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Review Article

Hepatitis C virus NS3 serine protease as a drug discovery target

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Abstract

Hepatitis C virus NS3 serine protease (HCV Pr) is an extensively studied enzyme for drug intervention. The target presented serious challenges in early screening efforts, however, with the lack of prominent active site features rendering traditional nonpeptidic serine protease inhibitor motifs and high-throughput screening campaigns ineffectual. In contrast, the peptidomimetic structure-based design approach has proven successful in the discovery of potent inhibitors of HCV Pr. Subsequent rational design efforts have led to the identification of an inhibitor that demonstrates efficacy in man, validating the years of research. This review summarizes why HCV Pr provides a viable drug discovery target despite the many obstacles, and details the breakthroughs in protein production and assay development that have facilitated inhibitor advances. The latest inhibitors in preclinical and clinical research and development are also presented, along with a discussion of how the recent HCV Pr clinical candidate challenges much of the dogma surrounding peptidomimetic design. In addition, future issues such as resistance, genotype coverage and HIV-HCV coinfected individuals are considered.

Introduction

Proteases have historically made excellent structure-based targets for inhibitor design. Over the last decade, clinical candidates have been identified for serine, aspartyl, cysteine and metalloproteases (1-6). With the well-documented success story of human immunodeficiency virus (HIV) aspartyl protease inhibitors, other viral proteases, including HCV NS3 protease (HCV Pr), have become attractive targets for drug intervention (7). Screening efforts were rapidly initiated upon identification of HCV Pr as a potential drug target (8, 9). The enzyme presented serious challenges, however, with the lack of prominent active site features rendering traditional non-peptidic serine protease inhibitor motifs and high-throughput screening campaigns ineffectual. As with HIV protease inhibitors, it has been the peptidomimetic structure-based design approach that has proved successful in the initial discovery of potent inhibitors of this enzyme (10, 11). In parallel with HCV Pr developments, the HCV field has entered an extremely dynamic period initiated by the development of a stable cell-based HCV replicon system (12). The ability to assess viral nonstructural protein replication has enabled evaluation of HCV Pr inhibitor efficacy with respect to viral replication as well as polyprotein processing. Utilizing rational design in conjunction with these advances, Lamarre's group at Boehringer Ingelheim selected the first HCV Pr inhibitor clinical candidate for evaluation in man (13). The efficacy of this inhibitor in humans (14) validates the years of research to identify a viable clinical candidate, and it will act as the benchmark for all HCV Pr inhibitors that follow. This review summarizes why HCV Pr provides a viable drug discovery target despite the many challenges, and details the breakthroughs in protein production and assay development that have facilitated HCV Pr inhibitor advances. The latest inhibitors in research and development, both preclinically and clinically, are also presented, along with a discussion of how the Boehringer Ingelheim candidate challenges much of the dogma regarding peptidomimetic design. In addition, future challenges such as resistance,

genotype coverage and HIV-HCV coinfected individuals are considered.

Background to hepatitis C virus

HCV is the major etiological agent of 90% of all cases of non-A, non-B hepatitis (15) infecting the liver. The incidence of HCV infection is becoming an increasingly severe public health concern with 2-15% of individuals infected worldwide (16), and prevalence as high as 17-26% reported in parts of Africa (Egypt) (17). While primary infection with HCV is often asymptomatic, most HCV infections progress to a chronic state that can persist for decades. Of those with chronic HCV infections, it is believed that about 20-50% will eventually develop cirrhosis of the liver and 20-30% of these cases will lead to liver failure or liver cancer (18). As the current HCV-infected population ages, the morbidity and mortality associated with HCV are expected to triple (19).

HCV is a Hepacivirus, a member of the Flaviviridae family of viruses (20). This family comprises small, enveloped, single-stranded, positive sense RNA viruses (21). Unlike the retrovirus HIV where the viral genome integrates into host cells and coexists while evading the host immune response, there exists the possibility to completely eliminate HCV infection from patients. In fact, an estimated 15-25% of individuals clear the virus naturally (22). Individuals with chronic infection have a moderate chance of responding to current treatments (23), although the probability of a successful response depends on the infecting genotype. Six major genotypes and several subtypes exist within each group for HCV (24, 25). While genotype 1 predominates in North America, Europe and Asia, genotypes 2 and 3 are also prevalent in significant numbers (26, 27). The other major genotypes, 4, 5 and 6, predominate in certain countries, but are only found sporadically elsewhere. With globalization, however, there is a possibility that the situation could change. For example, according to a report in 1999, patients in Southern Europe were infected predominantly with genotype 1 (75%) while 25% were infected with genotypes 2 and 3 (28). However, in a recent clinical trial in Spain, 24% of patients were positive for genotype 4 (29). The high turnover rate of the virus (30) and the lack of proofreading function of the HCV RNA-dependent RNA polymerase further increase heterogeneity of genotype sequences. Quasi-species of closely related, but distinct, HCV viral populations thus exist within an infected patient (31) and are speculated to be one mechanism by which HCV escapes immune surveillance and establishes persistent infection in the majority of infected individuals (27).

An approved treatment for HCV infection uses interferon alfa (IFN-α), which indirectly affects HCV infection by stimulating the host antiviral response. The limited efficacy of intravenous INF-α monotherapy is improved through coadministration with the oral, broad-spectrum antiviral nucleoside analog ribavirin, producing a 3- to 5-fold improvement in sustained antiviral response (32, 33). Further improvements in response have been achieved through the replacement of INF-α with pegylated INF-α (34, 35), resulting in an INF-α half-life increase through the lowering of renal clearance. As a consequence, a treatment regimen of 3 doses per week can be replaced by once weekly dosing. Nevertheless, despite the improved regimen and sustained viral responses equivalent to the prescribed INF-α/ribavirin combination therapy, pegylated INF-α is not well tolerated. INF-α-based therapies are often associated with adverse side effects that can be sufficiently severe to cause termination of therapy (36). They are also expensive and exhibit variable efficacy among HCV genotypes (23) with, for example, the predominant genotype 1 being less responsive than genotypes 2 and 3. Drugs inhibiting viral targets are not available for HCV and the heterogeneity of the virus does not bode well for vaccine development. Consequently, there is an unmet medical need for drugs that selectively inhibit the virus in HCV-infected patients, demonstrate improved toxicity profiles and have extended genotype coverage, most specifically genotype 1.

HCV Pr structure and function

A general strategy for the development of antiviral agents has been to inhibit virally encoded enzymes essential for viral replication, as observed with HIV (5, 37, 38). HCV employs four enzymes that are essential for viral replication. A brief description of the function of these enzymes is described in Figure 1. Mutagenesis of the respective enzyme function in infectious clones eradicates infectivity in a chimpanzee model (39), strongly suggesting that these enzymes are essential for virus replication and thus excellent drug discovery targets. While drug intervention for all viable HCV targets is ongoing (40-42), intensive effort has focused on the inhibition of the HCV Pr.

HCV Pr is classified as a serine protease (43), identified by a catalytic triad comprising histidine (His57), aspartate (Asp81) and serine (Ser139). This activity is encoded in the amino-terminal 180 amino acids of the nonstructural (NS)3 protein, while the remainder of the NS3 protein encodes an ATPase and helicase activity (44). The protease exhibits a chymotrypsin/trypsin-like fold consisting of two six-stranded β-barrels encompassing the oxyanion-stabilizing loop (residues 135 to 139), predicted by sequence homology and later confirmed by structural determination (45). The protease was subsequently crystallized in the presence of a peptide encompassing residues 21 to 34 of the NS4A cofactor (46-48). This region of the NS4A protein was established as crucial for efficient proteolytic cleavage of the HCV nonstructural polyprotein (49). Crystallographic and NMR studies (46, 50, 51) have highlighted the conformational changes that occur at the protease N-terminus upon the direct interaction of NS4A (52). The peptide causes a reorganization of the protein surface to adopt β-strand

