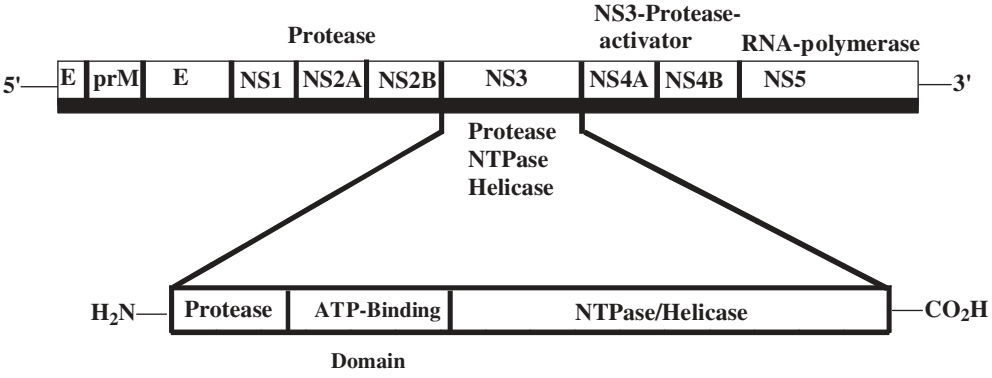


Fig. 9. Schematic representation of the structure of flaviviridae polyprotein with the expanded NS3 region. The enzymatic activities associated with the nonstructural proteins are shown.

(355,356). Helicases are capable of unwinding duplex RNA and DNA structures by disrupting the hydrogen bonds that keep the two strands together (359,360). This unwinding activity is essential for the virus replication. Recently reported "knock out" experiments demonstrated unambiguously that the switch-off of the helicase activity abolishes the virus propagation of bovine diarrhea virus (BVDV) and of dengue fever virus (DENV). According to the data, the inhibition of the helicase activity associated with NS3 protein may be an effective tool for reduction of virus replication. The NS5 region, on the other hand, is associated with the viral RNA-dependent RNA polymerase activity. The NTPase/helicase activities of NS3, along with the RNA-dependent RNA polymerase activity of NS5, are thought to be the essential components of the viral replicase complex (361), and therefore, are the potential targets for development of antiviral therapy.

5.6. Antiviral Therapy for HCV Infections. *Vaccines.* There is no vaccine for HCV and vaccines for hepatitis A and B do not provide immunity against hepatitis C (248,362–373). There are various strains of HCV and the virus undergoes mutations. Consequently, it will be difficult to develop a vaccine. Also, there is no effective immune globulin preparation. Furthermore, despite the discovery of HCV by molecular biological methods and the successful sequencing of the entire genome, a permissive cell culture system for propagating HCV has yet to be established. Although breakthroughs have been made recently in the development of model systems for studying viral RNA replication, no cell lines are yet available for producing the infectious virus. A non-primate animal model also does not exist. As a result, the production of specific drugs against HCV has been impeded although excellent diagnostic methods for it have been developed. An encouraging news, nevertheless, is the recent discovery that mutations in a protein of certain strains of HCV will allow these strains to replicate more vigorously in human cell culture. The *in vitro* assay based on this finding, called the HCV-replicon system (373), is a big step forward in improving an essential tool for studying the virus and suggests a starting point for the design of effective vaccines.

Currently Available Treatments. *(a) Interferons:* All current treatment protocols for hepatitis C are based on the use of various preparations of interferon alpha, which are administered by intramuscular or subcutaneous injection (323,374–384). Interferon alpha is a naturally occurring glycoprotein that is secreted by cells in response to viral infections. It exerts its effects by binding to a membrane receptor. Receptor binding initiates a series of intracellular signaling events that ultimately leads to enhanced expression of certain genes. This leads to the enhancement and induction of certain cellular activities including augmentation of target cell killing by lymphocytes and inhibition of virus replication in infected cells. Interferon alfa-2a (Roferon-A; Hoffmann-La Roche), inteferon alpha-2b (Intron-A; Schering-Plough) and interferon alfacon-1 (Infergen; Intermune) are all approved in the United States for the treatment of adults with chronic hepatitis C as single agents. More recently peginterferon alpha, sometimes called pegylated interferon, has been available for the treatment of chronic hepatitis C. There are two preparations of peginterferon alpha that have been studied in patients with hepatitis C: peginterferon alpha-2b (Peg-Intron; Schering-Plough) and peginterferon alpha-2a (Pegasys; Hoffmann-La Roche). The differences between these two preparations are subtle and most

