20 Antiviral agents

20.1 Viruses and viral diseases

Viruses are non-cellular, infectious agents which take over a host cell in order to survive and multiply. There are a large variety of different viruses which are capable of infecting bacterial, plant, and animal cells, with more than 400 different viruses known to infect humans.

Viruses can be transmitted in a variety of ways. Those responsible for diseases such as influenza (flu), chicken pox, measles, mumps, viral pneumonia, rubella, and smallpox can be transmitted through the air by an infected host sneezing or coughing. Other viruses can be transmitted by means of arthropods or ticks leading to diseases such as Colorado tick fever and yellow fever. Some viruses are unable to survive for long outside the host and are transmitted through physical contact. The viruses responsible for AIDS, cold sores, the common cold, genital herpes, certain leukaemias, and rabies are examples of this kind. Finally, food-borne or water-borne viruses can lead to hepatitis A and E, poliomyelitis, and viral gastroenteritis.

Historically, viral infections have proved devastating to human populations. It has been suggested that smallpox was responsible for the major epidemics that weakened the Roman Empire during the periods AD 165–180 and AD 251–266. Smallpox was also responsible for the decimation of indigenous tribes in both North and South America during European colonization. In some areas, it is estimated that 90% of the population died from the disease. Various flu epidemics and pandemics have proved devastating. The number of deaths worldwide due to the flu pandemic of 1918–1919 is estimated to be over 20 million, much greater than the number killed by military action in the First World War.

The African continent has its fair share of lethal viruses including Ebola and the virus responsible for Lassa fever. In the past, viral diseases such as these occurred in isolated communities and were easily contained. Nowadays, with cheap and readily available air travel, tourists are able to visit remote areas, thus increasing the chances of rare or new viral diseases spreading round the world. Therefore, it is important that world health authorities monitor potential risks and take appropriate action when required. The outbreak of severe acute respiratory syndrome (SARS) in the Far East during 2003 could have had a devastating effect worldwide if it had been ignored. Fortunately, the world community acted swiftly and the disease was brought under control relatively quickly. Nevertheless, the SARS outbreak serves as a timely warning of how dangerous viral infections can be. Scientists have warned of a nightmare scenario involving the possible evolution of a 'supervirus'. Such an agent would have a transmission mode and infection rate equivalent to flu, but a much higher mortality rate. There are already lethal viruses which can be spread rapidly and have a high mortality rate. Fortunately, the latency period between infection and detectable symptoms is short and so it is possible to contain the outbreak, especially if it is in isolated communities. If such viral infections evolved such that the latency period increased to that of AIDS, they could result in devastating pandemics equivalent to the plagues of the Middle Ages.

Considering the potential devastation that viruses can wreak on society, there are fears that terrorists might one day try to release lethal viral strains on civilian populations. This has been termed **bioterrorism**. To date, no terrorist group has carried out such an action, but it would be wrong to ignore the risk.

It is clear that research into effective antiviral drugs is a major priority in medicinal chemistry.

20.2 Structure of viruses

At their simplest, viruses can be viewed as protein packages transmitting foreign nucleic acid between host cells. The type of nucleic acid present depends on the virus concerned. All viruses contain one or more molecules

of either RNA or DNA, but not both. They can therefore be defined as RNA or DNA viruses. Most RNA viruses contain single-stranded RNA (ssRNA), but some viruses contain double-stranded RNA. If the base sequence of the RNA strand is identical to viral messenger RNA (mRNA), it is called the positive (+) strand. If it is complementary, it is called the negative (−) strand. Most DNA viruses contain double-stranded DNA, but a small number contain single-stranded DNA. The size of the nucleic acid varies widely, with the smallest viral genomes coding for 3–4 proteins and the largest coding for over 100 proteins.

The viral nucleic acid is contained and protected within a protein coat called the **capsid**. Capsids are usually made up of protein subunits called **protomers** which are generated in the host cell and can interact spontaneously to form the capsid in a process called **self-assembly**. Once the capsid contains the viral nucleic acid, the whole assembly is known as the **nucleocapsid**. In some viruses, the nucleocapsid may contain viral enzymes which are crucial to its replication in the host cell. For example, the flu virus contains an enzyme called **RNA-dependent RNA polymerase** within its nucleocapsid (Fig. 20.1).

Additional membranous layers of carbohydrates and lipids may be present surrounding the nucleocapsid, depending on the virus concerned. These are usually derived from the host cell, but they may also contain viral proteins which have been coded by viral genes.

The complete structure is known as a **virion** and this is the form that the virus takes when it is outside the host cell. The size of a virion can vary from 10 nm to 400 nm. As a result, most viruses are too small to be seen by a light microscope and require the use of an electron microscope.

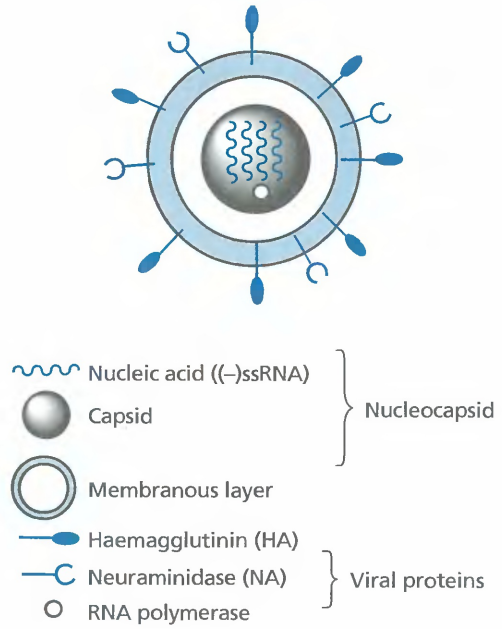


FIGURE 20.1 Diagrammatic representation of the flu virus.

20.3 Life cycle of viruses

The various stages involved in the life cycle of a virus are as follows (Fig. 20.2):

- **Adsorption:** a virion has to first bind to the outer surface of a host cell. This involves a specific molecule on the outer surface of the virion binding to a specific protein or carbohydrate present in the host cell membrane. The relevant molecule on the host cell can thus be viewed as a 'receptor' for the virion. Of course, the host cell has not produced this molecule to be a viral

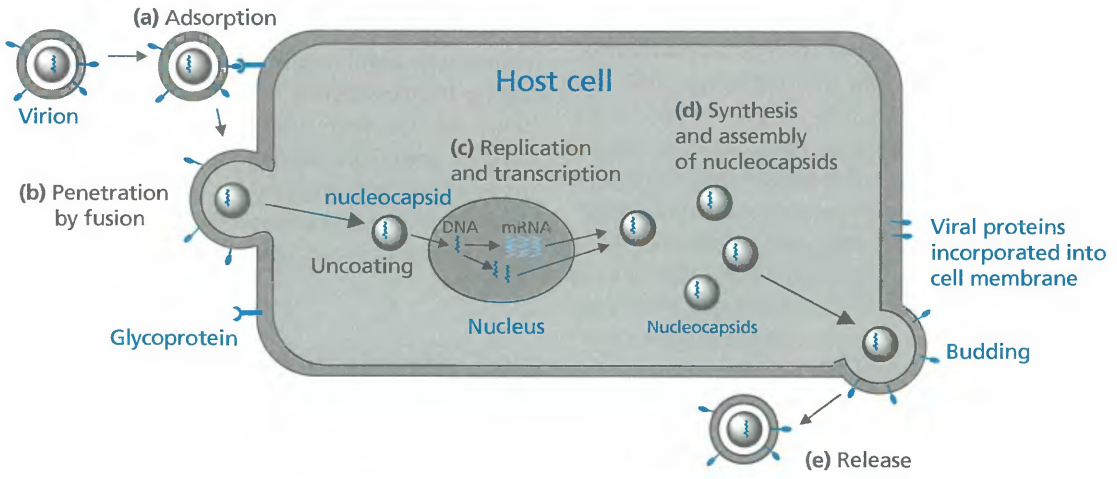


FIGURE 20.2 Life cycle of a DNA virus such as herpes simplex.

receptor. The molecules concerned are usually glycoproteins, which have crucial cellular functions such as the binding of hormones. The virion takes advantage of these, however, and once the virion is bound, the next stage can take place—introduction of the viral nucleic acid into the host cell.

- **Penetration and uncoating:** different viruses introduce their nucleic acid into the host cell by different methods. Some inject their nucleic acid through the cell membrane; others enter the cell intact and are then uncoated. This can also happen in a variety of ways. The viral envelope of some virions fuses with the plasma membrane and the nucleocapsid is then introduced into the cell (Fig. 20.2). Other virions are taken into the cell by endocytosis where the cell membrane wraps itself round the virion and is then pinched off to produce a vesicle called an endosome (Fig. 20.39). These vesicles then fuse with **lysosomes**, and host cell enzymes aid the virus in the uncoating process. Low endosomal pH also triggers uncoating. In some cases, the virus envelope fuses with the lysosome membrane and the nucleocapsid is released into the cell. Whatever the process, the end result is the release of viral nucleic acid into the cell.
- **Replication and transcription:** viral genes can be defined as *early* or *late*. Early genes take over the host cell such that viral DNA and/or RNA is synthesized. The mechanism involved varies from virus to virus. For example, viruses containing negative single-strand RNA use a viral enzyme called RNA-dependent RNA polymerase (or transcriptase) to synthesize mRNA, which then codes for viral proteins.
- **Synthesis and assembly of nucleocapsids:** late genes direct the synthesis of capsid proteins and these self-assemble to form the capsid. Viral nucleic acid is then taken into the capsid to form the nucleocapsid.
- **Virion release:** naked virions (those with no outer layers round the nucleocapsid) are released by cell lysis where the cell is destroyed. In contrast, viruses with envelopes are usually released by a process known as budding (Fig. 20.2). Viral proteins are first incorporated into the host cell's plasma membrane. The nucleocapsid then binds to the inner surface of the cell membrane and at the same time viral proteins collect at the site and host cell proteins are excluded. The plasma membrane containing the viral proteins then wraps itself round the nucleocapsid and is pinched off from the cell to release the mature virion.

The life cycle stages of herpes simplex, flu virus, and HIV are illustrated in Figs. 20.2, 20.12, and 20.39 respectively.

20.4 Vaccination

Vaccination is the preferred method of protection against viral disease and has proved extremely successful against childhood diseases such as polio, measles, and mumps, as well as historically serious diseases such as smallpox and yellow fever. The first successful vaccination was carried out by Edward Jenner in the eighteenth century. Having observed that a milkmaid had contracted the less virulent cowpox and had subsequently become immune to smallpox, he inoculated people with material from cowpox lesions and discovered that they too gained immunity from smallpox.

Vaccination works by introducing the body to foreign material which bears molecular similarity to some component of the virus, but which lacks its infectious nature or toxic effects. The body then has the opportunity to recognize the molecular fingerprint of the virus (i.e. specific antigens), and the immune system is primed to attack the virus should it infect the body. Usually a killed or weakened version of the virus is administered so that it does not lead to infection itself. Alternatively, fragments of the virus (subunit vaccines) can be used if they display a characteristic antigen. Vaccination is a preventive approach and is not usually effective on patients who have already become infected.

Vaccines are currently under investigation for the prevention or treatment of HIV, dengue fever, genital herpes and haemorrhagic fever caused by the Ebola virus. There are difficulties surrounding the HIV and flu viruses, however, because rapid gene mutation in these viruses results in constant changes to the amino acid composition of glycoproteins normally present on the viral surface. Since these glycoproteins are the important antigens that trigger the immune response, any changes in their structure 'disguise' the virus and the body's primed immune system fails to recognize it.

Another problem concerning vaccination relates to patients with a weakened immune response. The main categories of patients in this situation are cancer patients undergoing chemotherapy, patients undergoing organ transplants (where the immune system has been deliberately suppressed to prevent organ rejection), and AIDS patients. Vaccination in these patients is less likely to be effective and the weakened immune response also leads to increased chances of infections such as pneumonia.

In situations where infection has occurred and the immune system is unable to counter the invasion, antiviral drugs can help to bring the disease under control and allow the immune system to regain ascendancy.

20.5 Antiviral drugs: general principles

Antiviral drugs are useful in tackling viral diseases where there is a lack of an effective vaccine, or where infection has already taken place. The life cycle of a virus means that for most of its time in the body, it is within a host cell and is effectively disguised both from the immune system and from circulating drugs. Since it also uses the host cell's own biochemical mechanisms to multiply, the number of potential drug targets that are unique to the virus is more limited than those that can be identified for invading microorganisms. Thus, the search for effective antiviral drugs has proved more challenging than that for antibacterial drugs. Indeed, the first antiviral agents appeared relatively late on in the 1960s, and only three clinically useful antiviral drugs were in use during the early 1980s. Early antiviral drugs included **idoxuridine** and **vidarabine** for herpes infections, and **amantadine** for influenza A.

Since then, progress has accelerated for two principle reasons—the need to tackle the AIDS pandemic, and the increased understanding of viral infectious mechanisms resulting from viral genomic research.

In 1981, it was noticed that gay men were unusually susceptible to diseases such as pneumonia and fungal infections—ailments which were previously only associated with patients whose immune response had been weakened. The problem soon reached epidemic proportions and it was discovered that a virus (the **human immunodeficiency virus**—HIV) was responsible. It was found that this virus infected T-cells—cells which are crucial to the immune response—and was therefore directly attacking the immune system. With a weakened immune system, infected patients proved susceptible to a whole range of opportunistic secondary diseases resulting in the term **acquired immune deficiency syndrome** (AIDS). This discovery led to a major research effort into understanding the disease and counteracting it—an effort which kick started more general research into antiviral chemotherapy. Fortunately, the tools needed to carry out effective research appeared on the scene at about the same time, with the advent of viral genomics. The full genome of any virus can now be quickly determined and compared with those of other viruses, allowing the identification of how the genetic sequence is split into genes. Although the genetic sequence is unlikely to be identical from one virus to another, it is possible to identify similar genes coding for similar proteins with similar functions. These proteins can then be studied as potential drug targets. Standard genetic engineering methods allow the production of pure copies of the target protein by inserting the viral gene into a bacterial cell thus allowing sufficient quantities of the protein to be synthesized and isolated (section 6.4). The protein can be used for screening as well as for studying drug-protein interactions.

Good drug targets are proteins which are likely to have the following characteristics:

- They are important to the life cycle of the virus such that their inhibition or disruption has a major effect on infection.
- They bear little resemblance to human proteins, thus increasing the chances of good selectivity and minimal side effects.
- They are common to a variety of different viruses and have a specific region that is identical in its amino acid composition. This makes the chances of developing a drug with broad antiviral activity more likely.
- They are important to the early stages of the virus life cycle so that the virus has less chance of spreading through the body and producing symptoms.

Most antiviral drugs in use today act against HIV, herpes viruses (responsible for a variety of ailments including cold sores and encephalitis), hepatitis B, and hepatitis C. Diseases such as herpes and HIV are chronic in developed countries, and intensive research has been carried out to develop drugs to combat them. In contrast, less research is carried out on viral diseases prevalent in developing countries such as tropical (dengue) and haemorrhagic (Ebola) fevers.

Most antiviral drugs in use today disrupt critical stages of the virus life cycle or the synthesis of virus-specific nucleic acids. Excluding drugs developed for the treatment of HIV, more drugs are available for the treatment of DNA viruses than for RNA viruses. Few drugs show a broad activity against both DNA and RNA viruses.

Studies of the human genome are also likely to be useful for future research. The identification of human proteins which stimulate the body's immune response or the production of antibodies would provide useful leads for the development of drugs that would have an antiviral effect by acting as immunomodulators.

KEY POINTS

- Viruses pose a serious health threat, and there is a need for new antiviral agents.
- Viruses consist of a protein coat surrounding nucleic acid, which is either RNA or DNA. Some viruses have an outer membranous coat that is derived from the host cell.
- Viruses are unable to self-multiply and need to enter a host cell in order to do so.

- Vaccination is effective against many viruses, but is less effective against viruses that readily mutate.
- Research into antiviral drugs has increased in recent years as a result of the AIDS epidemic and the need to find drugs to combat it.
- Antiviral research has been aided by advances in viral genomics and genetic engineering, as well as the use of X-ray crystallography and molecular modelling.

20.6 Antiviral drugs used against DNA viruses

Most of the drugs which are active against DNA viruses have been developed against herpesviruses to combat diseases such as cold sores, genital herpes, chicken pox, shingles, eye diseases, mononucleosis, Burkitt's lymphoma, and Kaposi's sarcoma. Nucleoside analogues have been particularly effective.

20.6.1 Inhibitors of viral DNA polymerase

Aciclovir was discovered by compound screening and was introduced into the market in 1981. Aciclovir has a nucleoside like structure and contains the same nucleic acid base as deoxyguanosine, but lacks the complete sugar ring. In virally infected cells, it is phosphorylated in three stages to form a triphosphate which is the active agent, and so aciclovir itself is a prodrug (Fig. 20.3).

Nucleotide triphosphates are the building blocks for DNA replication where a new DNA strand is constructed using a DNA template—a process catalysed by the enzyme **DNA polymerase**. Aciclovir triphosphate prevents DNA replication in two ways. First, it is sufficiently similar to the normal deoxyguanosine triphosphate building block (Fig. 20.4) that it can bind to DNA polymerase and inhibit it. Second, DNA polymerase can catalyse the attachment of the aciclovir nucleotide to the growing DNA chain. Since the sugar unit is incomplete and lacks the required hydroxyl group normally present at position 3′ of the sugar ring, the nucleic acid chain cannot be extended any further. Thus, the drug acts as a chain terminator (see section 9.5).

However, what is to stop aciclovir triphosphate inhibiting DNA polymerase in normal, uninfected cells? The answer lies in the fact that aciclovir is only converted to the active triphosphate in infected cells. The explanation for this lies in the first phosphorylation reaction catalysed by the enzyme thymidine kinase. Although this enzyme is present in host cells, the herpes virus carries its own version. It turns out that viral thymidine kinase is 100 times more effective at converting aciclovir to its monophosphate than host cell thymidine kinase. Once formed, the monophosphate is converted to the active triphosphate by cellular enzymes. Therefore, in normal uninfected cells, aciclovir is a poor substrate for cellular thymidine kinase and remains as the prodrug. This, along with the fact that there is a selective uptake of aciclovir by infected cells, explains its excellent activity and much reduced toxicity relative to previous drugs. Another feature which enhances its safety is that aciclovir triphosphate shows a 50-fold selective action against viral DNA polymerases relative to cellular polymerases.

The oral bioavailability of aciclovir is quite low (15–30%) and to overcome this, various prodrugs were developed

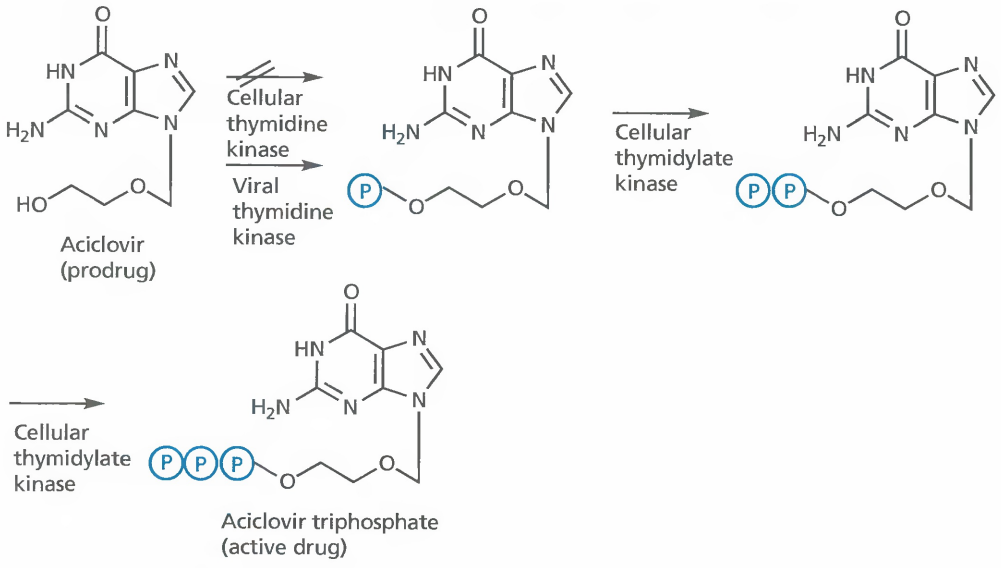


FIGURE 20.3 Activation of aciclovir. Ⓟ represents phosphate groups.

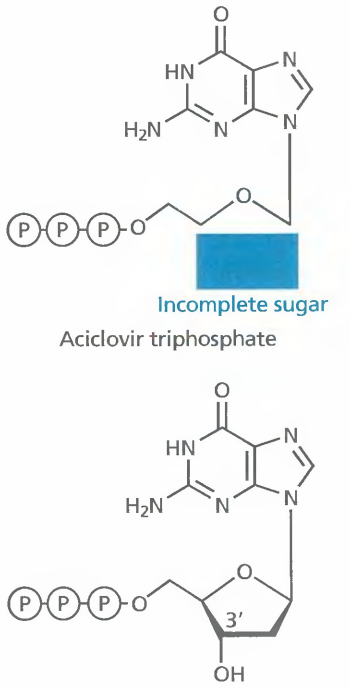


FIGURE 20.4 Comparison of aciclovir triphosphate and deoxyguanosine triphosphate.

to increase water solubility. **Valaciclovir** (Fig. 20.5) is an L-valyl ester prodrug that is absorbed from the gut far more effectively than aciclovir itself. However, the prodrug has similar polarity and ionization to aciclovir, and so the prodrug is no more able to cross the cell membranes of the gut wall by passive diffusion than aciclovir itself. Moreover, poorer absorption is observed if D-valine is used for the prodrug instead of L-valine, suggesting that a specific binding interaction is involved in the absorption process. This implies that the prodrug is actively transported by transport proteins in the gut, and that the valine allows the prodrug to be recognised and bound by these proteins. Transport proteins normally responsible for transporting dipeptides across the cell wall have been implicated in this process i.e. the human intestinal proton-dependent oligopeptide transporter (hPEPT-1) and human intestinal di-/tripeptide transporter (HPT-1). Once valaciclovir is absorbed, it is hydrolysed to aciclovir in the liver and gut wall. **Desciclovir** (Fig. 20.5) is a prodrug of aciclovir which lacks the carbonyl group at position 6 of the purine ring and is more water soluble. Once in the blood supply, metabolism by cellular xanthine oxidase oxidizes the 6-position to give aciclovir.

Ganciclovir (Fig. 20.5) is an analogue of aciclovir which bears an extra hydroxymethylene group, while **penciclovir** (Fig. 20.6) is an analogue of ganciclovir. **Famciclovir** (Fig. 20.6) is a prodrug of penciclovir where the two alcohol groups are masked as esters making the structure less polar, and leading to better absorption. Once absorbed, the acetyl groups are hydrolysed by esterases, and the purine ring is oxidized by aldehyde oxidase in the liver to generate penciclovir. Phosphorylation reactions then take place in virally infected cells as described previously.

Some viruses are immune from the action of the above antiviral agents because they lack the enzyme thymidine kinase. As a result, phosphorylation fails to take place. **Cidofovir** was designed to combat this problem (Fig. 20.7). It is an analogue of deoxycytidine 5-monophosphate, where the sugar and phosphate groups have been replaced by an acyclic group and a phosphonomethylene group, respectively. The latter group acts as a bioisostere for the phosphate group and is used because the phosphate group itself would be more susceptible to enzymatic hydrolysis. Since a phosphate equivalent is already present, the drug does not require thymidine kinase to become activated. Two more phosphorylations can now take place catalysed by cellular kinases to convert cidofovir to the active 'triphosphate'.