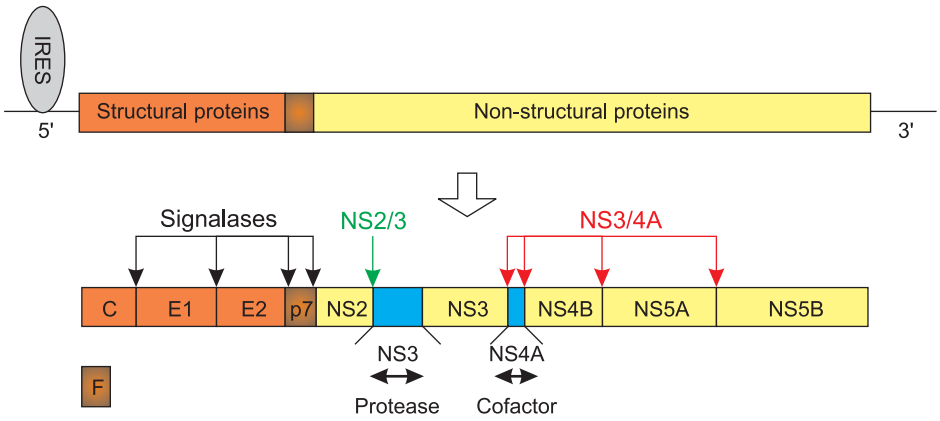

Fig. 1. Organization and function of HCV proteins in viral replication. The HCV genome is approximately 9500 nucleotides in length and is comprised of 5' and 3' non-translated regions (NTRs), and an open reading frame encoding the structural and nonstructural proteins (57). The 5' NTR comprises the internal ribosome-entry site (IRES) and is essential for RNA replication and protein synthesis while the 3' NTR is crucial for replication (184). The structural proteins include the core protein (C) and the envelope glycoproteins (E1 and E2). The p7 hydrophobic protein is speculated to be an ion channel (185) which may be required for the generation of infectious particles. Cleavage of these proteins, depicted by the black arrows, is by host signal peptidases. The nonstructural (NS) proteins include four enzyme activities and two proteins of unresolved function. Two proteases (NS2/3 and NS3) are responsible for nonstructural polyprotein processing. The NS2/3 protease displays characteristics of both a metallo and cysteine protease (186, 187) dependent on zinc to autocatalytically cleave between NS2 and NS3 (186, 188), as indicated by the green arrow. The NS3 serine protease resides in the N-terminus of the NS3 domain and, when complexed with its cofactor NS4A (in blue), is responsible for the four remaining cleavage events (red arrows). The first cleavage is autocatalytic, occurring in *cis* between NS3 and NS4A while subsequent cleavage events occur in *trans*. The C-terminus of the NS3 domain is comprised of a NTPase/RNA helicase activity which unwinds putative double-stranded replication intermediates in an ATP-dependent manner or removes regions of secondary structure allowing the NS5B RNA-dependent RNA polymerase (RdRp) to replicate positive and negative strands. The two remaining proteins, NS4B and NS5A, are involved in the replication complex although their functions are more ambiguous than the described enzyme activities. NS4B may be responsible for forming an ordered membranous compartment for template replication (189) while NS5A may modulate NS5B polymerase activity (190). An alternative reading frame encoding a frame-shift protein (F-protein) has recently been identified (191, 192).

and α-helix secondary structures, maximizing the surface area for substrate contact, while the catalytic triad occupies a conformation apparent in other chymotrypsin-like serine proteases. These conformational changes brought about by NS4A binding are thus speculated to stabilize the protease structure while enhancing proteolytic activity. The absolute role of NS4A on proteolytic activity is still under investigation. While kinetic studies clearly demonstrate an enhancement of enzyme activity in the presence of NS4A, a substrate induced-fit mechanism may be directly responsible for the observed enzyme activity in the absence of NS4A. Since NMR structures in the presence/absence of NS4A were similar with respect to the catalytic His/Asp residues, it has thus been suggested that NS4A may play a different and more subtle role in proteolysis. This was concluded from NMR studies where a product inhibitor P2 residue shielded the catalytic His/Asp interaction from solvent (53). These interactions, however, may depend on the inhibitor. More recently, another report studying protease/inhibitor interactions in solution observed differences in inhibitor binding affinity and catalytic residue position depending on the presence or absence of NS4A, and concluded a critical role of cofactor for substrate hydrolysis (54). NS4A also interacts with the protease N-terminus forming an α-helix with a very hydrophobic external face. It is postulated that this face anchors the NS3 protease to the endoplasmic reticulum membrane, the likely site of viral replication (55, 56).

HCV Pr is responsible for processing four cleavage events in the nonstructural polyprotein (Fig. 1). The first cleavage event occurs in *cis*, while the subsequent three are mediated by *trans*-cleavage (57). Sequence comparison of substrate sequences across the cleavage site revealed a conservation of residues at the P6, P1 and P1 prime (') sites (58). These preferences were later confirmed in substrate peptide studies assessing the catalytic efficiency of various HCV Pr recombinant forms (48, 59, 60), as well as in studies evaluating substitution effects on polyprotein processing in cells (61). The intermolecular consensus sequence differs from the intramolecular cleavage site by substituting the P1 cysteine (Cys) for threonine (Thr) (62, 63). Although substrate mapping experiments indicate Thr is an acceptable P1 residue, Cys is clearly preferred (59, 64, 65), explaining its presence in three of the four natural cleavage sites. Ser or Ala are tolerated at P1' while residues with bulky lipophilic side chains, such as leucine, tryptophan or tyrosine, are apparent at P4'. The other conserved position is P6, where a negatively charged residue generally resides although it is not a stringent requirement (59, 60, 64).

