Viral Proteases

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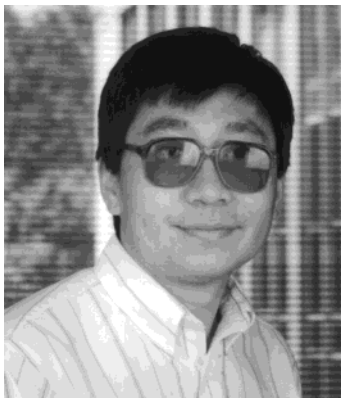
I. Introduction

Viruses are major pathogenic agents that can cause a variety of serious diseases in humans, other animals, and plants. Due to their clinical and scientific importance, viruses have been under intensive study ever since their first isolation about a century ago. The studies of viruses, their life cycles, and their interactions with the hosts have established many new areas of biological research over the years.

Viruses can be divided into two major categories: enveloped and nonenveloped viruses. Further classification can be based on the nature of the genetic material that is packaged by the virus, for example, single-stranded and double-stranded RNA (ssRNA and dsRNA) viruses and ssDNA and dsDNA viruses. The ssRNA genome can have positive or negative sense, depending on whether it can be directly translated to produce the viral protein. Finally, retroviruses contain a positive-sense RNA genome, but it is reverse-transcribed to DNA during the viral life cycle.

Well-known examples of positive-sense ssRNA viruses include picornaviruses (human rhinoviruses and foot-and-mouth disease virus, section III), togaviruses (alphaviruses, section II), and flaviviruses (hepatitis C virus, West Nile virus, and yellow fever virus, section II). Herpesviruses (section VI) and



Liang Tong is Associate Professor of Biological Sciences at Columbia University in New York, NY. He received his Ph.D. in 1989 in biophysical chemistry from the University of California at Berkeley, where he studied the structure and function of the proto-oncogene *ras* p21 in the laboratory of Prof. Sung-Hou Kim. He then did his postdoctoral research with Prof. Michael G. Rossmann at Purdue University, and determined the crystal structure of Sindbis virus capsid protein, which revealed a chymotrypsin-like serine protease. In 1992, he became Senior Scientist (and in 1996 Principal Scientist) at Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, CT, where he continued his research on viral proteases (HIV protease and HCMV protease) and other medically important proteins. He is the recipient of the first Boehringer Ingelheim (Worldwide) Research and Development Award. In 1997, he moved to Columbia University and established a vigorous research program on the structural biology of proteins involved in signal transduction as well as enzymes of medical/biochemical importance. In addition, he has maintained a strong and continuing interest in the development of new methodology and computer software for protein crystallography. He has been highly productive in his scientific research, and has so far published about 80 papers.

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adenoviruses (section VII) are familiar examples of enveloped and nonenveloped dsDNA viruses, respectively. Human immunodeficiency virus (HIV) is the most prominent example of retroviruses (section V). Finally, hepatitis B virus is another reverse-transcribing virus, but it contains a DNA genome.

The discovery and development of new antiviral agents is an important component of the research on viruses. This research has led to the clinical use of many drugs that are directed against enzymes and other processes that are crucial for the life cycles of a variety of viruses. A target that has proven to be useful against many viruses is the virally encoded polymerases, for example, the nucleoside and nonnucleoside inhibitors of HIV-1 reverse transcriptase. However, potent, clinically relevant polymerase inhibitors are not available against many medically important viruses, and viral resistance to the existing drugs is becoming an increasingly more serious problem. This points to the need for new antiviral agents and new targets for developing such agents.

Viral proteases represent an attractive target for the development of novel antiviral agents. Studies over the past 20 years have shown that many viruses encode one or more proteases.[1,2] These enzymes catalyze the processing of viral polyproteins or the maturational processing of precapsids, and their catalytic activity is required for the production of new, infectious virions. The clinical relevance of viral protease inhibitors was demonstrated recently with the successful application of HIV protease inhibitors in the treatment of acquired immunodeficiency syndrome (AIDS) patients. Moreover, the combination therapy of protease and reverse transcriptase inhibitors has proven to be the most effective regimen against HIV infections.

Viral and cellular proteases can be classified by their structures and/or their catalytic machinery.[3,4] This classification is now accessible via a Web-based database, MEROPS.[5] In this review, we will focus primarily on viral proteases for which structural information is currently available (Table 1). The review is therefore organized on the basis of the backbone folds of the proteases, starting with those whose folds display similarity to those of cellular proteases—chymotrypsin-like serine proteases (section II), chymotrypsin-like cysteine proteases (section III), papain-like cysteine proteases (section IV), and pepsin-like aspartic proteases (section V). This is followed by two viral proteases that have unique backbone folds—herpesvirus proteases (section VI) and adenovirus proteases (section VII). The description of these proteases will focus on their structures and biochemistry and the development of their inhibitors.

II. Chymotrypsin-like Serine Proteases

Chymotrypsin-like serine proteases have been found in vertebrates, bacteria, and also viruses. Examples of vertebrate proteases of this family include chymotrypsin, trypsin, thrombin, elastase, and kallikrein.[3] Examples of bacterial proteases of this family include *Streptomyces griseus* protease A (SGPA), SGPB, and α-lytic protease. Examples of

Table 1. Properties of Viral Proteases Discussed in This Review

protease	sect	fold	active site	substrate specificity	notes
Sindbis virus capsid protein	II.A	chymotrypsin	Ser215, His141, Asp163	(S,T)(E,V)XW↓(S,A)(A,L)	autocatalytic, autoinhibited
hepatitis C virus NS3 protease	II.B	chymotrypsin	Ser139, His57, Asp81	(D,E)XXXX(C,T)↓(S,A)XX(L,W, Y)	requires NS4A cofactor, also binds Zn^{2+}
picornavirus 3C protease	III	chymotrypsin	Cys147, His40, Glu71	X(V,T)XQ↓GP	
picornavirus 2A protease	III	chymotrypsin	Cys106, His18, Asp35	(L,I)XTX↓G	requires Zn^{2+}
picornavirus leader protease	IV	papain	Cys51, His148, Asp163	RKLK↓GAGS, (viral) ANLG↓RTTL (eIF4G)	
HIV protease	V	pepsin	Asp25, Asp25	(S/T)XN(F/Y)↓P	dimeric enzyme
HCMV protease	VI	unique	Ser132, His63, His157	(V,L,I)XA↓S	requires dimerization
adenovirus protease	VII	unique	Cys122, His54, Glu71	(M,L,I)XGX↓G, (M,L,I)XGG↓X	requires pVIc and DNA as cofactors

