Viral Protease Inhibitors

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Abstract This review provides an overview of the development of viral protease inhibitors as antiviral drugs. We concentrate on HIV-1 protease inhibitors, as these have made the most significant advances in the recent past. Thus, we discuss the biochemistry of HIV-1 protease, inhibitor development, clinical use of inhibitors, and evolution of resistance. Since many different viruses encode essential proteases, it is possible to envision the development of a potent protease inhibitor for other viruses if the processing site sequence and the catalytic mechanism are known. At this time, interest in developing inhibitors is limited to viruses that cause chronic disease, viruses that have the potential to cause large-scale epidemics, or viruses that are sufficiently ubiquitous that treating an acute infection would be

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beneficial even if the infection was ultimately self-limiting. Protease inhibitor development is most advanced for hepatitis C virus (HCV), and we also provide a review of HCV NS3/4A serine protease inhibitor development, including combination therapy and resistance. Finally, we discuss other viral proteases as potential drug targets, including those from Dengue virus, cytomegalovirus, rhinovirus, and coronavirus.

1 Introduction

This chapter reviews the development of inhibitors of viral proteases. A major focus will be on inhibitors of the HIV-1 protease, because this target has seen the greatest amount of work, and many of the biological lessons learned are directly applicable to other viral systems. A major limitation in antiviral therapy is the development of inhibitor resistance, and this topic will also be considered, while a more general review on antiviral resistance also referring to protease inhibitors is provided in Chap. 11 of this book. Space does not permit comprehensive referencing; therefore, we cite seminal initial reports, refer to comprehensive review articles, and discuss the more recently published literature. Finally, although many viruses encode essential proteases, we focus on viruses where drug development either has or is likely to progress to the clinic.

Viral proteases are part of a common strategy of the synthesis of large precursor proteins that are cleaved to generate mature active proteins. Most viral proteases are derived from well-known eukaryotic protease families, but have evolved their own specificities. Thus, the catalytic mechanisms of peptide bond cleavage follow well known chemistry catalyzed by canonical active site residues, but the specificity of the site of cleavage is determined by interactions between the protease and the amino acid side chains that flank the site of cleavage (i.e., the scissile bond). Drug design takes advantage of the known features of the catalytic mechanism and adds specificity based on the flanking interactions, guided in part by structural information. Given this starting point, the design of protease inhibitors has typically proceeded through the large-scale screening of substrate-like molecules.

The requirements of protease inhibitors as drugs in terms of potency, pharmacokinetics, and toxicity will vary depending on the nature of the infection and the goals of therapy. At one extreme is treatment of HIV-1, a chronic infection that requires life-long therapy and full suppression of viral replication. At the other extreme is the treatment of human rhinovirus (i.e., the cold virus), where short-term treatment to blunt viremia will likely be sufficient to reduce the unwanted symptoms of a cold. In all cases, viral proteases represent very attractive targets with familiar mechanisms of catalysis that frequently allow for the design of transition state analogs and with distinct specificities from host proteases.

2 Biochemistry of HIV-1 Protease and Development of Inhibitors

The main features of HIV-1 protease expression and processing have been reviewed (Swanstrom and Wills 1997). The protease is encoded in the viral *pro* gene, located downstream of the *gag* gene and upstream of the *pol* gene, which encodes the viral polymerase and integrase. It is expressed as part of a large polyprotein precursor, the Gag–Pro–Pol precursor. Given the dimeric nature of the viral protease, it is clear that this Gag–Pro–Pol precursor must dimerize to allow the protease to become active. The details of how the protease excises itself from the precursor are poorly understood, although the initial cleavage appears to occur in *cis* at a novel cleavage site (Pettit et al. 2004).

The HIV-1 protease, like other retroviral proteases, is a homodimeric aspartyl protease (see Fig. 1). The active site is formed at the dimer interface, with the two aspartic acids located at the base of the active site. The enzymatic mechanism is thought to be a classic acid–base catalysis involving a water molecule and what is called a "push–pull" mechanism. The water molecule is thought to transfer a proton to the dyad of the carboxyl groups of the aspartic acids, and then a proton from the dyad is transferred to the peptide bond that is being cleaved. In this mechanism, a tetrahedral intermediate transiently exists, which is nonconvalent and which is mimicked in most of the currently used FDA approved inhibitors.

A distinctive feature of the protease is the presence of a mobile beta turn in each subunit, which serves as a flap covering the active site. For substrate to get access to the active site, the flaps have to move away in what must be an ongoing dynamic

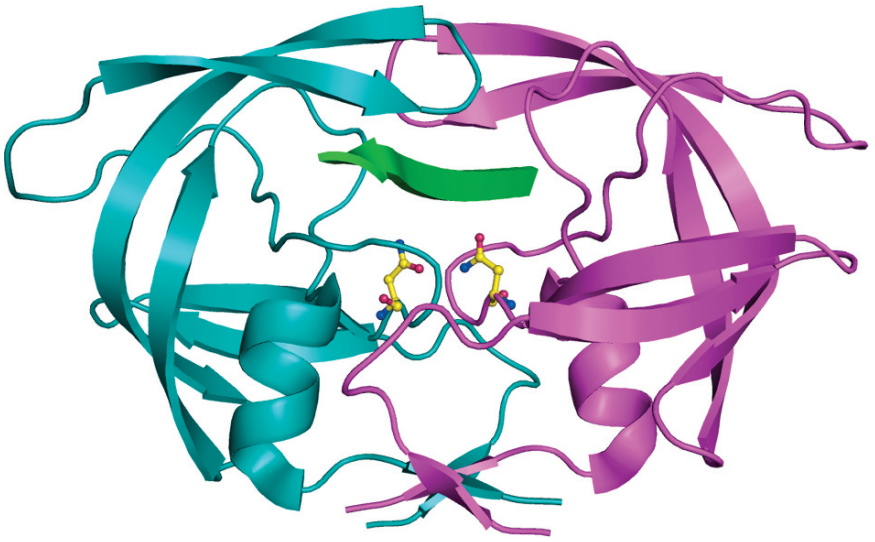

Fig. 1 A ribbon diagram of the crystal structure of a substrate complex of the homo-dimer HIV-1 protease (1kj7) (Prabu-Jeyabalan et al. 2002). Each monomer is shown in *cyan* and *pink*; the substrate is shown in *green*, and the catalytic aspartic acids are highlighted in *yellow*

process. Once the substrate is recognized and bound, the flaps move back over the active site and lock down over the bound substrate, completing the active site cavity and permitting substrate cleavage.