In contrast to aciclovir, **idoxuridine, trifluridine** and **vidarabine** (Fig. 20.8) are phosphorylated equally well by viral and cellular thymidine kinase, and so there is less selectivity for virally infected cells. As a result, these drugs have more toxic side effects. Idoxuridine, like

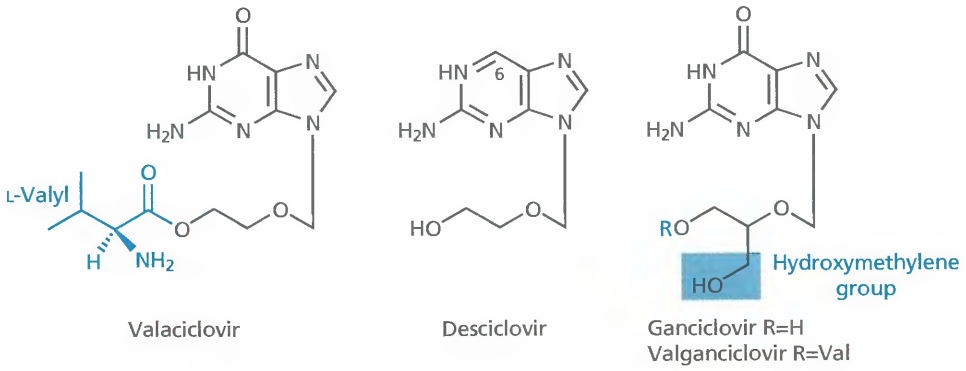


FIGURE 20.5 Prodrugs and analogues of aciclovir.

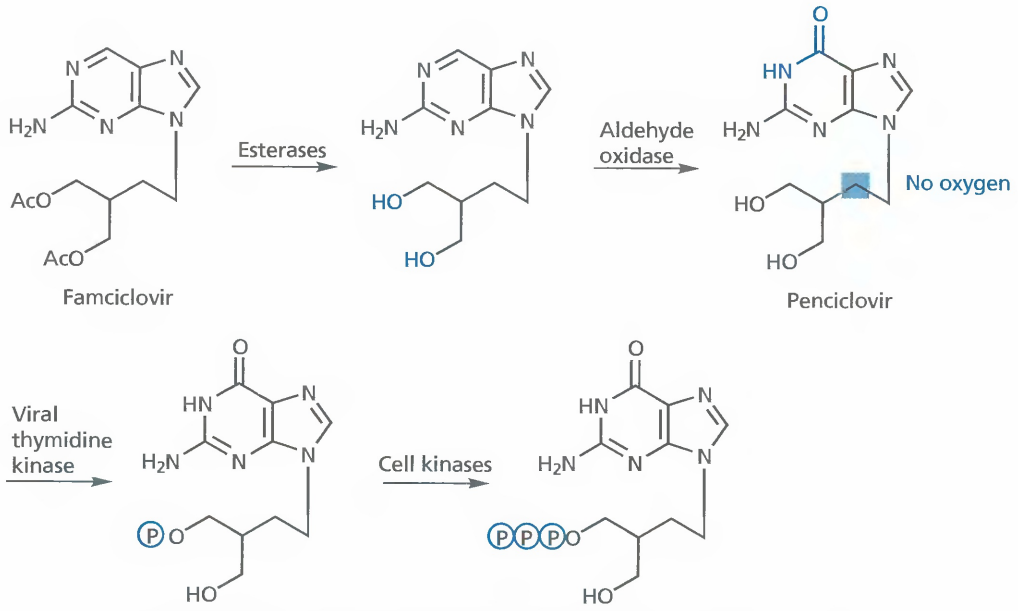


FIGURE 20.6 Penciclovir and famciclovir. Ⓟ represents a phosphate group.

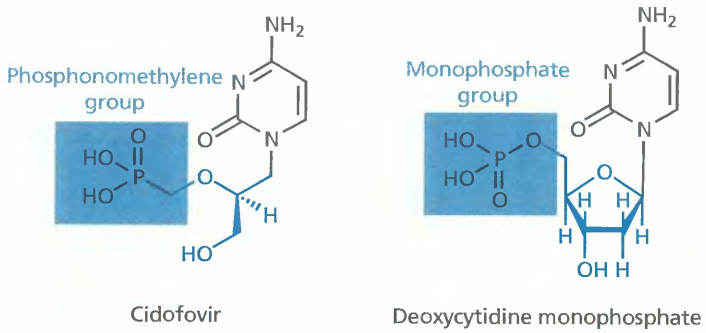


FIGURE 20.7 Comparison of cidofovir and deoxycytidine monophosphate.

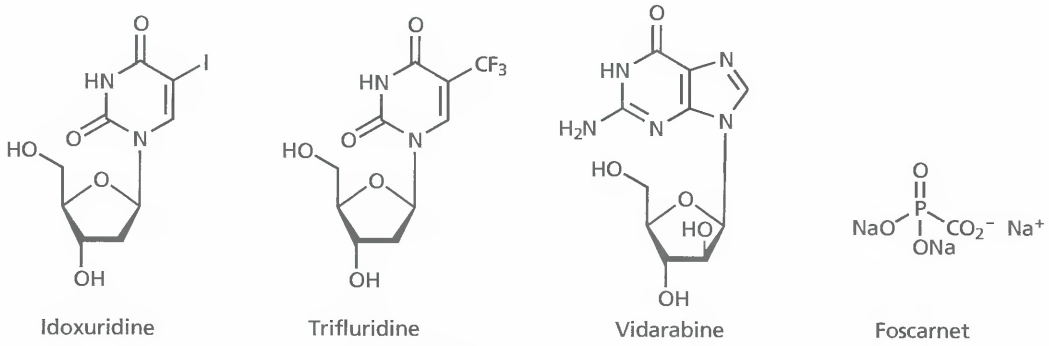


FIGURE 20.8 Miscellaneous antiviral agents.

trifluridine, is an analogue of deoxythymidine and was the first nucleoside-based antiviral agent licensed in the USA. The triphosphate inhibits viral DNA polymerase as well as thymidylate synthetase. **Vidarabine** (Fig. 20.8) is the purine counterpart of the pyrimidine nucleoside cytarabine (ara-C) and was an early antiviral drug with clinical applications. Aciclovir is now used in preference because of its lower toxicity.

Foscarnet (Fig. 20.8) was discovered in the 1960s, and inhibits viral DNA polymerase, However, it is

BOX 20.1 Clinical aspects of viral DNA polymerase inhibitors

Aciclovir represented a revolution in the treatment of herpes infections, being the first relatively safe, non-toxic drug to be used systemically. It is used for the treatment of infections due to herpes simplex 1 and 2 (i.e. herpes simplex encephalitis and genital herpes), as well as varicella-zoster viruses (VZV) (i.e. chickenpox and shingles). Unfortunately, strains of herpes are appearing which are resistant to aciclovir. This can arise due to mutations, either of the viral thymidine kinase enzyme such that it no longer phosphorylates aciclovir, or of viral DNA polymerase such that it no longer recognizes the activated drug. Aciclovir is not effective against all types of herpes virus. There are eight herpes viruses, which are divided into three subfamilies. Aciclovir is effective against the α-subfamily but not the β-subfamily, because the latter produces a different thymidine kinase that fails to phosphorylate the drug.

Valaciclovir is a valine prodrug of aciclovir, and is particularly useful in the treatment of VZV infections. When this prodrug is given orally, blood levels of aciclovir are obtained which are equivalent to those obtained by intravenous administration.

Desciclovir is another prodrug for aciclovir, but is somewhat more toxic, thus limiting its potential.

Ganciclovir is phosphorylated by thymidine kinases produced by both the α- and β-subfamilies of herpesvirus, and can be used against both viruses. Unfortunately, the drug is not as safe as aciclovir as it can be incorporated into cellular DNA. Nevertheless, it can be used for the treatment of cytomegalovirus (CMV) infections. This is a virus that causes eye infections and can lead to blindness. Aciclovir is not effective in this infection because CMV does not encode a viral thymidine kinase. Ganciclovir, on the other hand, can be converted to its monophosphate by kinases other than thymidine kinase. Since ganciclovir has a low oral bioavailability, the valine prodrug **valganciclovir** has been introduced for the treatment of CMV infections.

Penciclovir essentially has the same spectrum of activity as aciclovir, but has a better potency, a faster onset and a longer duration of action. It is used topically for the treatment of cold sores (HSV-1), and intravenously for the treatment of HSV in immunocompromised patients. Like aciclovir, penciclovir has poor oral bioavailability and is poorly absorbed from the gut due to its polarity. Therefore, **famciclovir** is used as a prodrug for better absorption.

Cidofovir is a broad-spectrum antiviral agent that shows selectivity for viral DNA polymerase, and is used to treat retinal inflammation caused by CMV. Unfortunately the drug is extremely polar and has a poor oral bioavailability (5%). It is also toxic to the kidneys, but this can be reduced by co-administering **probenecid** (Section 19.5.1.9).

Idoxuridine can be used for the topical treatment of herpes keratitis, but **trifluridine** is the drug of choice for this disease since it is effective at lower dose frequencies.

Foscarnet is used in the treatment of CMV retinitis where it is approximately equal in activity to ganciclovir. It can also be used in immunocompromised patients for the treatment of HSV and VZV strains, which prove resistant to aciclovir. It does not undergo metabolic activation.

non-selective and toxic. Moreover, since it is highly charged, it has difficulty crossing cell membranes.

20.6.2 Inhibitors of tubulin polymerization

The plant product **podophyllotoxin** (Fig. 20.9) has been used clinically to treat genital warts (caused by the DNA virus **papillomavirus**), but it is not as effective as **imiquimod** (section 20.10.4). It is a powerful inhibitor of tubulin polymerization (sections 2.7.1 and 10.2.2).

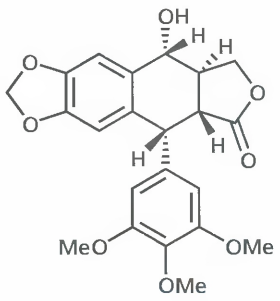


FIGURE 20.9
Podophyllotoxin.

20.6.3 Antisense therapy

Fomivirsen (Fig. 20.10) is the first, and so far the only, DNA antisense molecule that has been approved as an antiviral agent. It consists of 21 nucleotides and a phosphonothioate backbone rather than a phosphate backbone to increase the metabolic stability of the molecule (section 14.10). The drug blocks the translation of viral RNA and is used against retinal inflammation caused by the CMV in AIDS patients. Because of its high polarity, it is administered as an ocular injection (intravitreal injection).

d(*P*-thio)(G-C-G-T-T-T-G-C-T-C-T-T-C-T-T-C-T-T-G-C-G)

FIGURE 20.10 Fomivirsen.

KEY POINTS

- Nucleoside analogues have been effective antiviral agents used against DNA viruses, mainly herpes viruses.
- Nucleoside analogues are prodrugs, which require to be phosphorylated to a triphosphate in order to be active. They have a dual mechanism of action whereby they inhibit viral DNA polymerase and also act as DNA chain terminators.
- Nucleoside analogues show selectivity for virally infected cells over normal cells if viral thymidine kinase is required to catalyse the first of three phosphorylation steps. They are also taken up more effectively into virally infected cells, and their triphosphates inhibit viral DNA polymerases more effectively than cellular DNA polymerases.
- Agents containing a bioisostere for a phosphate group can be used against DNA viruses lacking thymidine kinase.
- Inhibitors of tubulin polymerization have been used against DNA viruses.
- A DNA antisense molecule has been designed as an antiviral agent.

20.7 Antiviral drugs acting against RNA viruses: HIV

20.7.1 Structure and life cycle of HIV

HIV (Fig. 20.11) is an example of a group of viruses known as the retroviruses. There are two variants of HIV. HIV-1 is responsible for AIDS in America, Europe and Asia, whereas HIV-2 occurs mainly in western Africa. HIV has been studied extensively over the last 20 years and a vast research effort has resulted in a variety of antiviral drugs which have proved successful in slowing down the disease, but not eradicating it. At present, most clinically useful antiviral drugs act against two targets—the viral enzymes **reverse transcriptase** and **protease.** There is a need to develop effective drugs against a third target, and a good knowledge of the life cycle of HIV is essential in identifying suitable targets (Fig. 20.11).

HIV is an RNA virus which contains two identical strands of (+) ssRNA within its capsid. Also present are the viral enzymes reverse transcriptase and **integrase,** as well as other proteins called p6 and

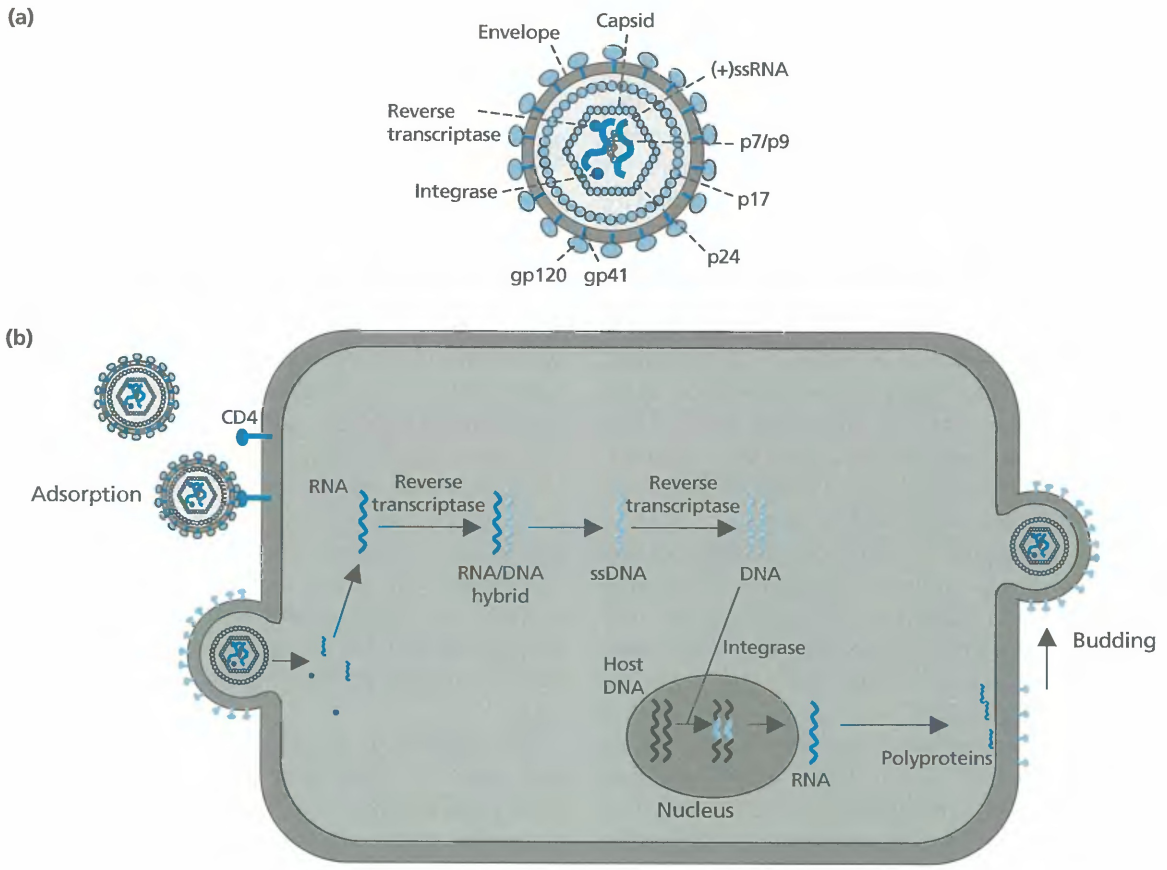


FIGURE 20.11 **(a)** Structure of virus particle and **(b)** life cycle of the human immunodeficiency virus (HIV).

p7. The capsid is made up of protein units known as p24, and surrounding the capsid there is a layer of matrix protein (p17), then a membranous envelope which originates from host cells and which contains the viral glycoproteins **gp120 and gp41**. Both of these proteins are crucial to the processes of adsorption and penetration. Gp41 traverses the envelope and is bound non-covalently to gp120, which projects from the surface. When the virus approaches the host cell, gp120 interacts and binds with a transmembrane protein called **CD4** which is present on host T-cells. The gp120 proteins then undergo a conformational change which allows them to bind simultaneously to chemokine receptors (CCR5 and CXCR4) on the host cell (not shown). Further conformational changes peel away the gp120 protein so that viral protein gp41 can reach the surface of the host cell and anchor the virus to the surface. The gp41 then undergoes a conformational change and pulls the virus and the cell together so that their membranes can fuse.

Once fusion has taken place, the HIV nucleocapsid enters the cell. Disintegration of the protein capsid then takes place, probably aided by the action of a viral enzyme called protease. Viral RNA and viral enzymes are then released into the cell cytoplasm. The released viral RNA is not capable of coding directly for viral proteins, or of self-replication. Instead, it is converted into DNA and incorporated into the host cell DNA. The conversion of RNA into DNA is not a process that occurs in human cells, so there are no host enzymes to catalyse the process. Therefore, HIV carries its own enzyme—reverse transcriptase—to do this. This enzyme is a member of a family of enzymes known as the DNA polymerases, but is unusual in that it can use an RNA strand as a template. The enzyme first catalyses the synthesis of a DNA strand using viral RNA as a template. This leads to a (+)RNA–(−)DNA hybrid. Reverse transcriptase catalyses the degradation of the RNA strand, then uses the remaining DNA strand as a template to catalyse the synthesis of double-stranded DNA (proviral DNA). Proviral DNA is now spliced into the host cell's DNA, a process catalysed by integrase—an enzyme also carried by the virion. Once the proviral DNA has been incorporated into host DNA, it is called the provirus and can remain dormant in host cell DNA until activated by cellular processes. When that occurs, transcription of the viral genes *env*, *gag* and *pol* takes place to produce viral RNA, some of which will be incorporated into new virions, and the rest of which is used in translation to produce three large non-functional polyproteins, one derived from the *env* gene, one from the *gag* gene, and the other from the *gag-pol* genes. The first of these polyproteins is cleaved by cellular proteinases and produces the viral glycoproteins (gp120 and gp41), which are incorporated into the cell membrane. The remaining two polypeptides (Pr55 and Pr160) are not split by cellular proteinases. Instead, they move to the inner membrane surface. The viral glycoproteins in the cell membrane also concentrate in this area, and cellular proteins are excluded. Budding then takes place to produce an immature membrane-bound virus particle. During the budding process a viral enzyme called protease is released from the gag-pol polypeptide. This is achieved by the protease enzyme autocatalysing the cleavage of susceptible peptide bonds linking it to the rest of the polypeptide. Once released, the protease enzyme dimerizes and cleaves the remaining polypeptide chains to release reverse transcriptase, integrase and viral structural proteins. The capsid proteins now self-assemble to form new nucleocapsids containing viral RNA, reverse transcriptase and integrase.

It has also been observed that a viral protein called Vpu has an important part to play in the budding process. Vpu binds to the host membrane protein CD4 and triggers a host enzyme to tag the CD4 protein with a protein called ubiquitin. Proteins that are tagged with ubiquitin are marked out for destruction by the host cell and so the CD4 proteins in the host cell are removed. This is important, as the CD4 proteins could complex with the newly synthesized viral proteins gp120 and prevent the assembly of the new viruses.

20.7.2 Antiviral therapy against HIV

Until 1987, no anti-HIV drug was available, but an understanding of the life cycle of HIV has led to the identification of several possible drug targets. At present, most drugs that have been developed act against the viral enzymes reverse transcriptase and protease. However, a serious problem with the treatment of HIV is the fact that the virus undergoes mutations extremely easily. This results in rapid resistance to antiviral drugs. Experience has shown that treatment of HIV with a single drug has a short-term benefit, but in the long term the drug serves only to select mutated viruses which are resistant. As a result, current therapy involves combinations of different drugs acting on both reverse transcriptase and protease. This has been successful in delaying the progression to AIDS and increasing survival rates, but there is a need to develop effective drugs against a third target.

The demands on any HIV drug are immense, especially since it is likely to be taken over long periods of time. It must have a high affinity for its target (in the picomolar range) and be effective in preventing the virus multiplying and spreading. It should show low activity for any similar host targets in the cell, and

BOX 20.2 Clinical aspects of antiviral drugs used against HIV

There is no cure for HIV infection, but anti-HIV drugs can halt or slow the rate at which the disease develops, leading to a significant increase in life expectancy. Unfortunately, the drugs used have toxic side effects, which is particularly significant since patients have to take these drugs for the rest of their lives. This means that patients have to be constantly monitored. Another problem with anti-HIV treatment is the ability of the virus to mutate and gain resistance against any one drug. It is preferable, therefore, to use a combination of drugs acting against two different enzyme targets—protease and reverse transcriptase. This is known as highly active antiretroviral therapy (**HAART**). When choosing which drugs to use, it is important to ensure that they have a synergistic or additive effect, and that they are compatible in terms of their toxic properties.

Currently, protease inhibitors (PIs) are used with reverse transcriptase inhibitors (divergent therapy) or with another protease inhibitor (convergent therapy). A combination of two NRTIs plus a protease inhibitor is recommended, but one can also use two PIs with an NRTI, or an NNRTI with two NRTIs. For example, the NNRTI efavirenz is used along with the NRTIs emtricitabine and tenofovir.

The NRTIs that can be used against HIV are zidovudine, didanosine, zalcitabine, stavudine, lamivudine, emtricitabine, tenofovir disoproxil, and abacavir.

NNRTIs used against HIV are nevirapine, delavirdine, and efavirenz.

PIs that are used are saquinavir, ritonavir, indinavir, nelfinavir, amprenavir, atazanavir, darunavir, fosamprenavir, lopinavir, and tipranavir.

The fusion inhibitor enfuvirtide has now been approved as an anti-HIV drug, and acts against a different target from the conventional anti-HIV drugs. It can be included alongside conventional drugs if the disease fails to respond to standard HAART therapy.

The first integrase inhibitor has also been approved for clinical use.

be safe and well tolerated. It must be active against as large a variety of viral isolates as possible, or else it only serves to select resistant variants. It needs to be synergistic with other drugs used to fight the disease and be compatible with other drugs used to treat opportunistic diseases and infections arising from the weakened immune response. The drug must stay above therapeutic levels within the infected cell and in the circulation. It must be capable of being taken orally and with a minimum frequency of doses, it should preferably be able to cross the blood–brain barrier in case the virus lurks in the brain. Finally, it must be inexpensive as it is likely to be used for the lifetime of the patient.

20.7.3 Inhibitors of viral reverse transcriptase

20.7.3.1 Nucleoside reverse transcriptase inhibitors

Since the enzyme reverse transcriptase is unique to HIV, it serves as an ideal drug target. Nevertheless, the enzyme is still a DNA polymerase and care has to be taken that inhibitors do not have a significant inhibitory effect on cellular DNA polymerases. Various nucleoside-like structures have proved useful as antiviral agents. The vast majority of these are not active themselves but are phosphorylated by three cellular enzymes to form an active nucleotide triphosphate. This is the same process previously described in section 20.6.1, but one important difference is the requirement for all three phosphorylations to be carried out by cellular enzymes as HIV does not produce a viral kinase.

Zidovudine (Fig. 20.12) was originally developed as an anticancer agent but was the first drug to be approved for use in the treatment of AIDS. It is an analogue of deoxythymidine where the sugar 3'-hydroxyl group has been replaced by an azido group. It inhibits reverse transcriptase as the triphosphate. Furthermore, the triphosphate is attached to the growing DNA chain. Since the sugar unit has an azide substituent at the 3' position of the sugar ring, the nucleic acid chain cannot be extended any further.

Didanosine (Fig. 20.12) was the second anti-HIV drug approved for use in the USA (1988). Its activity was unexpected since the nucleic acid base present is inosine—a base which is not naturally incorporated into DNA. However, a series of enzyme reactions converts this compound into 2',3'-dideoxyadenosine triphosphate which is the active drug. Studies of the enzyme's active site led to the development of **lamivudine** and **emtricitabine** (Fig. 20.12) (analogues of deoxycytidine where the 3' carbon has been replaced by sulfur). Other clinically useful NRTIs used against HIV and/or hepatitis B include **abacavir** (the only guanosine analogue), **stavudine,** and **zalcitabine** (Fig. 20.13). **Tenofovir disoproxil** and **adefovir dipivoxil** are prodrugs of modified nucleosides. Both structures contain a monophosphate group protected by two extended esters. Hydrolysis *in vivo* reveals the phosphate group which can then be phosphorylated to the triphosphate as described previously.