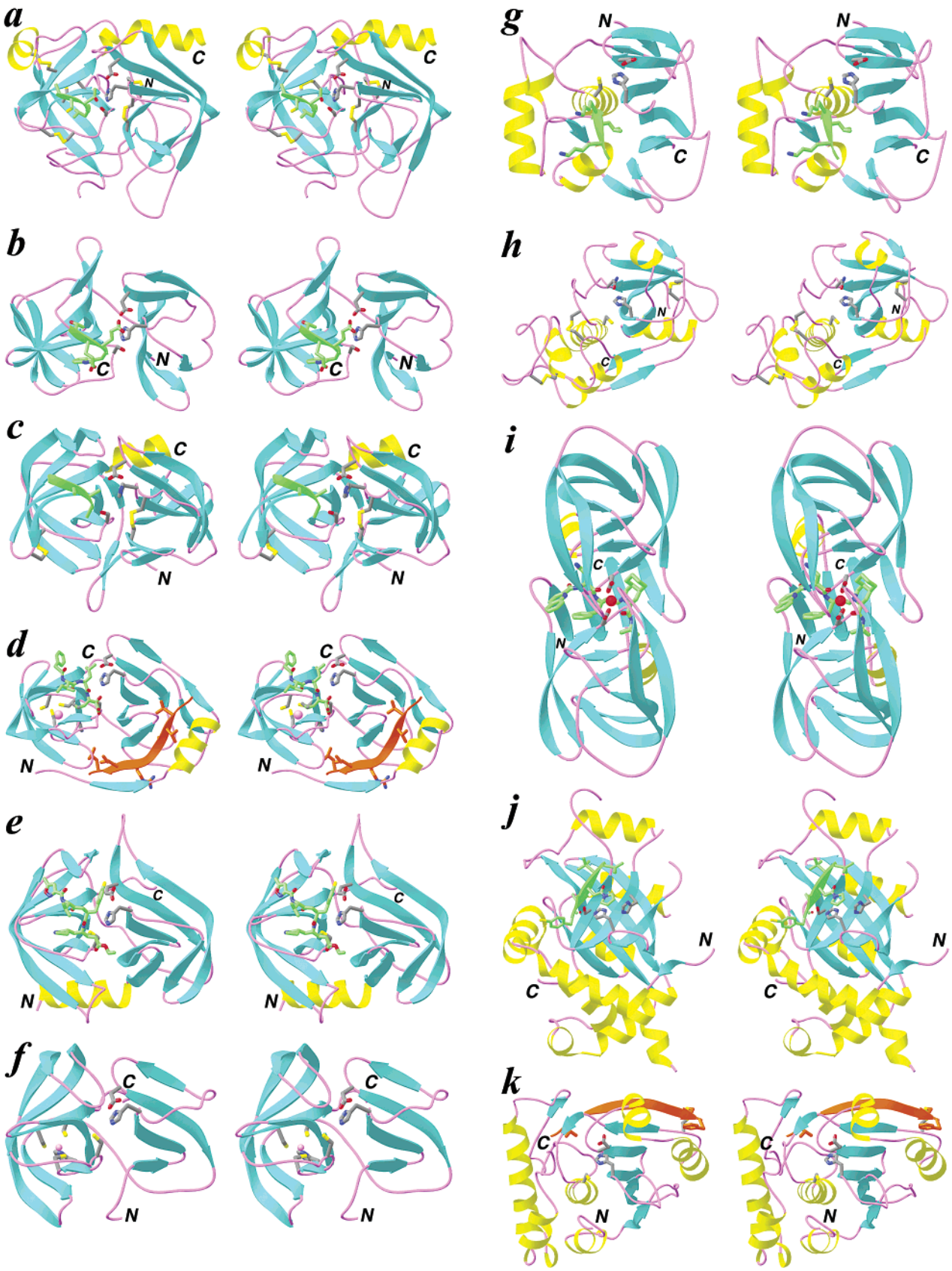

Figure 1. Crystal structures of cellular and viral proteases. Schematic drawings of the structures of (a) chymotrypsin, (b) Sindbis virus capsid protein, (c) SGPA, (d) HCV NS3 protease in complex with an inhibitor with F_2Abu at P_1, (e) HRV2 3C protease in complex with AG7088, (f) HRV2 2A protease, (g) FMDV leader protease, (h) papain, (i) HIV-1 protease in complex with saquinavir, (j) HCMV protease in complex with BILC 821, and (k) adenovirus protease. The β-strands of the protease are shown as arrowed ribbons in cyan, α-helices in yellow, and the connecting loops in purple. The catalytic triad residues and dilsulfide bridges are shown as stick models, in gray for carbon atoms. The inhibitors are shown in green, and the cofactor is shown in orange. Except for HCMV and HIV, the different proteases are shown with their catalytic triads in roughly the same orientation. The Zn^{2+} binding sites in the NS3 and 2A proteases are also shown.

viral proteases of this family include alphavirus capsid proteins and hepatitis C virus NS3 protease, which will be discussed in more detail in this section. In addition, picornavirus 2A and 3C proteases have a chymotrypsin-like fold, although they have a Cys residue as the active site nucleophile. These proteases will be discussed in the next section.

The structures of chymotrypsin-like serine proteases contain two "Greek-key" β-barrel domains, which are connected by a linker (Figure 1a). The active site of the proteases is made up of a catalytic triad of Ser195 as the catalytic nucleophile, His57 as the second member, and Asp102 as the third member in cellular chymotrypsin-like serine proteases. The residues of vertebrate and bacterial proteases are numbered on the basis of sequence and structural alignment to chymotrypsinogen, but the viral proteases are numbered using the natural sequence.

The active site is located at the interface between the two β-barrel domains, with His57 and Asp102 coming from the N-terminal β-barrel, and Ser195 coming from the C-terminal barrel (Figure 1a). The oxyanion hole, which stabilizes the oxyanion of the tetrahedral transition state, is formed by the main chain amides of residues 193 and 195. Peptide inhibitors/substrates are bound in the extended conformation, forming an antiparallel β-sheet with residues 214−216 of the protease (Figure 1a), and the substrate specificities of the proteases are determined by the shape/charge complementarity of the binding pockets.

A. Capsid Protein Autoprotease of Alphaviruses

Alphaviruses are small, mosquito-borne, enveloped viruses that infect humans and other vertebrates.[6,7] They can cause a variety of diseases in humans, such as encephalitis, fever, arthritis, and rash. Sindbis virus, originally identified from mosquitoes collected near the town of Sindbis, Egypt, is the prototype member of this family of viruses. The alphaviruses contain an icosahedral nucleocapsid with a diameter of about 400 Å, which is assembled from 240 copies of the capsid protein together with the positive-sense ssRNA genome.[8,9]

Alphaviruses produce two polyproteins in infected cells.[6] The nonstructural (ns) p270 polyprotein is translated directly from the viral genome, and is processed into four viral proteins, nsP1, nsP2, nsP3, and nsP4.[6] The protease that catalyzes this processing is located in the C-terminal domain of the nsP2 protein.[10] Mutagenesis and biochemical studies showed that this is a cysteine protease, with Cys481 and His558 of the Sindbis virus nsP2 protein in the active site of the enzyme.[11−13] The relative positioning of the Cys and His residues in the active site suggests that this may be a papain-like protease, although there is no sequence homology to papain at the amino acid level.

1. Functions of the Capsid Protein

The structural p130 polyprotein is translated from a subgenomic viral mRNA that is derived from the 3′-end of the viral genome. The capsid protein of the virus is located at the extreme N-terminus of this structural polyprotein, and it is released by a *cis* autoproteolysis from the polyprotein.[14−16] Processing at the other sites in the structural polyprotein is catalyzed by cellular proteases (signalase and Golgi protease).[9]

The capsid protein of alphaviruses has three functions—*cis* autocatalysis to release itself from the viral structural polyprotein, assembly with the viral RNA genome to form the capsid, and recognition of the viral glycoprotein tails to produce capsid envelopment. After the *cis* cleavage to release itself from the structural polyprotein, the catalytic activity of the capsid protein is turned off, and its further functions do not depend on the enzymatic activity.

The capsid protein of Sindbis virus has 264 amino acid residues. The sequences of the alphavirus capsid proteins contain a strictly conserved Gly-Asp-Ser-Gly motif, which is identical to the motif at the active site serine of the chymotrypsin-like cellular proteases.[17,18] Mutagenesis studies confirmed that the Ser215 residue in this motif is required for the autoproteolytic activity of the capsid protein.[19,20] In addition, mutagenesis studies identified residue His141 as the second member, and possibly Asp163 as the third member, of the catalytic triad.[19,21]

2. Structures of the Capsid Protein

The crystal structures of the Sindbis virus capsid protein, and the related Semliki Forest virus capsid protein, reveal that the protein has a backbone fold that is identical to that of chymotrypsin-like serine proteases (Figure 1b), and confirm that residues Ser215, His141, and Asp163 form the catalytic triad of the enzyme (Table 1).[22−27] Moreover, the structures show that the C-terminus of the capsid protein, residues 261−264 in Sindbis virus capsid protein, is situated in the active site of the enzyme, showing interactions that are equivalent to those observed for peptide substrates with other cellular chymotrypsin-like serine proteases (Figure 1b). The side chain of the last residue of the capsid protein, Trp264, is positioned in the S_1 pocket, and the protease requires a Trp residue here for efficient catalysis.[28] Therefore, the structural information clearly demonstrates the molecular mechanism for the *cis* autocatalysis as well as the autoinhibition of the alphavirus capsid proteins. The natural capsid protein is not truly an enzyme, as it has a turnover number of 1.

The part of the alphavirus capsid protein that forms this chymotrypsin-like fold only covers about 140 residues (residues 114−250 in Sindbis virus). In contrast, chymotrypsin, trypsin, thrombin, and other related mammalian cellular proteases have about 230 residues. Structural and sequence comparisons of the chymotrypsin-like serine proteases show that they can be roughly classified into three categories (Figure 1a−f (1) mammalian proteases with about 230 residues, which require activation from zymogens and contain many disulfide bonds (five in chymotrypsin, Figure 1a), (2) bacterial proteases with about 190 residues, which do not require activation and contain fewer disulfide bonds (two in SGPA, Figure 1c), and (3) viral proteases with about 140 residues,

which do not need activation and contain no disulfide bonds (Figure 1b,d).[23,29] The differences in the sizes of these proteases are reflected by the deletion of many surface features in the viral proteases as compared to chymotrypsin, especially in the N-terminal domain (Figure 1a−f).

The large size of the S_1 pocket in alphavirus capsid protein favors aromatic residues such as Trp at P_1, a substrate specificity similar to that of chymotrypsin. However, the S_1 pocket in the viral protease is partly exposed to the solvent due to the deletion of surface features. Similarly, the side chain of the third member of the catalytic triad, Asp163, is partly exposed in the viral protease, whereas it is completely shielded from solvent in the cellular proteases.[23] The burial of this charged side chain is believed to enhance its ability to polarize the second member His residue.

3. RNA Recognition and Capsid Envelopment

In addition to carrying out the autoproteolysis, the C-terminal 150 residues of the capsid protein are also crucial for interacting with viral glycoproteins in the envelope. A hydrophobic pocket on the surface of the capsid protein may be used to bind two hydrophobic side chains in the tail of the glycoprotein.[25,27,30−32]

The N-terminal 110 residues of the alphavirus capsid proteins are highly divergent, and their conformation was not observed in the crystal structure.[22,33] These residues are important for the recognition of the viral RNA and the initiation of capsid formation.[34] These residues are therefore likely to be located on the inside of the viral capsid.