The protease cleaves the Gag and Gag–Pro–Pol polyproteins at ten sites. The enzyme recognizes a series of eight residues as a substrate cleavage site, ranging from P4–P4′. Nearly 1,000 $Å^2$ of the substrate is buried within the active site (Prabu-Jeyabalan et al. 2002). The sequences of these ten substrate sites are quite diverse and are cleaved with different efficiencies. Most of the substrate sites have a branched amino acid residue at the P2 site, a hydrophobic residues at P1, and an aromatic or proline at P1′. However, there are exceptions to each of these patterns: the nucleocapsid-p1 cleavage site (which is the last and slowest site to be cleaved in the processing of Gag) has an alanine at P2 and an asparagine at P1. The different substrate sequences result in cleavage rates, with nearly 400-fold differences in cleavage efficiencies. These differences in rates coupled together with the three-dimensional topology of the polyproteins likely contribute to the sequential order in which the substrates are cleaved.

Crystallographic studies imply that although little sequence homology exists between the different protease cleavage sites, what is conserved is the shape that they adopt within the active site of the enzyme (Prabu-Jeyabalan et al. 2002). This shape has been termed the "substrate envelope" and represents the consensus volume overlapping the majority of the substrates. Most likely, HIV-1 protease recognizes a particular peptide sequence as being a substrate by a combination of accessibility and the shape the sequence can adopt.

The development of HIV-1 protease inhibitors (PI) represents a highly successful effort in drug discovery, with nine PI currently approved by FDA and several more in clinical trials. The initial leads for HIV-1 PI were found within the pharmaceutical companies' libraries, which had been developed for another pharmaceutically interesting aspartyl protease, renin. These initial lead compounds, knowledge of the cleavage site sequences, a known catalytic mechanism with a generalizable strategy to generate a transition state analog, and the ability to readily crystallize the protease with a bound inhibitor have provided all the tools necessary for this successful and ongoing drug development effort.

HIV-1 protease was the first therapeutic target where "structure-based drug design" was broadly used to optimize inhibitor design (Wlodawer and Erickson 1993). Potential lead compounds were cocrystallized in complex with the enzyme and new compounds were designed to better fit and complement interactions within the active site. In the race to develop HIV-1 PI, competing laboratories would cocrystallize compounds that their competitors patented in order to figure out alternative chemical scaffolds that would preserve the same contacts but with better pharmacokinetics or bioavailability. This resulted in many of the PI occupying and filling the active site in a highly overlapping manner. These inhibitors are fairly hydrophobic and generally fit into the P2–P2′ region of the active site, often with a hydrophobic cyclical side chain at P1 and a fairly bulky functional group at P1′. All nine FDA-approved PI are competitive active site inhibitors that bind with affinities to the purified enzyme ranging from low nanomolar to low picomolar (Fig. 2) (Chen et al. 1994; Kaldor

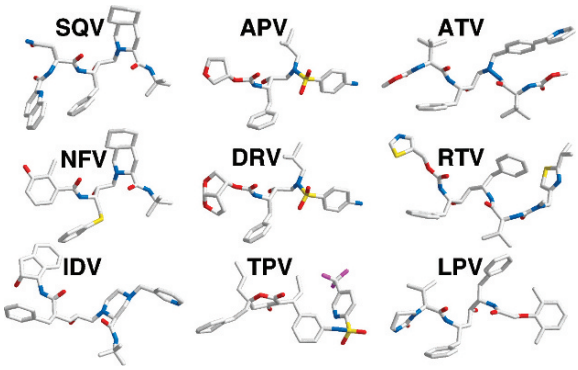

Fig. 2 The three-dimensional structures of the nine currently FDA-approved HIV-1 Protease inhibitors, colored by atom-type, *white*-carbon, *blue*-nitrogen, *red*-oxygen, *yellow*-sulfur, *purple*-florine. (Figure made in Pymol; (de-Lano 2002). *SQV* saquinavir, *APV* amprenavir, *ATV* atazanavir, *NFV* nelifinavir, *DRV* darunavir, *RTV* ritonavir, *IDV* indinavir, *TPV* tipranavir, *LPV* lopinavir

et al. 1997; Kempf et al. 1995; Krohn et al. 1991; Stoll et al. 2002; Thaisrivongs and Strohbach 1999). Seven of the nine inhibitors (except Tipranavir and Darunavir) are peptidomimetics that mimic the enzymatic transition state, containing a variety of noncleavable dipeptide isosteres as core scaffolds to mimic the transition state of substrate cleavage. Thus, although chemically different, many of the PI have very closely overlapping structures and interactions.

2.1 Clinical Use of HIV-1 Protease Inhibitors and Evolution of Resistance

The introduction of PI and the beginning of highly active antiretroviral therapy (HAART) in 1995 has had a profound impact on the paradigm of the treatment of HIV-1 infection: the potential to change an often progressive and fatal infection to a chronic and manageable disease. The continuing evolution of PI-based antiviral therapy has been due to numerous factors, including the ongoing need for more potent inhibitors with improved pharmacokinetics, decreased side effect profiles, and higher genetic barriers of resistance to combat multidrug resistant HIV. The nine PI compounds currently approved by the United States Food and Drug Administration for treatment of HIV-1 infection (Fig. 2) are as follows: saquinavir (SQV), ritonavir (RTV), indinavir (IDV), nelfinavir (NFV), lopinavir (LPV), atazanavir (ATV), fosamprenavir (FPV), tipranavir (TPV), and darunavir (DRV). We discuss the clinical use of each of the PIs in chronological order of FDA approval.

Saquinavir. SQV was first shown in vitro to have potent HIV-1 inhibition in acutely infected cells with an IC_{50} in the subnanomolar range and to inhibit viral maturation in chronically infected cells at 10 nM (Craig et al. 1991). Subsequently, clinical trials with SQV monotherapy in HIV-1 infected men at concentrations up to 600 mg three times a day for 16 weeks resulted in a decrease in HIV-1 RNA of 80% $(0.7\log_{10})$ (Kitchen et al. 1995). These and other data facilitated SQV in becoming the first FDA-approved PI in December 1995. In its original formulation,

SQV had a low bioavailability of 4%, due to extensive first-pass metabolism in the intestine and in the liver by cytochrome P450. Characterization of HIV-1 mutants with decreased sensitivity to SQV revealed that amino acid substitutions G48V and L90M significantly increased IC_{50} concentrations by approximately 40-fold (Jacobsen et al. 1995; Turriziani et al. 1994). Thus, the need for more potent PIs with improved pharmacokinetics and a higher genetic barrier to resistance quickly became apparent.