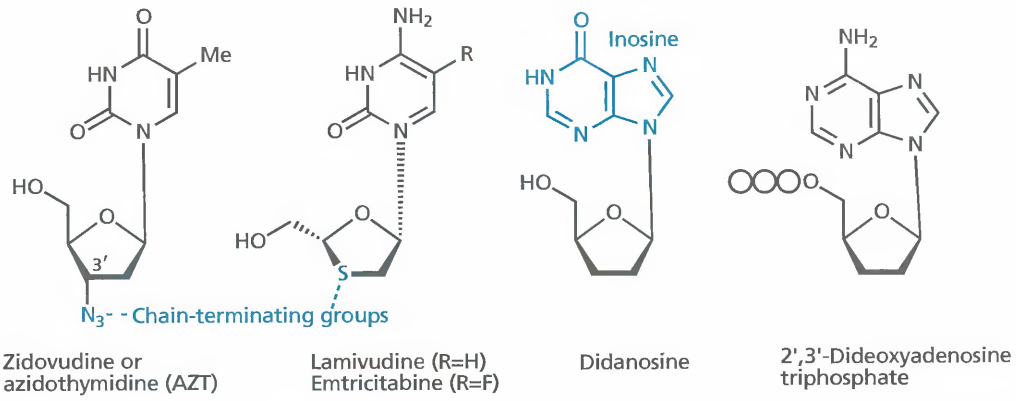


FIGURE 20.12 Inhibitors of viral reverse transcriptase.

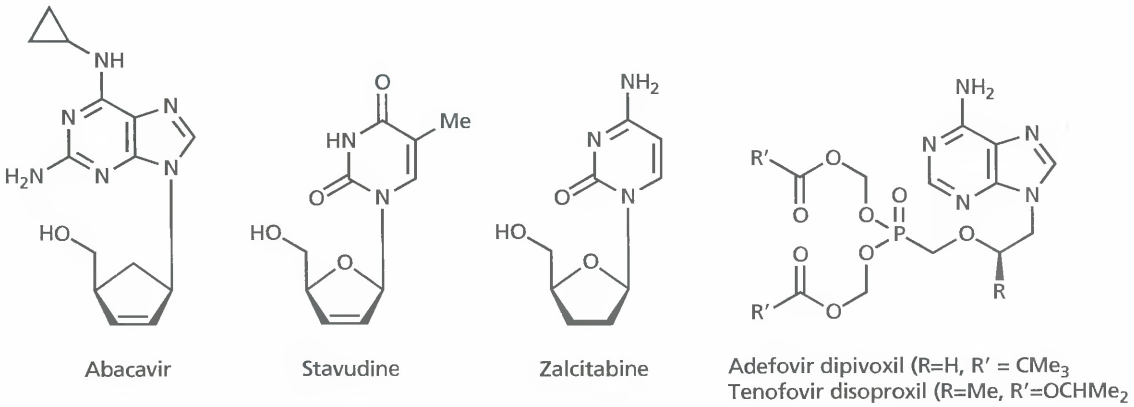


FIGURE 20.13 Further inhibitors of viral reverse transcriptase.

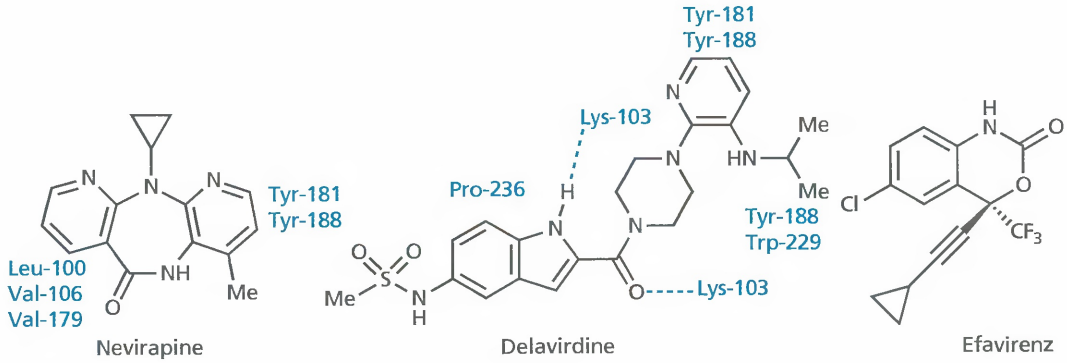


FIGURE 20.14 Non-nucleoside reverse transcriptase inhibitors in clinical use (interactions with amino acids in the binding site are shown in blue).

20.7.3.2 Non-nucleoside reverse transcriptase inhibitors

The NNRTIs (Fig. 20.14) are generally hydrophobic molecules that bind to an allosteric binding site which is hydrophobic in nature. Since the allosteric binding site is separate from the substrate binding site, the NNRTIs are non-competitive, reversible inhibitors. They include first-generation NNRTIs such as **nevirapine** and **delavirdine,** as well as second-generation drugs such as **efavirenz.** X-ray crystallographic studies on inhibitor–enzyme complexes show that the allosteric binding site is adjacent to the substrate binding site. Binding of an NNRTI to the allosteric site results in an induced fit which locks the neighbouring substrate-binding site into an inactive conformation. Unfortunately, rapid resistance emerges

due to mutations in the NNRTI binding site, the most common being the replacement of Lys-103 with asparagine. This mutation is called K103N and is defined as a **pan-class resistance mutation.** The resistance problem can be countered by combining an NNRTI with an NRTI from the start of treatment. The two types of drugs can be used together as the binding sites are distinct.

Nevirapine was developed from a lead compound discovered through a random screening programme, and has a rigid butterfly-like conformation that makes it chiral. One 'wing' interacts through hydrophobic and van der Waals interactions with aromatic residues in the binding site while the other wing interacts with aliphatic residues. The other NNRTI inhibitors bind to the same pocket and appear to function as π electron donors to aromatic side chain residues.

Delavirdine was developed from a lead compound discovered by a screening programme of 1500 structurally diverse compounds. It is larger than other NNRTIs and extends beyond the normal pocket such that it projects into surrounding solvent. The pyridine region and isopropylamine groups are the most deeply buried parts of the molecule and interact with tyrosine and tryptophan residues. There are also extensive hydrophobic contacts. Unlike other first-generation NNRTIs, there is hydrogen bonding to the main peptide chain next to Lys-103. The indole ring of delavirdine interacts with Pro-236, and mutations involving Pro-236 lead to resistance. Analogues having a pyrrole ring in place of indole might avoid this problem.

Second- and third-generation NNRTIs were developed specifically to find agents that were active against resistant variants as well as wild type virus. This development has been helped by X-ray crystallographic studies which show how the structures bind to the binding site. It has been shown from sequencing studies that in most of the mutations that cause resistance to first-generation NNRTIs, a large amino acid is replaced by a smaller one, implying that an important binding interaction has been lost. Interestingly, mutations that replace an amino acid with a larger amino acid appear to be detrimental to the activity of the enzyme, and no mutations have been found which block NNRTIs sterically from entering the binding site.

Efavirenz is a benzoxazinone structure and was the only second-generation NNRTI on the market in 2004. It has activity against many mutated variants but has less activity against the mutated variant K103N. Nevertheless, activity drops less than for nevirapine, and a study of X-ray structures of each complex revealed that the cyclopropyl group of efavirenz has fewer interactions with Tyr-181 and Tyr-188 than nevirapine does. Consequently, mutations of these amino acids have less effect on efavirenz than they do on nevirapine. Efavirenz is also a smaller structure and can shift its binding position when K103N mutation occurs, allowing it to form hydrogen bonds to the main peptide chain of the binding site.

X-ray crystallographic studies of enzyme complexes with second-generation NNRTIs reveal that these agents contain a non-aromatic moiety which interacts with the aromatic residues Tyr-181, Tyr-188 and Trp-229 at the top of the binding pocket. The ability to form hydrogen bonds to the main peptide chain, and a relatively small bulk, are important since they allow compounds to change their binding mode when mutations occur.

A large number of third-generation NNRTIs are being studied, including those shown in Fig. 20.15. **Emivirine** was developed from a lead compound found by screening structures similar to aciclovir. Resistance is slow to occur and requires two mutations to occur in the binding site. The isopropyl group at C-5 forces the aromatic residue of Tyr-181 into an orientation which allows an enhanced interaction with the aromatic substituent at C-6.

SJ3366 inhibits HIV-1 replication at a concentration below 1 nM with a therapeutic index greater than 4 million and, unlike other NNRTIs, inhibits HIV-2. **DPC 083** was developed from efavirenz.

Capravirine retains activity against mutants, including those with the K103N mutation. It has three hydrogen bonding interactions with the main chain of the protein active site, including one to Pro-236 which is not present in other inhibitors. It is suggested that hydrogen bonding to the main chain rather than to residues makes the molecule less vulnerable to mutations because binding to the polypeptide chain remains constant. The molecule is also quite flexible and this may allow it to adopt different orientations in mutated binding sites such that it can still bind. It is also larger than most other inhibitors and has potentially more interactions. This means that the loss of one interaction due to a mutation is less critical to the overall binding strength. So far, resistance to capravirine is found only after two mutations have occurred in the binding site. The chloro-substituted aromatic ring occupies the top of the binding pocket and makes more contact with the highly conserved Trp-229 indole side chain than the aromatic rings of first-generation compounds. Hydrophobic interactions also take place to the aromatic rings of Tyr-181 and Tyr-188. The pyridine ring interacts with the side chains of Phe-227 and Pro-236.

20.7.4 Protease inhibitors

In the mid-1990s, the use of X-ray crystallography and molecular modelling led to the structure-based design of a series of inhibitors which act on the viral enzyme HIV protease. Like the reverse transcriptase inhibitors, protease inhibitors (PIs) have a short-term benefit when they are used alone, but resistance soon develops.

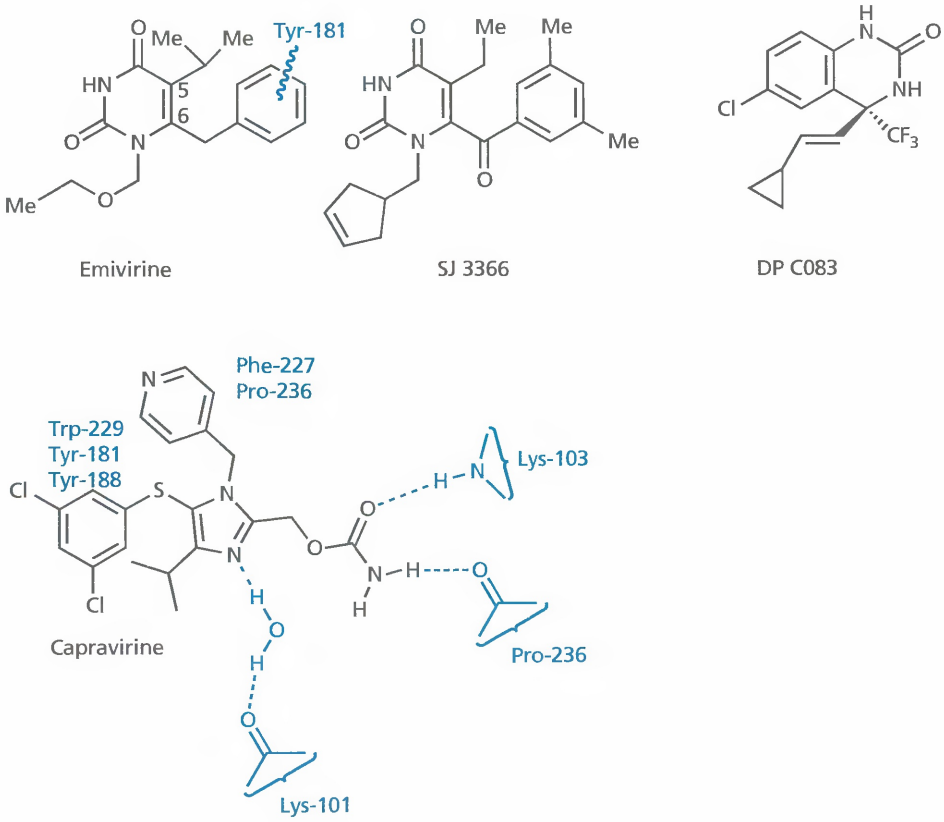


FIGURE 20.15 Examples of third-generation non-nucleoside reverse transcriptase inhibitors.

BOX 20.3 Clinical aspects of reverse transcriptase inhibitors

Nucleoside reverse transcriptase inhibitors

Nucleoside reverse transcriptase inhibitors (NRTIs) are currently used as part of the combination therapy for combating HIV. NRTIs generally have good oral bioavailability, are only minimally bound to plasma proteins, and are excreted through the kidneys. They also act against both HIV-1 and HIV-2. However, they are often associated with toxic side effects. **Zidovudine** was the first anti-HIV drug to reach the market, but can cause severe side effects such as anaemia. **Didanosine** was the second anti-HIV drug approved for use and reached the US market in 1988. However, there is a risk of toxicity to the pancreas. **Abacavir** was approved in 1998 and has been used successfully in children, in combination with the protease inhibitors nelfinavir and saquinavir. However, life-threatening hypersensitivity reactions have been reported in some patients. **Tenofovir disoproxil** was approved for HIV-1 treatment in 2001. It remains in infected cells longer than many other antiretroviral drugs, allowing for once-daily dosing, but can have toxic effects on the kidneys. It can be used alongside **emtricitabine** which is relatively free of toxic side effects. Other NRTIs used against HIV include **lamivudine** and **stavudine**. Lamivudine is less toxic than zidovudine and has also been approved for the treatment of hepatitis B.

Zalcitabine is an NRTI which acts against hepatitis B, but long-term toxicity means that it is unacceptable for the treatment of chronic viral diseases which are not life threatening. **Adefovir dipivoxil** was approved by the US FDA in 2002 for the treatment of chronic hepatitis B. It is also active on viruses such as CMV and herpes.

Non-nucleoside reverse transcriptase inhibitors

Compared to the NRTIs, the non-nucleoside reverse transcriptase inhibitors (NNRTIs) show a higher selectivity for HIV-1 reverse transcriptase over host DNA polymerases. As a result, NNRTIs are less toxic and have fewer side effects. Unfortunately, rapid resistance emerges if an NNRTI is used on its own, but this does not occur if the NNRTI is combined with an NRTI from the start of treatment. NNRTIs are restricted to HIV-1 activity and are generally metabolized by the liver. They can interact with other drugs and bind more strongly to plasma proteins. **Nevirapine** and **efavirenz** are currently prescribed NNRTIs in the treatment of HIV.

Consequently, combination therapy is now the accepted method of treating HIV infections. When protease and reverse transcriptase inhibitors are used together, the antiviral activity is enhanced and viral resistance is slower to develop.

Unlike the reverse transcriptase inhibitors, the PIs are not prodrugs and do not need to be activated. Therefore, it is possible to use *in vitro* assays involving virally infected cells in order to test their antiviral activity. The protease enzyme can also be isolated allowing enzyme assays to be carried out. In general, the latter are used to measure IC_{50} levels as a measure of how effective novel drugs are in inhibiting the protease enzyme. The IC_{50} is the concentration of drug required to inhibit the enzyme by 50%. Thus, the lower the IC_{50} value, the more potent the inhibitor. However, a good PI does not necessarily mean a good antiviral drug. In order to be effective, the drug has to cross the cell membrane of infected cells, and so *in vitro* whole-cell assays are often used alongside enzyme studies to check cell absorption. EC_{50} values are a measure of antiviral activity and represent the concentration of compounds required to inhibit 50% of the cytopathic effect of the virus in isolated lymphocytes. Another complication is the requirement for anti-HIV drugs to have a good oral bioavailability (i.e to be orally active). This is a particular problem with the PIs. As we shall see, most PIs are designed from peptide lead compounds. Peptides are well known to have poor pharmacokinetic properties (i.e. poor absorption, metabolic susceptibility, rapid excretion, limited access to the central nervous system and high plasma protein binding). This is due mainly to high molecular weight, poor water solubility, and susceptible peptide linkages. In the following examples, we will find that potent PIs were discovered relatively quickly, but that these had a high peptide character. Subsequent work was then needed to reduce the peptide character of these compounds in order to achieve high antiviral activity, alongside acceptable levels of oral bioavailability and half-life.

Clinically useful PIs are generally less well absorbed from the gastro-intestinal tract than reverse transcriptase inhibitors, and are also susceptible to first pass metabolic reactions involving the cytochrome P450 isozyme (CYP3A4). This metabolism can result in drug-drug interactions with many of the other drugs given to AIDS patients to combat opportunistic diseases (e.g. rifabutin, ketoconazole, rifampin, and astemizole).

20.7.4.1 The HIV protease enzyme

The HIV protease enzyme (Fig. 20.16) is an example of an enzyme family called the **aspartyl proteases**—enzymes which catalyse the cleavage of peptide bonds and which contain an aspartic acid in the active site that is crucial to the catalytic mechanism. The enzyme is relatively small and can be obtained by synthesis. Alternatively, it can be cloned and expressed in fast-growing cells, then purified in large quantities. The enzyme is easily crystallized with or without an inhibitor bound to the active site, and this has meant that it has proved an ideal candidate for structure-based drug design. This involves the X-ray crystallographic study of enzyme–inhibitor complexes and the design of novel inhibitors based on those studies.

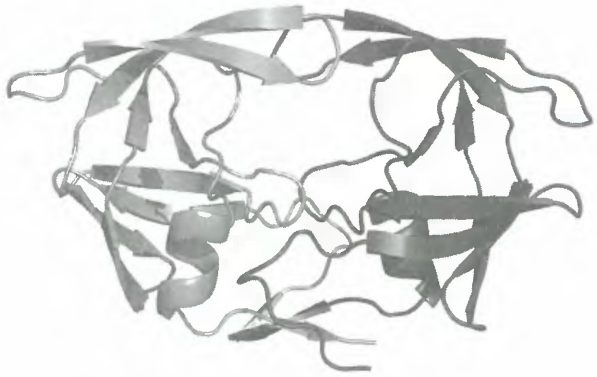


FIGURE 20.16 The HIV protease enzyme.

The HIV protease enzyme is a dimer made up of two identical protein units, each consisting of 99 amino acids. The active site is at the interface between the protein units and, like the overall dimer, it is symmetrical, with two-fold rotational (C2) symmetry. The amino acids Asp-25, Thr-26, and Gly-27 from each monomer are located on the floor of the active site, and each monomer provides a flap to act as the ceiling. The enzyme has a broad substrate specificity and can cleave a variety of peptide bonds in viral polypeptides, but crucially it can cleave bonds between a proline residue and an aromatic residue (phenylalanine or tyrosine) (Fig. 20.17). The cleavage of a peptide bond next to proline is unusual and does not occur with mammalian proteases such as renin, pepsin, or cathepsin D, and so the chances are good of achieving selectivity against HIV protease over mammalian proteases. Moreover, the symmetrical nature of the viral enzyme and its active site is not present in mammalian proteases, again suggesting the possibility of drug selectivity.

There are eight binding subsites in the enzyme, four on each protein unit, located on either side of the catalytic region (Fig. 20.17). These subsites accept the amino acid residues of the substrate and are numbered S1–S4 on one side and S1'–S4' on the other side. The relevant residues on the substrate are numbered P1–P4 and P1'–P4' (Fig. 20.18). The nitrogen and oxygen of each peptide bond in the substrate's peptide backbone is involved in hydrogen bonding interactions with the enzyme, as shown in Fig. 20.18. A water molecule is present in the active site which acts as a hydrogen bonding bridge to

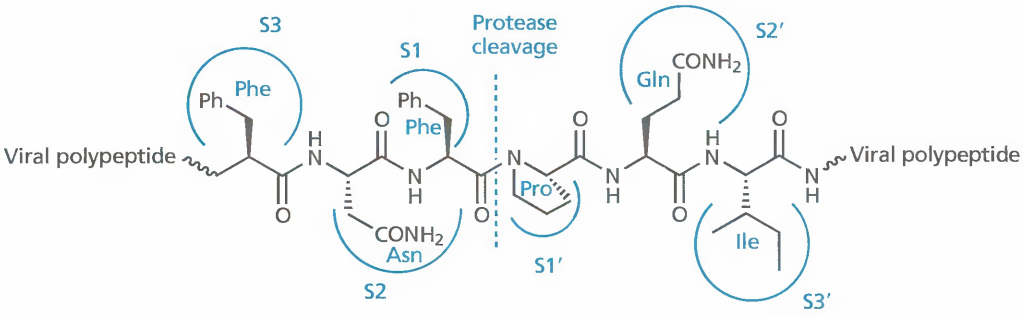


FIGURE 20.17 The aromatic-proline peptide bond that is cleaved by HIV protease (six of the eight binding subsites are shown).

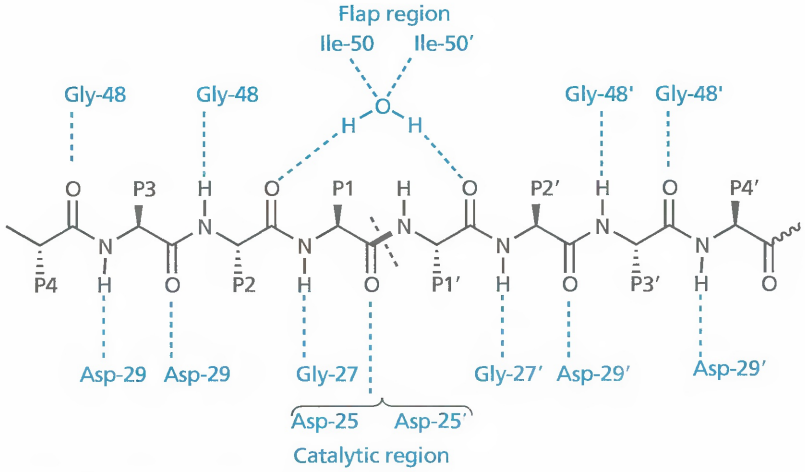


FIGURE 20.18 Interactions between the substrate's peptide backbone and the active site of HIV protease.

two isoleucine NH groups on the enzyme flaps. This hydrogen bonding network has the effect of closing the flaps over the active site once the substrate is bound.

There are two variants of HIV protease. The protease enzyme for HIV-2 shares 50% sequence identity with the protease enzyme for HIV-1. The greatest variation occurs outwith the active site, and so inhibitors are found to bind similarly to both enzymes.

The aspartic acids Asp-25 and Asp-25' are involved in the catalytic mechanism and are on the floor of the active site, each contributed from one of the protein subunits. The carboxylate residues of these aspartates and a bridging water molecule are involved in the mechanism by which the substrate's peptide bond is hydrolysed (Fig. 20.19).

20.7.4.2 Design of HIV protease inhibitors

A similar hydrolytic mechanism to that shown in Fig. 20.19 takes place for a mammalian aspartyl protease called **renin**. This enzyme was extensively studied before the discovery of HIV protease, and a variety of renin inhibitors were designed as antihypertensive agents (section 7.4). These agents act as **transition-state inhibitors** and many of the discoveries and strategies resulting from the development of renin inhibitors were adapted to the design of HIV PIs.

Transition-state inhibitors are designed to mimic the transition state of an enzyme catalysed reaction. The advantage of this approach is that the transition state is likely to be bound to the active site more strongly than either the substrate or product. Therefore, inhibitors resembling the transition state are also likely to be bound more strongly. In the case of the HIV protease catalysed reaction, the transition state resembles the tetrahedral intermediate shown in Fig. 20.19. Since such structures are inherently unstable, it is necessary to design an inhibitor which contains a **transition-state isostere**. Such an isostere would have a tetrahedral centre to mimic the tetrahedral centre of the transition state, yet be stable to hydrolysis. Fortunately, several such isosteres had already been developed in the design of renin inhibitors (Fig. 20.20). Thus, a large number of structures

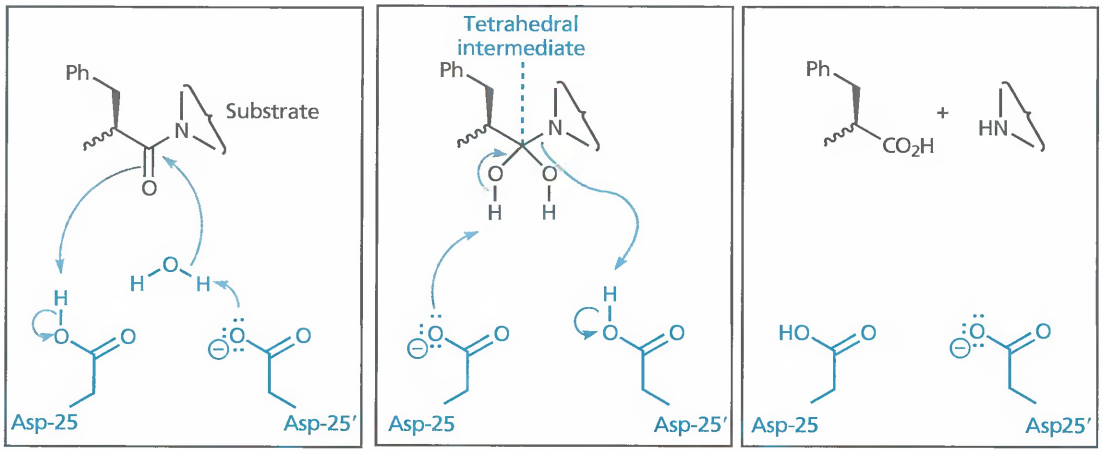


FIGURE 20.19 Mechanism of the reaction catalysed by HIV protease.