data suggest that they are equivalent with regards to efficacy and side effect profile. Peginterferon alphas differ from the older, unmodified interferon alphas in that a polyethylene glycol molecule is attached to the interferon molecule. As a result its elimination from the body is slowed and higher, more constant blood levels of interferon alpha are achieved with less frequent dosing. In contrast to unmodified interferon alpha, which must be injected three times a week to treat chronic hepatitis C, peginterferon alpha needs to be injected only once a week. With peginterferon alpha-2a alone, ~30–40% of patients achieve a sustained response to treatment for 24–48 weeks (332,385). *(b) Nucleoside Analogue Ribavirin and Combination Therapy:* As mentioned earlier under general description of nucleoside analogues as antiviral agents, ribavirin is a synthetic nucleoside containing a five-membered triazole ring, which has shown activity against a broad spectrum of viruses (77,79,90,376,382,383,386–389). In several studies, oral ribavirin was examined as a single agent for the treatment of adults with chronic hepatitis C. Although decreases in serum alanine transaminase (ALT) activities were seen with treatment (390–392), the overall results of these studies were discouraging as sustained-responses were rarely achieved. Therefore, FDA did not approve ribavirin alone for hepatitis C. In the United States, it was first approved in aerosol form for the treatment of a certain type of respiratory virus infection in children. Because of its partial effectiveness, ribavirin was studied in subsequent trials in combination with interferon alpha (376,383,384, 393–395). It was discovered that the addition of ribavirin to interferon alpha-2b is superior to interferon alpha-2b alone in the treatment of chronic hepatitis C, especially in achieving a sustained response in patients not previously treated with interferon. This led to FDA approval of this combination therapy of interferon alpha-ribavirin for this indication in December 1998. Most recently, the FDA has approved the combination of peginterferon alpha plus ribavirin for the treatment of chronic hepatitis C. For eligible patients with chronic hepatitis C, a peginterferon alpha plus ribavirin is likely to be the best treatment option for the near future. Clinical trials have show that the sustained response rate is ~50% of patients given this combination for 24–48 weeks.

The treatment using interferon alpha with or without ribavirin is, nevertheless, associated with may side effects. During treatment, patients must be monitored carefully for side effects including flu-like symptoms, depression, rashes, other unusual reactions and abnormal blood counts. Furthermore, ribavirin is associated with a significant risk of abnormal fetal development, and women of childbearing potential should not begin therapy until a report of a negative pregnancy test has been obtained and not become pregnant during treatment. In general, the patient probably needs to have blood tests approximately once a month, and somewhat more frequently at the beginning of treatment. In addition, patients considered for treatment with interferon alpha-2b plus ribavirin should not have the complications of serious liver dysfunction and such subjects should only be considered for treatment of hepatitis C in specialized studies. Thus, it appears that more research is needed to develop safer, more effective and cheaper drugs against HCV.

Current Research Trends in Mechanism-Based HCV Inhibitors. The current intensive effort to discover novel therapies to treat HCV infection is aimed primarily at specific processes that are essential to HCV replication.

These include viral RNA replication, which uses the NS3 helicase/NTPase and the NS5B RNA-dependent RNA polymerase (RdRp); virus translation, controlled by regulatory elements such as the 5′-nontranslated region (5′NTR) that contains the internal ribosome entry site (IRES); and processing of the viral protein by the NS2–NS3 and NS3–NS4A proteases.