Ritonavir. RTV was derived by optimization of the pharmacokinetic properties of a series of C2 symmetry-based PIs, and in vitro studies demonstrated RTV to be a potent antiviral agent with activity against a variety of HIV-1 strains with an IC_{50} of 10–40 nM (Kempf et al. 1995). Antiretroviral activity in vivo was demonstrated over a broad range of doses, with a peak decline in HIV-1 plasma viremia of 1.2 $\log_{10}$ (Danner et al. 1995; Markowitz et al. 1995b). Unfortunately, despite its inhibition of HIV-1, RTV often resulted in dose-dependent GI side effects that were frequently intolerable. These side effects are secondary to many unique features of RTV: high oral bioavailability of 80% (Kempf et al. 1995) and potent inhibition of cytochrome P450–3A4 (the most potent of all PIs) and 2D6 (Hoetelmans 1999). The fortuitous strong inhibition of the P450–3A4 metabolism pathway resulted in a strikingly long half-life for RTV. However, another limitation of RTV monotherapy was its low genetic barrier to resistance, with mutations at codons 82 or 84 substantially reducing viral susceptibility (Markowitz et al. 1995a). The selection of protease variants with reduced affinity for RTV is consistent with the hydrophobic interaction between RTV and the isopropyl side chain of Val 82 as observed by X-ray crystallography. Because of the ability of RTV to inhibit P450–3A4, the paradigm of PI-based therapies changed from using RTV as a single PI to "boosting" of other PIs with RTV to increase their half-lives (Kempf et al. 1997). This has become the standard and maintains prolonged blood levels of the boosted PI, increases potency, decreases the required dosage, and minimizes PI side effects. In a similar fashion, RTV boosting impacts the dosing requirements of other classes of antiviral agents, including the CCR5 coreceptor antagonist, Maraviroc.

Indinavir. The final design of IDV included elements to improve absorption and bioavailability by the addition of a basic amine into the backbone of a hydroxyethylene transition-state mimic compound. IDV demonstrated in vitro inhibition of a variety of HIV-1 and HIV-2 isolates with an IC_{95} in the 12–100 nM range (Vacca et al. 1994). Monotherapy trials demonstrated up to a 3.1 $\log_{10}$ decline in HIV-1 RNA levels (Stein et al. 1996). The genetic correlates of in vivo resistance revealed variable patterns of multiple substitutions, indicating that resistance evolved through multiple pathways during ongoing replication; however, resistant isolates typically had substitutions at codons 46 and/or 82 (Condra et al. 1996).

Nelfinavir. Using structure-based design in conjunction with predicted oral pharmacokinetics, NFV was identified and found to have potent inhibition of HIV-1 in vitro with an IC_{50} in the 2 nM range (Kaldor et al. 1997). Clinical trials of NFV revealed robust and sustained reductions in HIV-1 RNA with over half of all subjects attaining a persistent 1.6 $\log_{10}$ reduction at 12 months, in conjunction with a mean increase in CD4 cells of 180–200 per mm^3 (Markowitz et al. 1998). In subjects

with viral rebound, the active site mutation allowing for NFV resistance was often mediated by a unique D30N substitution; of note, this mutation does not confer phenotypic cross-resistance to other PIs.

Lopinavir. The many limitations of the first generation PIs (modest oral bioavailability, short plasma half-life, high binding to plasma proteins, strict dietary restrictions, and a low genetic barrier to resistance) led to the eventual discovery of LPV (Sham et al. 1998). In vitro studies of LPV demonstrated inhibition of wild type and mutant proteases with an IC_{50} in the subnanomolar range. Furthermore, LPV, like most PIs, is exquisitely sensitive to pharmacokinetic enhancement by codosing with ritonavir, producing sustained concentrations in the plasma that are $>$ 50-fold over the EC_{50}, which has led to the coformulation LPV/RTV (Kaletra). In vivo, potent and durable viral suppression has been examined in antiretroviral naïve patients receiving LPV/RTV in conjunction with two nucleoside reverse transcriptase inhibitors (NRTI), with 85% of patients having HIV-1 viral loads ≤ 400 copies/ml at 48 weeks (Murphy et al. 2001). In contrast to first generation PIs, Kaletra has been shown to have a much higher genetic barrier of resistance. Selection of highly resistant viral variants is due to the sequential appearance of multiple mutations, such as I84V-L10F-M46I-T91S-V32I-I47V (Carrillo et al. 1998). Moreover, mutations at 11 amino acid positions in protease (L10F/I/R/V, K20M/R, L24I, M46I/L, F53L, I54L/T/V, L63P, A71I/L/T/V, V82A/F/T, I84V, and L90M) are associated with reduced susceptibility, but these must appear in combinations. The median IC_{50} of LPV against isolates with 0–3, 4 or 5, 6 or 7, and 8–10 of the 11 mutations is 0.8-, 2.7-, 13.5-, and 44.0-fold higher, respectively, than the IC_{50} against wild-type (Kempf et al. 2001). The genotypic inhibitory quotient (relationship between LPV trough levels and the number of *pro* mutations) is the main independent predictor of response to LPV/RTV (Gonzalez de Requena et al. 2004). To further reinforce the potency and high resistance barrier, small-scale clinical trials have shown that two-thirds of patients on Kaletra alone are capable of maintaining suppression to below the limit of detection (50 HIV-1 RNA copies/ml) after 4 years of follow-up (Pulido et al. 2008).