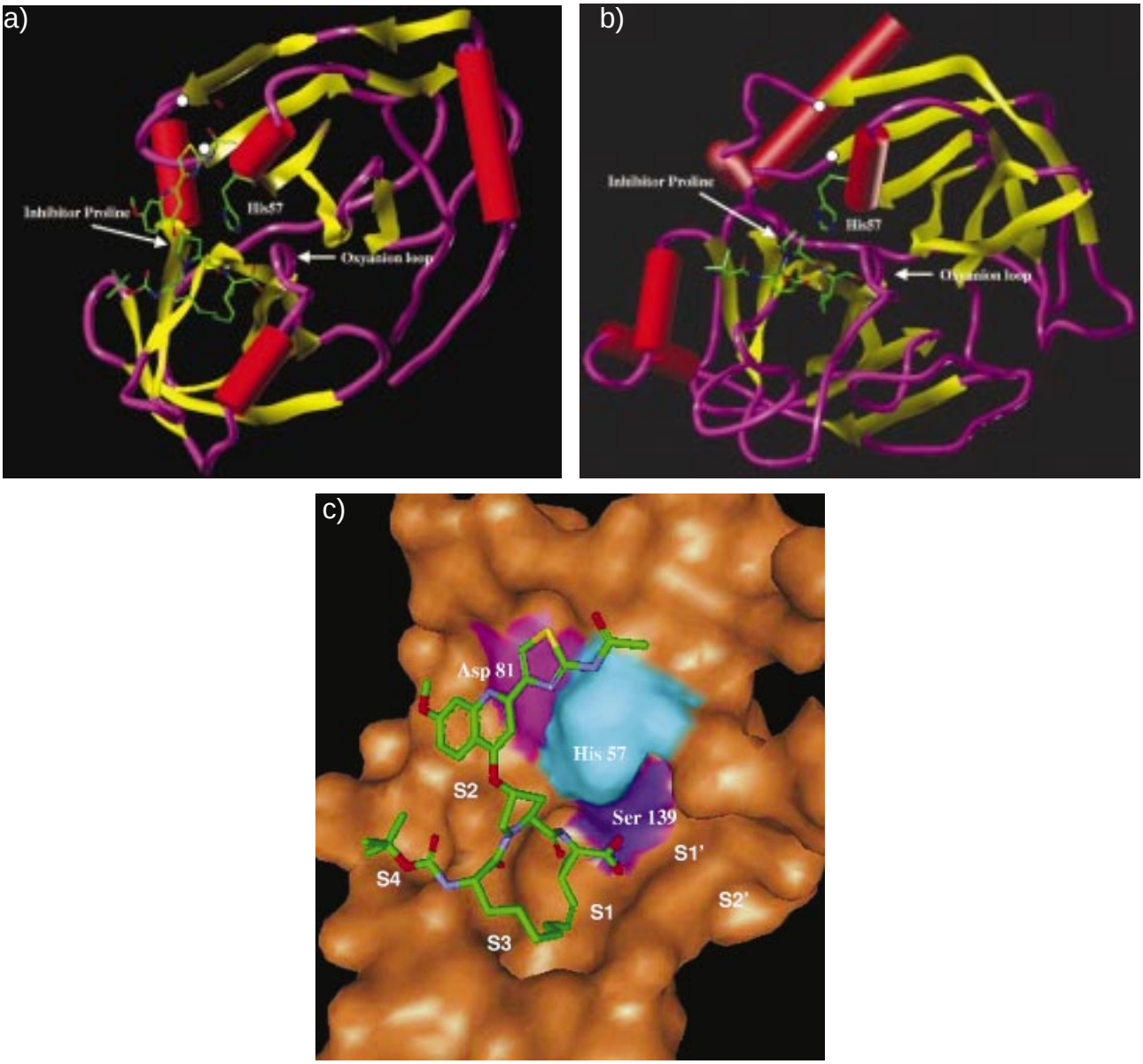

Fig. 2. Comparison of the HCV Pr and porcine pancreatic elastase (PPE) structures. A: Model of a Boehringer Ingelheim inhibitor (193) bound to HCV Pr (derived from PDB entry 1jxp) (56), tertiary structure highlighted. B: Model of PPE with inhibitor bound (PDB entry 1qix) (194). Structures are depicted in an equivalent orientation with the catalytic His labeled in both structures. The primary difference proximal to the active site lies in the loop above the catalytic Asp. The beginning and end of said loop is marked in each structure. In elastase the loop is considerably larger, shielding the catalytic Asp from solvent. In HCV Pr the loop is virtually nonexistent, leaving the Asp and surrounding region solvent exposed. Note the highlighted prolines share a common β-strand hydrogen bonding interaction (P2 position for each inhibitor). C: Model of HCV Pr with inhibitor (193, 195) with active site surface shown. Each site pocket occupied by the inhibitor is highlighted, together with the catalytic triad of the enzyme. Note that the Lys136 side chain has been truncated to methyl. This side chain is highly labile and obscures the primary binding interactions. Models and graphics for A and B were created in Sybyl 6.7 (a product of Tripos: www.tripos.com). Graphics for model C were created in Weblab ViewerPro 4.0 (a product of Accelrys: www.accelrys.com).

HCV Pr has structural and biochemical characteristics dissimilar to other mammalian serine proteases such as elastase or trypsin. Despite consisting of the characteristic two six-stranded β-barrels, the loops connecting the β-strands are relatively short (Fig. 2) (45).

Fewer amino acids comprising the two β-barrel motifs in HCV Pr results in the absence of several loops common to many other mammalian serine proteases. One of the loops absent in HCV Pr is positioned such that it can interact with residues on the P-side of the substrate.

The absence of this loop produces relatively featureless substrate binding pockets, providing an explanation for the apparent lack of substrate specificity over P2 to P5 (62), and why HCV Pr requires peptide substrates spanning residues P6 to P4' (60) for efficient cleavage. The most defined HCV Pr pocket is the S1 pocket, which is selective for Cys. It is smaller and very hydrophobic when compared with most other chymotrypsin-like serine proteases. The elastase S1 pocket is the most similar, preferring small aliphatic residues such as valine, while bulky and aromatic residues such as phenylalanine (Phe) or positively charged residues such as arginine (Arg) are favored in most other chymotrypsin-like family members. Another unique feature of HCV Pr is the three basic residues located in the vicinity of the active site. One of these residues, lysine (Lys) 136, appears to play a role in product inhibition (66). The extent to which product inhibition occurs is a phenomenon unique to HCV Pr and is proving to be an important element in strategies for drug design (10, 11). Interestingly, the three *trans*-cleavage products inhibit proteolytic activity whereas the *cis*-cleavage product exerts no inhibitory effect (67). The *cis*-cleavage product, with its naturally conserved suboptimal P1 Thr, is speculated to exist to prevent premature inhibition of subsequent cleavage events whereas the *trans*-cleavage products may play roles in regulation of polyprotein processing by reducing the availability of proteins for subsequent replication. Pertinent interactions between representative product inhibitors and the HCV Pr active site, observed by structural studies, have been adequately reviewed elsewhere (11, 67). However, some assumptions suggested with respect to active site preferences are deserving of further comment. The S2 pocket is rather featureless due to the absence of the β-E1 to β-F1 loop (45) (Fig. 2). Interactions with the basic side chain of Arg155 were initially postulated to result in acidic residues being favored in hexapeptide product inhibitors (67), with strongly basic residues proving to be detrimental to activity. Recent inhibitor disclosures have shown, however, that large aromatic and weakly basic residues can also interact in the S2 pocket with good potency (13, 68). These inhibitors extend beyond the enzyme active site typically occupied by the side chains of the natural substrate. Another point of interest relates to modifications to the prime site pockets that manifest themselves upon binding of NS4A. The S1' pocket is larger than observed in thrombin and elastase and, although Ser and Ala are required for efficient substrate cleavage (59, 60), bulkier aromatic moieties can be accommodated (65). These observations will be discussed further in the inhibitor design and development section.

Another characteristic of the HCV Pr structure is a zinc binding motif with the metal tetrahedrally coordinated by the two cysteines Cys97 and Cys99 from a linker between the two β-barrels, and Cys145 from the C-terminal barrel through a water molecule to His149 (46). Since the zinc binding site is on the opposite side of the protein to the active site, the site may support structural stabilization (45, 46) as is the case for other serine proteases such as elastase, which possess a disulfide bridge in a similar position.

The bulk of HCV Pr structural characteristics have been established from crystallographic and NMR studies of the catalytic domain. Given the unquestionable utility of these structures in inhibitor design, their biological relevance would appear to be vindicated. The recently published crystal structure of the full-length multifunctional NS3 revealed segregation of the protease and helicase domains via a flexible strand (56). Reassuringly, the two subdomain folds were equivalent to crystal structures of the isolated protease and helicase domains, again pointing to the probable relevance of the isolated catalytic domain. Nevertheless, significant differences in inhibition have been reported using product-based inhibitors on the HCV Pr catalytic domain *versus* full-length NS3 (69), leading the authors to conclude that the influence of the helicase domain is substantial. It should be noted that identical assay reagents were used for both recombinant protease forms. This raises the potential issue that activity differences may be the result of screening conditions, since optimal reagent concentrations often vary from one construct to the next. An example of this is evident in literature examples of full-length HCV Pr assays. Johansson *et al.* (69) utilized the catalytic domain and full-length NS3 with significantly different concentrations of sodium chloride and glycerol compared with Howe *et al.* (70), using full-length NS3 constructs with the NS4A either tethered at the N-terminus or connected at the C-terminus as in the virus. Consequently, although subtle differences may exist between protease forms, there is little doubt the catalytic domain provides a biologically relevant construct.

Reagents and technologies for discovery of HCV Pr inhibitors

Generation of protein

Extensive quantities of active and stable HCV Pr are essential for supplying screening efforts, especially when taking into account the assay requirements of combinatorial libraries and ever-expanding screening decks. Crystallographic and NMR structural studies also consume large amounts of protein. Consequently, identification of procedures capable of reliably generating high quality protein with good solubility, low aggregation and enzymatic activity are a crucial requirement for carrying out structure-based inhibitor design. The catalytic domain, encompassing at least the first 181 residues of the NS3 domain, was initially expressed in both *E. coli* and baculovirus systems providing material that led to a detailed understanding of activity and function (8, 62, 71). As interest turned toward structural characterization, better expression and purification protocols were developed (46, 60, 72). For crystallization, high protein concentrations are desirable, and this has been accomplished in the presence and absence of complex formation of the NS3 catalytic domain with exogenous

NS4A (45, 46). The addition of polypeptide tags, such as poly-His, fused to the C-terminus of the protein do not interfere with the conformation of the active site (46), but enhance stable protein expression, solubility and purification. An N-terminal polypeptide tag is also possible, as demonstrated in constructs where the essential NS4A residues are tethered at the N-terminus to the HCV Pr domain (73-76). Tethering of the NS3 catalytic domain to NS4A via its C-terminus has also been reported (77). Employing optimized bacterial codon usage with said constructs produce greater than 50 mg of purified protein per liter of bacterial culture, with solubilities in excess of 30 mg/ml in the absence of detergent (75). Such yields and characteristics are suitable for both crystallography and NMR studies.

Recent efforts have focused on obtaining full-length NS3 for *in vitro* studies. Full-length NS3 expressed from different hosts, (78-81) once complexed with NS4A, resulted in equivalent or enhanced activities to those reported for the catalytic domain. Considering the size of the protein, acceptable yields after purification were also achieved (80). Employment of constructs encoding a poly-His tagged NS3/4A with a C-terminus poly-Lys tail (82) or a poly-His tagged NS4A peptide fused to the full-length NS3 through a flexible tetra amino acid linker (83) further augmented yields. As with the tethered catalytic domain constructs, complex formation occurs during expression, resulting in better expression yields and enhanced activity compared with full-length NS3 alone. The resulting protein was also found to be crystallizable (84).