The alphavirus capsid protein autoprotease is likely to be under different evolutionary pressure as compared to its cellular homologues. It only has a turnover of 1 in its natural function. Therefore, optimal catalytic efficiency for this enzyme may not be crucial, and its major biological function may be the formation and envelopment of the capsid. The structural studies clearly show differences in the active site triad and the substrate binding pockets between the cellular and viral proteases as a result of the deletions in the viral protease. The small size of the viral protease may be due to possible evolutionary pressure of maintaining small genomes.[1] It would be very interesting to characterize the kinetic parameters of the alphavirus proteases and compare them with those of the cellular proteases. The original samples were purified from disrupted virus particles, and are therefore autoinhibited. However, the capsid proteins have recently been produced by recombinant expression in bacteria, thus opening the possibility of studying the kinetic properties of uninhibited capsid proteins.[25]

B. Hepatitis C Virus NS3 Protease

Hepatitis C virus (HCV) is a member of the *Flaviviridae* family,[35] which contains many other serious human pathogens, such as yellow fever virus (flavus means yellow in Latin), St. Louis encephalitis virus, and West Nile virus. The West Nile virus gained additional prominence during the recent outbreaks in the United States. HCV causes chronic infections of the affected individuals, and can lead to severe liver diseases including cirrhosis and hepatocellular carcinoma. There are roughly 170 million chronic HCV carriers in the world, or roughly about 3% of the world population.[35,36] HCV is an important public health threat, as 20−30% of the infected individuals will develop serious liver diseases. HCV infection is the primary cause for the need for liver transplantation, as currently there is no satisfactory therapy against this virus.

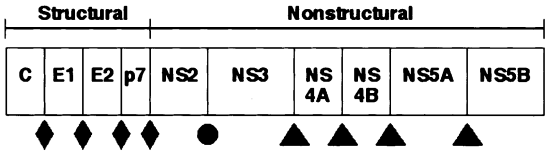

Figure 2. Polyprotein processing of hepatitis C virus. Cleavage sites of the NS3 protease are indicated by triangles, and the single cleavage site of the NS2/NS3 protease is indicated by the circle. Processing of the structural proteins is catalyzed by cellular proteases, indicated by the diamonds. The NS3 protease domain covers the N-terminal one-third of the NS3 protein, and the C-terminal two-thirds contains a helicase. Note that there is no cleavage site between the protease and the helicase domains.

The diameter of the HCV virus particles is about 500 Å.[35] It has a lipid envelope and a nucleocapsid containing a positive-sense ssRNA of 9500 bp. The viral genome encodes a single polyprotein of about 3000 amino acid residues. The structural proteins, located at the N-terminal end, are released from the polyprotein by cellular proteases (Figure 2).[35] Release of the nonstructural proteins from the polyprotein is predominantly catalyzed by the viral NS3 protease,[37] and the protease activity is required for the life cycle of the yellow fever virus.[38] Therefore, the NS3 protease of HCV has been studied intensively over the past few years as a target for new antiviral drugs.[39−42]

1. Structures of NS3 Protease

The amino acid sequences of NS3 proteases contain the Gly-X-Ser-Gly motif at the catalytic Ser residue, together with conserved His and Asp residues. This led to the suggestion that NS3 protease may be a chymotrypsin-like serine protease, with Ser139, His57, and Asp81 of HCV NS3 protease forming the catalytic triad of the enzyme (Table 1).[18,38,43,44] Mutagenesis of any of these residues disabled processing of the HCV polyprotein.[45−47] However, besides the Gly-X-Ser-Gly motif, there is no recognizable sequence homology between NS3 proteases and cellular chymotrypsin-like serine proteases.

Structural studies confirm that the NS3 protease is a chymotrypsin-like serine protease (Figure 1d).[48−53] The protease contains the two canonical Greek-key β-barrels, and it also has a unique 30-residue extension at the N-terminus (Figure 1d). Like the Sindbis virus capsid protein, the NS3 protease does not have many of the extended surface loops that are found in the cellular enzymes, as there are only about 140 residues in the two β-barrels. NMR studies showed that the N-terminal domain is significantly more flexible than the C-terminal domain in solution.[51]

The protease domain covers the N-terminal one-third of the NS3 protein, while the C-terminal two-

thirds of NS3 has helicase activity (Figure 2). The crystal structure of the full-length NS3 protein of HCV showed that the C-terminus of NS3 is located in the active site of the NS3 protease domain, confirming that the cleavage between NS3 and NS4 occurs in *cis*.[54] Processing at the other sites of the polyprotein is likely to occur in *trans*. The structure also contains a covalently linked NS4A cofactor (see below), by fusing the C-terminus of NS4A to the N-terminus of NS3 (Figure 1d). In addition, the structure showed that the helicase and the protease domains are segregated from each other, consistent with biochemical results that the domains can function separately.

The crystal structures revealed the binding site of zinc, which is known to activate the protease.[42,48,49] The ligands of the zinc atom include Cys97 and Cys99 from the linker between the two β-barrels, and Cys145 from the C-terminal barrel. The fourth ligand is a water molecule, which is hydrogen-bonded to His145. The zinc binding site is on the opposite side of the protease from the active site (Figure 1d), and its activating effect is likely due to stabilization of the protease. However, zinc binding may not be absolutely required for maintenance of the structure.[55]

2. NS4A Cofactor

NS4A contains 54 residues and is released from the N-terminus of the viral NS4 protein by the action of the NS3 protease (Figure 2). It is an amphipathic peptide, with a hydrophobic N-terminus and a hydrophilic C-terminus.[56] One of the functions of this peptide is the activation of the NS3 protease as a cofactor, and a synthetic peptide covering residues 21−34 of NS4A (GSVVIVGRIILSGR) is sufficient for this activation.[56−59] The NS4A cofactor is required for the cleavage at the NS3−NS4A and NS4B−NS5A junctions of the polyprotein (Figure 2), and enhances the processing at the other sites. The N-terminal 20 residues of the NS4A cofactor, which are highly hydrophobic, may help anchor the NS3/NS4A complex to the cell membrane.[50]

Crystal structures of the NS3/NS4A complex showed that the NS4A peptide forms a β-strand and is hydrogen-bonded to the first strand of the N-terminal β-barrel (Figure 1d).[49,50,54] On the other side, the NS4A cofactor is hydrogen-bonded to a β-strand in the unique N-terminal extension of the protease. As a result, the N-terminal barrel of the NS3/NS4A complex has eight β-strands. Residues that are buried in the NS3−NS4A interface (Figure 1d) have been shown to be important for the interactions on the basis of mutagenesis studies.[56−59] NMR studies suggested that the N-terminal extension of NS3 is mostly disordered in the NS4A complex,[60] although biochemical studies showed that this unique extension is crucial for the activation by NS4A.[49,61−63]

The NS4A binding site is more than 10 Å away from the active site of the NS3 protease (Figure 1d). Therefore, it is likely that NS4A mostly plays an indirect role, by stabilizing the N-terminal domain of the protease, especially the conformation of His57 and Asp81 residues in the catalytic triad, so that it is more catalytically efficient.[49,60] However, the NS4A cofactor may influence the binding of the P′ side of the substrates/inhibitors.[64] The presence of NS4A can produce up to 1000-fold activation of the protease, with the k_{cat}/K_m of the complex approaching 200000 M^{-1} s^{-1}.[65]

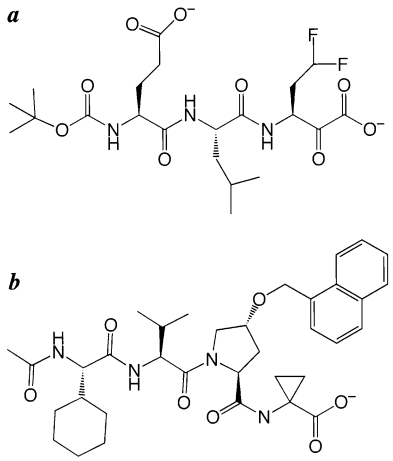

Figure 3. Chemical structures of inhibitors of HCV NS3 protease: (a) an α-ketoacid inhibitor with F_2Abu at the P_1 position;[68] (b) an inhibitor with 1-aminocyclopropylcarboxylic acid at the P_1 position.[69]

3. Inhibitors of the Protease

The natural substrates of the NS3 protease of HCV predominantly prefer a Cys residue at P_1 and Ser at P_1', and efficient hydrolysis requires the presence of P_6 to P_4' residues (Table 1).[42,66] The crystal structures suggest a shallow, nonpolar S_1 binding pocket, and Phe154 in the pocket may be crucial for the sequence specificity.