Atazanavir. Designed to simplify drug dosing regimens, ATV was approved in 2003 and became the first once daily PI. In vitro, ATV has robust anti-HIV activity with an IC_{50} less than 5 nM and an IC_{90} of 9–15 nM in cell culture (Robinson et al. 2000). A phase 2 clinical trial showed efficacy of ATV in combination with didanosine and stavudine in ART-naïve patients after 48 weeks, with a mean reduction from baseline HIV-1 RNA (> 2.33 $\log_{10}$) and over half of all patients with HIV-1 viral loads < 400 copies/ml (Sanne et al. 2003). In contrast to other PIs, especially when boosted with RTV, ATV has not been associated with clinically relevant dyslipidemia or insulin resistance. Other large scale, phase 3 trials have confirmed these findings and revealed noninferiority between ATV vs. a nonnucleoside reverse transcriptase inhibitor (NNRTI) (efavirenz) in conjunction with two NRTIs (combivir) (Squires et al. 2004) or ATV/RTV vs. LPV/RTV (Johnson 2006). ATV has a distinct resistance profile relative to other PIs, with susceptibility maintained against 86% of isolates resistant to 1–2 PIs (Colonno et al. 2004); however, a unique I50L substitution has been shown to be the signature mutation for resistance to ATV.

Fosamprenavir. FPV is an inactive, highly water soluble phosphate ester prodrug of amprenavir (APV), a PI that includes a sulfonamide moiety. FPV was synthesized to allow decreased pill burden and improve patient compliance, and is completely hydrolyzed to APV in the gut endothelium. Although APV was FDA-approved in April 1999, production was discontinued after FPV became available in October 2003. Initial in vitro studies of APV demonstrated potent HIV-1 inhibition, with a mean IC_{50} of 12 nM against six HIV clinical isolates (St Clair et al. 1996). Two pivotal large scale clinical trials demonstrated superior of FPV vs. NFV to contribute to the suppression of viral RNA load are the NEAT (Rodriguez-French et al. 2004) and SOLO trials (MacManus et al. 2004). Like other second generation PIs, FPV can select for an unusual in vitro resistance profile, with multiple substitutions at codons 46, 47, and 50 needed to confer high-level resistance (Partaledis et al. 1995).

Tipranavir. In contrast to the previously approved PIs that are classified as peptidomimetics and transition state analogs, that is, structurally similar to the tetrahedral intermediate that forms during hydrolytic cleavage of a peptide bond of the natural substrate (Randolph and DeGoey 2004), TPV was the first of the nonpeptidic PIs of the dihydropyrone sulfonafamide class that are structurally similar to coumadin. Initial in vitro data demonstrated that TPV was a promising candidate for further development (Poppe et al. 1997). Further in vitro studies demonstrated potent inhibition by TPV against clinical isolates resistant to multiple PIs, due to its molecular flexibility, allowing TPV to fit into the active site of PR that has become resistant to other PIs (Plosker and Figgitt 2003; Rusconi et al. 2000). Phase II clinical trials have shown as many as 16–20 *pro* mutations, including at least three PI resistance-associated mutations are needed to confer decreased susceptibility (Plosker and Figgitt 2003). The Randomized Evaluation of Strategic Intervention in Multi-Drug Resistant Patients [RESIST 1 AND RESIST 2 trials (Cahn et al. 2006; Gathe et al. 2006)] assessed the safety and efficacy of boosted TPV vs. other boosted PIs (LPV, SQV, APV, and IDV) in the setting of optimized background regimens. At week 24, half of the patients with fewer than 12 PI mutations vs. 30% in the comparator PI-containing arm responded to therapy (≥ 1 $\log_{10}$ decline in baseline HIV-1 viral RNA) ($p = 0.025$). At week 48, the proportion of patients with undetectable viral loads (≤ 400 copies/ml) was significantly greater in the TPV arm vs. the comparator arm (30.4% vs. 13.8%, respectively, $p < 0.001$).

Darunavir. DRV is the second nonpeptidic PI and was synthesized by replacing the tetrahydrofuranyl (THF) urethane moiety of amprenavir with a bis-THF component (Koh et al. 2003). Additional hydrogen bonding between the bis-THF ring and the PI backbone results in activity against a broad range of clinical isolates with an IC_{50} in the nanomolar range, including multidrug-resistant HIV-1. Phase IIb clinical trials [Performance of TMC114/r when evaluated in treatment experienced patients with PI resistance (POWER1 and 2)] have examined highly treatment-experienced patients randomly assigned to either DRV/RTV or comparator boosted PIs (SQV, APV, SQV, or ATV). The proportion of patients achieving viral suppression (HIV-1 RNA ≤ 400 copies/ml) was 63% vs. 19% in the DRV vs. comparator arm, respectively. Subset analyses revealed a significantly greater proportion of patients receiving DRV, who had up to eight PI resistance mutations, achieved HIV-1 viral loads

≤ 50 copies/ml at 24 weeks vs. patients receiving a comparator PI (47% vs. 25%, respectively). Viral load suppression was sustained until at least week 48, with 61% of DRV/RTV patients vs. 15% of comparator patients having viral load reductions ≥ 1 $\log_{10}$ copies/ml (Clotet et al. 2007). Surface plasmon resonance technology has been used to analyze the unique binding kinetics of DRV and its substrate to explain the potent antiviral activity; the dissociative half-life of DRV was found to be significantly greater than other analyzed PIs (FPV, ATV, LPV, and TPV) and binding affinity of DRV was >2 orders of magnitude higher vs. other PIs (Dierynck et al. 2007).

2.2 The Structural and Mechanistic Basis of HIV-1 Protease Inhibitor Resistance

Nearly 70% of the 99 residues in HIV-1 protease are known to mutate (Hoffman et al. 2003; Johnston et al. 2004; Rhee et al. 2005; Wu et al. 2003). Many of these mutate with a higher frequency in patients undergoing antiretroviral therapy. Although some sites such as D30N, G48V, I50V/L, V82A, V84I, and L90M are considered primary drug-resistant mutations, they occur in complex interdependent combinations with many other sites throughout the enzyme. These sites of mutation are both within and outside the active site. In addition, specific coevolution of the sequences of particular cleavage sites has become evident. The residues that are conserved appear to be primarily for enzymatic activity, or structural reasons, that is, the dimer interface, flexibility, or key glycine residues. The extensiveness of the mutational patterns in both the enzyme and the substrates indicates that this is a very plastic and adaptable target. Chapter 11 provides more detailed information about resistance development in HIV-1 antiviral therapy.