Recombinant enzyme in vitro assays

Protein supply and knowledge of the cleavage sites are the key requirements for generation and optimization of a protease assay. The value of a recombinant enzyme assay is in the demonstration of a potential inhibitor directly interfering with the target, assuming the correct folding of the protein. In addition, inhibitor mechanism of action studies against the target can be evaluated with relative ease. Furthermore, the recombinant enzyme assay provides a clean and uncomplicated method for appraising structure-activity relationships (SARs) as inhibitors are optimized. The protease exerts different kinetic parameters on peptide substrates portraying each of the cleavage sites, with the peptide representing the NS5A/B cleavage site being hydrolyzed more efficiently than the NS4A/B site (60, 65). Reported kinetic parameters on similar peptide sequences have differed considerably depending on the enzyme form utilized and the reaction buffer components; however, these discrepancies are inconsequential for relative inhibitory comparisons. Over the past few years, many low- and high-throughput screening assays have been described for HCV Pr. Protease inhibitor effects can be evaluated by high performance liquid chromatography (HPLC) (60, 85) by measuring substrate and cleaved product. This method is adequate for exploratory studies or as a secondary assay but time-consuming for lead identification/optimization. In the HIV protease field, fluorescence resonance energy transfer (FRET)-based substrates were routinely utilized in the early 90s for continuous monitoring of proteolytic activity in the presence of inhibitors (86) using 96-well formats. Taliani *et al*. (87) designed a HCV Pr fluorogenic substrate loosely based on the HCV NS4A/B cleavage site. An ester linkage was incorporated at the cleavage site, enabling rapid turnover of the peptide (κ_{cat}/K_m = 345000 $M^{-1}s^{-1}$). Not only is this assay sensitive, enabling detection with as low as 250 pM enzyme, it provides a means of evaluating inhibitor potencies in minutes. Other groups have reported the design of FRET-based and chromogenic substrates established around the various cleavage site sequences (88-92), suggesting better stability toward nonenzymatic hydrolysis (89, 90) and enhancement of assay sensitivity (89). A caveat with fluorescent-based assays is the possibility of false positives as a result of potential inhibitors quenching the signal. It is thus advisable to have a secondary assay at hand to confirm protease inhibitory effects.

The FRET-based continuous assay is convenient for a medium-throughput approach, or as a secondary assay for compound characterization. For high-throughput screening (HTS) campaigns, fully automated, rapid and robust assays conducive to 96-well and 384-well assay formats have been described. Two different scintillation proximity assays (SPAs) have been designed using radiolabeled peptides mimicking HCV Pr cleavage sites, monitoring either an increase (93) or decrease (94) of signal depending upon hit identification. Fowler *et al.* (93) immobilized a biotinylated tritiated peptide on streptavidin-coated SPA beads. Upon proteolytic cleavage, a decrease in signal was recorded whereas in the presence of an inhibitor, the signal was greater. Steinkuhler *et al.* (94) immobilized the protease domain to SPA beads via a biotin recognition sequence fused at the C-terminus of the protease. Interaction of the protease with a tritiated peptide was detected by scintillation counting. In the presence of an inhibitor, the radioligand was displaced, reducing the radioligand readout. More recently, Berdichevsky *et al.* (95) described an HTS system whereby a green fluorescent protein is fused to a cellulose-binding domain via the NS5A/B protease cleavage site. Cleavage of substrate results in emission of fluorescent light that is detected and quantified by fluorimetry. The above mentioned authors reported using 20-40 nM of protease per reaction, which is significantly greater than quantities required in the continuous FRET-based assay (87). Assay sensitivity is not a major concern in the discovery of an early hit, however, since the probability of identifying low nanomolar hits from an HCV Pr HTS screening campaign is negligible.

Cell-based assays

Currently, the effects of antiviral inhibitors on the HCV life cycle are poorly understood due to the lack of a

reliable cell culture system allowing viral infection and easily accessible animal models. While this is the case, HCV Pr inhibitors can be evaluated in alternative cell-based systems. The advantage of a cell-based assay over a recombinant enzyme is the demonstration of an inhibitor to penetrate the cell membrane and exert its effects on the target under near physiological conditions.

Establishment of HCV Pr cell-based assays is relatively straightforward since replicating viral systems are not essential for evaluating proteolytic activity. To date, numerous cell systems have been described in the literature. Their applicability to reliable low- to medium-throughput assessment, however, can vary significantly. Focusing on the cell systems employing NS3/4A constructs, Bansal and Pasquinelli (96) illustrate an assay utilizing Western immunoblotting as a method for detecting proteolytic cleavage. This is useful for establishing proof of principle for inhibitors acting on polyprotein cleavage, but not as an efficient, dependable screen. A number of groups describe reporter systems where luminescence from a microtiter plate is the readout of proteolytic activity (97-100). Characterized systems use either a reporter fused to a specific peptide cleavage sequence (97, 100, 101) or to the polyprotein (98, 99, 102). The former allows *trans*-cleavage events to be evaluated whereas the latter measures both *cis*- and *trans*-cleavage events simultaneously in the context of the natural polyprotein. Reporters utilized include secreted alkaline phosphatase (SEAP), luciferase, chloramphenicol acetyl transferase (CAT) and β-galactosidase (β-Gal). There are advantages and disadvantages for each system. SEAP, unlike the other systems, has the advantage of being secreted into the media, thus cells are not lysed and continuous measurement of activity over time is feasible. However, the assay is slower and there is a media transfer step. For ease and quickness of assay, luciferase and β-Gal signals are measured directly from the screen plates without transfer and are conducive to 384-well plate format. The β-Gal is cheap but advances in the luciferase reporter (103) provide a more stable option.

Evaluation of potential HCV Pr inhibitors on viral infectivity has been limited as a consequence of inadequate infectious HCV cell systems. Alternative approaches have included the establishment of chimeric viruses, with either closely related viruses such as bovine viral diarrhea virus (BVDV) (104) and hepatitis G (GBV-B) (105) or with well-studied viruses such as Sindbis (106) and poliovirus (107). The assay turn-around time of these systems is not as rapid as with simple reporter assays and there is greater variation in the data. Nevertheless, they permit the potential evaluation of protease inhibitors on viral infection and provide a tool for drug resistance assessment. In each of these systems, variants of the NS3 or NS3/4A complex were introduced, either by replacing respective viral protease and cleavage sites with those of HCV Pr, replacing non-essential sequences for viral infectivity, or fusing protease sequence to a structural polyprotein gene, making propagation of infectious viruses reliant on HCV Pr-mediated proteolysis. Representative BVDV, GBV-B and Sindbis HCV NS3/4A chimeras were identified with similar viral proliferation characteristics to parent virus. It should be noted that BVDV and Sindbis HCV NS3/4A chimeras were prone to deletion and mutation during viral passage although the actual protease domain was not affected and neither was its activity. Small animal models exist for the above parent viruses (108-110); thus, these viral chimera systems are potentially beneficial for *in vivo* testing of HCV Pr inhibitors as well as cell-based studies. The poliovirus NS3 chimera was hindered with respect to growth kinetics and viral titer when compared with parent virus, probably due to inefficient cleavage of the substrate. Moreover, inclusion of the HCV NS4A cofactor for proteolytic enhancement yielded no virus. Exclusion of the NS4A cofactor makes this system potentially suboptimal for HCV Pr inhibitor evaluation due to anticipated protease conformational variations relative to the NS3/4A complex.

All cell systems discussed thus far are specific for examining the effects of inhibitors directly on HCV Pr activity, whether via the inhibition of peptide *trans*-cleavage or polyprotein processing. Moreover, these systems can be modified to evaluate potential inhibitors across a spectrum of HCV genotypes. Assessment of protease inhibitor effects on HCV viral replication, however, was not possible until the advent of the replicon system (12). The replicon contains all of the essential HCV proteins required for RNA replication (5' and 3' NTR, NS3 to NS5B; Fig. 1). Inhibitory effects on HCV RNA replication can be examined by measuring HCV RNA, which is quantified using real-time polymerase chain reaction (RT-PCR) techniques (39). Although the RT-PCR approach is valuable for ascertaining effects on RNA production, it is not optimal as an inhibitor screening mode because of high reagent costs, assay variability and labor time. The first constructed replicon reporter system was assessed in transient experiments (111). More recently, stable cell lines expressing replicon reporter systems have been described. The first example encodes β-lactamase upstream of the selectable neomycin marker, and is expressed in conjunction with replicon replication (112). The second system employs stable cells that coexpress SEAP under the control of the HIV long terminal repeat promoter (LTR), and a modified replicon construct encoding the HIV tat transactivator protein (113). Replication of subgenomic RNA replicons leads to the subsequent induction of SEAP synthesis followed by secretion to the media where it is readily quantified. These replicon systems currently allow evaluation of HCV Pr inhibitors on both polyprotein processing and RNA replication of 1b genotype variants, and could be established for the recently described 1a genotype (114). Low nanomolar protease inhibitors have been reported to inhibit replication of HCV replicons representing both of these genotypes (13). With the identification of new cell culture adaptive mutations (115, 116), production of more robust and sensitive screening assays should result, although their relevance for viable infectious HCV genomes is open to debate (117).