Based on the solved crystal structure of HCV RNA NTPase/helicase and of DNA NTPase/helicases from *Escherichia coli* and *Bacillus stearothermophilus*, two alternative mechanisms of the duplex unwinding reaction (see above) have been postulated (359,396–398). Both models predict that the enzymes bind and hydrolyse NTP by a well characterized NTP binding pocket. The energy released is used for the "march" of the enzyme along the DNA or RNA structures and the unwinding reaction results from capturing single strand (ss) regions that arise due to thermal fluctuations at the fork (359,396). Alternatively, the energy could be transferred to the fork and used for disruption of the hydrogen bonds that keep the strains together (359,396). Consistent with the proposed models, the following mechanisms of inhibition of the helicase activity could be considered: (*a*) inhibition of the NTPase activity by interference with NTP binding (399,400), (*b*) inhibition of NTPase activity by an allosteric mechanism (399), and (*c*) inhibition of the coupling of NTP hydrolysis to unwinding reaction (400). Additional mechanistic possibilities include interference in the interaction of helicase with its RNA or DNA substrate via (*d*), competitive blockade of substrate binding site (401), or by (*e*) inhibition of the unwinding by steric inhibition of translocation of the enzyme along the polynucleotide chain (402). There are even more mechanistic possibilities by which the helicase activity could be inhibited. Binding studies of Porter and coworkers (403,404) revealed a putative nucleoside-binding site within the HCV NTPase/helicase molecule. The function and location of the second binding site remains unknown. Nevertheless, there is accumulating evidence that the NTPase and helicase activities of the viral super family II (SFII) enzymes might be modulated by occupation of these putative nucleoside-binding sites. For example, ribavirin-5′-triphosphate (RTP), which is a potent, classical, competitive inhibitor of the NTPase activity of the WNV and HCV NTPase/helicases at lower ATP concentrations ($<K_M$), failed to inhibit the ATPase activity at higher ATP concentrations ($\gg K_M$), and instead, even stimulated the enzyme activity (400,405). By contrast, the RTP inhibits moderately the helicase activity of both enzymes by a mechanism that is independent of the ATP concentrations (405). The phenomenon results most probably from occupation of a second nucleoside binding site by RTP (400). Thus, intense research efforts are currently being directed at designing inhibitors of HCV helicase and NTPase.

HCV RNA-dependent RNA polymerase (RdRp) is also a good target for anti-HCV therapy in that its activity is essential for viral replication and infectivity. The biochemical properties of NS5B have been characterized extensively (406). A detailed view of HCV NS5B was revealed by the crystal structures of the RdRp (407,408). Although canonical polymerase features exist, HCV NS5B adopts a unique molecular structure that resembles a "thumb–palm–finger" that is different from other known DNA and RNA polymerases, highlighting the attractiveness of the HCV polymerase as a drug target. Recently, a benzo-1,2,4-thiadiazine derivative has been reported to be a potent inhibitor of HCV RdRp (409). As

nucleosides and nucleotides are anticipated to modulate the activities of HCV helicase, NTPase, as well as poymerase, the future drugs against HCV are likely to be based on analogues of nucleosides/-tides.

The highly conserved regions in the internal ribosome entry site (IRES) of the HCV RNA genome, its distinctive translational–initiation mechanism and its essential role in mediating the unusual translational–initiation and replication processes of HCV make these elements an attractive target for compounds that inhibit transcription and translation of the HCV RNA. The specific sites and subdomains for interfering with IRES function have been identified. As the tertiary structures of these important subdomains are now available, it is possible to apply structure-based methods for the discovery of inhibitors of HCV protein synthesis and replication. Recently, it has been reported that Vitamin B12 stalls the 80 S ribosomal complex on HCV IRES (410).

The metal-dependent cysteine protease NS2–NS3 catalyzes cleavage between NS2 and NS3 in an autoproteolytic manner (370). The amino-terminal portion of NS2 is responsible for membrane association, whereas its carboxy terminus, which overlaps with NS3, is believed to catalyze the cleavage of the NS2–NS3 site. The activity of the chymotrypsin-like serine protease that is encoded within the amino-terminal 180 amino acids of NS3 is indispensable for HCV infectivity in the chimpanzee model (411). The structure of the protease domain and the full-length NS3 protein were solved by X-ray crystallography (412). Efficient processing requires the NS3 protease in combination with the NS4A cofactor and a structural zinc molecule (370). The NS3 protease is prone to inhibition by specific penta- or hexapeptides derived from the amino-terminal NS3 cleavage products, which have provided the basis for lead optimization of peptidomimetic inhibitors (413–415). This class of optimized compounds has shown submicromolar potencies in in vitro enzymatic assays, as well as in the HCV-replicon system.

Another class of compounds being developed as HCV inhibitors are ribozymes, which inhibit viral replication by cleavage of the target HCV genomic RNA (416,417). Ribozymes are naturally occurring, short RNA molecules with endoribonuclease activity that can catalyze sequence-specific cleavage of RNA. Antisense oligonucleotides have been employed as an alternative to selectively target the HCV RNA genome. The target RNA is cleaved RNA by an RNaseH at the site of oligonucleotide hybridization, and results in inhibition of gene expression. A number of antisense oligonucleotides have been designed to bind to the stem–loop structures in the HCV IRES, and have been shown to be effective in inhibiting HCV replication in cell-culture assays (418,419). ISIS 14803 is a 20-mer, 5′-methylcytidine phosphorothioate antisense oligonucleotide that is in a Phase II clinical trial at present in patients with chronic HCV infections (420).