At a fundamental level, mutations can confer only drug resistance if they maintain function. For HIV-1 protease, this implies that the enzyme continues to recognize and cleave its diverse set of substrate sequences permitting viral maturation. Since all the protease inhibitors are competitive active site inhibitors with the substrates, how resistance can occur without impacting substrate recognition was somewhat of a dilemma. In comparing the crystal structures of the substrate complexes with the crystal structures of the inhibitor complexes, it was found (King et al. 2004a) that the inhibitors protruded from the "substrate envelope" at specific locations. The positions at which each inhibitor protruded away from the substrates and contacted HIV-1 protease corresponded very closely with the site of drug resistant mutations (King et al. 2004a; Prabu-Jeyabalan et al. 2006). In fact, inhibitors that fit more tightly within the substrate envelope have a higher threshold for drug resistance such as APV and DRV (King et al. 2004b). Thus, drug resistance can potentially be greatly limited by restricting the inhibitors to fit within the substrate envelope region. Testing of this as an inhibitor design strategy has been recently utilized, resulting in a number of high affinity, novel HIV-1 protease inhibitors that retain a flat binding profile to drug-resistant protease variants (Altman et al.

2008; Chellappan et al. 2007). However, other resistance-associated mutations exist throughout the enzyme and these likely have a role in maintaining the activity or fitness of the enzyme.

3 The Flavivirus NS3/4A Serine Protease

Several viruses in the Flaviviridae family are targets for antiviral development. Hepatitis C Virus (HCV), of the *Hepacivirus* genus, has the greatest impact on the human population. Dengue Virus of the *Flavivirus* genus also represents an important human pathogen for which no vaccine is available. These are enveloped viruses with a plus-stranded RNA genome of approximately 10 kb. The genome encodes a single large precursor protein of approximately 3,000 amino acids, which includes all the structural and nonstructural viral proteins. Viral replication takes place in the cytoplasm of the infected cell.

3.1 Discovery and Structure of the HCV NS3/4A Serine Protease

The early characterization of HCV polyprotein processing and the enzymes involved has been reviewed (Bartenschlager 1999). The presence of a chymotrypsin-like serine protease was first inferred based on sequence comparisons, with its presence placed within the amino-terminal third of the NS3 protein and the C-terminal two-thirds of NS3 encoding a helicase activity. This protease activity is responsible for four of the ten cleavages that occur in the large precursor protein. The immediate downstream product, NS4A, is a subunit of the protease that greatly increases the level of catalytic activity, giving rise to its name of NS3/4A.

The extensive work understanding the nature of the NS3/4A protease culminated with the determination of the structure at atomic resolution (Kim et al. 1996; Love et al. 1996). The structure confirmed the relationship to members of the chymotrypsin family, with the overall structure being a double β-barrel. One of the structures (Kim et al. 1996) included a peptide representing the NS4A cofactor. The NS4A peptide forms one of the eight β-strands that make up the N-terminal β-barrel of the NS3/4A protease whose presence helps to form the expected catalytic triad (His57, Asp81, Ser139) juxtaposed in the structure to form the active site; the structure also revealed a relatively shallow substrate binding cleft (Fig. 3).

Comparison of the NS3/4A protease cleavage sites shows only three positions that are conserved: E/DXXXXC/T-S/A (Bartenschlager 1999). The availability of a robust recombinant enzyme assay revealed that the enzyme recognizes a ten amino acid stretch of the substrate, from P6 to P4′ (Steinkuhler et al. 1996; Zhang et al. 1997), with the P1 Cys being the major determinant of cleavage site recognition (Bartenschlager 1999). The structures confirmed earlier modeling studies that placed a Phe residue at the bottom of the S1 subsite in position to allow van der

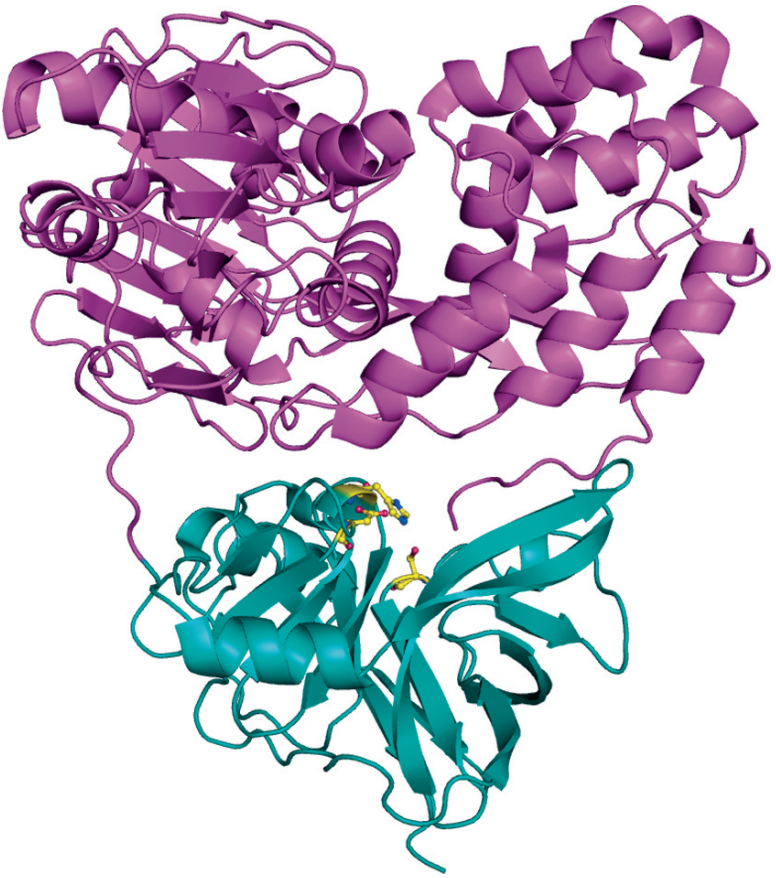

Fig. 3 A ribbon diagram of the HCV NS3/4A protease 1CU1 (Yao et al. 1999). The serine protease domain is shown in *cyan* with the catalytic triad highlighted in *yellow*, and the helicase domain is in *magenta*

Waals interactions with the P1 Cys, and also located basic Arg and Lys residues in position to interact with the P6 acidic residue. More detail of the binding of the N-terminal (i.e., P residues) part of the substrate was subsequently inferred from NMR studies showing a β-sheet interaction between this N-terminal segment of the substrate and enzyme largely confined to within the C-terminal β-barrel (Cicero et al. 1999).