The age of reliable cell systems permissive to HCV infection may be fast approaching. Sung *et al.* recently reported the establishment of B-cell lymphoma cell lines persistently infected with HCV (118). Furthermore, virus particles produced from these cell lines were shown to be infectious to unexposed B-cells, as well as primary human hepatocytes. Unlike the replicon systems where HCV genotype 1a and 1b systems were developed, the B-cell system was infected with genotype 2b. It will be interesting to ascertain whether other genotype representatives are capable of producing persistent infections in the B-cell system. If virus production can be maintained in immortalized cells, as suggested, efficacy of potential inhibitors on viral infectivity and evaluation of drug resistance in the context of the whole virus will become a reality.

Animal model systems

As outlined above, an array of assays and technologies exist for evaluation of protease inhibitors. These systems permit optimization of an inhibitor but do not provide an indication of efficacy in a complex *in vivo* environment. Advances in HCV therapy have been hampered by the absence of a convenient animal model system that supports HCV infection. Although the lack of replicating systems has not prevented the discovery of HCV Pr inhibitors, a good animal model would provide added confidence for advancing a potential drug to the clinic, especially since antivirals specific for HCV targets have yet to be approved. While elevated costs have limited its use, the chimpanzee model of HCV infection was instrumental in observing the clinical course of infection, determining the physical properties of the virus and eventual cloning of the HCV nucleic acid (119). Two HCV mouse models were developed recently in an effort to create a more generally usable system. One was established by transplanting human hepatocytes into homozygous, severe combined immune deficient (SCID) mice overexpressing a urokinase plasminogen activator transgene from an albumin promoter (Alb-uPA) (120). These mice developed persistent HCV infections with high viral titers 35 weeks after inoculation with serum from HCV-infected patients. The second “HCV-Trimera” model was developed by using γ-irradiated mice reconstituted with SCID mouse bone marrow cells, followed by transplantation of HCV-infected human liver fragments (121). Although viremia was recorded, levels were lower than those reported for the Alb-uPA model and persisted for approximately 1 month. Both models established successful infections with a range of genotypes. Furthermore, the validity of these murine models in drug discovery was achieved by demonstrating a reduction in viral titer in response to either interferon treatment (122) or a small-molecule inhibitor of the HCV internal ribosomal entry site (121).

Despite the value of such models for demonstration of drug efficacy and assessment of drug pharmacokinetic properties, the best reassurance can only be obtained by advancing a drug candidate to human clinical trials. Upon determination of efficacy in the clinics, a retrospective correlation can be established.

Design and development of HCV Pr inhibitors

The search for an HCV Pr inhibitor has proven challenging. Reports suggesting success with efforts employing high-throughput screening approaches have not surfaced. Consequently, efforts have focused on structure-based rational drug design (67, 123, 124). This approach has been utilized successfully with other serine proteases such as the coagulation pathway enzymes factors VIIa (125), Xa (126) and thrombin (127). HCV Pr inhibitor development has recently focused on the optimization of peptide mimetics, despite a long-standing stigma attached to this inhibitor class. Historically, issues such as proteolytic cleavage of amide bonds as well as suboptimal pharmacokinetic (PK) properties have compromised the utility of such inhibitors. The latter has generally been attributed to high molecular weight (MW), although high logP and excess number of free hydrogen bond components have been raised additionally as potential disadvantages (128). Recent studies, however, have questioned the relevance of the relationship between MW and bioavailability (129). In addition, advances in peptidomimetic design have illustrated that reducing amide bond count or shielding them with lipophilic moieties can dramatically improve PK properties. In the HIV protease arena, many oral drugs exist that display MWs significantly greater than 500 and, nonetheless, exhibit acceptable PK properties (130). These examples challenge many of the tenets related to molecular weight. However, it should be noted that optimization of PK properties was achieved by thorough studies assessing the effects of minor modifications to the molecule as exemplified in the development of ritonavir (131). In the case of atazanavir, such investigations (132) have led to a peptide inhibitor with a PK profile acceptable for once-daily administration. Preliminary phase II clinical results for the HCV Pr inhibitor BILN-2061 suggest acceptable exposure with a twice-daily dosing regimen (14). BILN-2061 has both a higher MW than most of the HIV protease drugs and also possesses hydrogen bond donor/acceptor counts in excess of the limits set by the “rule of 5” (128). The compound provides an example of successful inhibitor design that runs contrary to conventional thinking on the subject of drug-likeness.

HCV Pr has proven a particular challenge primarily due to the featureless nature of the active site. As detailed in the structure-function section (and highlighted in Fig. 2), the shape of the active site lacks definition compared with other serine proteases. This is in part due to a missing loop above the S2 subsite. In addition, HCV Pr possesses an S1 (Cys) recognition pocket, which is small compared with the large basic/hydrophobic (Arg/Phe) trenches typically found in chymotrypsin-like

serine proteases. These issues are highlighted by the fact that HCV Pr requires peptide substrates spanning residues P6 to P4' for efficient cleavage (60).

The P-side hexapeptide motif dominated much of the early research in inhibitor development until the important work of Llinas-Brunet *et al.* (10), who determined that inhibitory activity could be modulated through substituent variation at P2. Hexapeptide inhibitor **1** (Fig. 3), derived from the N-terminal product of NS5A/B cleavage, highlights an example of this SAR. This discovery has enabled a reduction of the peptidic character through N-terminal truncation of the P4-P6 residues. It offers no apparent advantages regarding molecular weight reduction, however, as large hydrophobic aromatic substituents are preferred. As shown in Figure 3, 4*R*-substitution of the P2 proline ring significantly increases the potency with, for example, benzyloxy analog **2** exhibiting 20-fold more potency than **1** (10). Increasing substituent hydrophobicity further enhances the inhibitory activity. For example, the naphthalen-1-ylmethoxy derivative **3** is 18-fold more potent than **2**. P2 substitution potential is extensive, reaching well beyond the substrate subsite into solvent exposed regions of the enzyme. This region can accommodate many different chemical moieties, and consequently offers plenty of opportunity to modulate the physicochemical properties of the inhibitor. The ability to bind to this region of the enzyme is highly dependent on chemotype, however, and as described in the sections below, some inhibitors are devoid of such capability. The solution structure of the carboxylic acid **2** bound to the protease domain as revealed by NMR studies (124) shows that **2** binds to the protease in a well-defined, extended conformation that is consistent with the usual binding mode of peptidic serine protease inhibitors. It also shows that the P3, P5 and P6 side chains have a lesser effect on direct binding to the protease. The bulky P3 side chain appears to play a critical role in rigidifying and preorganizing an extended conformation in the unbound state, with a Cα *tert*-butyl substituent being well tolerated at this position. Accordingly, reduction of the size of the peptide by N-terminal truncation of the acidic P5 and P6 residues is possible, and this provides tetrapeptide **4** (Fig. 3) that represents a minimally charged HCV Pr inhibitor (133, 134). This series of inhibitors with a C-terminal carboxylic acid is highly selective for HCV Pr compared to related serine proteases. For example, **4** does not significantly inhibit human leukocyte elastase (IC_{50} >300 μM). As cysteine is the consensus P1 residue among all the natural *trans*-cleavage sites and a determinant of specificity, it has been necessary to identify a viable replacement for the sulfhydryl group if *in vivo* oxidative metabolism of this group (including disulfide formation) is to be prevented. It has previously been shown that norvaline is a feasible replacement for the P1 cysteine of the hexapeptide product inhibitor (135).