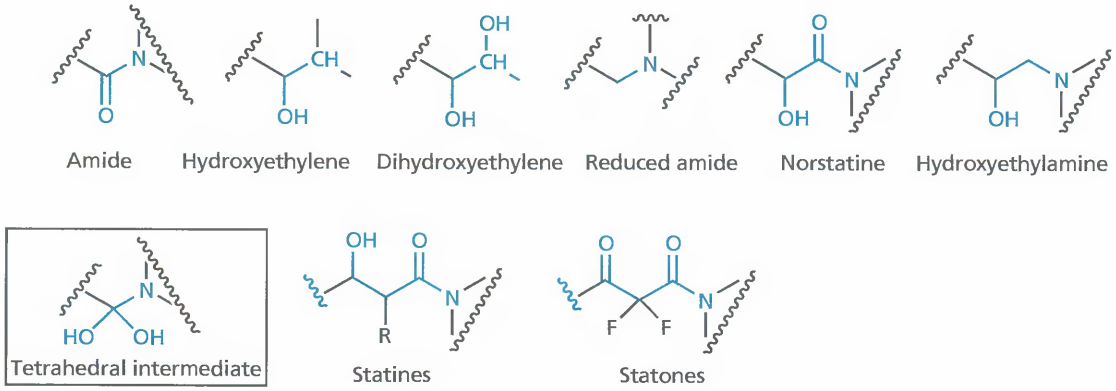


FIGURE 20.20 Transition-state isosteres.

were synthesized incorporating these isosteres, with the hydroxyethylamine isostere proving particularly effective. This isostere has a hydroxyl group which mimics one of the hydroxyl groups of the tetrahedral intermediate and binds to the aspartate residues in the active site. It is also found that the stereochemistry of this group is important to activity, with the *R*-configuration generally being preferred. This preference is determined by the nature of the P1' group that is present.

Having identified suitable transition-state isosteres, inhibitors were designed based on the enzyme's natural peptide substrates, since these contain amino acid residues which fit the eight subsites and allow a good binding interaction between the substrate and the enzyme. In theory, it might make sense to design inhibitors such that all eight subsites are filled to allow stronger interactions. However, this leads to structures with a high molecular weight and consequently poor oral bioavailability.

Therefore, most of the PIs were designed to have a core unit spanning the S1 to S1' subsites. Further substituents were then added at either end to fit into the S2/S3 and S2'/S3' subsites. Early inhibitors such as saquinavir (see below) have amino acid residues at P2 and P2'. Unfortunately, these compounds have a high molecular weight and a high peptide character leading to poor pharmacokinetic properties. More recent inhibitors contain a variety of novel P2 and P2' groups which were designed to reduce the molecular weight of the compound as well as its peptide character, in order to increase aqueous solubility and oral bioavailability. The S2 and S2' subsites of the protease enzyme appear to contain both polar (Asp-29, Asp-30) and hydrophobic (Val-32, Ile-50, Ile-84) amino acids, allowing the design of drugs that contain hydrophobic P2 groups capable of hydrogen bonding. It has also been possible to design a P1 group that can span both the S1 and S3 subsites, allowing the removal of the P3 moiety, thus lowering the molecular weight. The P2 group is usually attached to P1 by an acyl link, because the carbonyl oxygen concerned acts as an important hydrogen bond acceptor to the bridging water molecule described previously (Fig. 20.18).

We shall now look at how these strategies were used to design individual PIs.

20.7.4.3 Saquinavir

Saquinavir was developed by Roche, and as the first PI to reach the market it serves as the benchmark for all other PIs. The design of saquinavir started by considering a viral polypeptide substrate (pol, see section 20.7.1) and identifying a region of the polypeptide which contains a phenylalanine–proline peptide link. A pentapeptide sequence Leu-Asn-Phe-Pro-Ile was identified and served as the basis for inhibitor design. The peptide link normally hydrolysed in this sequence is between Phe and Pro and so this link was replaced by a hydroxyethylamine transition-state isostere to give a structure which successfully inhibited the enzyme (Fig. 20.21). The amino acid residues for Leu-Asn-Phe-Pro-Ile are retained in this structure and bind to the five subsites S3-S2'. Despite that, enzyme inhibition is relatively weak. The compound also has a high molecular weight and high peptide-like character, both of which are detrimental to oral bioavailability.

Consequently, the Roche team set out to identify a smaller inhibitor, starting from the simplest possible substrate for the enzyme—the dipeptide Phe-Pro (Fig. 20.22). The peptide link was replaced by the hydroxylamine transition-state isostere and the resulting *N*- and *C*-protected structure (I) was tested and found to have weak inhibitory activity. The inclusion of an asparagine group (structure II) to occupy the S2 subsite resulted in a 40-fold increase in activity, and a level of activity greater than the pentapeptide analogue (Fig. 20.21). This might seem an unexpected result as the latter occupies more binding subsites. However, it has been found that the crucial interaction of inhibitors is in the core region S2-S2'. If the addition of extra groups designed to bind to other subsites weakens the interaction to the core subsites, it can lead to an overall drop in activity. For example, addition of leucine to structure II resulted in a drop in activity, despite the fact that leucine can occupy the S3 subsite.

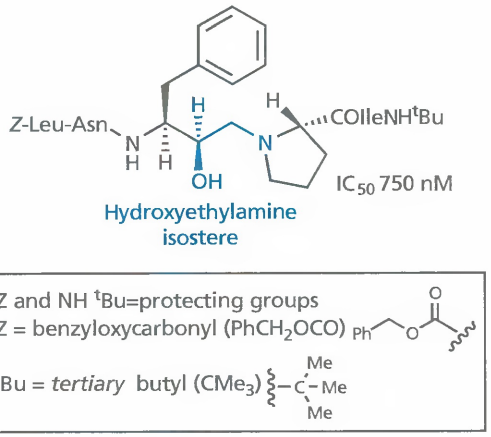


FIGURE 20.21 Pentapeptide analogue incorporating a hydroxyethylamine transition-state isostere.

Structure II was adopted as the new lead compound and the residues P1 and P2 were varied to find the optimum groups for the S1 and S2 subsites. As it turned out, the benzyl group and the asparagine side chain were already the optimum groups. An X-ray crystallographic study of the enzyme–inhibitor complex was carried out and revealed that the protecting group (Z) occupied the S3 subsite which proved to be a large hydrophobic pocket. Therefore, the protecting group was replaced with a larger quinoline ring system which could occupy the subsite more fully and this led to a sixfold increase in activity (structure III). Variations were also carried out on the carboxyl half of the molecule. Proline fits into the S1' pocket but it was found that it could be replaced by a bulkier decahydroisoquinoline ring system. The *t*-butyl ester protecting group was found to occupy the S2' subsite and could be replaced by a *t*-butylamide group which proved more stable in animal studies. The resulting structure was saquinavir having a further 60-fold increase in activity. The *R*-stereochemistry of the transition state hydroxyl group is essential. If the configuration is *S*, all activity is lost.

X-ray crystallography of the enzyme-saquinavir complex (Figs. 20.22 and 20.23) demonstrated the following:

- The substituents on the drug occupy the five subsites S3-S2'.
- The *t*-butylamine nitrogen is positioned in such a way that further *N*-substituents would be incapable of reaching the S3' subsite.
- There are hydrogen-bonding interactions between the hydroxyl group of the hydroxyethylamine moiety and the catalytic aspartates (Asp-25 and Asp-25').
- The carbonyl groups on either side of the transition-state isostere act as hydrogen bond acceptors to a bridging water molecule. The latter forms hydrogen bonds to the isoleucine groups in the enzyme's flap region in a similar manner to that shown in Fig. 20.18.

Saquinivir is still used clinically but suffers from poor oral bioavailability and susceptibility to drug resistance. Various efforts have been made to design simpler analogues of saquinavir which have lower molecular weight, less peptide character, and consequently better oral bioavailability.

20.7.4.4 Ritonavir and lopinavir

Ritonavir was developed by Abbott Pharmaceuticals to take advantage of the symmetrical properties of the protease enzyme and its active site. Since the active site has

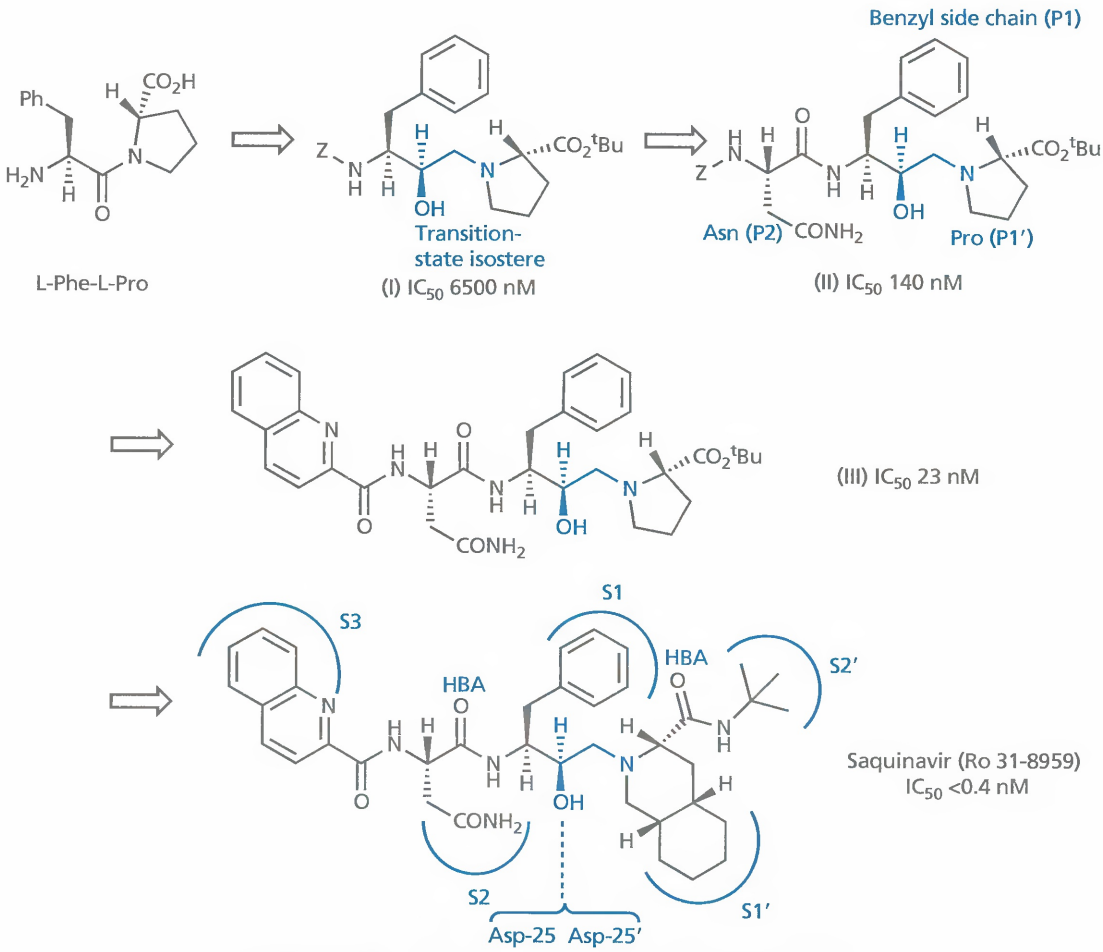


FIGURE 20.22 Development of saquinavir (Z=$PhCH_2OCO$).

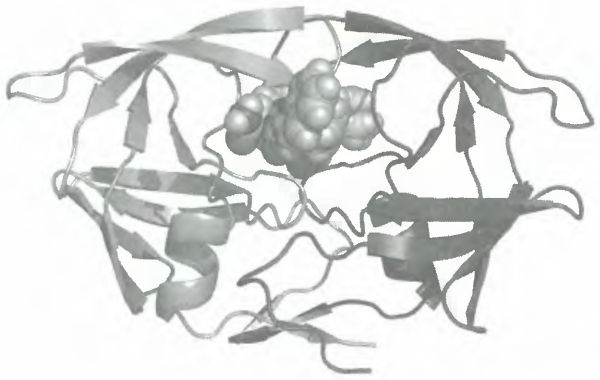


FIGURE 20.23 Saquinavir bound to the active site of HIV protease.

C2 symmetry, a substrate is capable of binding 'left to right' or 'right to left' as the binding subsites S1–S4 are identical to subsites S1'–S4'. This implies that it should be possible to design inhibitors having C2 symmetry. That should have several advantages. First, symmetrical inhibitors should show greater selectivity for the viral protease over mammalian aspartyl proteases, since the active sites of the latter are not symmetrical. Second, symmetrical molecules might be less recognizable to peptidases, resulting in improved oral bioavailability. Third, the development of saquinavir showed that a benzyl residue was the optimum binding group for the S1 subsite. Since the S1' subsite is identical to S1, a symmetrical inhibitor having benzyl groups fitting both S1 and S1' subsites should bind more strongly and have improved activity. This argument could also be extended for the binding groups fitting the S2/S2' subsites and so on.

Since there was no lead compound having C2 symmetry, one had to be designed which not only had the necessary C2 symmetry, but which also matched up with the C2 symmetry of the active site once it was bound. The first lead compound was designed by considering the tetrahedral reaction intermediate derived from the natural substrate. It was assumed that the axis of C2 symmetry for the active site passed through the reaction centre of this intermediate (Fig. 20.24). Since the benzyl group was known to be optimum for binding to the S1 subsite, the

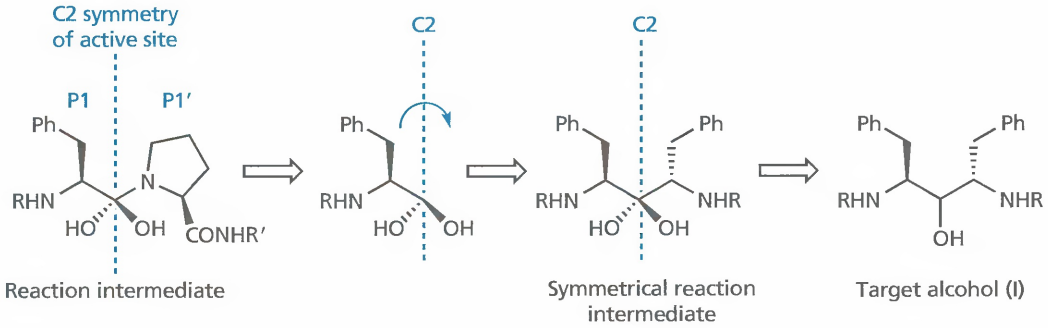


FIGURE 20.24 *De novo* design of a symmetrical lead compound acting as an inhibitor.

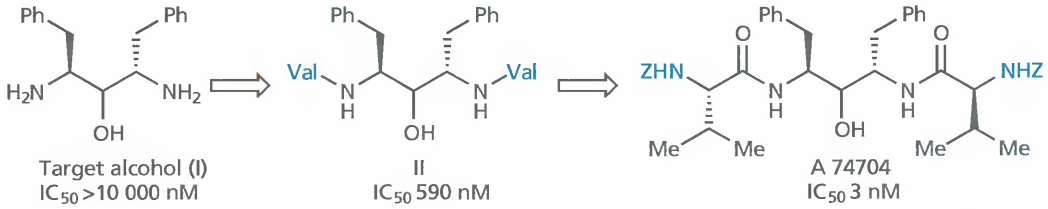


FIGURE 20.25 Development of A 74704 (Z=PhCH$_2$OCO).

left-hand portion of the molecule was retained and the right-hand portion was deleted. The left-hand moiety was then rotated such that two benzyl residues were present in the correct orientation for C2 symmetry. The resulting geminal diol is inherently unstable, so one of the alcohols was removed leading to the simplest target alcohol (I; R=H). In order to check whether this target molecule would match the C2 symmetry of the active site when bound, a molecular modelling experiment was carried out whereby the inhibitor was constructed in the active site. The results of this analysis were favourable and so the target alcohol was synthesized. Although it only had weak activity as an enzyme inhibitor, and was inactive against the virus *in vitro*, it still served as a lead compound designed by *de novo* techniques (section 17.15).

The next stage was to extend the molecule to take advantage of the S2 and S2' subsites. A variety of structures was synthesized and tested, revealing vastly improved activity when valine was added, and further improvement when the valines had *N*-protecting groups (A 74704; Fig. 20.25). A 74704 also showed *in vitro* activity against HIV itself, and was resistant to proteolytic degradation. The structure was co-crystallized with recombinant protease enzyme, and studied by X-ray crystallography to reveal a symmetrical pattern of hydrogen bonding between the inhibitor and the enzyme (Fig. 20.26). It was also found that a water molecule (Wat-301) still acted as a hydrogen bonding bridge between the carbonyl groups of P2 and P2', and the NH groups of Ile-50 and Ile-50' on the flaps of the enzyme. The C2 symmetry axes of the inhibitor and the active site passed within 0.2 Å of each other and deviated by an angle of only 6°, demonstrating that the design philosophy was valid.

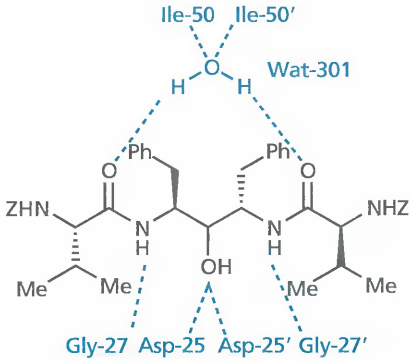


FIGURE 20.26 Binding interactions between the backbone of A 74704 and the active site of HIV protease (Z=PhCH$_2$OCO).

Further analysis of the crystal structure suggested that the NH groups on the inhibitor were binding to Gly-27 and Gly-27' but were too close to each other to allow optimum hydrogen bonding. To address this, it was decided to design symmetrical inhibitors where the relevant NH groups would be separated by an extra bond. In order to achieve this, the axis of C2 symmetry was placed through the centre of the susceptible bond. The same exercise described above was then carried out leading to the target diol shown in Fig. 20.27.

Diol structures analogous to the alcohols previously described were synthesized. Curiously, it was found that

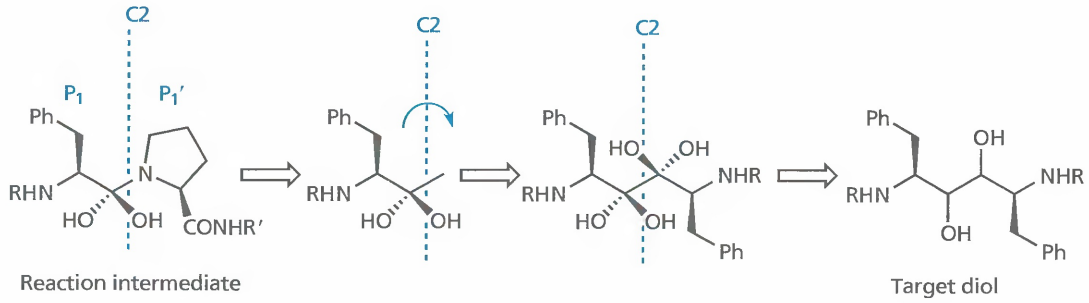


FIGURE 20.27 *De novo* design of a symmetrical diol inhibitor.

the absolute configuration of the diol centres had little effect on activity, and that the activity of the diols was generally better than the corresponding alcohols. For example, the diol equivalent of A 74704 (Fig. 20.28) had a 10-fold better level of activity. Unfortunately, this compound had poor water solubility. A crystal structure of the enzyme-inhibitor complex was studied, which revealed that the terminal portions of the molecule were exposed to solvation. This meant that more polar groups could be added at those positions without affecting binding. Consequently, the terminal phenyl groups were replaced by more polar pyridine rings. The urethane groups near the terminals were also replaced by urea groups, leading to A 77003, which had improved water solubility. Unfortunately, the oral bioavailability was still unsatisfactory and so the structure entered clinical trials as an intravenous antiviral agent rather than an oral one.

Modelling studies of how A 77003 might bind to the active site suggested two possible binding modes, one where each of the diol hydroxyl groups formed symmetrical hydrogen bonds to each of the aspartate residues, and one where only one of the hydroxyl groups hydrogen bonded to both aspartate groups. To investigate this further, X-ray crystallography was carried out on the enzyme-inhibitor complex, revealing that asymmetric binding was taking place, whereby the (R)-OH took part in hydrogen bonding with both aspartate residues, and the (S)-OH was only able to form a single hydrogen bonding interaction. This analysis also showed that the increased separation of the amide NHs failed to improve the geometry of the hydrogen bonding interactions with Gly-27 and Gly-27'. Thus, the improved activity of the diols over the alcohols was due to reasons other than those proposed. Results such as these are not totally unexpected when carrying out *de novo* design, since flexible molecules often bind differently from the manner predicted. The better activity for the diols may in fact be due to better binding of the P' groups to the S' subsites.

The fact that the (S)-hydroxyl group makes only one hydrogen bonding interaction suggested that it might be worth removing it, as the energy gained from only one hydrogen bonding interaction might be less than the energy required to desolvate the hydroxyl group before binding. This led to A 78791, which had improved activity and was shown by X-ray crystallography to bind in the same manner as A 77003.

A study was then carried out to investigate what effect variations of molecular size, aqueous solubility, and hydrogen bonding would have on the pharmacokinetics and activity of these agents. This led to A 80987, where the P2' valine was removed, and the urea groups near the ends were replaced by urethane groups. In general, it was found that the presence of *N*-methylureas was good for water solubility and bioavailability, whereas the presence of urethanes (or carbamates) was good for plasma half-life and overall potency. Thus, it was possible to fine-tune these properties by a suitable choice of group at either end of the molecule.