3.2 Development and Clinical Testing of HCV Protease Inhibitors

Characterization of peptide substrates led to the observation that the N-terminal peptide product was able to inhibit the enzyme (Llinas-Brunet et al. 1998; Steinkuhler

et al. 1998). This proved to be a useful starting point for inhibitor design, first by optimizing the sequence of a peptide inhibitor (Ingallinella et al. 1998) and subsequently using nonpeptidic substituents. A detailed review of HCV protease inhibitor development can be found in Lin (2006) and De Francesco and Carfi (2007).

A major limitation in the development of anti-HCV compounds was the lack of a virus replication system. This was finally overcome with the development of a novel replicon system that directed persistent replication in a cell culture format (Lohmann et al. 1999). Using such a system, it was possible to demonstrate antiviral activity of an NS3/4A inhibitor in a cell culture assay, and demonstrate potency on par with treatment with interferon-α (Pause et al. 2003).

The first inhibitor to be developed to the point of testing in humans was BILN 2061 [celuprevir; (Lamarre et al. 2003)]. This inhibitor is a tripeptide mimetic of substrate residues P1–P3, which included rigidifying the structure by linking the P1 and P3 equivalent side chains in a macrocyclic structure. It is a competitive inhibitor with a Ki in the range of 0.5 nM, with the EC_{50} in cell culture about tenfold higher. In a short-term clinical trial, a 2–3 log decrease in viral RNA load was observed with just two days of drug administration, followed by virus rebound after cessation. These results were confirmed in a larger group of subjects infected with HCV genotype1 (Hinrichsen et al. 2004); however, decreases were more variable in subjects infected with genotypes 2 and 3 (Reiser et al. 2005). Unfortunately, development of this compound has been suspended due to toxicity.

The second NS3/4A protease inhibitor developed to the point of clinical trials was VX-950/telaprevir/TVR. This is a mimetic of P1–P4 peptide representing the cleavage site at NS5A/5B. The novel feature of its design is the inclusion of an α-keto-amide group at the C terminus. In this position the keto carbonyl is able to form a reversible covalent bond with the Ser139 nucleophile at the active site (Perni et al. 2006). This inhibitor has a Ki of 7 nM and an EC_{50} about 50-fold higher in cell culture replicon assays. In an initial phase 1 dosing study with administration over a 2 week period, there was a 3 log drop in viral RNA load in the blood in 90% of subjects (Reesink et al. 2006), although the maximum drop was seen between 3 and 7 days followed by rebound of virus in the blood. Coadministration of telaprevir and interferon-α blocked the rebound within this 2 week period (Forestier et al. 2007).

The third NS3/4A inhibitor developed was SCH 503034 (boceprevir). This inhibitor also uses the keto-amide strategy to form a covalent bond with Ser139, and in this case representing a P1–P3 mimetic, although the P3 capping group occupies S4 (Venkatraman et al. 2006). An initial phase 1 dosing study showed potency in humans which was enhanced with coadministration of interferon-α (Sarrazin et al. 2007).

Other NS3/4A inhibitors are in earlier stages of development. TMC435350, a macrocyclic inhibitor, has shown good potency in vitro and high bioavailability (Simmen et al. 2007). ITMN-191 is another macrocyclic inhibitor that is entering early clinical trials. It appears to have a two-step binding mechanism that results in noncovalent binding but slow dissociation (Rajagopalan et al. 2007).

3.3 Combination Therapy

Treatment of HCV has strong parallels with the treatment of HIV-1, specifically the need to suppress viral replication. The major advantage with HCV treatment compared to HIV-1 treatment is that sustained suppression of HCV can lead to the complete clearance of virus, that is, a cure. As with HIV-1 treatment, the use of several agents together gives better sustained viral response compared with the use of single agents. The current therapy of ribavirin and interferon-α has a 70% success rate with 24 weeks of treatment in people with genotypes 2 and 3, while this treatment is successful in only 50% of subjects with genotype 1 and after 48 weeks of treatment (http://digestive.niddk.nih.gov/ddiseases/pubs/chronichepc/). Future studies will initially combine new agents with the approved therapies (ribavirin and interferon-α); however, the use of combinations of new agents with known virus-specific targets is a major goal. Experimental evidence generated using inhibition of a replicon in a cell culture-based assay supports the use of agents with different viral targets (Wyles et al. 2007). Important milestones will be to avoid the use of interferon-α and to reduce the time on therapy to reach sustained viral response.

Interim results from the PROVE1 and PROVE2 phase 2 trials have been reported in subjects infected with genotype 1 HCV. The design of PROVE1 is to dose telaprevir (VX950) with interferon-α and ribavirin for 12 weeks with a variable therapy tail of interferon-α and ribavirin. At the initial 12 week time point, viral RNA was suppressed in 70% of subjects, compared to 39% receiving only interferon-α and ribavirin (Jacobson et al. 2007); in addition, six of nine subjects receiving all three drugs for 12 weeks but without the tail therapy achieved sustained viral response, with the other three showing evidence of selection of telaprevir resistance. In PROVE2, the 12 week responses were 79% viral RNA suppression for treatment with telaprevir plus IFN-α and ribavirin, but only 63% in dual therapy with telaprevir and IFN-α, compared to 43% with IFN-α and ribavirin (Zeuzem et al. 2007). These results are consistent with the idea that adding combinations of agents will result in improved responses, although an effect on sustained viral response rates has not yet been determined.

Interim results from the SPRINT-1 phase 2 trial of boceprevir (SCH 503034) have been released. In subjects who received boceprevir plus interferon-α and ribavirin, viral RNA loads were suppressed at week 12 in between 70 and 79% of subjects infected with genotype 1 HCV, compared with only 34% in the interferon-α/ribavirin standard of care arm (www.sch-plough.com/schering_plough/news/release.jsp?releaseID = 1064540). However, it is not yet known if this enhanced early response will translate into sustained response.

3.4 Resistance

Resistance to NS3/4A inhibitors was primarily tested using the replicon system in tissue culture and closely parallels what was seen with resistance to HIV-1 PI.

Specifically, incomplete suppression of viral replication allows for the selection of variants with mutations in NS3 that have reduced sensitivity to the inhibitor, and this also holds true in clinical testing. An unresolved question is the extent to which different protease inhibitors select for the same or different resistance mutations. This question is complicated by the fact that different levels of a drug can select for different mutations, and resistance can go through different pathways giving a stochastic feature to the appearance of resistance. Cell culture selection schemes generally involve a relatively small sample size, again adding to the stochastic nature of the results, and resistance evolving in vivo may include low-level variants that are not detected but potentially available to seed the rapid outgrowth of resistant variants with a therapy switch. The lesson to date with HIV-1 protease inhibitors is that improved drug potency has been a much more effective strategy in dealing with resistance within the protease inhibitor drug class than attempts to design drugs that elicit nonoverlapping resistance patterns. Chapter 11 provides more detailed information about resistance development in HCV antiviral therapy.