Peptidomimetic inhibitors of the protease have been developed on the basis of the preferred sequences of the natural substrates.[42,64] Extremely potent decapeptide inhibitors covering the P_6 to P_4' positions, with IC_{50} values of less than 200 pM, have been reported.[67] Norvaline, difluoroaminobutyric acid (F_2Abu), and 1-aminocyclopropylcarboxylic acid have been found to be good surrogates for the P_1 Cys residue (Figure 3).[68,69] Compounds containing α-ketoacids are potent, slow-binding inhibitors of the protease, with an overall K_i of 67 nM for a compound covering the P_4 to P_1 positions (Figure 3).[68] Modifications of the P_2 Pro residue in the natural substrate also afforded potent inhibitors of the protease, with an IC_{50} of 3.5 μM for a compound covering the P_4 to P_1 positions (Figure 3).[69]

Structures of the protease in complex with inhibitors covering the P_4 to P_1 positions and containing F_2Abu at P_1 have been determined by both crystallographic and NMR methods.[55,70] The inhibitors are bound in the extended conformation, forming an antiparallel β-sheet with the protease, similar to the binding mode observed for chymotrypsin inhibitors (Figure 1a,d). The P_1 group interacts with the side chains of Phe154, Val132, Leu135, and the aliphatic portion of Lys136. NMR studies also confirm that the F_2Abu side chain is close to the phenyl ring of Phe154.[70] The structures revealed tight hydrogen-bonding between the His57 and Asp81 side chains

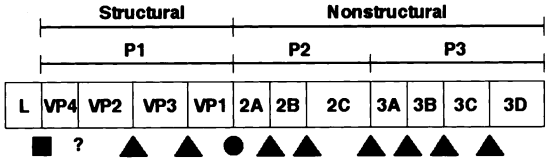

Figure 4. Polyprotein processing of picornaviruses. Cleavage sites of the 3C, 2A, and leader proteases are indicated by triangles, a circle, and a square, respectively. The enzyme catalyzing the cleavage between VP4 and VP2 is currently unknown. The polyprotein is divided into three segments, P1, P2, and P3. The 2A and leader proteases are present in only some of the picornaviruses. L represents the leader protease.

in the triad, and it has been suggested that the protease undergoes an induced fit in the presence of inhibitors and substrates.[70] The NS4A cofactor preorganizes the structure of the protease, and inhibitor binding induces further, small changes in the protease.[71] Transfer NOE and differential line-broadening techniques have also been used to study the interactions between NS3 protease and inhibitors.[72,73] Inhibitor binding to the protease has also been studied by fluorescence resonance energy transfer (FRET) techniques, using a dansylated hexapeptide inhibitor.[74] The preference for acidic residues at the P_6 position of the substrate (Table 1) may be related to a positive surface patch in the S_5/S_6 pockets.[75]

Besides peptidomimetic compounds, natural product inhibitors[76,77] and potent RNA aptamer inhibitors[78] of the NS3 protease have also been identified. An antibody that recognizes the region near Asp81, the third member of the triad, is also a potent, competitive inhibitor of the protease.[79,80] In a different approach, a cyclic hexapeptide lead compound was developed on the basis of the inhibition of the NS3 protease by minibodies (minimized antibody variable domains).[81] A bisbenzimidazole-based, Zn^{2+}-dependent inhibitor has also been described.[82]

III. Chymotrypsin-like Cysteine Proteases, Picornavirus 2A and 3C Proteases

Picornaviruses are small, nonenveloped viruses containing a positive-sense ssRNA genome.[83] The name picorna is derived from "pico" (small) and "RNA". This family of viruses contains many well-known human and animal pathogens, such as polioviruses (poliomyelitis), rhinoviruses (common cold), foot-and-mouth disease virus (FMDV), and hepatitis A virus.

The icosahedral virions of picornaviruses have diameters of about 300 Å. The viral genome of about 8000 bases encodes a single polyprotein, which is processed cotranslationally by virally encoded proteases, including the 3C, 2A and leader proteases (Figure 4).[1,83−86] Most of the processing of this polyprotein is catalyzed by the 3C protease or its precursor 3CD, and all picornaviruses encode this enzyme. On the other hand, the 2A and leader proteases are present only in some of the picornaviruses. The 2A protease catalyzes the release of the structural polyprotein, and the cleavage site is located at the N-terminus of the protease (Figure 4).[87] The leader protease catalyzes the release of itself from the polyprotein, with the cleavage site at its C-terminus (Figure 4).[88]

The 2A and leader proteases also play an important role in inhibiting host cell protein synthesis by cleaving the eukaryotic initiation factor 4G (eIF4G). This cleavage blocks translation from the 5′-capped mRNA of the host cells, whereas translation from the viral mRNA is not affected as it is initiated from an internal ribosome entry segment (IRES).[83] Recent studies suggest however that the cleaved eIF4G can still support translation from capped mRNA, although not as efficiently as from viral RNA.[89] In addition, other enzymes can also cleave eIF4G, including the 3C protease of FMDV,[90] HIV protease,[91] and cellular caspases and other proteases.[92] The leader protease is a papain-like cysteine protease, and will be discussed in more detail in the next section.

A. Structures of the Proteases

Both the 3C and the 2A proteases are cysteine proteases.[1] Sequence analysis predicted that these two proteases have backbone folds that are similar to those of chymotrypsin, a serine protease, rather than to those of other cysteine proteases, even though the overall sequence identity between the viral protease and chymotrypsin is below 20%.[22,29,93] The 2A and 3C proteases contain the Gly-X-Cys-Gly motif that is reminiscent of the Gly-Asp-Ser-Gly motif at the active site of the chymotrypsin-like serine proteases. Crystal structures of these proteases from several different picornaviruses (human rhinoviruses 2 and 14, hepatitis A virus, and poliovirus) confirmed that the 2A and 3C proteases are chymotrypsin-like cysteine proteases.[94−99]

There are only 182 residues in the 3C protease of human rhinovirus 14 (HRV14). Excluding residues in an N-terminal α-helix that is unique to the 3C proteases, only about 170 residues form the double-barrel chymotrypsin-like fold (Figure 1e). Therefore, the surface features of this enzyme are more similar to those of the bacterial proteases, with about 190 residues (Figure 1c).

In comparison, the 2A protease of HRV2 has 142 residues, even fewer than the 3C protease. The N-terminal domain of the 2A protease contains only a part of the Greek-key β-barrel, in the form of a four-stranded antiparallel β-sheet (Figure 1f).[98] This represents additional deletions in the fold of the chymotrypsin-like proteases, and the 2A protease is the smallest enzyme known in this family. Another feature of the 2A protease is that it contains a tightly bound Zn ion near the beginning of the C-terminal domain (Figure 1f). The Zn ion is coordinated tetrahedrally by the side chains of three Cys residues and one His residue, which are highly conserved among the 2A proteases. Removing the Zn ion from the protease requires denaturation of the enzyme.[100,101] Structural and biochemical analyses suggest that Zn binding may be important for the stability of the enzyme, possibly to compensate for the instability due to the small N-terminal domain.[98] This binding site is equivalent, but not identical, to that in the NS3 protease of hepatitis C viruses (see section II.B and

Figure 1d), although the binding affinity in HCV is much weaker.

B. Active Sites of the Proteases

The catalytic triad of the 3C protease of HRV14 contains residues Cys147, His40, and Glu71 (Table 1). In hepatitis A virus (HAV) 3C protease, the residue that is equivalent to Glu71 is Asp84, but its side chain is pointed away from the second member His44 residue.[94] It has been suggested that the Tyr143 may function as the third member in HAV 3C protease.[94,96]

The natural substrates of 3C proteases generally have Gln at the P_1 position, Gly at the P_1' position, and a small aliphatic side chain at P_4 (Table 1).[99] The crystal structures suggest that the P_1 Gln side chain is recognized by the conserved His161 and Thr142 residues in the S_1 pocket of the HRV14 3C protease,[94,95] confirming earlier predictions based on sequence analysis.[29,93] Assays with recombinant 3C proteases in vitro showed that the P_5 through P_2' residues of the substrate are absolutely required for cleavage.[102,103] As both the N- and C-termini of the protease are far from the active site (Figure 1e), the 3C protease is likely to function exclusively in *trans* in its cleavage of the viral polyprotein. The 3C protease can be activated by the presence of 0.8 M Na_2SO_4, suggesting possible induced-fit behavior for this enzyme.[104]

The catalytic triad of the 2A protease of HRV2 contains Cys106, His18, and Asp35 (Table 1),[98] which is also supported by mutagenesis studies.[105,106] Besides interacting with His18, the third member Asp35 is also involved in a large network of hydrogen-bonding interactions. It has been proposed that the Cys and His residues exist as an imidazolium−thiolate ion pair, similar to that of papain.[107] However, the activity of the 2A protease is limited to a much smaller pH range as compared to that of papain,[107] possibly due to the misalignment of the Cys−His residue pair in the active site.[98]

The substrate preference of the 2A protease is mostly defined by residues at the P_4, P_2, and P_1' positions (Table 1).[108,109] A Thr residue at P_2 is strongly preferred for cleavage by the protease, and a model of the enzyme/substrate complex suggests this residue may hydrogen-bond with Ser83 of the protease.[98] In comparison, the S_1 pocket appears to be rather open and can accommodate a variety of side chains.[98,108] The substrate preference of the protease was also confirmed by yeast two-hybrid screening, which revealed a Leu-X-Thr-Z motif (X for any residue, Z for a hydrophobic residue) for binding to the protease.[110]

It is believed that the 2A processing of the viral polyprotein occurs in *cis*, meaning that the enzyme can catalyze a cleavage at its own N-terminus (Figure 4).[87] By adjusting the main chain conformation of the first five residues, it is possible to bring the N-terminus into the active site, giving support to the *cis* cleavage by this enzyme.[98]

C. Inhibitors of the Proteases

Due to their important roles in the processing of the viral polyprotein, the 2A and especially the 3C proteases are attractive targets for the design and development of new chemotherapeutic agents against picornavirus infections.[39,111−113] This is especially relevant for rhinovirus, as the presence of more than 100 serotypes of this virus essentially precluded the development of a successful vaccine. In comparison, poliovirus infection was practically eradicated through vaccination programs.