Despite being smaller, A 80987 retained activity and had improved oral bioavailability. However, it had a relative short plasma lifetime, was bound strongly to plasma proteins, and it was difficult to maintain therapeutically high levels. Metabolic studies then showed that A 80987 was *N*-oxidized at either or both pyridine rings, and that the resulting metabolites were excreted mainly in the bile. In an attempt to counter this, stereoelectronic design strategies were carried out. First, alkyl groups were placed on the pyridine ring at the vacant position *ortho* to the nitrogen. These were intended to act as a steric shield, but proved ineffective in preventing metabolism. It was then proposed that metabolism might be reduced if the pyridine rings were less electron rich, and so methoxy or amino substituents were added as electron-withdrawing groups. However, this too failed to prevent metabolism. Finally, the pyridine ring at P3 was replaced by a variety of heterocycles in an attempt to find a different ring system which would act as a bioisostere, but would be less susceptible to metabolism. The best results were obtained using the more electron-deficient 4-thiazolyl ring. Although water solubility decreased, it could be restored by reintroducing an *N*-methylurea group in

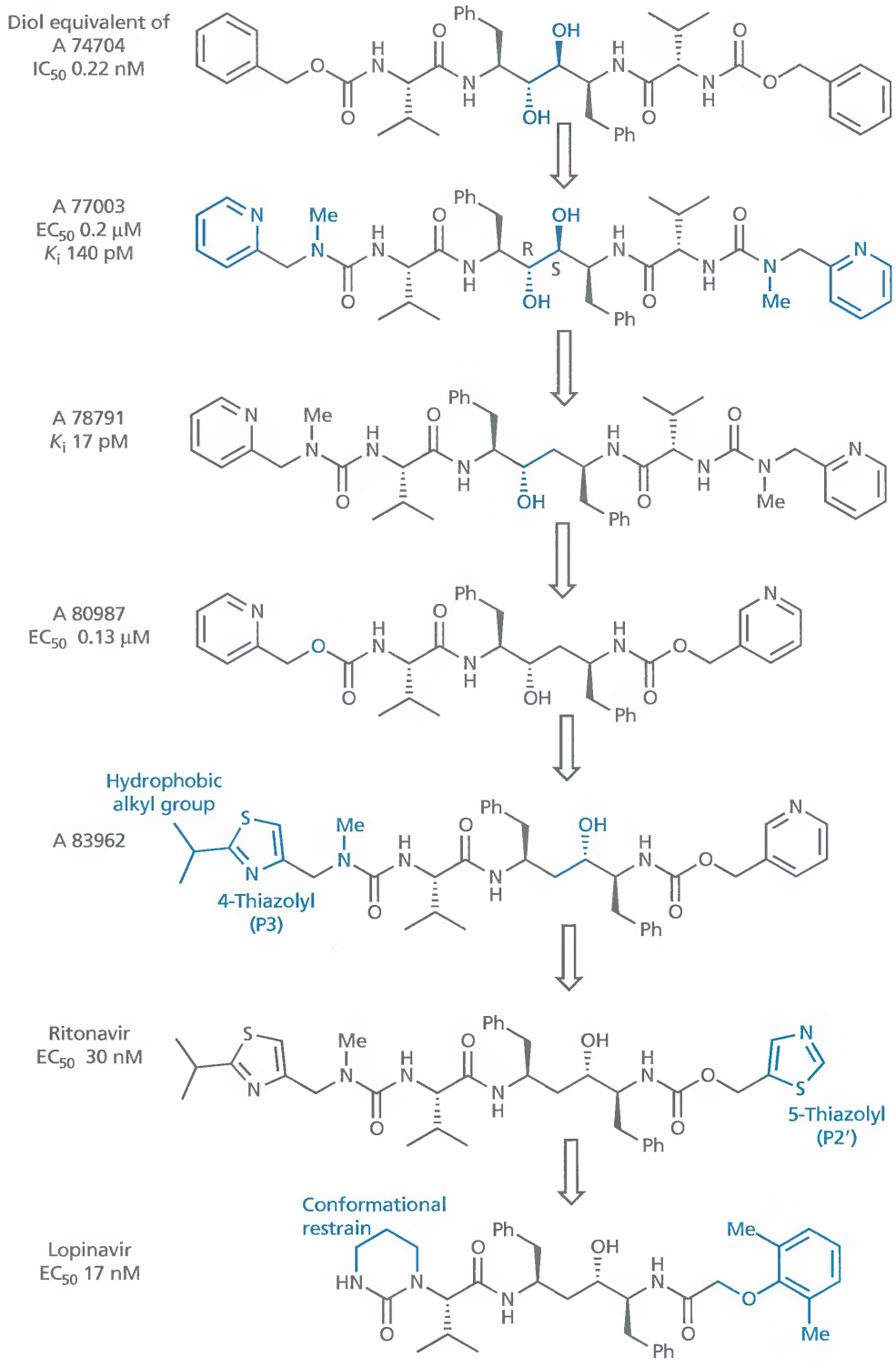


FIGURE 20.28 Development of ritonavir (ABT 538) and lopinavir (ABT 378).

place of one of the urethanes. Further improvements in activity were obtained by placing hydrophobic alkyl groups at the 2-position of the thiazole ring (P3), and by subsequently altering the position of the hydroxyl group in the transition-state isostere. This led to A 83962, which showed an 8-fold increase in potency over A 80987.

Attention now turned to the pyridine group at P2' which was replaced by a 5-thiazolyl group to give

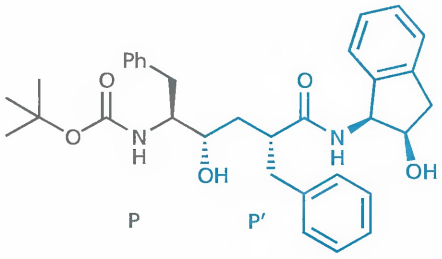
ritonavir, with good activity and oral bioavailability. The good activity indicated that a hydrogen bonding interaction was taking place between the thiazolyl N and Asp-30 (specifically the NH of the peptide backbone). This matched a similar hydrogen bonding interaction involving the pyridine N in A 80987. The improved bioavailability is due principally to better metabolic stability (20 times more stable than A 80987), and it was possible to get therapeutic plasma levels of the drug lasting 24 hours following oral administration.

Resistant strains of the virus have developed when ritonavir is used on its own. These arise from a mutation of valine at position 82 of the enzyme to either alanine, threonine or phenylalanine. X-ray crystallography shows that there is an important hydrophobic interaction between the isopropyl substituent on the P3 thiazolyl group of ritonavir and the isopropyl side chain of Val-82 which is lost as a result of this mutation. Further drug development led to lopinavir (Fig. 20.29) where the P3 thiazolyl group was removed and a cyclic urea group was incorporated to introduce conformational constraint. This allowed enhanced hydrogen bonding interactions with the S2 subsite, which balanced out the loss of binding due to the removal of the thiazolyl group. As this structure does not have any interactions with Val-82, it is active against the ritonavir resistant strain.

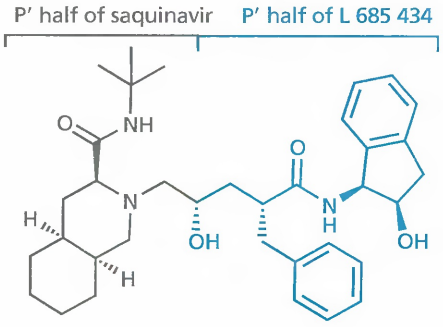
20.7.4.5 Indinavir

The design of indinavir included an interesting hybridization strategy (Fig. 20.29). Merck had designed a potent PI that included a hydroxyethylene transition-state isostere (L 685,434). Unfortunately, it suffered from poor bioavailability and liver toxicity. At this point, the Merck workers concluded that it might be possible to take advantage of the symmetrical nature of the active site. Since the S and S' subsites are equivalent, it should be possible to combine half of one PI with half of another to give a structurally distinct hybrid inhibitor. A modelling study was carried out to check the hypothesis and the Merck team decided to combine the P' half of L 685434 with the P' half of saquinavir. The P' moiety of saquinavir was chosen for its solubility enhancing potential, and the P' moiety of L 685434 is attractive for its lack of peptide character. The resulting hybrid structure (L 704,486) was less active as an inhibitor, but was still potent. Moreover, the presence of the decahydroisoquinoline ring system resulted in better water solubility and oral bioavailability (15%) as intended.

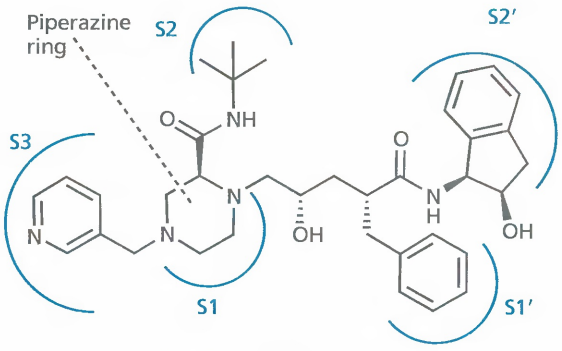
Further modifications were aimed at improving binding interactions, aqueous solubility and oral bioavailability. The decahydroisoquinoline ring was replaced by a piperazine



L 685 434 IC_{50} 0.3 nM



L 704 486; IC_{50} 7.6 nM



Indinavir IC_{50} 0.56 nM

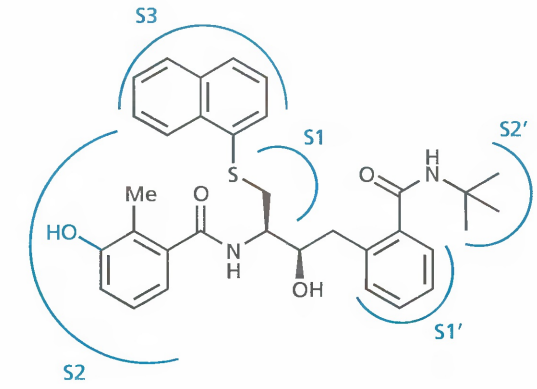
FIGURE 20.29 Development of indinavir.

ring, the additional nitrogen helping to improve aqueous solubility and oral bioavailability. A pyridine substituent was then added to access the S3 subsite and to improve binding. This resulted in indinavir, which reached the market in 1996.

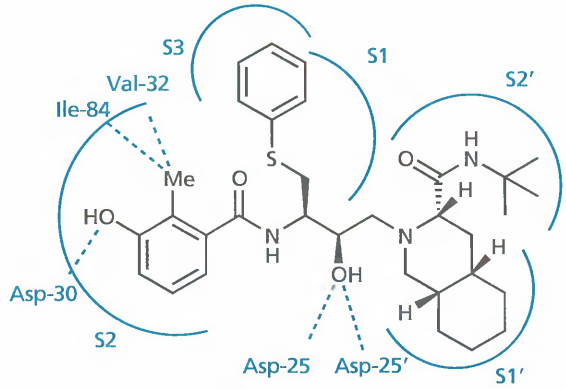
20.7.4.6 Nelfinavir

The development of nelfinavir was based on work carried out by the Lilly company, aimed at reducing the molecular weight and peptide character of PIs. Structure-based drug design had been used to develop AG1254 (Fig. 20.30), which contains an extended substituent at P1, capable of spanning and binding to both of the S1 and S3 subsites of the enzyme. This did away with the need for a separate P3 group and allowed the design of compounds with a lower molecular weight. They also designed a new P2 group to replace an asparagine residue which had been present in their lead compound. This group was designed to bind effectively to the S2 subsite and since it was different from any amino acid residue, the peptide character of the compound was reduced. Unfortunately, the antiviral activity of AG 1254 was not sufficiently high and the compound had poor aqueous solubility.

The company decided to switch direction and see what effect their newly designed substituents would have if they were incorporated into saquinavir, and this led ultimately to nelfinavir. A crystal structure of nelfinavir bound to the enzyme showed that the molecule is bound in an extended conformation where the binding interactions involving the molecular backbone are similar to saquinavir. A tightly bound water molecule serves as a hydrogen bonding bridge between the two amide carbonyls of the inhibitor and the flap region of the enzyme in a similar manner to other enzyme-inhibitor complexes. The crystal structure also showed that the *S*-phenyl group resides mainly in the S1 site and partially extends into the S3 site. The substituted benzamide group occupies the S2 pocket with the methyl substituent interacting with valine and isoleucine through van der Waals interactions, and the phenol interacting with Asp-30 through hydrogen bonding.



AG 1254
K_i 3 nm



Nelfinavir
K_i 2.0 nM EC_{50} 0.008–0.02 μM

FIGURE 20.30 AG 1254 and nelfinavir.

20.7.4.7 Palinavir

Palinavir (Fig. 20.31) is a highly potent and specific inhibitor of HIV-1 and HIV-2 proteases. The left-hand or P half of the molecule is similar to saquinavir and the molecule contains the same hydroxyethylamine transition-state mimic. The right-hand (P') side is different and was designed using the same kind of extension strategy used in nelfinavir. In this case, the P1' substituent was extended to occupy the S1' and S3' subsites. This was achieved by replacing the original proline group at P1' with 4-hydroxypipecolinic acid and adding a pyridine containing substituent to access the S3' subsite.

The crystal structure of the enzyme-inhibitor complex shows that the binding pockets S3–S3' are all occupied. Two carbonyl groups interact via the bridging water molecule to isoleucines in the enzyme flaps. The hydroxyl group interacts with both catalytic aspartate residues. Finally, the oxygen atoms and nitrogen atoms of all the amides are capable of hydrogen bonding to complementary groups in the active site. Work is currently in progress to simplify palinavir by introducing a single group that will span two binding subsites, thus allowing the removal of the P3 binding group.

20.7.4.8 Amprenavir and darunavir

Amprenavir (Fig. 20.32) was designed by Vertex Pharmaceuticals as a non-peptide-like PI using saquinavir as the lead compound. Saquinavir suffers from having a

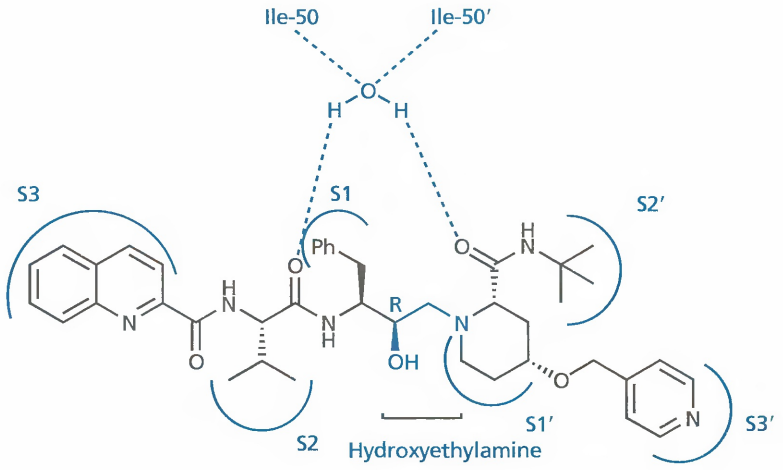


FIGURE 20.31 Palinavir and binding interactions.

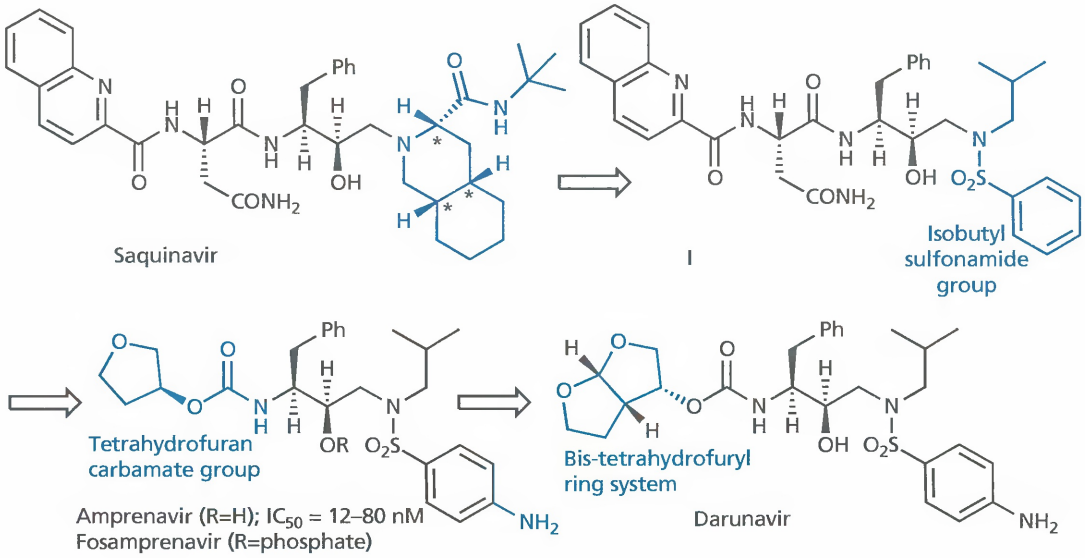


FIGURE 20.32 Development of amprenavir and darunavir.

high molecular weight and a high peptide character, both of which are detrimental to oral bioavailability. Therefore, it was decided to design a simpler analogue with a lower molecular weight and less peptide character, but retaining good activity. First, the decahydroisoquinoline group in saquinavir was replaced by an isobutyl sulfonamide group to give structure I. This had the advantage of reducing the number of asymmetric centres from six to three, allowing easier synthesis of analogues. Further simplification and reduction of peptide character was carried out by replacing the P2 and P3 groups with a tetrahydrofuran (THF) carbamate which had been previously found by Merck to be a good binding group for the S2 subsite. Finally an amino group was introduced on the phenylsulfonamide group to increase water solubility and to enhance oral absorption. Fosamprenavir is a phosphate prodrug for amprenavir.

Further work has shown that a fused bis-tetrahydrofuryl ring system is a better binding group for the hydrophobic S2 pocket than a single THF ring, because it fills the pocket more completely and forms hydrogen bonding interactions between the ring oxygens and the peptide backbone of the enzyme. Since these interactions are with the protein backbone rather than the amino acid residues, mutations are less likely to lead to drug resistance. Darunavir is a second-generation PI which contains this feature, but there are several other compounds currently being studied.

20.7.4.9 Atazanavir

Atazanavir (Fig. 20.33) was approved in June 2003 as the first once daily HIV-1 PI to be used as part of a combination therapy. It is similar to the early compounds leading towards ritonavir.

20.7.4.10 Tipranavir

Tipranavir (Fig. 20.34) is an example of a PI that was designed from a non-peptide lead compound. High throughput screening of 5000 structurally diverse compounds from a compound bank led to the discovery that the anticoagulant **warfarin** was a weak PI with antiviral activity. Various warfarin analogues were then tested leading to the discovery that **phenprocoumon** (Fig. 20.34) was a more potent competitive enzyme inhibitor with weak antiviral activity. Both these structures are used therapeutically for other purposes and have high oral bioavailability. Therefore they served as promising lead compounds for non-peptide like antiviral agents with good oral bioavailability.

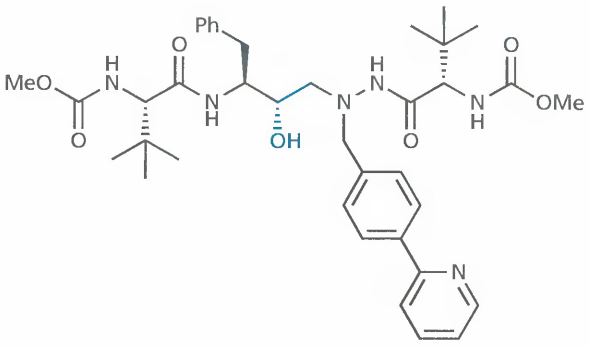


FIGURE 20.33 Atazanavir.

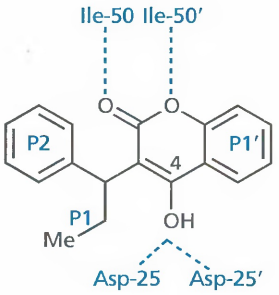
A crystal structure of the enzyme inhibitor complex was determined showing that the 4-OH group could form hydrogen bonds with the catalytic aspartate residues, while the two lactone oxygens could form hydrogen bonds directly to the isoleucine groups (Ile-50 and Ile-50' in the enzyme flaps. Unlike all the previous PIs, there was no bridging water involved in this interaction. Therefore, these compounds represented a new class of inhibitors with a novel pharmacophore of hydrogen bonding interactions. The crystal structure also showed that the ethyl and phenyl groups fitted the S1 and S2 subsites respectively, while the benzene ring of the coumarin ring system fitted the S1 subsite. Phenprocoumon was used as the lead compound for further development (see OUP website) and resulted in the discovery of tipranavir which is now used clinically.

20.7.4.11 Alternative methods of inhibition

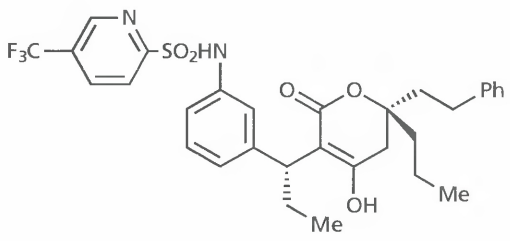
An alternative approach to inhibiting the protease enzyme would be to prevent its formation in the first place. Studies are in progress to design protein-protein binding inhibitors that will prevent the association of the two protein subunits that make it up (section 10.5).

20.7.5 Inhibitors of other targets

Antisense agents are being developed to block the production of the HIV protein Tat, which is needed for the transcription of other HIV genes. **Trecovirsen** (Fig. 20.35) is a phosphorothioate oligonucleotide containing 25 nucleotides and has been designed to hybridize with the mRNA derived from the HIV gene *gag*, to prevent its translation into HIV proteins. It was withdrawn from clinical trials due to toxicity but a similar oligonucleotide (**GEM92**) with increased stability is currently in clinical trials.



Phenprocoumon
K_i 1 μM; ED_{50} 100–300 μM



Tipranavir (PNU-140690)
K_i 8pM; IC_{50} 30 nM

FIGURE 20.34 Phenprocoumon and tipranavir.

BOX 20.4 Clinical aspects of protease inhibitors

Protease inhibitors (PIs) are an important component of the drug cocktail used to treat HIV. Care has to be taken when administering the agents to haemophiliacs and diabetics since the agents can increase the risk of bleeding, and lower blood sugar levels.

Saquinivir was the first PI to reach the market in 1995. It shows a 100-fold selectivity for both HIV-1 and HIV-2 proteases over human proteases. Approximately 45% of patients develop clinical resistance to the drug over a 1-year period, but resistance can be delayed if it is given in combination with reverse transcriptase inhibitors. The oral bioavailability of saquinavir is only 4% in animal studies, although this is improved if the drug is taken with meals. The compound is also highly bound to plasma proteins (98%). As a result, the drug has to be taken in high doses to maintain therapeutically high plasma levels. A curious problem related to saquinavir is that its plasma levels can be reduced if the patient takes garlic.

Ritonavir reached the market in 1996. It is active against both HIV-1 and HIV-2 proteases and shows selectivity for HIV proteases over mammalian proteases. Despite the fact that ritonavir is highly plasma bound (99%), has a high molecular weight, and has a peptide-like nature, it has better bioavailability than many other PIs. This is due to greater stability to drug metabolism, and it is possible to get therapeutic plasma levels of the drug which last 24 hours following oral administration. The metabolic stability of the agent is a result of the drug's ability to act as a potent inhibitor of the cytochrome P450 enzyme CYP3A4 which means that it shuts down its own metabolism. Care has to be taken when drugs affected by CYP3A4 are taken alongside ritonavir, and doses of the latter should be adjusted accordingly. On the other hand, ritonavir's ability to inhibit CYP3A4 is useful when it is used alongside other PIs that are normally metabolized by this enzyme (e.g. saquinavir, indinavir, nelfinavir, and amprenavir). Since ritonavir inhibits CYP3A4, the lifetime and plasma levels of other PIs can be increased. For this reason, it is often administered in small doses alongside other PIs. If it is intended to be used as an anti-HIV drug in its own right, it is administered with nucleoside reverse transcriptase inhibitors.

Lopinavir is active against ritonavir-resistant strains of HIV, and is administered with ritonavir as a single capsule combination called Kaletra. Each capsule contains 133 mg of lopinavir and 33 mg of ritonavir with the latter serving as a cytochrome P450 inhibitor to increase the level of lopinavir present in the blood supply.

Indinavir has better oral bioavailability than saquinavir and is less highly bound to plasma proteins (60%). It is usually administered alongside nucleoside reverse transcriptase inhibitors such as didanosine.

Nelfinavir was marketed in 1997 and is used as part of a four-drug combination therapy. Like indinavir and ritonavir, nelfinavir is more potent than saquinavir because of its better pharmacokinetic profile. Compared to saquinavir, it has a lower molecular weight and log*P*, and an enhanced aqueous solubility, resulting in enhanced oral bioavailability. It can inhibit the metabolic enzyme CYP3A4 and thus affects the plasma levels of other drugs metabolized by this enzyme. It is 98% bound to plasma proteins.

Amprenavir was licensed to GlaxoWellcome and was approved in 1999. It is reasonably specific for the viral protease relative to mammalian proteases, and is about 90% protein bound. It has good oral bioavailability (40–70% in animal studies). **Fosamprenavir** is a phosphate prodrug for amprenavir, and was approved by the US FDA and EMRA in 2003 and 2004 respectively. The prodrug acts as a slow-release version of amprenavir, reducing the number of pills required. It is usually administered with ritonavir. **Darunavir** is a second-generation PI developed by Tibotec, and was approved by the US FDA in 2006 as the first treatment of drug-resistant HIV. It is usually administered with ritonavir.

Atazanavir was approved in June 2003 as the first once-daily HIV-1 PI to be used as part of a combination therapy. It is usually administered with ritonavir.