3.5 The Dengue Virus NS2B/NS3 Serine Protease

Dengue Virus is a member of the *Flavivirus* genus of the Flaviviridae family. Because of the number of people at risk for Dengue Virus infection (2.5 billion), with the attendant risk of progressing to dengue hemorrhagic fever, and the lack of an available vaccine, the development of antivirals against the Dengue Virus protease is an important goal. Unlike HCV, the activating subunit of the Dengue Virus protease comes from a proteolytic fragment of the upstream protein, that is, the NS2B fragment (Arias et al. 1993; Chambers et al. 1993). The recent crystal structure of the dengue virus protease has clarified a number of issues, especially when compared to the structure of the West Nile Virus NS2B/NS3 protease with a bound inhibitor (Erbel et al. 2006). The protease has a similar double β-barrel structure, with the NS2B fragment contributing part of a β sheet. In the inhibitor-bound state, the NS2B fragment has extensive contacts around the NS3 protease domain and also contributes to the substrate binding site. This more intimate interaction helps explain the profound requirement of the NS2B fragment for significant activity (Yusof et al. 2000). A distinctive feature of the protease target cleavage site is the presence of Arg residues at both P1 and P2 (reviewed in Melino and Paci 2007). However, the S1 subsite has a strong stacking interaction with the P1 side chain, while the interaction between P2 and S2 is based on electrostatic interactions (Erbel et al. 2006). Given the known substrates and the catalytic mechanism, drug discovery efforts have been undertaken, although none has progressed to clinical trials (Melino and Paci 2007).

4 Other Viral Proteases

Since many different viruses encode essential proteases, it is possible to envision the development of a potent protease inhibitor for any of these viruses, given the knowledge of the processing site sequence and the catalytic mechanism. At this time, interest in developing inhibitors is limited to settings of viruses that cause chronic disease, viruses that have the potential to cause large scale epidemics, or viruses that are sufficiently ubiquitous that treating an acute infection would be beneficial even if the infection is ultimately self-limiting. Later we review one example of each of these cases.

4.1 The Human Cytomegalovirus Protease

Human Cytomegalovirus (HCMV, Human herpesvirus 5/HHV-5) is in the Herpesviridae family, Betaherpesvirinae subfamily, *Cytomegalovirus* genus, and is a large, enveloped, double-stranded DNA virus. Infection rates in the general population are 80–90%, with asymptomatic infections in healthy individuals. However, becoming immunocompromised or immunosuppressed can result in enhanced replication, leading to pneumonia and retinitis; in addition, congenital infections of newborns can lead to neurologic abnormalities. The available therapies are based on nucleoside analogs and have significant toxicity.

The herpesvirus protease was first described for HSV-1 and HCMV (Liu and Roizman 1991; Welch et al. 1991). The protease, called assemblin, is encoded in the HCMV gene UL80 as the amino-terminal part of a precursor protein. The C-terminal domain of the precursor is the viral assembly protein and cleavage events within the precursor are required for the packaging of viral DNA (reviewed in Brignole and Gibson 2007). The crystal structures of the HCMV protease (Chen et al. 1996; Qiu et al. 1996; Shieh et al. 1996; Tong et al. 1996) and bound to inhibitors (Khayat et al. 2003; Tong et al. 1998) have revealed a number of features of this target (reviewed in Tong 2002). The core of the enzyme is a seven stranded β-barrel structure that represents a novel fold for a serine protease. In addition, the catalytic triad is Ser–His–His, rather than the more familiar Ser–His–Asp, and, combined with the novel fold, represent a third independent evolution of a serine protease (in addition to the trypsin and subtilisin families). The protease crystallized as a dimer, which is the active form of the enzyme (Darke et al. 1996; Margosiak et al. 1996). However, the two active sites in the dimer are displaced from each other and dimerization appears to stabilize the structure of the active site at a distance. The structure changes further when binding substrate (by induced fit) with the substrate/inhibitor binding in an extended conformation and forming an anti-parallel β-sheet to align the scissile bond with the active site. P2 and P4 are solvent exposed while the remaining side chains from $P5-P1'$ interact with the protease.

Given a known mechanism of catalysis, that is, a serine nucleophile, and a consensus substrate sequence (V,L,I)XA/S, it has been possible to carry out drug

discovery efforts. A series of inhibitors based on different chemical backbones have been described, all sharing an activated carbonyl to react chemically with the nucleophile (reviewed in Tong 2002). However, to date, none of these compounds has made it into clinical development. Given this outcome, alternative screens have been developed that will allow high throughput screens in cell-based assays as an alternative to screens based on inhibition of enzymatic activity (for example, Cottier et al. 2006). Finally, the potential for similar drug development efforts exist for other herpesviruses such as HSV-1, HSV-2, and KSHV, although little work has been done to date. An alternative approach to develop an inhibitor has been explored with the KSHV protease by targeting the dimer interface to block the essential step of dimerization (Shimba et al. 2004).

4.2 The Rhinovirus 3C Protease

Rhinoviruses cause a significant fraction of the common colds suffered by the human population. However, members of the *Rhinovirus* genus (Picornaviridae family) include 100 different serotypes that infect humans, making a vaccine strategy impractical. Thus, alternative strategies are needed to intervene in these non-life-threatening but inconvenient infections.

Rhinovirus, like poliovirus, synthesizes a large precursor protein from which all of the mature viral proteins are generated. Two viral proteases are involved in these cleavages: 2A protease cleaves the polyprotein precursor at its own N terminus, while the 3C protease is responsible for additional cleavage events to generate the mature viral proteins. Both proteases can release themselves from the polyprotein precursor. Cleavage by 3C occurs between Gln/Gly, but flanking sequences affect efficiency (reviewed in Racaniello 2001).

Structural analysis of the rhinovirus and the hepatitis A virus 3C proteases (Allaire et al. 1994; Matthews et al. 1994) confirmed earlier predictions that the picornavirus 3C proteases are similar to chymotrypsin-like serine proteases in their fold. An important difference is that the serine nucleophile of serine proteases is replaced with a cysteine; however, the 3C protease is structurally distinct from the eukaryotic cysteine protease class of enzymes.