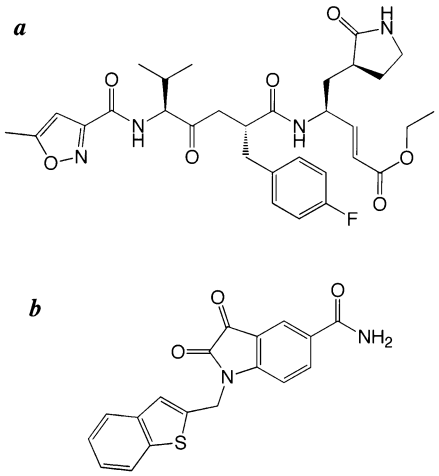

Figure 5. Chemical structures of inhibitors of rhinovirus 3C protease: (a) AG7088, currently in clinical trials;[112,131] (b) a cyclic α-ketoamide isatin inhibitor (this scaffold was identified from structure-based design and screening[124,127]).

A variety of mechanism-based covalent inhibitors of the 3C protease have been reported over the years. These include iodoacetyl peptides,[114,115] peptide aldehyde inhibitors,[116−118] peptide inhibitors with azaglutamine derivatives as analogues of the P_1 glutamine residue,[119] and peptide inhibitors containing activated ketones.[120−122] Nitric oxide is an inhibitor of the 3C protease by covalently modifying the active site cysteine residue.[123] Homophthalimides, originally identified from screening, are nonpeptidic inhibitors of both the 3C and 2A proteases.[124,125] A natural product isolated from a Chinese herb, 2-methoxystypandrone, is a moderately selective inhibitor of the 3C protease.[126]

The design and development of novel 3C protease inhibitors have been aided substantially by the availability of the crystal structures of the protease, both in the free enzyme form and in complex with various inhibitors.[112] The peptidomimetic inhibitors are bound in the extended conformation, forming an antiparallel β-sheet with the protease (Figure 1e). Examination of the binding modes of the P_2, P_1, and P_1' residues led to the design of reversible, nonpeptidic, cyclic α-ketoamide isatin inhibitors, with K_i values against HRV14 3C protease as low as 2 nM (Figure 5).[127] The binding mode of the designed inhibitors was confirmed by structural studies of the enzyme complex. Interestingly, the isatin scaffold was also identified from an inhibitor screening effort.[124]

Structural information was also crucial in the design and optimization of inhibitors containing Michael acceptors.[128,129] Incorporation of *trans*-α,β-unsaturated esters into substrate-based peptide inhibitors allows the attack by the active site Cys

residue, leading to covalent, irreversible inhibition of the 3C protease. Crystal structures of the protease in complex with such inhibitors confirm the mechanism of inhibition,[128] and structure-based design helped the development of a large collection of inhibitors containing Michael acceptors.[130] The modifications include methylation of the P_2 amide nitrogen,[131] and the replacement of this nitrogen by an oxygen[132] or methylene group.[133] This amide nitrogen is exposed to the solvent in the complex and is therefore amenable for modifications.

One of the most potent compounds in this series is AG7088 (Figure 5), with a $k_{obs}/[I]$ of 1.47×10^6 M^{-1} s^{-1} and an EC_{90} of less than 0.1 μM against 48 different serotypes of HRV.[112,131] This compound is currently in clinical trials. It contains a lactam moiety as a mimic for the P_1 glutamine side chain of the natural substrate. Crystal structures showed that the carbonyl oxygen of the Gln side chain is recognized by the side chain of His161, and the amide nitrogen is hydrogen-bonded to the main chain carbonyl of Thr142. However, part of this amide group is exposed to the solvent, which makes it possible for the incorporation of lactams as mimics for this side chain. The P_2 amide nitrogen has been replaced by a methylene group.[133] The structure of this inhibitor in complex with the 3C protease of HRV2 has also been determined (Figure 1e).[112] In addition to lactams, benzamides have also been developed as analogues of the glutamine residue.[134]

IV. Papain-like Cysteine Proteases, Picornavirus Leader Protease

Besides the 3C and 2A proteases (see the previous section), some of the picornaviruses (including foot-and-mouth disease virus, FMDV) also encode another protease, known as the leader protease as it is the first product in the viral polyprotein (Figure 4).[83] Like the 2A protease, this protease contributes to the inhibition of host protein synthesis by cleaving eIF4G, although at a different site than that for 2A. In addition, the leader protease catalyzes its own release from the viral polyprotein, likely via an intramolecular (*cis*) reaction.[135] Interestingly, the sequence at this cleavage site appears to be rather different from that in eIF4G (Table 1).

Crystal structures of the FMDV leader protease confirm earlier studies that the protease is a papain-like enzyme, with Cys51, His148, and Asp163 forming the catalytic triad (Figure 1g).[99,136−140] In comparison, the catalytic triad in papain has an Asn residue as the third member (Cys25, His159, Asn175, Figure 1h). However, the part of the leader protease that forms the papain-like fold contains only about 150 residues, whereas papain has 210 residues. Therefore, the structure of the FMDV leader protease lacks most of the long surface loops as compared to papain (Figure 1g,h),[139] a situation that is reminiscent of the viral chymotrypsin-like serine proteases (Figure 1a−f). The third member Asn175 is completely buried in the papain structure, whereas the Asp163 side chain is exposed to the solvent in the leader protease.

Detailed structural and sequence differences between the leader protease and papain have important impacts on the catalytic properties of these enzymes. The leader protease appears to be rather sensitive to cation concentration and pH variations.[140] In addition, the substrate specificity of the two proteases is different, with leader protease unable to cleave standard papain substrates while papain can cleave leader protease substrates.[140]

At the C-terminus, the FMDV leader protease has a long extension that enables it to reach the active site of the enzyme.[139] This structural feature is absent in papain, but allows the *cis* self-processing of the leader protease from the viral polyprotein.[135] In some of the crystals studied, this C-terminal extension is docked into the active site of a neighboring molecule, allowing an examination of the binding mode of the substrate (Figure 1g).[139] The C-terminal peptide assumes an extended conformation, and interactions with the leader protease are similar to those observed for papain/peptide complexes. The P_1 and P_2 residues make the most extensive interactions with the protease. The P_1 Lys side chain is bound in a narrow cleft, and its ammonium group interacts with a negatively charged surface patch. The P_2 Leu is buried in a hydrophobic pocket, and a hydrophobic residue is required at this position in both types of substrates (Table 1).[135,139] Similarly, the presence of negatively charged surface patches in the S′ sites may explain the preference for Arg at the P_1' position of the eIF4G substrates.

V. HIV Protease

HIV is an enveloped retrovirus with a positive-sense RNA genome.[141] Upon infection of the host cell, the viral RNA is reverse-transcribed to DNA, which is then integrated into the host genome to produce the provirus. The mature HIV particles are spherical, about 1000 Å in diameter. The nucleocapsid of the virus is conical in shape. A dimer of the RNA genome, about 13 kbp in length, is contained in the nucleocapsid. HIV is the etiological agent for the AIDS epidemic, and roughly 36 million people worldwide are infected by this virus.[142]

The genome of HIV encodes an aspartic protease, HIV protease, that is crucial for the processing of the viral polyprotein and the maturation of the virus particles.[143] Therefore, the protease is an attractive target for the design of anti-HIV agents, making HIV-1 protease one of the best studied enzymes. The reader is referred to the many excellent reviews cited here for more detailed information.[141,144−148]

The HIV-1 protease is a homodimer, with each monomer having 99 amino acid residues. The structure of the protease is related to that of cellular aspartic proteases such as pepsin (Figure 1i), with the distinction that the cellular proteases are monomers, with two domains that are structurally equivalent to the monomers of HIV protease.[149,150] The active site of HIV protease is located at the dimer interface, with each monomer contributing one of the catalytic Asp residues (Asp25 and Asp25′, Table 1). The active site and the bound substrate/inhibitor are