Tipranavir is used to treat HIV infections that are resistant to other PIs. However, there have been cases of life-threatening liver toxicity.

d(*P*-thio)(T-C-T-T-C-C-T-C-T-C-T-C-T-A-C-C-C-A-C-G-C-T-C-T-C)

FIGURE 20.35 Trecovirsen.

Other agents under study for the treatment of HIV include integrase inhibitors, and cell entry inhibitors. Blocking entry of a virus into a host cell is particularly desirable, as it is so early in the life cycle. **Enfuvirtide** was approved in March 2003 as the first member of a new class of fusion inhibitors. It is a polypeptide consisting of 36 amino acids which matches the C-terminal end of the viral protein gp41. It works by forming an α-helix and binding to a group of three similar α-helices belonging to the gp41 protein. This association prevents the process by which the virus enters the host cell. In order to bring about fusion, the gp41 protein anchors the virus to the cell membrane

of the host cell. It then undergoes a conformational change where it builds a grouping of six helices using the three already present as the focus for that grouping (Fig. 20.36). This pulls the membranes of the virion and the host cell together so that they can fuse. By binding to the group of three helices, enfuvirtide blocks formation of the required hexamer and prevents fusion.

The manufacture of enfuvirtide involves 106 steps, which makes it expensive and may limit its use. A smaller compound (**BMS 378806**) is being investigated which binds to gp120 and prevents the initial binding of the virus to CD4 on the cell surface.

***N*-Butyldeoxynojirimycin** (Fig. 20.37) is a carbohydrate that inhibits **glycosidases**—enzymes that catalyse the trimming of carbohydrate moieties which are linked to viral proteins. If this process is inhibited, too many carbohydrate groups end up attached to a protein, resulting in the protein adopting a different conformation. It is thought that the gp120 protein is affected in this way and cannot be peeled away as described in section 20.7.1 to reveal the gp41 protein.

Bicyclams such as **JM 3100** (Fig. 20.37) block the CCR5 chemokine receptor and are under investigation as drugs which will prevent membrane fusion and cell entry.

Maraviroc (Fig. 20.38) was approved as a CCR5 antagonist in 2007, and is the first anti-HIV agent to act on a molecular target on the host cell rather than the virus. It was developed from a compound which also had

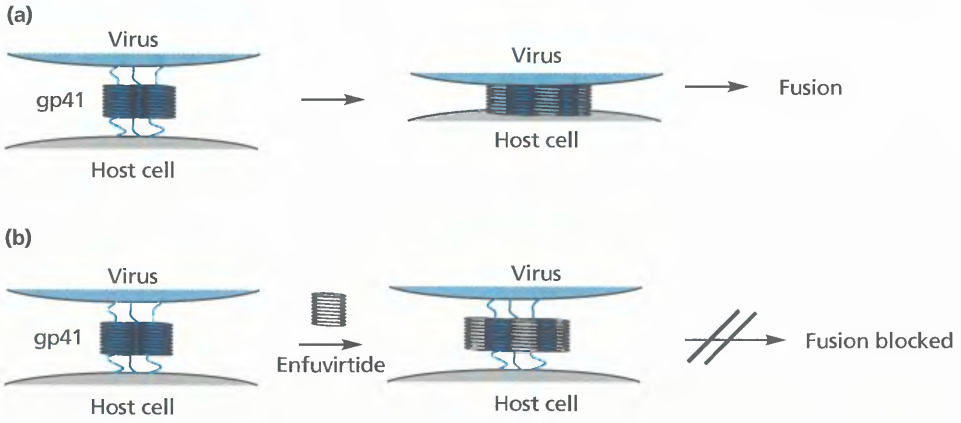
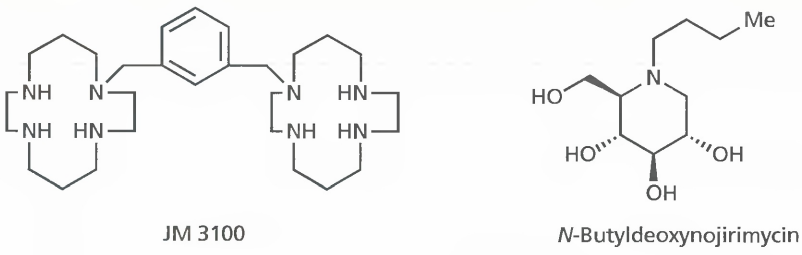
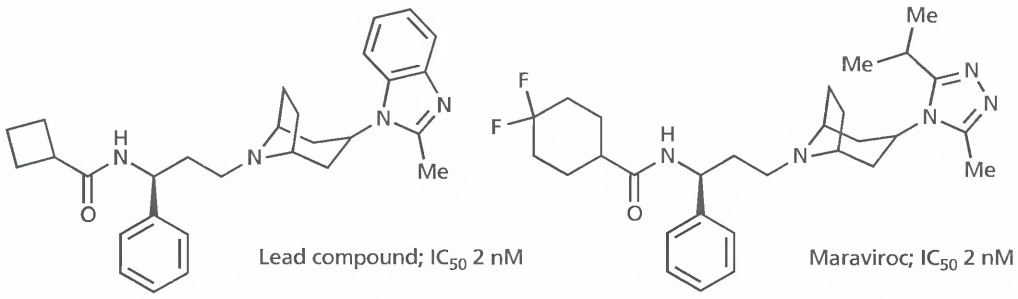


FIGURE 20.36 **(a)** Normal mechanism of fusion. **(b)** Enfuvirtide acting as a fusion inhibitor.



JM 3100 *N*-Butyldeoxynojirimycin

FIGURE 20.37 Agents that inhibit cell entry.



Lead compound; IC_{50} 2 nM Maraviroc; IC_{50} 2 nM

FIGURE 20.38 Comparison of meraviroc and the lead compound from which it was developed.

potent activity, but which blocked HERG ion channels (Box 12.3). Agents which block these channels often have toxic cardiac side effects, and so a large number of analogues were synthesized to find a potent compound which did not block the HERG ion channels. Maraviroc was the result. It is an example of an agent that works by blocking protein-protein interactions between a viral protein and a host cell protein (section 10.5).

The first integrase inhibitor to reach the market in 2007 was **raltegravir** (Fig. 20.39). The keto-enol system is important for activity since it acts as a chelating group for two magnesium ion cofactors in the enzyme's active site.

KEY POINTS

- HIV is a retrovirus containing RNA as its genetic material, and is responsible for AIDS.
- The two main viral targets for anti-HIV drugs are the enzymes reverse transcriptase and protease. Combination therapy is the favoured treatment, but there is a need to develop drugs that are effective against a third target.
- The potency and safety demands for anti-HIV drugs are high, as they are likely to be used for the lifetime of the patient.
- Reverse transcriptase is a DNA polymerase which catalyses the conversion of single-stranded RNA to double-stranded DNA. No such biochemical process occurs in normal cells.
- Nucleoside reverse transcriptase inhibitors are prodrugs that are converted by cellular enzymes to active triphosphates, which act as enzyme inhibitors and chain terminators.
- Non-nucleoside reverse transcriptase inhibitors act as enzyme inhibitors by binding to an allosteric binding site.
- The protease enzyme is a symmetrical dimeric structure consisting of two identical protein subunits. An aspartic acid residue from each subunit is involved in the catalytic mechanism.
- The protease enzyme is distinct from mammalian proteases in being symmetrical and being able to catalyse the cleavage of peptide bonds between proline and aromatic amino acids.
- Protease inhibitors are designed to act as transition-state inhibitors. They contain a transition-state isostere that is tetrahedral but stable to hydrolysis. Suitable substituents are added to fill various binding pockets usually occupied by the amino acid residues of polypeptide substrates.
- To obtain an orally active PI, it is important to maximize the binding interactions with the enzyme, while minimizing the molecular weight and peptide character of the molecule.
- Cell fusion inhibitors have been developed, one of which has reached the market.
- The first integrase inhibitor has been approved for the market.

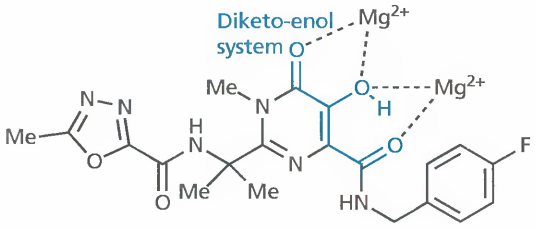


FIGURE 20.39 The integrase inhibitor raltegravir acting as a metal ion chelator.

20.8 Antiviral drugs acting against RNA viruses: flu virus

20.8.1 Structure and life cycle of the influenza virus

Influenza (or flu) is an airborne, respiratory disease caused by an RNA virus which infects the epithelial cells of the upper respiratory tract. It is a major cause of mortality, especially among the elderly, or among patients with weak immune systems. The most serious pandemic occurred in 1918 with the death of at least 20 million people worldwide caused by the Spanish flu virus. Epidemics then occurred in 1957 (Asian flu), 1968 (Hong Kong flu), and 1977 (Russian flu). Despite the names given to these flus, it is likely that they all derived from China where families are in close proximity to poultry and pigs, increasing the chances of viral infections crossing from one species to another.[1] In 1997, there was an outbreak of flu in Hong Kong which killed 6 out of 18 people. This was contained by slaughtering infected chickens, duck and geese which had been the source of the problem. If action had not been swift, it is possible that this flu variant could have become a pandemic and wiped out 30% of the world's population. This emphasizes the need for effective antiviral therapies to combat flu.

The nucleocapsid of the flu virus contains (−) single-stranded RNA and a viral enzyme called **RNA polymerase** (Fig. 20.1). Surrounding the nucleocapsid, there is a membranous envelope derived from host cells which contains two viral glycoproteins called **neuraminidase** (NA) and **haemagglutinin** (HA) (which acquired its name because it can bind virions to red blood cells and

[1] On the other hand, there has been a recent theory that the 1918 pandemic originated in army transit camps in France. The living conditions in these camps were similar to communities in China in the sense that large numbers of soldiers were camping in close proximity to pigs and poultry used as food stocks. The return of the forces to all parts of the globe following the First World War could explain the rapid spread of the virus.

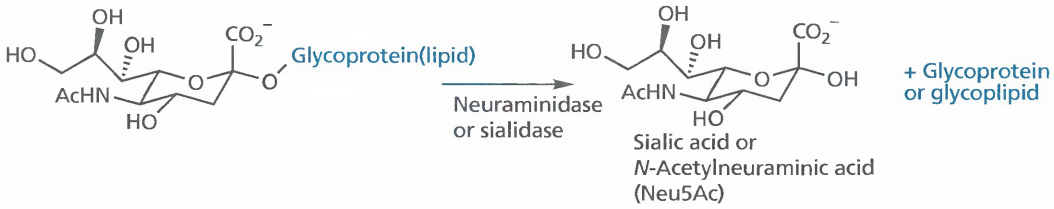


FIGURE 20.40 Action of neuraminidase (sialidase).

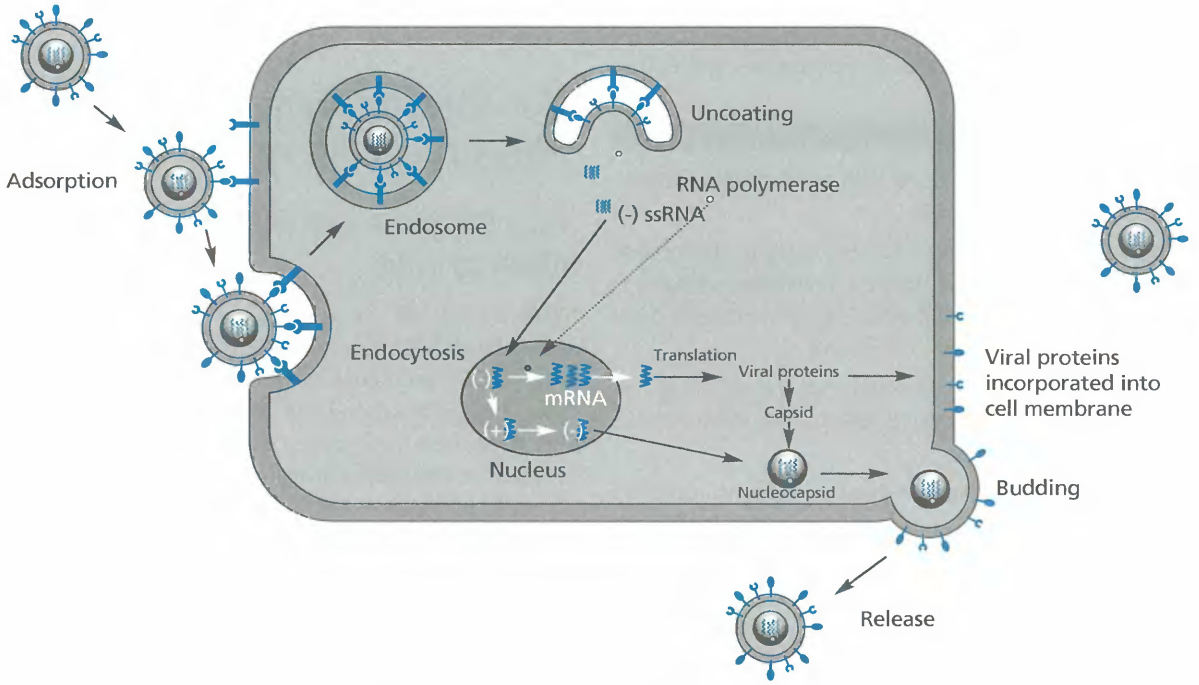


FIGURE 20.41 Life cycle of the influenza virus.

cause haemagglutination). These glycoproteins are spike-like objects which project about 10 nm from the surface and are crucial to the infectious process.

In order to reach the epithelial host cells of the upper respiratory tract, the virus has to negotiate a layer of protective mucus, and it is thought that the viral protein NA is instrumental in achieving this. The mucosal secretions are rich in glycoproteins and glycolipids which bear a terminal sugar substituent called **sialic acid** (also called *N*-acetylneuraminic acid). Neuraminidase (also called sialidase) is an enzyme which is capable of cleaving the sialic acid sugar moiety from these glycoproteins and glycolipids (Fig. 20.40), thus degrading the mucus layer and allowing the virus to reach the surface of epithelial cells.

Once the virus reaches the epithelial cell, adsorption takes place whereby the virus binds to cellular glycoconjugates which are present in the host cell membrane, and which have a terminal sialic acid moiety. The viral protein HA is crucial to this process. Like NA, it recognizes sialic acid but instead of catalysing the cleavage of the sialic acid from the glycoconjugate, HA binds to it (Fig. 20.41). Once the virion has been adsorbed, the cell membrane bulges inwards taking the virion with it to form a vesicle called an endosome—a process called **receptor mediated endocytosis**. The pH in the endosome then decreases, causing HA in the virus envelope to undergo a dramatic conformational change whereby the hydrophobic ends of the protein spring outward and extend towards the endosomal membrane. After contact, fusion occurs and the RNA nucleocapsid is released into the cytoplasm of the host cell. Disintegration of the nucleocapsid releases viral RNA and viral RNA polymerase which both enter the cell nucleus.

Viral RNA polymerase now catalyses the copying of (−) viral RNA to produce (+) viral RNA, which departs the nucleus and acts as the mRNA required for the translation of viral proteins. Copies of (−) viral RNA are also produced in the nucleus and exported out of the nucleus.

Capsid proteins spontaneously self-assemble in the cytoplasm with incorporation of (−) RNA and newly produced RNA polymerase to form new nucleocapsids. Meanwhile, the freshly synthesized viral proteins HA and NA are incorporated into the membrane of the host cell. Newly formed nucleocapsids then move to the cell membrane and attach to the inner surface. HA and NA move through the cell membrane to these areas and at the same time, host cell proteins are excluded. Budding then takes place and a new virion is released. NA aids this release by hydrolysing any interactions that take place between HA on the virus and sialic acid conjugates on the host membrane.

There is an important balance between the rate of desialylation by NA (to aid the virion's departure from the host cell) and the rate of attachment by HA to sialylated glycoconjugates (to allow access to the cell). If NA was too active, it would hinder infection of the cell by destroying the receptors recognized by HA. On the other hand, if the enzyme activity of NA was too weak, the newly formed virions would remain adsorbed to the host cell after budding, preventing them from infecting other cells. It is noticeable that the amino acids present in the active site of NA are highly conserved, unlike amino acids elsewhere in the protein. This demonstrates the importance of the enzyme's activity level.

Since HA and NA are on the outer surface of the virion, they can act as antigens (i.e. molecules which can potentially be recognized by antibodies and the body's defence systems). In theory, it should be possible to prepare vaccines which will allow the body to gain immunity from the flu virus. Such vaccinations are available, but they are not totally protective and they lose what protective effect they have with time. This is because the flu virus is adept at varying the amino acids present in HA and NA, thus making these antigens unrecognizable to the antibodies which originally recognized them—a process called **antigenic variation**. The reason it takes place can be traced back to the RNA polymerase enzyme which is a relatively error-prone enzyme and means that the viral RNA which codes for HA and NA is not consistent. Variations in the code lead to changes in the amino acids present in NA and HA which results in different types of flu virus based on the antigenic properties of their NA and HA. For example, there are nine antigenic variants of NA.

There are three groups of flu virus, classified as A, B and C. Antigenic variation does not appear to take place with influenza C, and occurs slowly with influenza B. With influenza A, however, variation occurs almost yearly. If the variation is small, it is called **antigenic drift**. If it is large, it is called **antigenic shift** and it is this that can lead to the more serious epidemics and pandemics. There are two influenza A virus subtypes which are epidemic in humans—those with H1N1 and H3N2 antigens (where H and N stand for HA and NA respectively). A major aim in designing effective antiviral drugs is to find a drug which will be effective against the influenza A virus, and remain effective despite antigenic variations. In general, vaccination is the preferred method of preventing flu, but antiviral drugs also have their place, for both the prevention and treatment of flu when vaccination proves unsuccessful.

20.8.2 Ion channel disrupters: adamantanes

The adamantanes (Fig. 20.42) were discovered by random screening and are the earliest antiviral drugs used clinically against flu, decreasing the incidence of the disease by 50–70%. **Amantadine** and **rimantadine** (Fig. 20.42) are related adamantanes with similar mechanisms of action and can inhibit viral infection in two ways. At low concentration (<1 μg/ml), they inhibit the replication of influenza A viruses by blocking a viral ion channel protein called matrix (M2) protein. At higher concentration (>50 μg/ml)), the basic nature of the compounds becomes important and they buffer the pH of endosomes and prevent the acidic environment needed for HA to fuse the viral membrane with that of the endosome. These mechanisms inhibit penetration and uncoating of the virus.

Unfortunately, the virus can mutate in the presence of amantadine to form resistant variants. Amantadine binds to a specific region of the M2 ion channel, and resistant variants have mutations which alter the width of the channel. Research carried out to find analogues which might still bind to these mutants proved unsuccessful. Work has also been carried out in an attempt to find an analogue which might affect the ion channel and pH levels at comparable concentrations. This has focused on secondary and tertiary amines with increased basicity, as well as alteration of the structure to reduce activity for the ion channel. The rationale is that resistant flu variants are less likely to be produced if the drug acts on two different targets at the same time. Rimantadine was approved in 1993 as a less toxic alternative to amantadine for the treatment of influenza A. Unfortunately, neither agent is effective against influenza B since this virus does not contain the matrix (M2) protein. Side effects are also a problem, possibly due to effects on host cell ion channels.

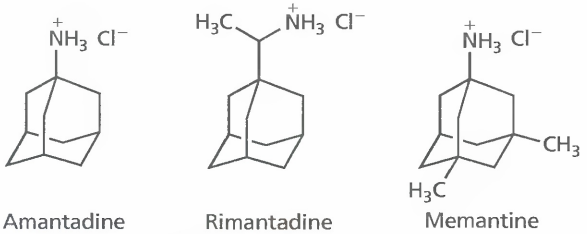


FIGURE 20.42 The adamantanes.

20.8.3 Neuraminidase inhibitors

20.8.3.1 Structure and mechanism of neuraminidase

Since NA plays two crucial roles in the infectious process (section 20.8.1), it is a promising target for potential antiviral agents. Indeed, a screening program for NA inhibitors was carried out as early as 1966 although without success. Following on from this, researchers set out to design a mechanism-based transition-state inhibitor. This work progressed slowly until the enzyme was isolated and its crystal structure studied by X-ray crystallography and molecular modelling.

Neuraminidase is a mushroom shaped tetrameric glycoprotein anchored to the viral membrane by a single hydrophobic sequence of some 29 amino acids. As a result, the enzyme can be split enzymatically from the surface and studied without loss of antigenic or enzymic activity. X-ray crystallographic studies have shown that the active site is a deep pocket located centrally on each protein subunit. There are two main types of the enzyme (corresponding to the influenza viruses A and B) and various subtypes. Due to the ease with which mutations occur, there is a wide diversity of amino acids making up the various types and subtypes of the enzyme. However, the 18 amino acids making up the active site itself are constant. As mentioned previously, the absolute activity of the enzyme is crucial to the infectious process, and any variation that affects the active site is likely to affect the activity of the enzyme. This in turn will adversely affect the infectious process. Since the active site remains constant, any inhibitor designed to fit it has a good chance of inhibiting all strains of the flu virus. Moreover, it has been observed that the active site is quite different in structure from the active sites of comparable bacterial or mammalian enzymes, so there is a strong possibility that inhibitors can be designed that are selective antiviral drugs.

The enzyme has been crystallized with sialic acid (the product of the enzyme catalysed reaction) bound to the active site, and the structure determined by X-ray crystallography. A molecular model of the complex was created which resembled the observed crystal structure as closely as possible. From this it was calculated that sialic acid was bound to the active site through a network of hydrogen bonds and ionic interactions as shown in Fig. 20.43.

The most important interactions involve the carboxylate ion of sialic acid, which is involved in ionic interactions and hydrogen bonds with three arginine residues, particularly with Arg-371. In order to achieve these interactions, the sialic acid has to be distorted from its most stable chair conformation (where the carboxylate ion is in the axial position) to a pseudo-boat conformation where the carboxylate ion is equatorial.

There are three other important binding regions or pockets within the active site. The glycerol side chain of sialic acid fills one of these pockets, interacting with glutamate residues and a water molecule by hydrogen bonding. The hydroxyl group at C-4 of sialic acid is situated in another binding pocket, interacting with a glutamate residue. Finally, the acetamido substituent of sialic acid fits into a hydrophobic pocket which is important for molecular recognition. This pocket includes the hydrophobic residues Trp-178 and Ile-222 which lie close to the methyl carbon (C-11) of sialic acid, as well as the hydrocarbon backbone of the glycerol side chain.

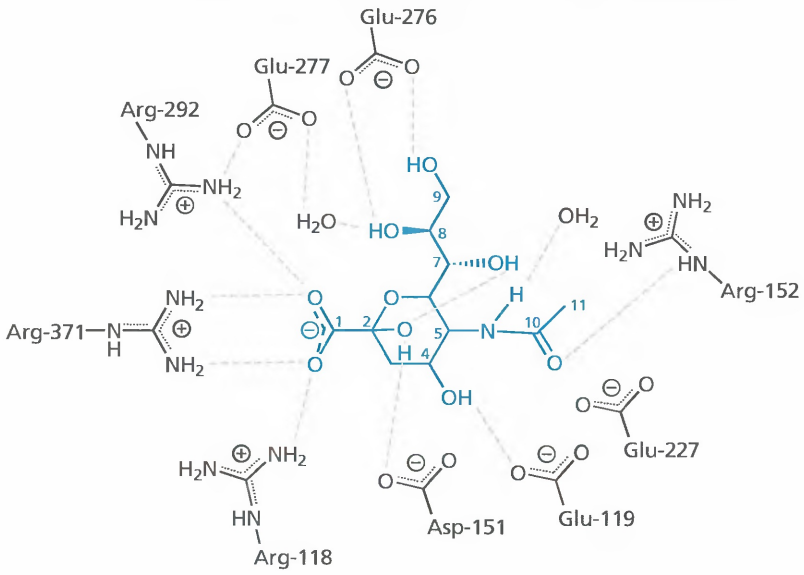


FIGURE 20.43 Hydrogen bonding interactions between sialic acid and the active site of neuraminidase.

It was further established that the distorted pyranose ring binds to the floor of the active site cavity through its hydrophobic face. The glycosidic OH at C-2 is also shifted from its normal equatorial position to an axial position where it points out of the active site and can form a hydrogen bond to Asp-151 as well as an intramolecular hydrogen bond to the hydroxyl group at C-7.