The propyl side chain of the P1 norvaline was further optimized to a cyclopropyl group, based on the observations that the Cα-hydrogen and the δ-CH_3 are in close proximity when bound to the protease, as determined by transferred NOESY studies on tetrapeptide inhibitor **7** (136). As shown in Figure 3, the P1 cyclopropyl derivative **5** is 5-fold more potent than the acyclic norvaline derivative **4**. Substitutions on the cyclopropyl ring fill the S1 pocket more completely and, in the case of the vinyl group shown in compound **6**, leads to a 10-fold increase in potency. Extensive SAR studies on P2 hydroxyproline substituents have been performed by incorporating functional groups (*e.g.*, in **8-10**, Fig. 3) (133, 134, 137) that seek to balance potency, aqueous solubility and membrane permeability as measured by a Caco-2 assay. For example, a large increase in potency is observed when the naphthalen-1-ylmethoxy group (as in **6**, IC_{50} = 0.36 μM) was replaced with a 2-phenyl-7-methoxyquinoline moiety (as in **8**, IC_{50} = 2 nM). Compound permeability can also be substantially enhanced without significantly sacrificing aqueous solubility when the 2-phenyl moiety of **9a** is replaced with an aminothiazole moiety. For example, tripeptide analogs **9a** and **9b** are equipotent (IC_{50} = 25 nM) and exhibit a less than 2-fold difference in solubility. However, **9b** is 80-fold more permeable than **9a** (133, 134). These potent tripeptidic inhibitors were obtained via deletion of the P4 residue, which is made possible by maximizing the conformational control induced by the P3 residue and by taking advantage of the potency imparted by the large P2 substituent. The N-terminal capping at P3 has been optimized further to capture hydrophobic interactions at S4 to obtain highly potent inhibitors of HCV Pr, such as **10** (IC_{50} = 6 nM) (68, 133, 134). NMR studies on **7** have also revealed conformational differences in the bound and unbound state for the P1 NH, P1 cyclopropyl and P2 β-H (136), suggesting the potential for chemical rigidification of the P1 bound conformation. This is exemplified by the series of P1-P3 macrocyclic inhibitors (138), of which **11** is representative (see also Fig. 2), and from which the clinical candidate BILN-2061 was ultimately identified. The characteristic substituted quinoline ring extends beyond the S2 site that is occupied by the hydroxyproline and above the α_1-helix that contains the catalytic His57. The carboxylate moiety sits in the oxyanion hole and the vinylcyclopropyl occupies the S1 pocket. The 5-carbon methylene chain slips into the channel connecting S1 to S3 and fills the S3 pocket, while the *tert*-butyl group covers the S4 pocket.

Bristol-Myers Squibb has recently claimed a series of structurally related tripeptide inhibitors, which contain a C-terminal acylsulfonamide (*e.g.*, **12,** Fig. 4) (139). Bulky aromatic groups appear to be well tolerated in this position. This is an interesting modification in contrast to the observations that conversion of the C-terminal carboxylic acid to alternative functionalities (*e.g.*, ester and amide) greatly reduces potency, and selectivity against other serine proteases (*e.g.*, elastase) (135).

IRBM/Merck sought to take advantage of the more soluble NS4A/B N-terminal cleavage product (Ac-DEMEEC-OH, K_i = 0.6 μM), rather than the NS5A/B N-terminal cleavage product, as an entry point for the design of HCV Pr inhibitors. Systematic optimization of the P2 to P5 residues was performed producing

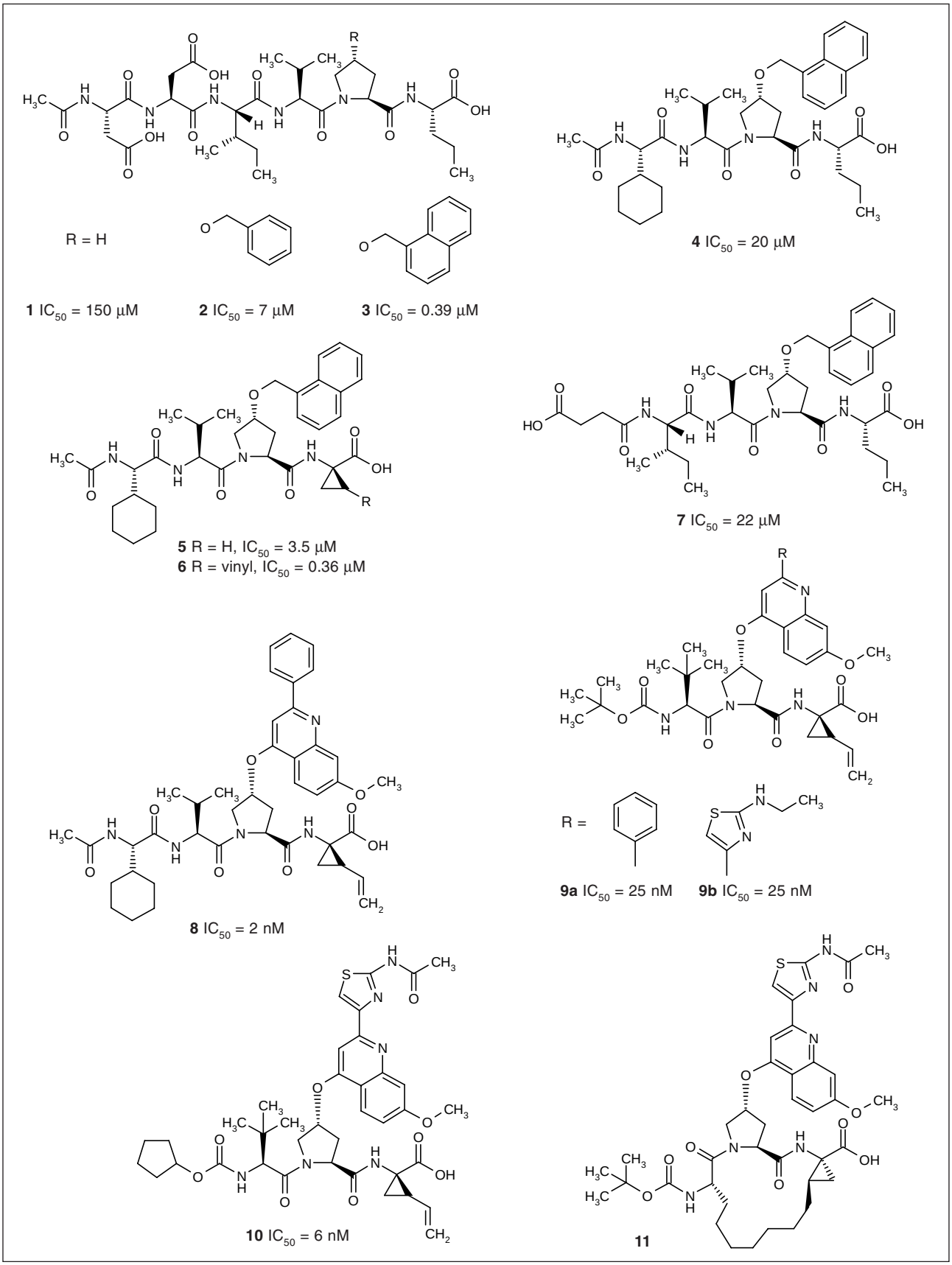

Fig. 3. Peptidic HCV Pr inhibitors that bear substitutions on the P2 hydroxyproline.

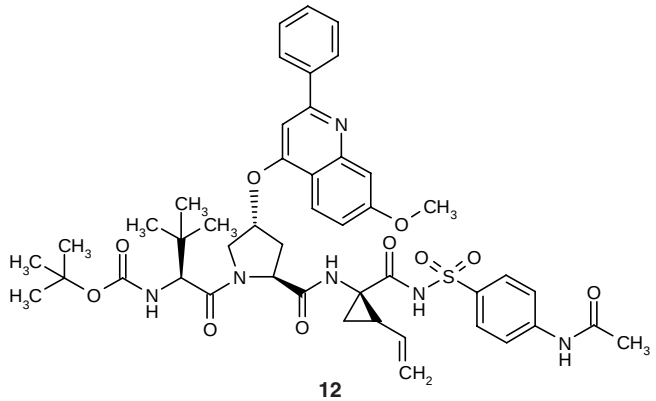

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Fig. 4. Peptidic HCV Pr inhibitor that incorporates acylsulfonamide at the C-terminal.