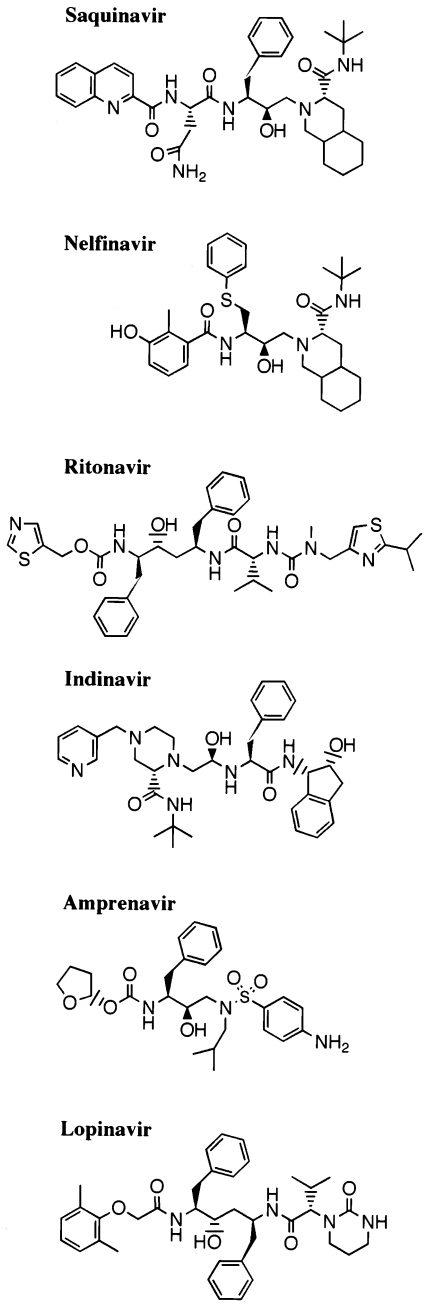

Figure 6. Chemical structures of HIV protease inhibitors approved for clinical use. These include saquinavir, ritonavir, indinavir, nelfinavir, amprenavir, and lopinavir.

shielded from the solvent by two flaps in the structure, one from each monomer (Figure 1i).

The significant amount of research on HIV-1 protease and its inhibitors has led to the approval of several protease inhibitors for clinical use in the treatment of AIDS, including saquinavir, ritonavir, indinavir, nelfinavir, amprenavir, and lopinavir (Figure 6).[39,145,151−158] Moreover, HIV protease inhibitors are the first peptidomimetic compounds that have advanced into the clinic. The highly active antiretroviral therapy (HAART), a combination therapy of HIV protease and reverse transcriptase inhibitors, has dramatically changed the landscape of HIV therapy. Gaining efficacy against resistant viruses is the challenge for the development of new anti-HIV agents.[159−165]

The design and development of these inhibitors have been aided significantly by the large amount of structural information on this enzyme, and there are hundreds of crystal structures of the protease in complex with a variety of different inhibitors.[145,148,166,167] This wealth of structural information has aided inhibitor optimization as well as the design of completely novel inhibitors. As the protease is a homodimer, inhibitors with internal 2-fold symmetry have been successfully designed. The structural studies reveal the presence of a conserved water molecule near the active site in all complexes with peptidomimetic inhibitors. The information was used in the design of novel, nonpeptidic inhibitors that replace this molecule, and one such inhibitor is currently in clinical trials.[145,148,168] Overall, structure-based drug design played a crucial role in the design and development of many HIV protease inhibitors and their rapid advancement into the clinic.

VI. Herpesvirus Proteases, a New Class of Serine Proteases

Herpesviruses are enveloped dsDNA viruses.[169] The icosahedral nucleocapsids of herpesviruses have diameters of about 1250 Å,[170] enclosing DNA genomes of between 130 and 250 kbp. The sizes of the enveloped virions are much larger, with diameters of up to 3000 Å, depending on the thickness of an amorphous protein layer (known as the tegument or matrix) between the nucleocapsid and the envelope.

Herpesviruses afflict most species of the animal kingdom, and each animal generally can be infected by several different herpesviruses.[169] For example, nine herpesviruses are currently known to infect humans, and they have been classified into three subfamilies. The α herpesviruses include herpes simplex virus type 1 (HSV-1), HSV-2, and varicellar-zoster virus (VZV). The β herpesviruses include human cytomegalovirus (HCMV), human herpes virus 6A (HHV6A), HHV6B, and HHV7. The γ herpesviruses include Epstein−Barr virus (EBV) and Kaposi-sarcoma-associated herpesvirus (KSHV, also known as HHV8). Despite their common name, the herpesviruses of the three subfamilies have substantial differences in their biology and genome content and the sequences of the viral proteins.[169]

Herpesvirus infections can cause severe health problems in humans and other animals. Symptoms from HSV infections were noted by the ancient Greeks, and the skin lesions caused by the infections led to the name herpes for these viruses.[171] HSV-2 causes primary and recurrent genital infections, making it a serious pathogen for sexually transmitted diseases. Infection by HCMV is widespread in the general public, with up to 90% of the urban population carrying this virus, although most of these infections are clinically asymptomatic. On the other hand, HCMV is a leading opportunistic infectious pathogen in individuals with suppressed or compromised immune systems, causing severe health problems such as pneumonia, retinitis, and death in these patients. The clinical importance of HCMV has increased substantially over the past 30 years due

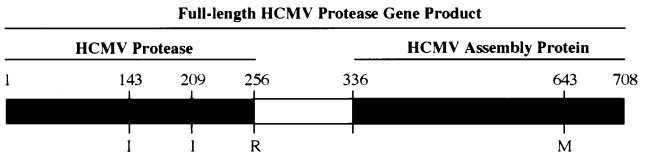

Figure 7. Maturational processing of herpesvirus protease and assembly protein. The assembly protein is identical to the C-terminal domain of the full-length protease gene product. The maturation (M), release (R), and internal (I) cleavage sites are indicated. The protease activity is fully contained within the N-terminal domain, shown in gray.

to the advent of organ transplantation and the emergence of the AIDS epidemic.[172]

Current chemotherapeutic agents for herpesvirus infections are targeted against the viral DNA polymerases.[171] For example, acyclovir, a guanine nucleoside analogue, is an efficacious agent for the treatment of HSV-1 and HSV-2 infections. However, acyclovir has little potency against HCMV infections, due to the significant differences in the biology of α and β herpesviruses. Moreover, viral resistance to current drugs is becoming an increasingly more serious problem. Herpesvirus protease has been studied intensively over the past few years as it represents an alternative target for the development of novel antiherpes chemotherapeutic agents.[39,173−177]

A. Herpesvirus Protease Is Required for the Viral Life Cycle

Herpesvirus protease was first identified in 1991 from studies on HSV-1 and HCMV.[178,179] The protease is required for the life cycle of the virus, as it carries out the maturational processing of the viral assembly protein (Figure 7). The formation of the herpesvirus capsid requires a scaffold that is built from the assembly protein. The subsequent packaging of the viral DNA genome is dependent on the proteolytic processing of the assembly protein. An HSV-1 temperature-sensitive mutant that has a defect in this processing cannot package the viral genome, and can only produce aberrant empty capsids at the nonpermissive temperature,[180,181] confirming the functional requirement of the protease.

The herpesvirus protease is the only protease that has been identified so far in the herpesvirus genome. The protease and the assembly protein are encoded by overlapping genes.[176] The gene for the assembly protein uses the 3′-portion of the gene for the protease (Figure 7). The maturational processing of the assembly protein occurs at the M site, near the C-terminus of the assembly protein. In addition, the protease catalyzes a cleavage at the R site, which releases an N-terminal fragment of about 250 residues from the full-length protease gene product. This fragment retains all the catalytic activity of the full-length protease protein, and is generally referred to as the herpesvirus protease (Figure 7). In the case of HCMV protease, the enzyme catalyzes two additional cleavages within the protease itself, at residues 143 and 209 (Figure 7). The cleavage at residue 143 produces a two-chain form of the protease that is still catalytically active.[182,183]

Table 2. Summary of Kinetic Parameters for HCMV Protease[a]

enzyme	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (s^{-1} M^{-1})
wild type	0.033	10.1	3300
H157E	0.0041 (8)	14.5 (0.7)	283 (12)
H157A	0.0033 (10)	21.8 (0.5)	151 (22)
H157A, S134A	0.00069 (48)	35.6 (0.3)	19 (170)
trypsin (D102N)	0.0011 (38000)	90 (1.6)	12 (24000)
subtilisin (D32A)	0.0023 (19000)	480 (0.4)	4.8 (52000)

[a] Data from ref 193. The numbers in parentheses are the ratios between the wild-type and mutant values. Values for the trypsin and subtilisin mutants are from the literature.[191,192]

B. Structures of Herpesvirus Proteases

The proteases within the individual herpesvirus subfamilies are highly conserved. For example, the proteases of HSV-1 and HSV-2 share 90% amino acid sequence identity. In contrast, the conservation among different subfamilies is much weaker, with HSV-2 and HCMV proteases sharing only 28% sequence identity. The sequences of herpesvirus proteases do not share any homology with other proteins in the database, and a cellular homologue of this protease has so far not been found.

The crystal structures of the proteases from HCMV, VZV, HSV-2, and KSHV have been reported.[184−190] Consistent with their unique amino acid sequences, the crystal structures show that herpesvirus proteases have a novel polypeptide backbone fold (Figure 1j), therefore establishing them as a new class of serine proteases.[3] The structure contains a central, mostly antiparallel seven-stranded β-barrel, which is surrounded by eight helices (Figure 1j). This β-barrel core is the most conserved structural feature among the proteases of the three herpesvirus subfamilies. In contrast, the conformations of the loops and helices on the surface have large variations among the different proteases, reflecting both sequence divergence and inherent structural flexibility of these enzymes.[177] Several of the surface loops are actually disordered in the crystal structures (Figure 1j).