Based on these results, a mechanism of hydrolysis was proposed which consists of four major steps (Fig. 20.44). The first step involves the binding of the substrate (sialoside)

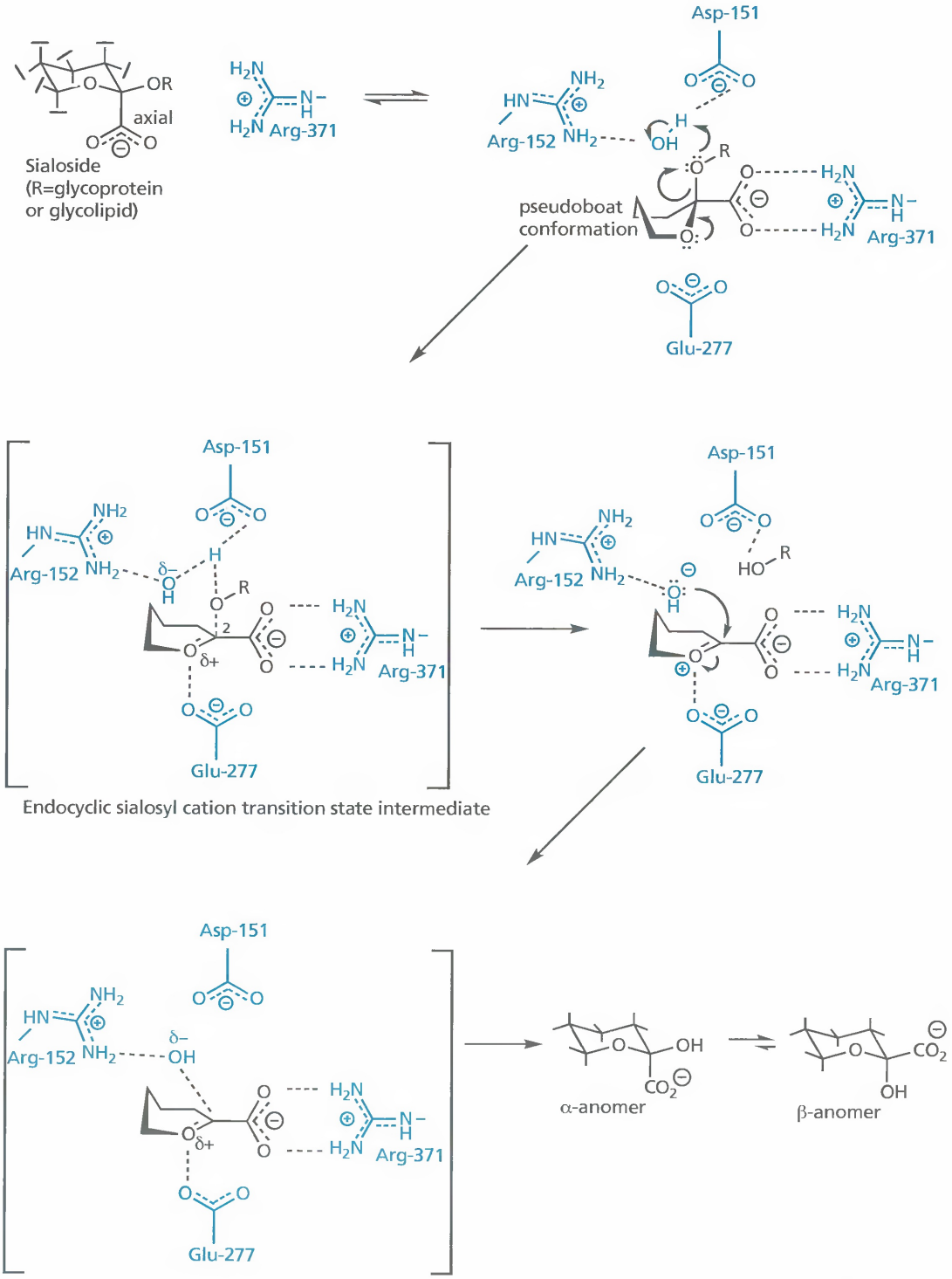


FIGURE 20.44 Proposed mechanism for the enzyme-catalysed hydrolysis of sialic acid.

as described above. The second step involves proton donation from an activated water facilitated by the negatively charged Asp-151, and formation of an endocyclic sialosyl cation transition-state intermediate. Glu-277 is proposed to stabilize the developing positive charge on the glycosidic oxygen as the mechanism proceeds.

The final two steps of the mechanism are formation and release of sialic acid. Support for the proposed mechanism comes from kinetic isotope studies which indicate that it is an S_N1 nucleophilic substitution. NMR studies have also been carried out which indicate that sialic acid is released as the α-anomer. This is consistent with an S_N1 mechanism having a high degree of stereofacial selectivity. Possibly expulsion of the product from the active site is favoured by mutarotation to the more stable β-anomer.

Finally, site directed mutagenesis studies have shown that the activity of the enzyme is lost if Arg-152 is replaced by lysine and Glu-277 by aspartate. These replacement amino acids contain similarly charged residues but have a shorter residue chain. As a result, the charged residues are unable to reach the required area of space in order to stabilize the intermediate.

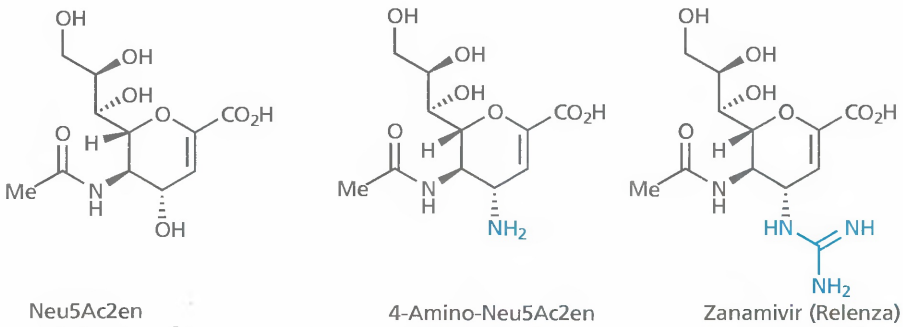
20.8.3.2 Transition state inhibitors: development of zanamivir (Relenza)

The transition state shown in Fig. 20.45 has a planar trigonal centre at C-2 and so sialic acid analogues containing a double bond between positions C-2 and C-3 were synthesized to achieve that same trigonal geometry at C-2. This resulted in the discovery of the inhibitor 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (Neu5Ac2en) (Fig. 20.45). In order to achieve the required double bond, the hydroxyl group originally present at C-2 of sialic acid had to be omitted, which resulted in lower hydrogen bonding interactions with the active site. On the other hand, the inhibitor does not need to distort from a favourable chair shape in order to bind, and the energy saved by this more than compensates for the loss of one hydrogen bonding interaction. The inhibitor was crystallized with the enzyme and studied by X-ray crystallography and molecular modelling to show that the same binding interactions were taking place with the exception of the missing hydroxyl group at C-2. Unfortunately, this compound also inhibited bacterial and mammalian sialidases and could not be used therapeutically. Moreover, it was inactive *in vivo*.

Following the development of a model active site, the search for new inhibitors centred around the use of **GRID** molecular modelling software to evaluate likely binding regions within the model active site. This involved setting up a series of grid points within the active site and placing probe atoms at each point to measure interactions between the probe and the active site (section 17.7.5). Different atomic probes were used to represent various functional groups. These included the oxygen of a carboxylate group, the nitrogen of an ammonium cation, the oxygen of a hydroxyl group and the carbon of a methyl group. Multi-atom probes were also used. A multi-atom probe is positioned such that one atom of the probe is placed at the grid point and then energy calculations are performed for all the atoms within the probe and a total probe energy is assigned to that grid point. The probe is then rotated such that each possible hydrogen bonding orientation is considered and the most favourable interaction energy accepted.

The most important result from these studies was the discovery that the region normally occupied by the 4-OH of sialic acid could interact with an ammonium or guanidinium ion. As a result, sialic acid analogues, having an amino or guanidinyl group at C-4 instead of a hydroxyl group, were modelled in the active site to study the binding interactions and to check whether there was room for the groups to fit.

These modelling studies were favourable and so the relevant structures were synthesized and tested for activity. 4-Amino-Neu5Ac2en (Fig. 20.46) contains the ammonium group and was found to be more potent than Neu5Ac2en. Moreover, it was active in animal studies



Neu5Ac2en
K_i (M) 4×10^{-6}; IC_{50} 5–10 μM

4-Amino-Neu5Ac2en
K_i (M) 4×10^{-8}

Zanamivir (Relenza)
K_i (M) 3×10^{-11}

FIGURE 20.45 Transition state inhibitors for the enzyme neuraminidase.

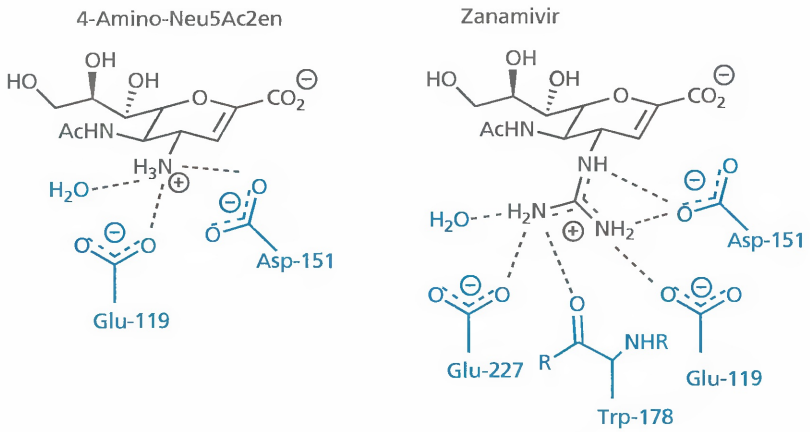


FIGURE 20.46 Binding interactions of ammonium and guanidinium moieties at C-4 with the active site of neuraminidase.

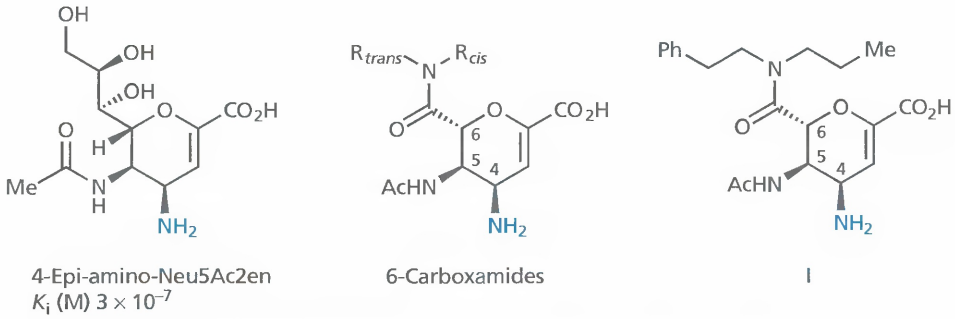


FIGURE 20.47 4-Epi-amino-Neu5Ac2en and carboxamides.

and showed selectivity against the viral enzyme, implying that the region of the active site which normally binds the 4-hydroxyl group of the substrate is different in the viral enzyme from comparable bacterial or mammalian enzymes. A crystal structure of the inhibitor bound to the enzyme confirmed the binding pattern predicted by the molecular modelling (Fig. 20.46).

Molecular modelling studies had suggested that the larger guanidinium group would be capable of even greater hydrogen bonding interactions, as well as favourable van der Waals interactions. The relevant structure (zanamivir; Fig. 20.45) was indeed found to be a more potent inhibitor having a 100-fold increase in activity. X-ray crystallographic studies of the enzyme-inhibitor complex demonstrated the expected binding interactions (Fig. 20.46). Moreover, the larger guanidino group was found to expel a water molecule from this binding pocket, and this is thought to contribute a beneficial entropic effect. Zanamivir is a slow-binding inhibitor with a high binding affinity to influenza A neuraminidase. It was approved by the US FDA in 1999 for the treatment of influenza A and B, and was marketed by Glaxo Wellcome and Biota. Unfortunately, the polar nature of the molecule means it has poor oral bioavailability (< 5%), and it is administered by inhalation.

Following on from the success of these studies, 4-epi-amino-Neu5Ac2en (Fig. 20.47) was synthesized to place the amino group in another binding region predicted by the GRID analysis. This structure proved to be a better inhibitor than Neu5Ac2en, but not as good as zanamivir. The pocket into which this amino group fits is small and there is no room for larger groups.

20.8.3.3 Transition state inhibitors: 6-carboxamides

A problem with the inhibitors described in section 20.8.3.2 is their polar nature. The glycerol side chain is particularly polar and has important binding interactions with the active site. However, it was found that it could be replaced by a carboxamide side chain with retention of activity (Fig. 20.47).

A series of 6-carboxamide analogues was prepared to explore their structure–activity relationships. Secondary carboxamides (where R_{cis}=H) showed similar weak inhibition against both A and B forms of the neuraminidase

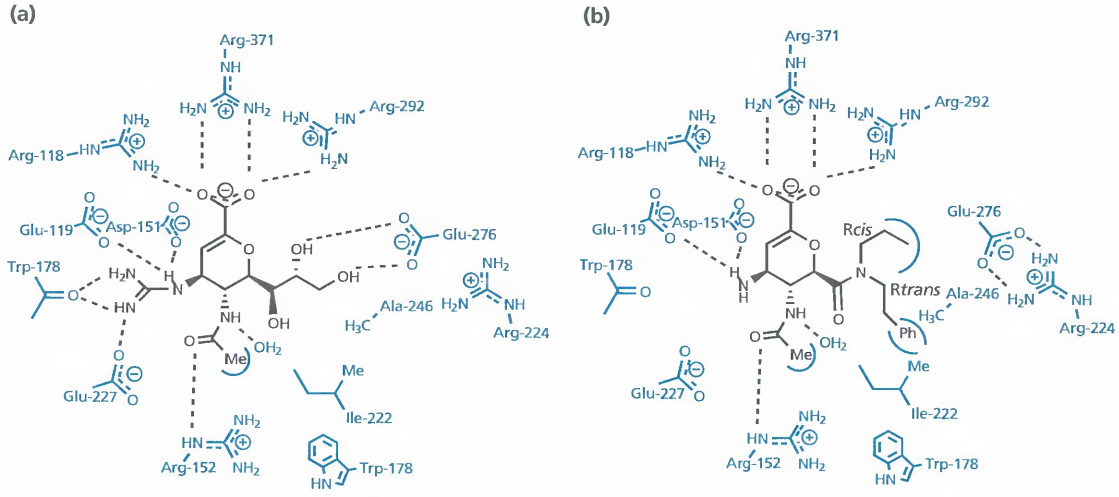


FIGURE 20.48 Binding interactions of zanamivir and carboxamides; **(a)** binding of zanamivir to the active site; **(b)** binding of carboxamide (I) to the active site.

enzyme. Tertiary amides having an alkyl substituent at the *cis* position resulted in a pronounced improvement against the A form of the enzyme with relatively little effect on the activity against the B form. Thus, tertiary amides showed a marked selectivity of 30–1000-fold for the A form of the enzyme. Good activity was related to a variety of different sized R_{trans} substituents larger than methyl, but the size of the R_{cis} group was more restricted, and optimum activity was achieved when R_{cis} was ethyl or *n*-propyl.

The 4-guanidino analogues are more active than corresponding 4-amino analogues but the improvement is slightly less than that observed for the glycerol series, especially where the 4-amino analogue is already highly active.

Crystal structures of the carboxamide (I in Fig. 20.47) bound to both enzymes A and B were determined by X-ray crystallography (Fig. 20.48). The dihydropyran portion of the carboxamide (I) binds to both the A and B forms of the enzyme in essentially the same manner as observed for zanamivir. The important binding interactions involve the carboxylate ion, the 4-amino group and the 5-acetamido group—the latter occupying a hydrophobic pocket lined by Trp-178 and Ile-222.

A significant difference, though, is in the region occupied by the carboxamide side chain. In the sialic acid analogues, the glycerol side chain forms intermolecular hydrogen bonds to Glu-276. These interactions are not possible for the carboxamide side chain. Instead, the Glu-276 side chain changes conformation and forms a salt bridge with the guanidino side chain of Arg-224, and reveals a lipophilic pocket into which the R_{cis} *n*-propyl substituent can fit. The size of this pocket is optimal for an ethyl or propyl group which matches the structure–activity (SAR) results. The R_{trans} phenethyl group lies in an extended lipophilic cleft on the enzyme surface formed between Ile-222 and Ala-246. This region can accept a variety of substituents, again consistent with SAR results.

Comparison of the X-ray crystal structures of the native A and B enzymes shows close similarity of position and orientation of the conserved active site residues except in the region occupied normally by the glycerol side chain, particularly as regards Glu-276. Zanamivir can bind to both A and B forms with little or no distortion of the native structures. Binding of the carboxamide (I) to the A form is associated with a change in torsion angles of the Glu-276 side chain such that the residue can form the salt bridge to Arg-224, but there is little distortion of the protein backbone in order to achieve this. In contrast, when the carboxamide binds to the B form of the enzyme, there is a significant distortion of the protein backbone required before the salt bridge is formed. Distortion in the B enzyme structure also arises around the phenethyl substituent. This implies that binding of the carboxamide to the B form involves more energy expenditure than to A and this can explain the observed specificity.

20.8.3.4 Carbocyclic analogues: development of oseltamivir (Tamiflu)

The dihydropyran oxygen of Neu5Ac2en and related inhibitors has no important role to play in binding these structures to the active site of neuraminidase. Therefore, it should be possible to replace it with a methylene isostere to form carbocyclic analogues such as structure I in Fig. 20.49. This would have the advantage of removing a polar oxygen atom which would increase hydrophobicity

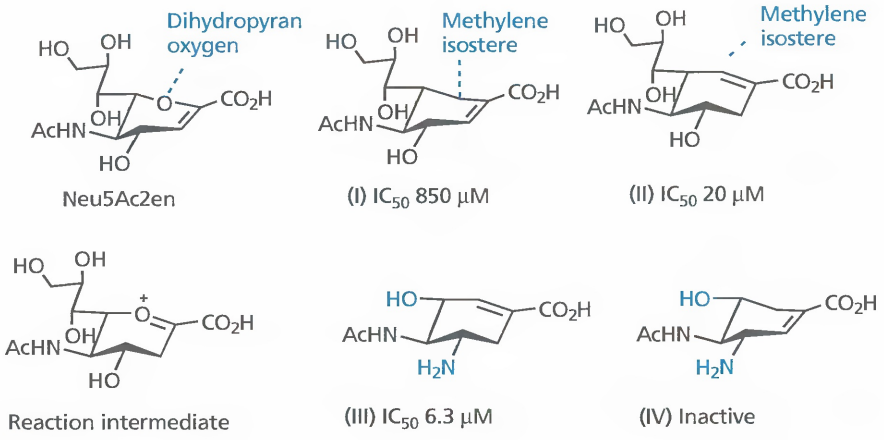
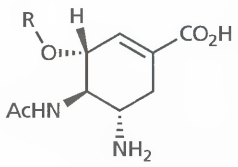


FIGURE 20.49 Comparison of Neu5Ac2en, reaction intermediate and carbocyclic structures.



R	IC_{50} (μM)
Me	3.70
Et	2.00
n-Pr	0.18
n-Bu	0.30

R	IC_{50} (μM)
CH_2CHMe_2	0.200
$CH(Me)CH_2CH_3$	0.010
$CH(Et)_2$	0.001

R	IC_{50} (μM)
CH_2OMe	2.00
$CH_2CH_2CF_3$	0.20
$CH_2CH{=}CH_2$	2.20
cyclopentyl	0.02
cyclohexyl	0.06
phenyl	0.53

FIGURE 20.50 Alkoxy analogues.

and potentially increase oral bioavailability. Moreover, it would be possible to synthesize cyclohexene analogues such as structure II, which more closely match the stereochemistry of the reaction's transition state than previous inhibitors—compare the reaction intermediate in Fig. 20.49 which can be viewed as a transition state mimic. Such agents might be expected to bind more strongly and be more potent inhibitors.

Structures I and II were synthesized to test this theory, and it was discovered that structure II was 40 times more potent than structure I as an inhibitor. Since the substituents are the same, this indicates that the conformation of the ring is crucial for inhibitory activity. Both structures have half chair conformations but these are different due to the position of the double bond.

It was now planned to replace the hydroxyl group on the ring with an amino group to improve binding interactions (compare section 20.8.3.2), and to remove the glycerol side chain to reduce polarity. In its place, a hydroxyl group was introduced for two reasons. First, the oxonium double bond in the transition state is highly polarized and electron deficient, whereas the double bond in the carbocyclic structures is electron rich. Introducing the hydroxyl substituent in place of the glycerol side chain means that the oxygen will have an inductive electron-withdrawing effect on the carbocyclic double bond and reduce its electron density. The second reason for adding the hydroxyl group was that it would be possible to synthesize ether analogues which would allow the addition of hydrophobic groups to fill the binding pocket previously occupied by the glycerol side chain (compare section 20.8.3.3). The resultant structure III was synthesized and proved to be a potent inhibitor. In contrast, the isomer IV failed to show any inhibitory activity.

A series of alkoxy analogues of structure III was now synthesized in order to maximize hydrophobic interactions in the region of the active site previously occupied by the glycerol side chain (Fig. 20.50). For linear alkyl chains, potency increased as the carbon chain length increased from methyl to *n*-propyl. Beyond that, activity was relatively constant (150–300 nM) up to and including *n*-nonyl, after which activity dropped. Although longer chains than propyl increase hydrophobic interactions, there is a downside in that there is partial exposure of the side chain to water outside of the active site.

Branching of the optimal propyl group was investigated. There was no increase in activity when methyl branching was at the β-position, but the addition of a methyl group at the α-position increased activity by 20-fold. Introduction of an α-methyl group introduces an asymmetric centre, but both isomers were found to have similar activity indicating two separate hydrophobic pockets. The optimal side chain proved to be a pentyloxy side chain ($R{=}CH(Et)_2$).

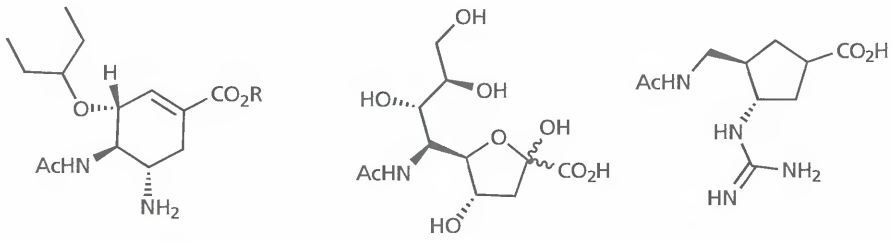


FIGURE 20.51 Oseltamivir and other ring systems.

The *N*-acetyl group is required for activity and there is a large drop in activity without it. The binding region for the *N*-acetyl group has limitations on the functionality and size of groups which it can accept. Any variations tend to reduce activity. This was also observed with sialic acid analogues.

Replacing the amino group with a guanidine group improves activity, as with the sialic acid series. However, the improvement in activity depends on the type of alkyl group present on the side chain, indicating that individual substituent contributions may not be purely additive.

The most potent of the above analogues was the pentyloxy derivative (GS 4071) (Fig. 20.51). This was co-crystallized with the enzyme and the complex was studied by X-ray crystallography, revealing that the alkoxy side chain makes several hydrophobic contacts in the region of the active site normally occupied by the glycerol side chain. In order to achieve this, the carboxylate group of Glu-276 is forced to orientate outwards from the hydrophobic pocket as observed with the carboxamides. The overall gain in binding energy from these interactions appears to be substantial, as a guanidino group is not required to achieve low nanomolar inhibition. Interactions elsewhere are similar to those observed with previous inhibitors.

Oseltamivir (Tamiflu) (Fig. 20.51) is the ethyl ester prodrug of GS 4071 and was approved in 1999 for the treatment of influenza A and B. The drug is marketed by Hoffman La Roche and Gilead Sciences. It is taken orally and is converted to GS4071 by esterases in the gastrointestinal tract.