nanomolar hexapeptide product inhibitors (*e.g.*, **13**, K_i = 40 nM) (Fig. 5) (140). Based on the fact that the CF_2H and SH groups have similar calculated steric and electrostatic features, difluoroaminobutyric acid was designed as a cysteine mimic (141). The hexapeptide analog **14,** containing a di-F-Abu P1 moiety, is as potent as the cysteine analogue **13**. In these studies, a number of mechanism-based inhibitors have been prepared with alpha-ketoacid **15** emerging as the most potent hexapeptide inhibitor identified (142). Truncation of the acidic P5-P6 residues produced compound **16**, which was further refined to the tripeptide **17** in which a benzyloxycarbonyl group mimics the P4 residue (a *tert*-butyloxycarbonyl was equally potent). Tripeptide **18** represents the minimal pharmacophore for this series in which the ketoacid moiety is deemed essential, since the corresponding aldehyde, ketoester, hydroxyacid and ketotetrazole are all inactive. Interestingly and surprisingly, the X-ray crystal structures of **17** and **18** bound to HCV Pr revealed that the ketoacid moiety bound in an unusual manner relative to the complexes known to form between classic serine hydroxyl traps and mammalian serine proteases, with the carboxylate bound in the oxyanion hole (143). Given the potential for enzyme conformational change on binding of mechanism-based inhibitors, the reasons for this preferred binding mode are not entirely clear. It has been hypothesized, however, that the overall conformation of the tripeptide in the initial preacylated complex with the enzyme, coupled with the electronic effect of the 1,1-difluoroethyl Cα (P1) substituent, do not favorably dispose the carbonyl for a nucleophilic attack by the Ser139 hydroxyl group. This is consistent with the 2-4 log decrease in the rate constants for both the initial complex and the covalent complex formation when compared to those for the ketoacid/trypsin (or thrombin) complex. Kinetic studies suggest a larger energy barrier for both the formation and the dissociation of the covalent complex. Unlike the series of tripeptide inhibitors derived from hexapeptide **1** (Fig. 3), this series of compounds only accepts relatively small hydrophobic non-β-branched alkyl side chains at P2, with Val and Phe 5-to 10-fold less active. Thus, extension beyond the S2 site via further branching of the P2 side chain may prove problematic. The crystal structure also showed that the lipophilic P2 Leu side chain covered the His57-Asp81 catalytic ion pair, shielding it from solvent. Both electrostatic and hydrophobic interactions between the P3 residue and the side chain of the labile Lys136 were evident. Further optimization was undertaken to capitalize on the hydrophobic interaction at S3 and hence minimize the charged character of the ketoacid inhibitors. This exercise produced the P3 cyclopentyl analog **19** (IC_{50} = 0.38 μM, Fig. 5), which is as potent as the diacid **18**. In an effort to further reduce the peptidic nature of this class of inhibitor, the capped dipeptide **20** was derived from tripeptide **19** (144). Interestingly, molecular docking experiments suggested that the preferred bound conformation of **20** favors an intramolecular hydrogen bond between the NH and the hydroxyl oxygen atom such that the phenyl ring projects into the S3 pocket and interacts with the side chain of Lys136.

Key electrostatic and hydrophobic interactions with the Lys136 side chain (also Arg109) have been further exploited in the design of inhibitors that extend into the S2' site. For example, when the C-terminal of the tripeptide is converted to a substituted amide, active compounds are obtained when appropriately disposed complimentary functionality is introduced (145, 146). The phenethyl amide derivative **21** (Fig. 5) that contains a *para*-substituted benzoic acid is 10-fold more potent than the parent compound as a result of close contacts between the carboxylic acids and the charged groups of Lys136 and Arg109. In contrast to α-ketoacids (*e.g.*, **18**), these amides do not require the reactive carbonyl moiety that confers potency by trapping the serine139 hydroxyl group. Taking advantage of these observations, inhibitors of HCV Pr that rely solely on S' site and oxyanion hole interactions were designed, for example **22** (147).

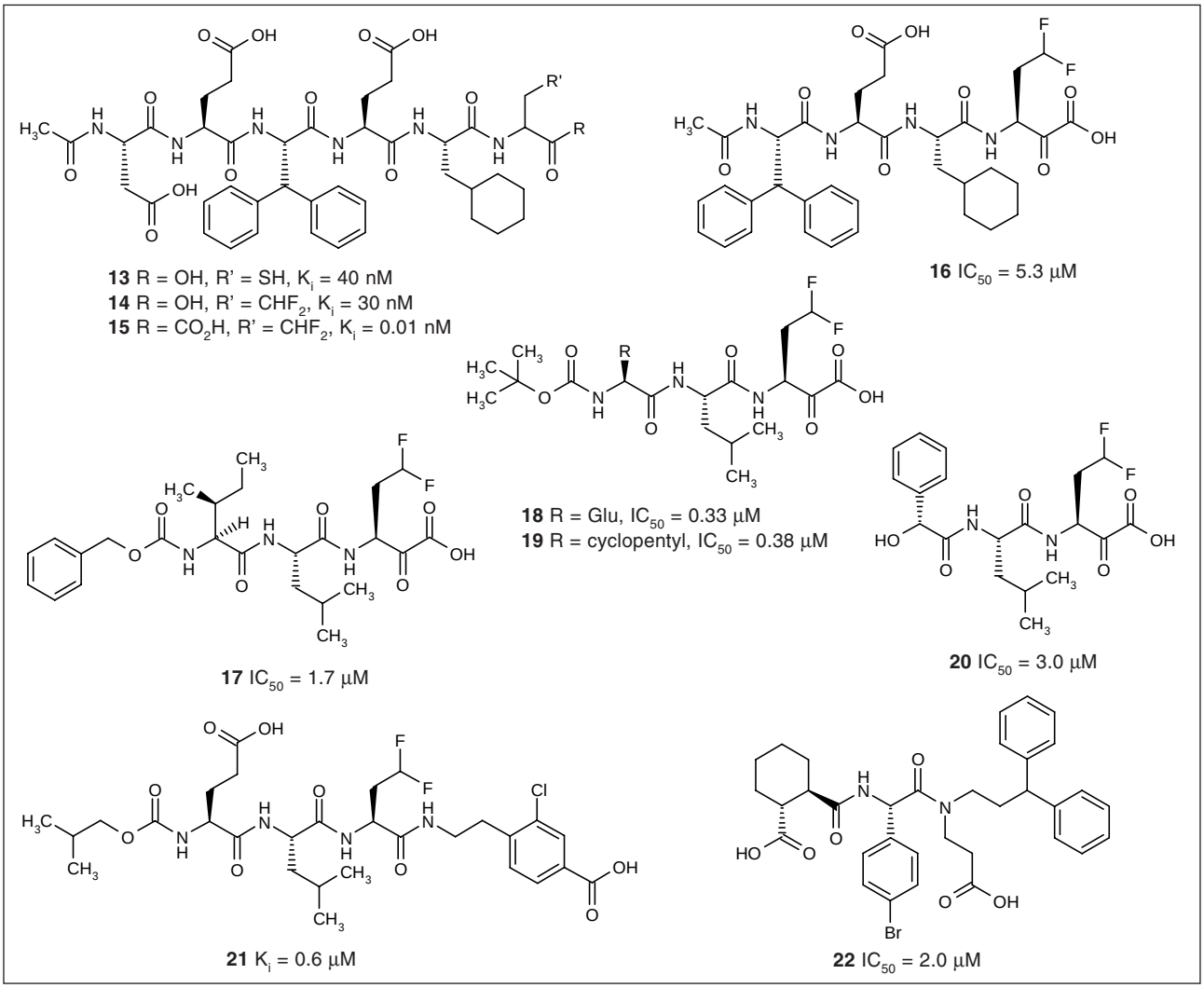
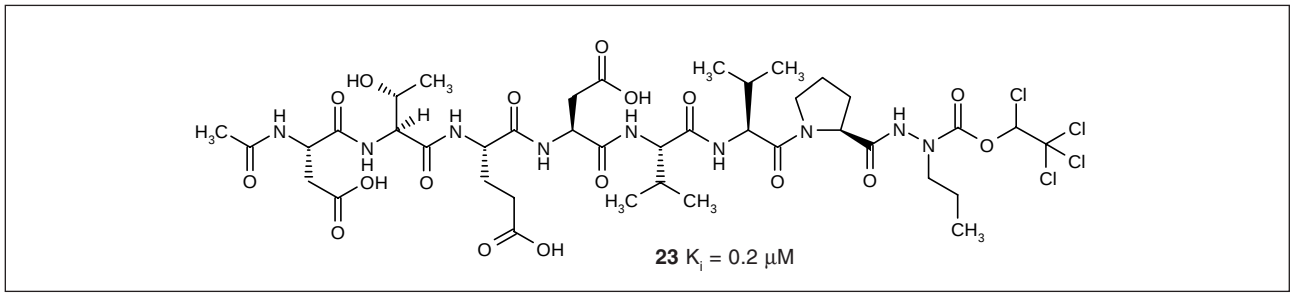


Fig. 5. Carboxylic acid and α-ketoacid HCV Pr inhibitors (**13, 14, 15-20**) and peptidic inhibitors (**21** and **22**) that utilize prime site interactions.

Although these studies have, to date, provided compounds with only low micromolar potencies, they may serve as a foundation for the development of inhibitors that exhibit a different binding mode relative to the more conventional product-based inhibitors described earlier. This could be of importance in addressing the issue of drug resistance associated with chemotherapy using protease inhibitors by combining compounds with complementary protease binding profiles.