C. A Novel Ser-His-His Catalytic Triad

The active site of the herpesvirus protease is located on the surface of the β-barrel and contains a novel Ser132-His63-His157 catalytic triad (residue numbering according to HCMV protease) (Table 1, Figure 1j). The presence of Ser132 and His63 in the active site of herpesvirus proteases has been determined from biochemical and mutagenesis studies.[173] The presence of a second His residue in the catalytic triad is unprecedented, as most classical serine proteases contain an acidic residue (Asp or Glu) as the third member. Nonetheless, the His157 side chain is positioned similar to that of the acidic third member in other proteases (Figure 1), giving support for the Ser-His-His catalytic triad in herpesvirus proteases.[177]

The exact role of the third member His157 residue is still not well understood. Removing the third member in classical serine proteases can produce greater than 20000-fold loss in catalytic activity (k_{cat}) (Table 2).[191,192] In contrast, the effect of removing the

third member on HCMV protease is much smaller, only about a 10-fold loss for the H157A mutant.[193] The H157D and H157E mutants, which would produce the classical catalytic triad in herpesvirus proteases, actually have about a 3-fold loss in activity relative to the wild-type enzyme (Table 2). Structural studies on the H157A mutant showed that the activity is partially rescued by a water molecule that mimics the His157 side chain.[193] The S134A/H157A double mutant, which removes the contribution of this water, has a 48-fold drop in activity (k_{cat}), which is still much smaller than the loss for the classical serine proteases.

An interesting observation from these studies is that the k_{cat} values of the mutants that contain only a functional diad are actually similar among the different serine proteases, about 0.001 s^{-1} (Table 2). This suggests that the activity of the catalytic diad in herpesvirus protease is similar to that in classical serine proteases (chymotrypsin and subtilisin). The His157 third member makes a much smaller contribution to the catalysis by herpesvirus proteases, possibly due to its weaker hydrogen-bonding capability and its partial exposure to the solvent in the structure.[193] As a consequence, herpesvirus proteases are slow enzymes. The k_{cat} with the best peptide substrate is about 0.1 s^{-1},[176,194] whereas that for chymotrypsin and subtilisin is about 60 s^{-1} (Table 2).

D. Inhibitors of Herpesvirus Proteases

As a target for the development of new antiherpes agents, a large number of inhibitors against herpesvirus proteases have been reported over the past few years.[39,174−176] These compounds can be roughly divided into three categories—active site inhibitors, cysteine modifiers, and natural products. The cysteine modifiers make covalent changes to Cys161 and Cys138 of HCMV protease,[195,196] and inhibit the protease possibly by the creation of steric hindrance in the active site as Cys161 is likely to be in the S_1' pocket. Surprisingly, a compound that is believed to modify Cys202, which is located in a flexible loop far away from the active site, can also irreversibly inhibit the protease.[197] The natural products are identified from high-throughput screening,[198,199] but their mechanism of inhibition is currently unknown.

The active site inhibitors include peptidomimetic compounds,[200,201] lactams,[202−208] oxazinones,[209−213] and benzothiopyran-4-ones.[214] A common feature among these inhibitors is the presence of an activated carbonyl, for example, α-ketoamide or trifluoromethyl ketone groups in the case of peptidomimetic compounds (Figure 8).[201] X-ray and NMR studies confirm the expectation that these compounds modify the Ser132 nucleophile, forming a reversible, tetrahedral intermediate (Figure 1j).[215,216] The oxyanion of this intermediate is hydrogen-bonded to the main chain amide of Arg165, confirming that it is the oxyanion hole of the enzyme. Both Arg165 and Arg166 are strictly conserved among herpesvirus proteases, and the R166A mutant has a 1500-fold loss in the k_{cat} of the enzyme.[217] The Arg166 side chain may contribute indirectly to the oxyanion hole of the enzyme, via two

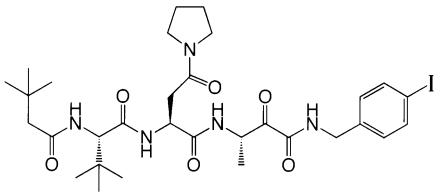

Figure 8. Chemical structure of BILC 821, a peptidomimetic inhibitor of HCMV protease. It contains an α-ketoamide as the activated carbonyl.[201,215]

structural water molecules at the bottom of the S_1 binding pocket.[189,193] It will be interesting to see whether one or both of these waters can be replaced by atoms in well-designed inhibitor molecules, as has been done successfully in the design of novel and potent inhibitors of HIV-1 protease.[168]

E. Inhibitor Binding and Induced-Fit Behavior

Despite the unique backbone fold, herpesvirus proteases recognize their peptide inhibitors (and possibly substrates) using a molecular mechanism that is conserved with the classical serine proteases, establishing herpesvirus proteases as another example of convergent evolution for serine proteases.[215] The peptide molecule is bound in an extended conformation, also shown by NMR studies,[218] making hydrogen bonds to a β-strand of the protease via the P_3 and P_1 residues (Figure 1j). In contrast to the classical serine proteases, where the nuleophilic serine residue comes from a different region of the structure, the catalytic Ser132 in herpesvirus proteases is located in the same β-strand that hydrogen-bonds to the substrate/inhibitor peptides.[215] This therefore represents a much more compact arrangement of the catalytically essential residues of the protease.

While the classical serine proteases behave mostly as lock-and-key enzymes, structural and biochemical studies show that herpesvirus proteases behave in an induced-fit manner,[219] with large conformational changes upon the binding of the peptide inhibitors.[177,215] These changes help define the S_3 and S_1 binding pockets of the protease, which are absent in the free enzyme structures. Fluorescence experiments in solution indicated a blue shift in the tryptophan emission spectra upon inhibitor binding, suggesting a conformational change in the protease.[216] The reporter for this fluorescence change is Trp42, consistent with the structural studies.

The structural studies reveal a small S_1 pocket, in agreement with the preference for Ala residues at the P_1 position of the substrates (Table 1).[173] The P_2 side chain is exposed on the surface of the structure, and the natural substrates generally have a hydrophilic residue at this position. The natural substrates,[173] and the structure−activity relationships of peptidomimetic inhibitors,[201] consistently indicate the preference for aliphatic side chains at the P_3 position. On the other hand, the crystal structures show a rather hydrophilic S_3 binding pocket, with the side chain of the strictly conserved Arg166 buried at the bottom of this pocket.[177] Interestingly, introducing acidic side

chains at the P_3 position abolished the activities of the inhibitors.

F. Dimerization Is Required for Catalytic Activity

Herpesvirus proteases exist in a monomer−dimer equilibrium in solution, but only the dimer form of the enzyme is catalytically active.[176,220−224] Kosmotropic agents can significantly activate herpesvirus proteases, possibly due to the stabilization of the dimer of the enzyme.[225,226] In the crystal structures, only the dimer forms of the enzymes are observed, but there is a complete active site in each monomer of the dimer, with the two active sites far from each other and the dimer interface.[177] As another manifestation of the differences among the three subfamilies of herpesviruses, there are significant differences in the dimer organization of their proteases.[177]

The molecular basis for the dimerization requirement of herpesvirus proteases was revealed from mutagenesis, biophysical, and structural studies.[227] Mutations in the dimer interface can produce severe reductions (1000-fold) in the catalytic activity of the enzyme, but the mutants are still dimeric in solution. This suggests that dimerization in itself is not sufficient for the activity of herpesvirus proteases. Structural studies of the dimer-interface mutant show a large reorganization of the dimer interface, which indirectly causes the disordering the oxyanion hole of the enzyme. Therefore, it appears that dimerization is required to stabilize the oxyanion hole of herpesvirus proteases.

VII. Adenovirus Protease, a Novel Cysteine Protease

Adenoviruses are dsDNA viruses that infect humans, other mammals, and birds.[228] They have icosahedral virions with diameters of about 1000 Å, and the viral genome contains about 36000 base pairs. Their name is derived from the fact the first viruses were isolated from the adenoids. In addition to the adenoids, they can infect other sites in the respiratory tract, as well as the eye and the gastrointestinal tract. Adenoviruses can cause acute respiratory disease, pneumonia, gastroenteritis, and other disorders in humans, especially children, although many adenovirus infections are clinically asymptomatic.[229] As a vector for the delivery of foreign DNAs in gene therapy, adenovirus has received significant attention over the past few years.

A. The Protease Is Essential for Producing Infectious Virions

The adenovirus protease catalyzes the maturational processing of six proteins in newly assembled viral capsids, and this processing is essential for the production of infectious virus particles.[230,231] The protease was originally identified from genetic studies of a temperature-sensitive (ts) mutant of the virus.[232−234] At the nonpermissive temperature, the ts mutant cannot produce mature infectious particles due to a defect in the processing of virion proteins. Recent studies show that this protease may also be required for the cellular entry process of the virus.[235,236]

The protease has about 200 amino acid residues (molecular weight of 23000) and is highly conserved among the different adenoviruses. However, there is no sequence homology between the protease and other proteins in the database. It was classified as a cysteine protease on the basis of biochemical and mutagenesis studies,[237,238] and it is sensitive to common active site cysteine protease inhibitors, such as Zn^{2+} and E64.[239] Cys122, one of two strictly conserved Cys residues among the proteases, is the active site nucleophile.[240,241] The strictly conserved His54 is the second member of the catalytic machinery (Table 1).[242] The order of these residues in the primary sequence (His54-Cys122) is different from that of the archetypical cysteine protease papain (Cys25-His159).