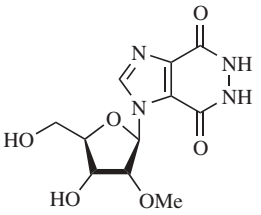
5.7. West Nile Virus (WNV). With an alarming increase in the number of cases of infection in wild birds, horses, pets, and humans, the WNV is currently gaining a wide attention in the United States and the western hemisphere (421–425). A number of Science Focus and News Focus articles have appeared in recent issues of Science magazine (426), in addition to countless news stories in popular magazines and newspapers. Three years after the 1999 outbreak of the WNV in New York City, which sickened 62 people, most of them elderly, and killed 7, the virus has been detected in >60 bird species and about a dozen mammals, and has spread to 44 states and the District of Columbia. As of September

2002, the Centers for Disease Control and Prevention have verified 1295 human cases of WNV, resulting in 54 deaths. WNV is mainly a bird virus that is spread by mosquitoes. Humans, horses, as well as a dozen other mammals are its dead-end hosts. Crows are the virus's most conspicuous hosts as they have been dying en masse with WNV infection. Most humans infected with WNV do not even know it, or they experience only mild, flu-like symptoms. Those over 65, and individuals with weakened immune systems, are especially vulnerable to WNV although recent cases have brought down the age barrier to as low as 50. Three months after the initial outbreak, 70% of the survivors still reported muscle weakness, 75% suffered from memory loss, 60% from confusion, and more than one-half could no longer live at home, although most were healthy, active, and lived normal lives before the WNV attack. Many of the patients end up with lingering neurological damage as often occurs with encephalitic infections.

Since there are currently no approved drugs or vaccines against WNV infection, the focus has been mainly on prevention. Given that mosquitoes are associated with WNV transmission, the key to preventing or controlling future outbreaks of WNV among horses and other animals is to control mosquito populations. Because horses and pets could be infected the same way people are, the key to prevention is to prevent mosquito bites. Products to prevent fleas and ticks have no effect on mosquitoes. There are over-the-counter products, however, available to repel mosquitoes. Similar recommendations would apply for other pets, livestock, or poultry should illness due to WNV in those types of animals come to be commonly recognized. In 2001, a license was issued by the USDA–APHIS Center for Veterinary Biologics, Inc. for an equine WNV vaccine, and so, vaccination is now available as an option for horses (427).

As noted above, both WNV and HCV belong to the same family of viruses called *Flaviviridae*. However, unlike HCV, WNV can be isolated from clinical specimens by tissue culture methods, and therefore, is used as a close mimic of HCV in experimental models. Also, since the structures of the viral genome, the encoded polyprotein, and the protease-processed structural and NS protein fragments are also very similar to those of HCV, the same viral targets as described above for HCV are currently being investigated by several research groups including ours (428,429). These targets include the WNV NTPase/helicase of the NS3 region as well as the viral RNA-dependent RNA polymerase of the NS5 region.

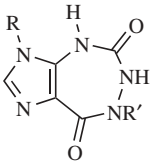
5.8. Antiviral Therapy for WNV Infections. There are no currently approved drugs or vaccines for treating or preventing the disease in humans, although a vaccine has recently been approved for horses as described above. Although ribavirin was initially reported to halt the viral replication, the need to use very high doses of the drug proved too toxic to be clinically useful (430). Furthermore, since WNV is still a rare virus affecting humans, there is not enough incentive for drug companies to develop anti-WNV drugs, but this scenario is likely to change as more and more cases of infection emerge. In a recent study, we have demonstrated that some imidazo[4,5-*d*]pyridazine nucleoside analogues act as inhibitors of WNV NTPase/helicase, and moderately reduce the unwinding activity of the enzyme (428). A comparable inhibitory potency was also observed in tissue culture systems, suggesting that this enzyme is indeed a viable target for inhibition of WNV replication. We have also recently



I

Imidazo[4,5-*d*]pyridazine

(IC_{50} = 30 μM)

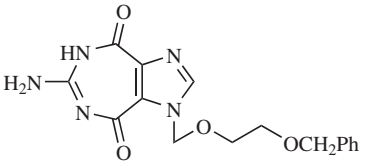


II

Imidazo[4,5-*e*][1,2,4]triazepine

(IC_{50} = 1.3–3.5 μM)