A variety of designs have been explored in developing inhibitors to the rhinovirus 3C protease (reviewed in Tong 2002). Inhibitor design was aided by structural studies of bound inhibitors (Matthews et al. 1999). One successful design of a 3C inhibitor included an unsaturated ethyl ester to allow the formation of a covalent bond with the protease active site Cys, known as a Michael Acceptor. Using this strategy of an irreversible inhibitor, one compound, rupintrivir, was developed and showed efficacy in the setting of experimentally induced human rhinovirus infections (Hayden et al. 2003). The ability to select for resistance to rupintrivir validated that the 3C protease was the drug target, with mutations at position 130 in the S2 subsite being the most common (Binford et al. 2007). However, rupintrivir failed to show efficacy in the setting of natural infection (Patick et al. 2005). A related

compound (compound 1) was identified that had significant bioavailability when taken orally, but this compound has not been taken into efficacy trials (reviewed in Patick 2006). Limitations in the rapid identification of the infecting agent makes the development of agent-specific therapeutics a challenge, given that a number of viruses can give cold-like symptoms, which likely can be ameliorated only with early intervention.

4.3 The SARS Coronavirus Protease

A new coronavirus was quickly identified after the outbreak of an atypical pneumonia in southern China early in 2003. The new virus eventually caused 8,000 infections with approximately 800 deaths in 29 countries. The condition was named Severe Acute Respiratory Syndrome, SARS, and the causative coronavirus named SARS-CoV. The zoonotic nature of the infection came with the identification of a similar virus in bats (Poon et al. 2005), although it is possible that the bat virus passed through other animal hosts and recombined with other SARS-like coronaviruses prior to infecting humans (Hon et al. 2008). SARS-CoV is not currently circulating in the human population; however, the mysterious appearance and rapid spread of this virus emphasized how vulnerable the human population is to such respiratory infections. This has spurred interest in the development of antivirals that could be used either in treatment or as prophylaxis to complement public health measures in curbing future outbreaks.

The SARS-CoV is classified in the Nidovirales Order, Coronaviridae Family, and *Coronavirus* Genus (www.virustaxonomyonline.com). The genome is approximately 30 kb, and gene expression is accomplished by the generation of 3′ co-terminal subgenomic messages. The replicase gene is encoded in two large overlapping reading frames, termed pp1a and pp1b. The encoded polyproteins undergo extensive proteolytic processing by two viral proteases, with a majority of the cleavage events (11) mediated by the viral 3C-like (3CL, also called main) protease (reviewed in Liang 2006). Cleavage sites are most conserved in the P1 position with Gln, then Ser, Ala, or Gly in the P1′ position, Leu, Met, or Phe in the P2 position, and Ala, Val, Pro, or Thr in the P4 position.

The suggested similarities between the coronavirus 3CL and picornavirus 3C protease was confirmed and extended when the structures of the 3CL protease were determined for several coronaviruses, including SARS (Anand et al. 2002, 2003; Lee et al. 2005; Xue et al. 2008; Yang et al. 2003). The 3CL protease contains three domains, with the two N-terminal domains comprised of a two β-barrel chymotrypsin fold, similar to trypsin/chymotrypsin and the picornavirus 3C protease. The C-terminal domain III is globular, composed of a series of α-helices, and has an unknown function. The crystal structure also confirmed earlier biochemical work that the enzyme is dimeric, with the unusual arrangement of the subunits being perpendicular to each other. Like the picornavirus 3C protease, the 3CL protease replaces the chymotrypsin active site serine with cysteine (Cys145 in the SARS 3CL

protease), which along with His41 makes a catalytic dyad rather than the prototypic catalytic triad. The S1 subsite accommodates Gln, with a specific interaction with a histidine placed at the bottom of the subsite. The hydrophobic P2 side chain is accommodated in a largely hydrophobic S2 subsite. The P3 side chain, in keeping with its poor conservation, is oriented toward the solvent, and the P4 side chain must be a small amino acid to fit into the shallow S4 subsite.

The initial search for an inhibitor of the SARS 3CL protease focused on preexisting drugs and compounds, some tested empirically and others selected based on modeling. The HIV-1 PI nelfinavir and lopinavir/ritonavir have been considered, with the latter actually used clinically in SARS CoV-infected subjects. The rhinovirus inhibitor ruprintrivir and related compounds have also been tested (reviewed in Fear et al. 2007). However, in the absence of any elements of specificity, these preexisting compounds would be expected to have low potency.

More focused efforts on drug development have used either lead compounds identified in the initial screens or started with substrate mimetics. Many of the approaches have used inhibitors that bind irreversibly to the active site Cys and occupy the enzyme subsites, with the most complete studies analyzing enzyme inhibition, inhibition of viral replication, and structural studies of inhibitor bound to enzyme. These studies have included a substrate-based inhibitor with a chloromethyl ketone group (Anand et al. 2003; Yang et al. 2003), substrate-based inhibitors with a Michael Acceptor (Xue et al. 2008; Yang et al. 2005), a peptidomimetic with an aldehyde acceptor (Yang et al. 2006), peptidomimetic inhibitors with a reactive phthalhydrazide group (Yin et al. 2007), and a substrate-based inhibitor with an epoxyketone reactive group (Goetz et al. 2007). This tally represents only a sampling of the drug discovery efforts directed at the SARS CoV 3CL protease.

The appearance of the SARS CoV revealed a new threat to human health. It is now clear that similar viruses, or recombinants of viruses, could emerge from a wide range of animals, analogous to the recurring introductions of influenza virus from migratory bird populations. Thus there may be some advantage to designing less potent inhibitors that have a broad spectrum against coronaviruses, since drug development is a very slow process compared with a spreading epidemic of a respiratory virus. It is likely that being able to blunt the initial acute viremia may be sufficient to provide clinical benefit. Toxicity and potency may be less critical in a short course of therapy compared to treating chronic infections like HIV-1 or HCV. The tools appear to be in place to develop 3CL protease inhibitors that will allow for more targeted clinical intervention in future outbreaks. However, challenge experiments to demonstrate efficacy are not possible, and so potency in model systems and dosing studies in healthy volunteers will likely replace the more lengthy clinical efficacy trials required of typical drugs. In this scenario, SARS CoV inhibitors would be made available during the next epidemic on a compassionate use basis.

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