B. Structure of Adenovirus Protease

The crystal structure confirms that the adenovirus protease belongs to a new class of cysteine proteases (Figure 1k).[4,243] The protease has a novel backbone fold, consistent with its unique amino acid sequences. The structure contains a central mixed five-stranded β-sheet that is surrounded by helices on both sides. Remarkably, despite its unique fold, the arrangement of the catalytic residues of adenovirus protease is similar to that in papain, including the catalytic triad (Cys122-His54-Glu71) as well as the oxyanion hole (Gln115) of the protease (Figure 1h,k). Moreover, the overall organization of the structure (Figure 1k) is actually similar to that of papain (Figure 1h) and picornavirus leader protease (Figure 1g), even though the positions of the structural features are different in the primary sequences of the proteases. The structural conservation of the active site suggests that adenovirus protease is likely to have the same catalytic mechanism as papain.[243]

A model for the structure of a substrate complex of the protease, built on the basis of the structural conservation in the active site region with papain,[243] can explain the substrate preferences of the adenovirus protease (Table 1).[244] The P_4 and P_2 residues are the major determinants of specificity, with P_2 being a Gly residue. In the model, the substrate is in an extended conformation and forms three hydrogen bonds with the protease. The P_2 and P_4 residues make contacts with the protease, whereas the P_1 and P_3 side chains are pointed away. Specifically, the Gly residue at P_2 lies over the indole ring of the strictly conserved Trp55 residue, just after the His54 residue in the catalytic triad.

C. Cofactors of Adenovirus Protease

The activity of adenovirus proteases can be enhanced significantly by the presence of a peptide cofactor,[238,245] which produces a 120-fold increase in the k_{cat} and 10-fold decrease in the K_m of the enzyme (Table 3).[246] The search for cofactors of the protease was spurred by the observation that protease samples purified recombinantly have much lower activity compared to those isolated from disrupted viruses, especially with artificial substrates.[247] The peptide cofactor comes from the C-terminus of virion protein VI. This 11-residue fragment, known as pVIc, has the

Table 3. Summary of Kinetic Parameters for Adenovirus Protease[a]

enzyme	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
protease alone	94.8	0.0023	24
protease + pVIc	9.9	0.27	27400
protease + DNA	9.2	0.025	2700
protease + pVIc + DNA	3.4	2.78	828000

[a] Data from ref 246. The assays were performed using a fluorogenic substrate, (Leu-Arg-Gly-Gly-NH)$_2$-rhodamine.[245]

sequence GVQSLKRRRCF.[248] It is likely that the peptide is released from the virion protein VI by the action of the protease itself, as the cleavage site (IVGL↓GVQS) matches the substrate preference of the protease.

In addition to the pVIc peptide, viral DNA is also a cofactor that can activate the enzyme,[245,248] giving rise to a 3−10-fold increase in k_{cat} (Table 3).[246,249] The DNA cofactor also has an impact on the binding of the peptide cofactor, lowering the K_d of the protease/peptide complex from 4 to 0.09 μM.[249] There does not appear to be any specific recognition of the actual base sequences of the DNA, as the protease can be activated by the presence of polyanions in general.[245] There are four positively charged patches on the surface of the protease, consistent with the binding of polyanions.[243] Detailed binding assays show that six protease molecules can be bound to a 36-mer ss- or dsDNA, whereas only one protease molecule can be bound to a 12-mer DNA, with the K_d of the interactions in the low nanomolar range.[246]

D. Mechanism of Activation by the Peptide Cofactor

The interaction between the adenovirus protease and the pVIc peptide has been visualized from the crystal structure of the complex.[243,250] The peptide is bound in an extended conformation, forming another strand for the central β-sheet of the enzyme (Figure 1k). The side chains of the peptide also have important interactions with the protease. The Val2 side chain is completely buried in the complex, and modifications near the N-terminus of the peptide have deleterious effects on the binding and activation.[249,251] A disulfide bond is observed between Cys10 of the peptide and Cys104 of the enzyme (Figure 1k), the other strictly conserved Cys residues among adenovirus proteases. Biochemical data also suggested the requirement of this disulfide bond.[238,241,252] A recent report showed however that the presence of DNA greatly suppresses the functional importance of this disulfide.[253]

However, the pVIc peptide is located far from the active site of the enzyme, suggesting an indirect mechanism in the protease activation by this peptide. The dominant effect of the peptide cofactor on k_{cat} (Table 3) suggests that its function may be to help organize the catalytic residues of the enzyme. Binding of the peptide cofactor causes a change in the fluorescence emission from Trp residues in the protease,[241] consistent with a conformational change. Unfortunately, the crystal structure of the protease in the absence of the peptide cofactor is currently not available.

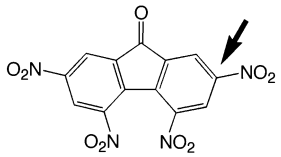

Figure 9. Chemical structure of TNFN, an inhibitor of adenovirus protease. The site of attack by the active site Cys residue of the protease is indicated by the arrow.[254]

The structure of the complex suggests that the peptide cofactor may stabilize the conformation of Gln115, the oxyanion hole of the enzyme. The Val2 side chain of the peptide is in van der Waals contact with that of Val114, which is highly conserved among the adenovirus proteases. A peptide missing the first two residues of pVIc cannot activate the protease and does not produce a change in the Trp emission spectra,[251] and mutagenesis studies also showed the functional importance of the Val2 residue.[249]

E. Inhibitors of Adenovirus Protease

Because of its crucial role in the life cycle of the virus, adenovirus protease is a target for the design and development of antiviral agents against infections by this virus. From in silico screening, the compound 2,4,5,7-tetranitro-9-fluorenone (TNFN) was selected as an active site inhibitor of the protease (Figure 9).[254] Enzymatic assays confirm that TNFN is a selective and irreversible inhibitor of adenovirus protease, with a K_i of 3 μM for binding and a rate constant of 0.006 s^{-1} for reacting with the active site Cys residue. Crystal structure analysis at 3 Å resolution of the protease/TNFN complex showed the presence of additional electron density in the active site, supporting the mechanism of action of the compound. The unique biochemical property of the protease has led to the proposal of a novel triple combination therapy, with inhibitors targeted against three different sites (the active site, the peptide binding site, and the DNA binding site) of the same virally encoded protein.[255]

VIII. Concluding Remarks

Studies on viral proteases have significantly increased our understanding of the life cycle of viruses, the mechanism of proteolytic processing, and the regulation of cellular processes. Detailed structural, biochemical, and mechanistic studies of this large collection of enzymes have revealed Nature's amazing versatility in evolving proteolytic machineries.[2,5]

A recurring theme from the structural and sequence analyses of the viral proteases is the remarkable compactness of these enzymes. In most cases where cellular structural homologues exist, the viral proteases are generally the smallest examples of the proteases currently known. This was seen with the alphavirus capsid protein autoprotease, the 3C, 2A, and leader proteases of picornaviruses, and the NS3 protease of hepatitis C virus. Another striking example is the retroviral aspartic proteases, where a gene encoding only 99 amino acid residues is suf-

ficient to produce a (dimeric) protease. This compactness may be related to the evolutionary pressure of maintaining small genomes for these viruses.[1,23] Nonetheless, these observations raise important questions about the evolution of these enzymes, and the overall folding of these structures.

In addition to their compact size, most of the viral proteases contain no disulfide bridges, in contrast to many classical cellular proteases. The studies show that cofactors are frequently used to stabilize the viral proteases, for example, the zinc binding sites in the 2A and NS3 proteases, and the peptide cofactors in NS3 and adenovirus proteases. More generally speaking, the second monomer of the HCMV protease dimer could also be considered as a cofactor, as it helps to stabilize the oxyanion hole of the enzyme.

Most viral proteases have little sequence homology to cellular proteins, even when they share the same backbone fold. In fact, cellular homologues of many viral proteases are currently not known. As a consequence of their unique sequences and compact sizes, viral proteases generally have distinct substrate specificity, which has significant implications for the design and development of their inhibitors. Compounds that are specific for the viral enzymes are less likely to have undesirable cross reactivity against cellular enzymes. At the same time, inhibitor binding to the viral proteases oftentimes requires the establishment of many weak interactions, mostly on the surface of the enzyme. This significantly complicates the development of potent, small, bioavailable inhibitors, and one current solution to this problem involves the formation of covalent interactions with the protease.

The clinical relevance of drugs directed against viral proteases has been validated by the success of HIV protease inhibitors. This has sparked a tremendous amount of interest in other viral proteases, and some of their inhibitors are now entering clinical trials. It can be expected that the significant amount of research and development effort that is now being focused on these enzymes will lead to many more exciting discoveries, and hopefully new antiviral therapeutic agents.

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