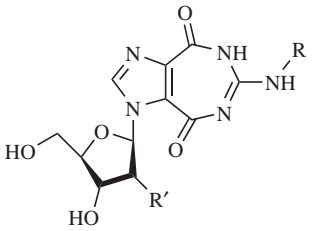
(a) R = H, R′ = CH_2Ph
(b) R = R′ = CH_2Ph



III

Imidazo[4,5-*e*][1,3]diazepine

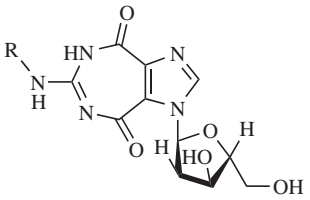
(IC_{50} = 5.0–11.0 μM)



IV

Imidazo[4,5-*e*][1,3]diazepine

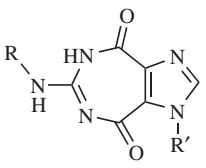
(a) R = $(CH_2)_{11}CH_3$, R′ = OH (IC_{50} = 1.0–3.0 μM)
(b) R = $(CH_2)_{13}CH_3$, R′ = OH (IC_{50} = 3.0–10.0 μM)
(c) R = $(CH_2)_{17}CH_3$, R′ = OH (IC_{50} = 5.0 μM)
(d) R = $(CH_2)_{11}CH_3$, R′ = H (IC_{50} = 3.0–10 μM)



V

Imidazo[4,5-*e*][1,3]diazepine

(a) R = $(CH_2)_{11}CH_3$
(IC_{50} = 3–10 μM)
(b) R = H (IC_{50} = 20–50 μM)



VI

Imidazo[4,5-*e*][1,3]diazepine

(a) R = $(CH_2)_{11}CH_3$, R′ = H
(IC_{50} = 3–10 μM)
(b) R = $(CH_2)_{11}CH_3$,
R′ = CH_2Ph-*p*-OMe
(IC_{50} = 5.0 μM)

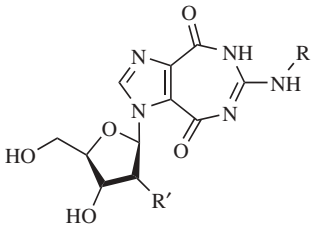
Fig. 10. *In vitro* inhibitors of the helicase activity of WNV NTPase/helicase, containing the imidazopyridazine, imidazodiazepine, and imidazotriazepine ring systems. The term IC_{50} represents the concentration of the inhibitor required to reduce the unwinding activity of the enzyme by 50% of that observed in the absence of the inhibitor.

discovered (429) that a variety of 5:7 fused heterocyclic bases, nucleosides, and nucleotides resembling ring-expanded (fat) purine structure are excellent *in vitro* inhibitors of the helicase activity of WNV NTPase/helicase and/or WNV RNA-dependent RNA polymerase (RdRp). Listed in Figure 10 are a few such inhibitors of the helicase activity of WNV NTPase/helicase, along with their respective IC_{50} values. The helicase activity was assessed, using a DNA substrate and ATP, as a function of increasing concentration of inhibitors. The term IC_{50} represents the concentration of the inhibitor required to lower the original unwinding activity observed in the absence of the inhibitor by 50%. Likewise, listed in Figure 11 are the structures and the corresponding IC_{50} values of inhibitors of the WNV RdRp activity *in vitro*. The term IC_{50} here reflects the concentration of the inhibitor required to reduce the WNV polymerase activity by 50%.

As is evident from IC_{50} values in Figure 10, both ring-expanded heterocyclic bases as well as nucleosides possess potent anti-WNV activity. Since compounds of general formula **IV** containing shorter than the C-12 side chain at position-6 failed to exhibit any significant activity, the presence of an adequately hydrophobic group at this junction appears to be necessary for activity. Most surprising was the fact that the sugar moiety is not absolutely necessary for activity as heterocyclic bases with the appropriately hydrophobic functionalities at either the seven- or the five-membered ring were just as or even more active than their nucleoside counterparts, as revealed by the activities of compounds of general formula **II**, **III**, and **VI**. The relatively somewhat less critical role of the sugar moiety in the observed anti-WNV activity was further revealed by the compounds of general formula **V**, which possess the unnatural α-anomeric configuration at the base–sugar junction. Finally, in view of the observed tight complex between nucleoside **IVd** and a DNA substrate that was completely stable in the presence of 0.5% sodium dodecyl sulfate (SDS), the mechanism of action of ring-expanded heterocyclic bases and nucleosides is currently believed to involve their interaction with the nucleic acid substrate of WNV helicase through binding to the major or minor groove of the double helix, and the consequent modulation of the enzyme activity. The substrate binding can result in either the inhibition or the enhancement of helicase activity, which was indeed found to be the case as a few other ring-expanded nucleosides tested were found to be the activators, rather than inhibitors, of the helicase activity of WNV NTPase/helicase.