20.8.3.5 Other ring systems

Work has been carried out to develop new NA inhibitors where different ring systems act as scaffolds for the important binding groups (Fig. 20.51).

The five-membered tetrahydrofuran (I) is known to inhibit neuraminidase with a potency similar to Neu5Ac2en. It has the same substituents as Neu5Ac2en, although their arrangement on the ring is very different. Nevertheless, a crystal structure of (I) bound to the enzyme shows that the important binding groups (carboxylate, glycerol, acetamido and C-4-OH) can fit into the required pockets. The central ring or scaffold is significantly displaced from the position occupied by the pyranose ring of Neu5Ac2en in order to allow this. This indicates that the position of the central ring is not crucial to activity, and that the relative position of the four important binding groups is more important.

Five-membered carbocyclic rings have also been studied as suitable scaffolds. Structure II (Fig. 20.51) was designed such that the guanidine group would fit the negatively charged binding pocket previously described. A crystal structure of the inhibitor with the enzyme showed that the guanidine group occupies the desired pocket and displaces the water molecule originally present. It is involved in charge-based interactions with Asp-151, Glu-119, and Glu-227, analogous to zanamivir.

Modelling studies suggested that the addition of a butyl chain to the structure would allow van der Waals interactions with a small hydrophobic surface in the binding site. The target structure now has four asymmetric centres, and a synthetic route was used which controlled the configuration of two of these. As a result, four racemates or eight isomers were prepared as a mixture (Fig. 20.52). Neuraminidase crystals were used to select the most active isomer of the mixture by soaking a crystal of the enzyme in the solution of isomers for a day and then collecting X-ray diffraction data from the crystal. This showed the active isomer to be structure I in Fig. 20.53. The structure binds to the active sites of both influenza A and B neuraminidases

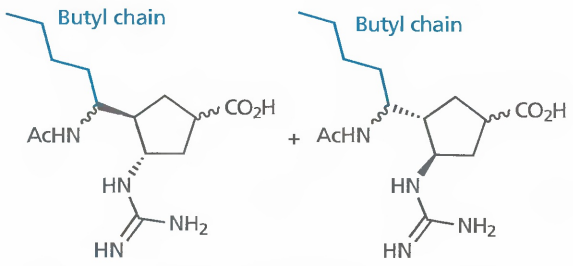


FIGURE 20.52 Mixture of isomers tested for their binding affinity to crystals of the neuraminidase enzyme.

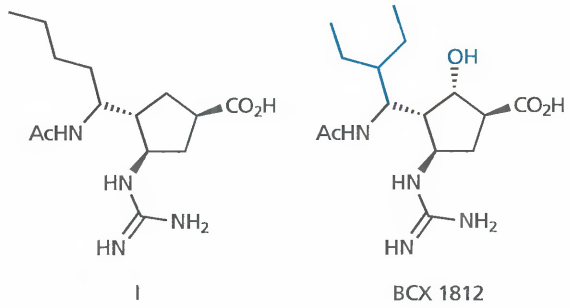


FIGURE 20.53 Development of BCX 1812.

with the *n*-butyl side chain adopting two different binding modes. In the influenza B neuraminidase, the side chain is positioned against a hydrophobic surface formed by Ala-246, Ile-222, and Arg-224. In the A version, the chain is in a region formed by the reorientation of the side chain of Glu-276.

BCX 1812 (Fig. 20.53) was designed to take advantage of both hydrophobic pockets in the active site. It was prepared as a racemic mixture, and a crystal of the neuraminidase enzyme was used to bind to the active isomer. Once identified, this was then prepared by a stereospecific synthesis. The relative stereochemistry of the substituents was the same as in structure (I).

In vitro tests of BCX-1812 versus strains of influenza A and B show it to be as active as zanamivir and GS4071. It is also four orders of magnitude less active against bacterial and mammalian neuraminidases, making it a potent and highly specific inhibitor of flu virus neuraminidase. *In vivo* tests carried out on mice showed it to be orally active and the compound is undergoing clinical trials.

20.8.3.6 Resistance studies

Studies have been carried out to investigate the likelihood of viruses acquiring resistance to the drugs mentioned above. This is done by culturing the viruses in the presence of the antiviral agents to see if mutation leads to a resistant strain.

Zanamivir has a broad spectrum efficacy against all type A and B strains tested, and interacts only with conserved residues in the active site of NA. Thus, in order to gain resistance, one of these important amino acids has to mutate. A variant has been observed where Glu-119 has been mutated to glycine. This has reduced affinity for zanamivir, and the virus can replicate in the presence of the drug. Removing Glu-119 affects the binding interactions with the 4-guanidinium group of zanamivir without affecting interactions with sialic acid. Zanamivir-resistant mutations were also found where a mutation occurred in HA around the sialic acid binding site. This mutation weakened affinity for sialic acid and so lowered binding. Thus, mutant viruses were able to escape more easily from the infected cell after budding. No such mutations have appeared during clinical trials, however.

Another mutation has been observed where Arg-292 is replaced by lysine. In wild-type NA, Arg-292 binds to the carboxylate group of the inhibitor and is partly responsible for distorting the pyranose ring from the chair to the boat conformation. In the mutant structure, the amino group of Lys-292 forms an ionic interaction with Glu-276 which normally binds the 8 and 9 hydroxyl groups of the glycerol side chain. This results in a weaker interaction with inhibitors and substrate alike, leading to a weaker enzyme.

One conclusion that has arisen from studies on easily mutatable targets is the desirability to find an inhibitor which is modified as little as possible from the normal substrate, and which uses the same interactions for binding.

KEY POINTS

- The flu virus contains (−) ssRNA and has two glycoproteins called haemagglutinin (HA) and neuraminidase (NA) in its outer membrane.
- HA binds to the sialic acid moiety of glycoconjugates on the outer surface of host cells leading to adsorption and cell uptake.
- NA catalyses the cleavage of sialic acid from glycoconjugates. It aids the movement of the virus through mucus and releases the virus from infected cells after budding.
- HA and NA act as antigens for flu vaccines. However, the influenza A virus readily mutates these proteins, requiring new flu vaccines each year.
- The adamantanes are antiviral agents that inhibit influenza A by blocking a viral ion channel called the matrix (M2) protein. At high concentration they buffer the pH of endosomes. They are ineffective against influenza B, which lacks the matrix (M2) protein.
- Neuraminidase has an active site which remains constant for the various types and subtypes of the enzyme and which is different from the active sites of comparable mammalian enzymes.
- There are four important binding pockets in the active site. The sialic acid moiety is distorted from its normal chair conformation when it is bound.
- The mechanism of reaction is proposed to go through an endocyclic sialosyl cation transition state. Inhibitors were designed to mimic this state by introducing an endocyclic double bond.
- Successful antiviral agents have been developed using structure-based drug design.
- Different scaffolds can be used to hold the four important binding groups.
- There is an advantage in designing drugs that use the same binding interactions as the natural ligand when the target undergoes facile mutations.

20.9 Antiviral drugs acting against RNA viruses: cold virus

The agents used against flu are ineffective against colds, as these infections are caused by a different kind of virus called a rhinovirus. Colds are less serious than flus. Nevertheless, research has taken place to find drugs which can combat them.

There are at least 89 serotypes of **human rhinoviruses** (HRV) and they belong to a group of viruses called the **picornaviruses** which include the polio, hepatitis A, and foot and mouth disease viruses. They are among the smallest of the animal RNA viruses, containing a positive strand of RNA coated by an icosahedral shell made up of 60 copies of 4 distinct proteins, VP1-VP4 (Fig. 20.54). The proteins VP1-VP3 make up the surface of the virion. The smaller VP4 protein lies underneath to form the inner surface and is in contact with the viral RNA. At the junction between each VP1 and VP3 protein, there is a broad canyon 25 Å deep and this is where attachment takes place between the virus and the host cell. On the canyon floor, there is a pore which opens into a hydrophobic pocket within the VP1 protein. This pocket is either empty or occupied by a small molecule called a **pocket factor**. So far the identity of the pocket factor has not been determined but it is known from X-ray crystallographic studies that it is a fatty acid containing seven carbon atoms.

When the virus becomes attached to the host cell, a receptor molecule on the host cell fits into the canyon and induces conformational changes which cause the VP4 protein and the *N*-terminus of VP1 to move to the exterior of the virus—a process called externalization. This is thought to be important to the process by which the virus is uncoated and releases its RNA into the host cell. It is thought that the pocket factor regulates the stability of the virion. When it is bound to the pocket, it stabilizes the capsid and prevents the conformational changes that are needed to cause infection.

A variety of drugs having antiviral activity are thought to mimic the pocket factor by displacing it and binding to the same hydrophobic pocket. The drugs concerned are called capsid-binding agents and are characteristically long-chain hydrophobic molecules. Like the pocket factor, they stabilize the capsid by locking it into a stable conformation and prevent the conformational changes required for uncoating. They also raise the canyon floor and prevent the receptor on the host cell from fitting the canyon and inducing uncoating (Fig. 20.55).

Pleconaril (Fig. 20.56) is one such drug that has undergone phase III clinical trials which demonstrate that it has an effect on the common cold. It is an orally active, broad-spectrum agent which can cross the blood–brain barrier. The drug may also be useful against the enteroviruses which cause diarrhoea, viral meningitis,

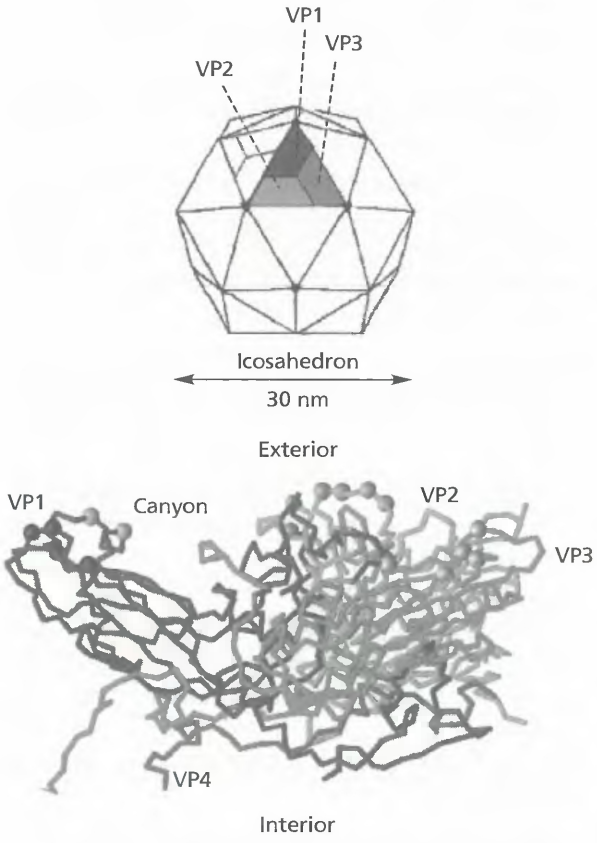


FIGURE 20.54 Structure of human rhinovirus and the proteins VP1-VP4.

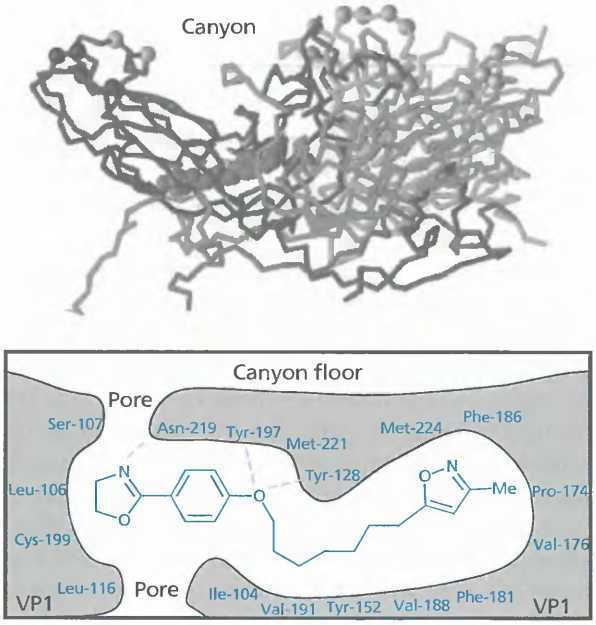


FIGURE 20.55 Binding of disoxaril (possible hydrogen bonds shown as dashed lines).

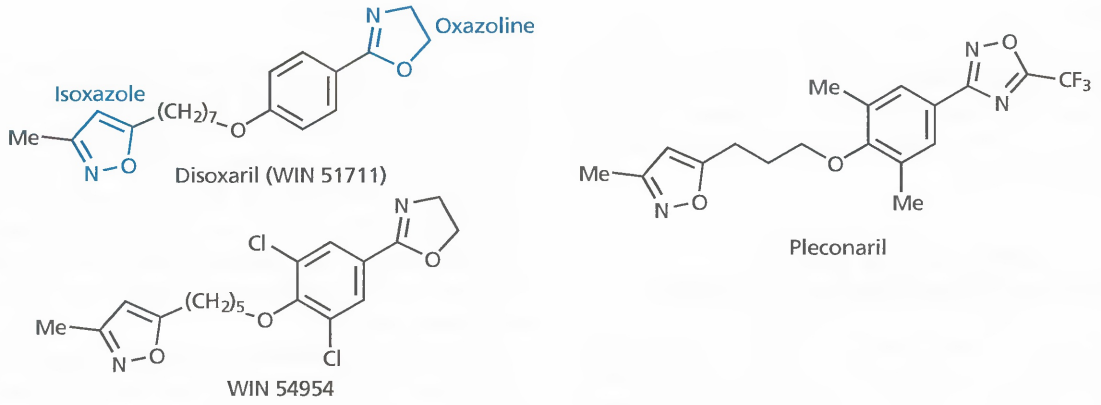


FIGURE 20.56 Capsid binding agents.

conjunctivitis, and encephalitis since they are similar in structure to the rhinoviruses.

The development of pleconaril started when a series of isoxazoles was found to have antiviral activity. This led to the discovery of **disoxaril** (Fig. 20.56) which entered phase I clinical trials, but proved to be too toxic. X-ray crystallographic studies of VP1-drug complexes involving disoxaril and its analogues showed that the oxazoline and phenyl rings were roughly coplanar and were located in a hydrophilic region of the pocket near the pore leading into the centre of the virion (Fig. 20.55). The hydrophobic isoxazole ring binds into the heart of the hydrophobic pocket and the chain provides sufficient flexibility for the molecule to bend round a corner in the pocket. Binding moves Met-221 which normally seals off the pocket, and this also causes conformational changes in the canyon floor. Structure-based drug design was carried out to find safer and more effective antiviral agents. For example, the chain cannot be too short or too long, or else there are steric interactions. Placing additional hydrophobic groups on to the phenyl ring improves activity against the HRV2 strain, since increased interactions are possible with a phenylalanine residue at position 116 rather than leucine. The structure **WIN 54954** was developed and entered clinical trials, but results were disappointing because extensive metabolism resulted in 18 different metabolic products due mainly to hydrolysis of the oxazoline ring. Further structure-based drug design led to modifications of the phenyl and oxazoline moieties. This included the introduction of a trifluoromethyl group to block metabolism, resulting in pleconaril, which has 70% oral bioavailability.

20.10 Broad-spectrum antiviral agents

Very few clinically useful, broad-spectrum antiviral agents that act on specific targets have reached the clinic. The following are some examples.

20.10.1 Agents acting against cytidine triphosphate synthetase

Cytidine triphosphate is an important building block for RNA synthesis, and so blocking its synthesis inhibits the synthesis of viral mRNA. The final stage in the biosynthesis of cytidine triphosphate is the amination of uridine triphosphate—a process that is catalysed by the enzyme cytidine triphosphate synthetase. **Cyclopentenyl**

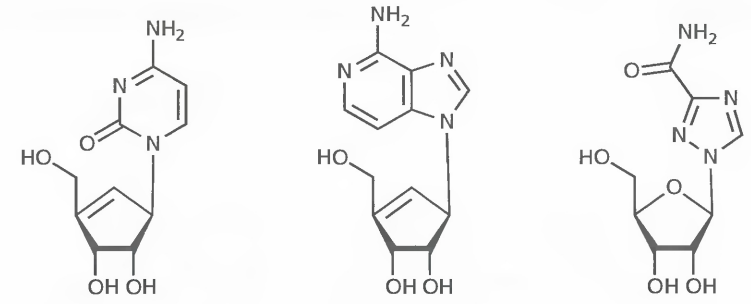


FIGURE 20.57 Broad-spectrum antiviral agents.

cytosine (Fig. 20.57) is a carbocyclic nucleoside that is converted in the cell to the triphosphate which then inhibits this final enzyme in the biosynthetic pathway. The drug has broad antiviral activity against more than 20 RNA and DNA viruses, and has also been studied as an anticancer drug.

20.10.2 Agents acting against *S*-adenosylhomocysteine hydrolase

The 5'-end of a newly transcribed mRNA is capped with a methyl group in order to stabilize it against phosphatases and nucleases, as well as enhancing its translation. *S*-Adenosylhomocysteine hydrolase is an intracellular enzyme that catalyses this reaction, and many viruses need it to cap their own viral mRNA. **3-Deazaneplanocin A** (Fig. 20.57) is an analogue of cyclopentenyl cytosine, and acts against a range of RNA and DNA viruses by inhibiting *S*-adenosylhomocysteine hydrolase.

20.10.3 Ribavirin (or virazole)

Ribavirin (Fig. 20.57) is a synthetic nucleoside that induces mutations in viral genes and is used against hepatitis C infection. It was the first synthetic, non-interferon-inducing broad-spectrum antiviral nucleoside, and can inhibit both RNA and DNA viruses by a variety of mechanisms, although it is only licensed for hepatitis C and respiratory syncytial virus. Nevertheless, it has been used in developing countries for the treatment of tropical and haemorrhagic fevers such as Lassa fever when there is no alternative effective treatment. Tests show that it is useful in combination with other drugs such as rimantadine. Its dominant mechanism of action appears to be depletion of intracellular pools of GTP by inhibiting inosine-5'-monophosphate dehydrogenase. Phosphorylation of ribavirin results in a triphosphate which inhibits guanyl transferase and prevents the 5' capping of mRNAs. The triphosphate can also inhibit viral RNA-dependent RNA polymerase. Due to these multiple mechanisms of action, resistance is rare. The drug's main side effect is anaemia, and it is a suspected teratogen.

20.10.4 Interferons

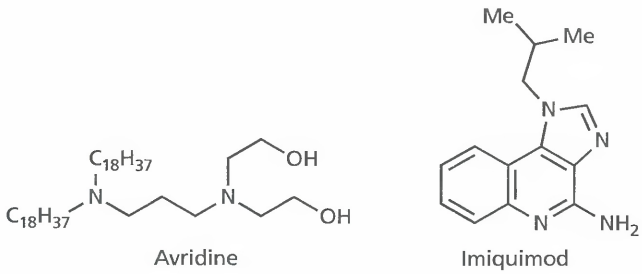
Interferons are small natural proteins which were discovered in 1957, and which are produced by host cells as a response to 'foreign invaders'. Once produced, interferons inhibit protein synthesis and other aspects of viral replication in infected cells. In other words, they shut the cell down. This can be described as an intracellular immune response. Administering interferons to patients has been seen as a possible approach to treating flu, hepatitis, herpes, and colds.

There are several interferons, which are named according to their source: α-interferons from lymphocytes, β-interferons from fibroblasts and γ-interferons from T-cells. α-Interferon (also called **alferon** or IFN-alpha) is the most widely used of the three types. In the past, isolating interferons from their natural cells was difficult and expensive, but recombinant DNA techniques allow the production of genetically engineered interferons in larger quantities (section 6.4). Recombinant α-interferon is produced in three main forms. The α-2a and α-2b are natural forms, and **alfacon-1** is the unnatural form. They have proved successful therapeutically, but can have serious toxic side effects. At present, α-interferon is used clinically against hepatitis B infections. It is also used with ribavirin against hepatitis C infections.

Interferon production in the body can also be induced by agents known as **immunomodulators**. One such example is **avridine** (Fig. 20.58), which is used as a vaccine adjuvant for the treatment of animal diseases such as foot and mouth. **Imiquimod** (Fig. 20.58) also induces the production of α-interferon, as well as other cytokines that stimulate the immune system. It is effective against genital warts.

20.10.5 Antibodies and ribozymes

Antibodies that recognize a virion-specific antigen will bind to that antigen and mark the virus out for destruction by the body's immune system. **Palivizumab** is a humanized monoclonal antibody which was approved in 1998. It is used for the treatment of respiratory syncytial infection in babies and blocks viral spread from cell to



Avridine Imiquimod

FIGURE 20.58 Immunomodulators.

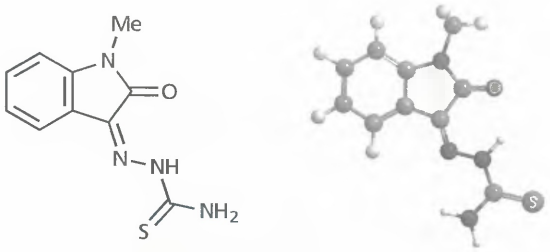


FIGURE 20.59 Methisazone.

cell by targeting a specific protein of the virus. Another monoclonal antibody is being tested for the treatment of hepatitis B.

It has been possible to identify sites in viral RNA that are susceptible to cutting by ribozymes—enzymatic forms of RNA. One such ribozyme is being tested in patients with hepatitis C and HIV. Ribozymes could be generated in the cell by introducing genes into infected cells—a form of gene therapy. Other gene therapy projects are looking at genes that would code for specialized antibodies capable of seeking out targets inside infected cells, or that would code for proteins which would latch on to viral gene sequences within the cell.

20.11 Bioterrorism and smallpox

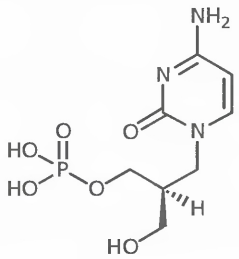
The first effective antiviral drug to be used clinically was an agent called **methisazone** (Fig. 20.59) which was used against smallpox in the 1960s. The drug was no longer required once the disease was eradicated through worldwide vaccination. In recent years, however, there have been growing worries that terrorists might acquire smallpox and unleash it on a world no longer immunized against the disease. As a result, there has been a regeneration of research into finding novel antiviral agents which are effective against this disease.

KEY POINTS

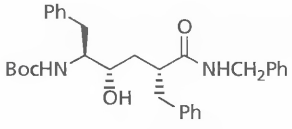
- There are few broad-spectrum antiviral agents currently available.
- The best broad-spectrum antiviral agents appear to work on a variety of targets, reducing the chances of resistance.
- Interferons are chemicals produced in the body, which shut down infected host cells and limit the spread of virus.
- Antibodies and ribozymes are under investigation as antiviral agents.

QUESTIONS

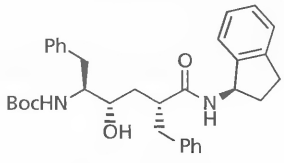
1. Consider the structures of the PIs given in section 20.7.4 and suggest a hybrid structure that might also act as a PI.
2. Consider the structure of the PIs in section 20.7.4 and suggest a novel structure with an extended subsite ligand.
3. What disadvantage might the following structure have as an antiviral agent compared with cidofovir?



4. Zanamivir has a polar glycerol side chain that has good interactions with a binding pocket through hydrogen bonding, yet carboxamides and oseltamivir have hydrophobic substituents which bind more strongly to this pocket. How is this possible?
5. Show the mechanism by which the prodrugs tenofovir disoproxil and adefovir dipivoxil are converted to their active forms. Why are extended esters used as prodrugs for these compounds?
6. Capravirine (Fig. 20.15) has a side chain that takes part in important hydrogen bonding to Lys-103 and Pro-236 in the allosteric binding site of reverse transcriptase, yet the side chain has a carbonyl group. Discuss whether this is prone to enzymatic hydrolysis and inactivation.
7. Most PIs bind to the active site with a water molecule acting as a hydrogen bonding bridge to the enzyme flaps. Suggest what relevance this information might have in the design of novel PIs.
8. The following structures were synthesized during the development of L 685434. Identify the differences between the two structures and suggest why one is more active than the other.



(IC_{50} = 111 nM)



(IC_{50} = 21 nM)

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Titles for general further reading are listed on p. 725.