The observed inhibition of WNV RdRp activity by ring-expanded heterocycles and nucleosides listed in Figure 11 also exhibited a parallel trend in that the presence, type, or configuration of the sugar moiety was relatively less critical as opposed to the type and location of the substituent on the heterocyclic ring. Thus, compounds **IVe** and **IVf**, which contained shorter than the C-12 side chain at position-6, and which were inactive against the helicase activity of WNV NTPase/helicase, are now found to be active against the polymerase activity of WNV RdRp. Once again, both the α-anomeric nucleoside **V** and the heterocycles **VI** with the appropriate hydrophobic substituents at position 1 and/or 6 exhibited remarkable inhibition of WNV RdRp activity.

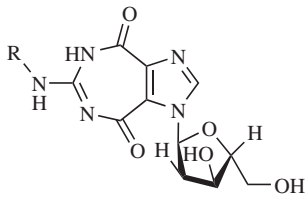
Recently, Chu and co-workers reported (431) the anti-WNV activity of L-neplanocin analogues in tissue culture systems. While the parent L-neplanocin



IV

Imidazo[4,5-*e*][1,3]diazepine

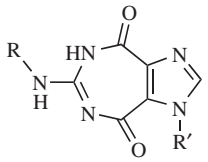
(e) R = $(CH_2)_3CH_3$, R′ = OH (IC_{50} = 10–30 μM)
(f) R = $(CH_2)_9CH_3$, R′ = OH (IC_{50} = <<3.0 μM)



V

Imidazo[4,5-*e*][1,3]diazepine

R = $(CH_2)_{11}CH_3$ (IC_{50} <<3.0 μM)



VI

Imidazo[4,5-*e*][1,3]diazepine

(a) R = $(CH_2)_{11}CH_3$, R′ = H
(IC_{50} << 3.0 μM)
(b) R = $(CH_2)_{11}CH_3$,
R′ = CH_2Ph-*p*-OMe
(IC_{50} << 3.0 μM)

Fig. 11. *In vitro* inhibitors of the polymerase activity of WNV RNA-dependent RNA polymerase (RdRp) activity. The term IC_{50} represents the inhibitor concentration required to reduce the polymerase activity by 50%.

itself was inactive, both its cytosine ($EC_{50} = 0.06$ μ*M*; $IC_{50} = 0.08$ μ*M* in CEM cells) and 5-fluorocytosine ($EC_{50} = 5.34$ μ*M*; $IC_{50} = 51.4$ μ*M* in CEM cells) analogues exhibited potent anti-WNV activity, but unfortunately, the compounds also suffered from significant cellular toxicity.

6. Conclusion

The molecular structure, life cycle, mode of infection, and replication process of four major viruses of current health scare, including HIV, HBV, HCV, and WNV, have been discussed at length with cursory references to other human viruses. Also elaborated on are the prophylactic as well as postinfection remedies that are both currently approved and under clinical development, along with viable, mechanism-based targets for future development of antiviral therapies. While no

vaccine nor total cure is yet available against HIV infection, great strides have been made in antiviral therapy to enable classification of AIDS as a manageable illness from that of an "absolute death sentence" only a few years ago. With regard to HBV infection, a vaccine is now available, but the initial clinical trials with a number of nucleoside analogues as anti-HBV agents were disappointing in light of severe toxicities associated with them. Nevertheless, a number of other nucleoside analogues belonging to the family of unnatural L-nucleosides and RENs that are currently under development appear to be promising. The ultimate success in treating HBV may lie in the combination drug therapy similar to the successful HAART therapy applied against HIV infection. Unfortunately, neither vaccines nor good drugs are currently available for treating HCV or WNV, the two viruses belonging to the family of *flaviviridae*, but a vast array of information is being rapidly accumulated on the structural biology and molecular virology of the two viruses to afford development of suitable antiviral therapies against them in the near future.

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