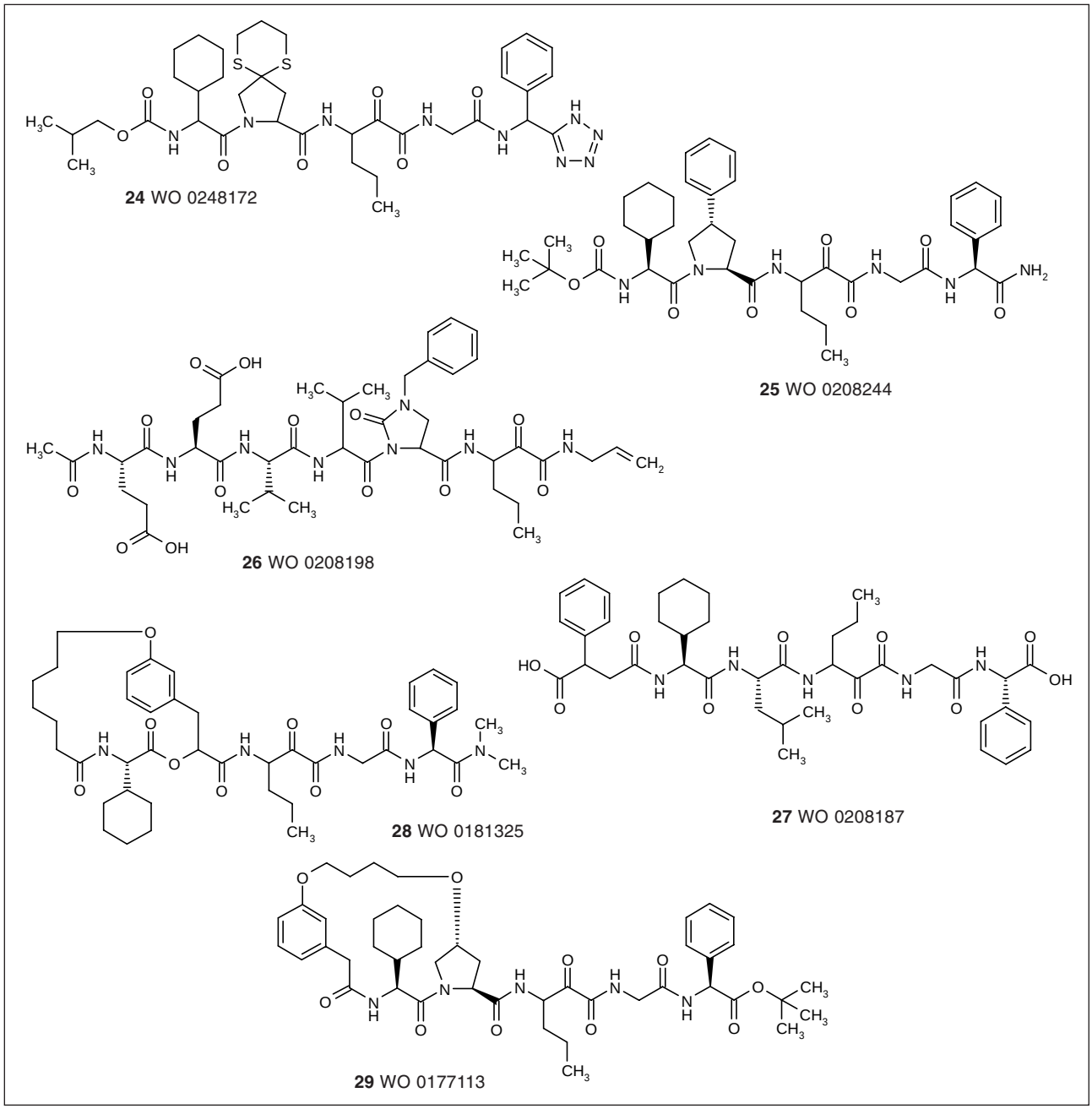
As discussed above, the structure of the ketoacid/NS3 protease complex has unveiled an inhibitor binding mode that is different from that anticipated, based on conventional knowledge. Unexpected results were also obtained when a library of azapeptides with different electrophilic leaving groups, as represented by compound **23** (Fig. 6), were evaluated (148). Incorporation of an aza-amino acid at P1 reduced the protease k_{cat} by about 200-fold, while the K_m was not affected. However, kinetic studies on these compounds found them to be competitive inhibitors with less than 10% of activity due to time-dependent inactivation by acylation.

In connection with the α-ketoacids, α-ketoamides, extending into the prime site via the amide linkage, have been extensively investigated by a number of groups. For example, Schering Plough/Corvas have disclosed multiple series of ketoamide derivatives that incorporate a variety of P2 moieties or replacements allowing access to structurally diverse P2 substitutions (**24-27**) (Fig. 7). Macrocycles obtained by joining P2 and P4 residues (*e.g.*, **28** and **29**) were also disclosed. The best characterized ketoamides described to date are a series of tetrapeptides derived from the NS5A/B cleavage site, of which **30** and **31** are representative (Fig. 8). These cyclopropylamides are an offspring of the prototype **32**. Notable features are a novel, *cis*-fused bicyclic proline at P2 and basic heterocyclic capping group at the P3 N-terminal. These analogs have been evaluated in the replicon assay, in combination with INF-α and/or ribavirin,



23 K_i = 0.2 μM

Fig. 6. Electrophilic azapeptide HCV Pr inhibitor.



24 WO 0248172

25 WO 0208244

26 WO 0208198

27 WO 0208187

28 WO 0181325

29 WO 0177113

Fig. 7. α-Ketoamide HCV Pr inhibitors disclosed by Schering Plough/Corvas.

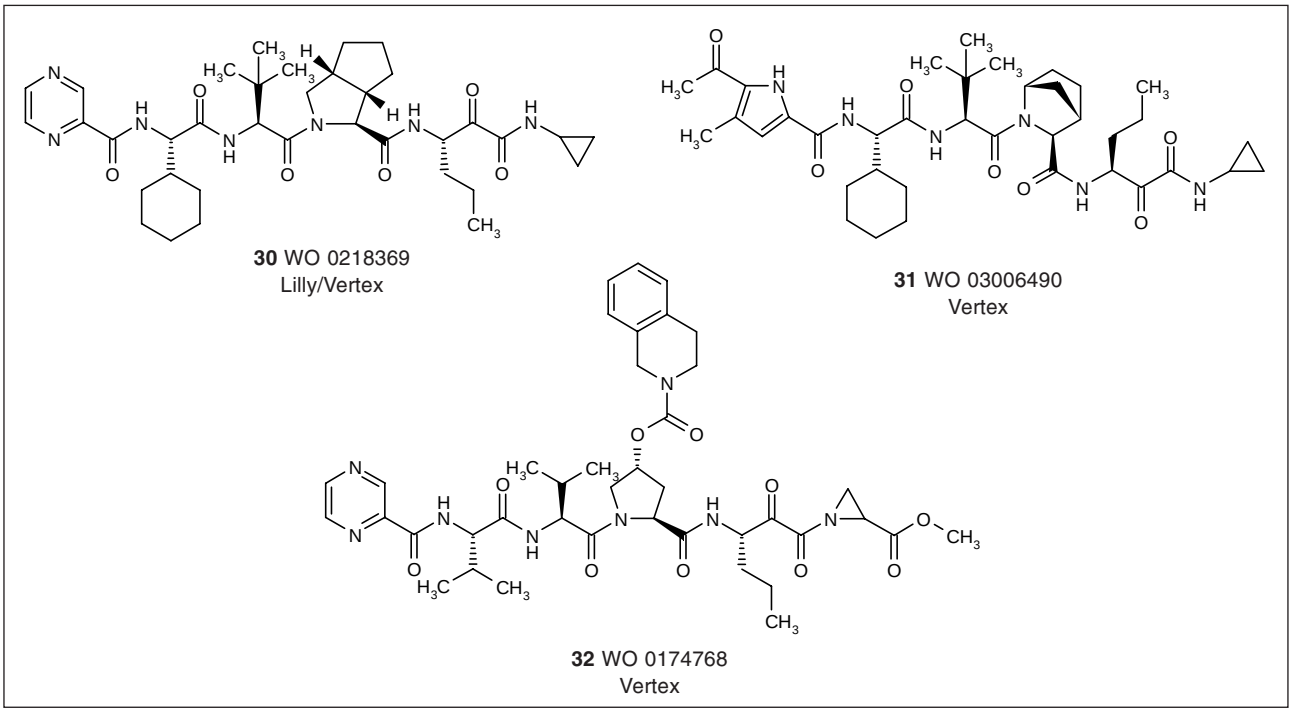


Fig. 8. α-Ketoamide HCV Pr inhibitors disclosed by Lilly and Vertex.

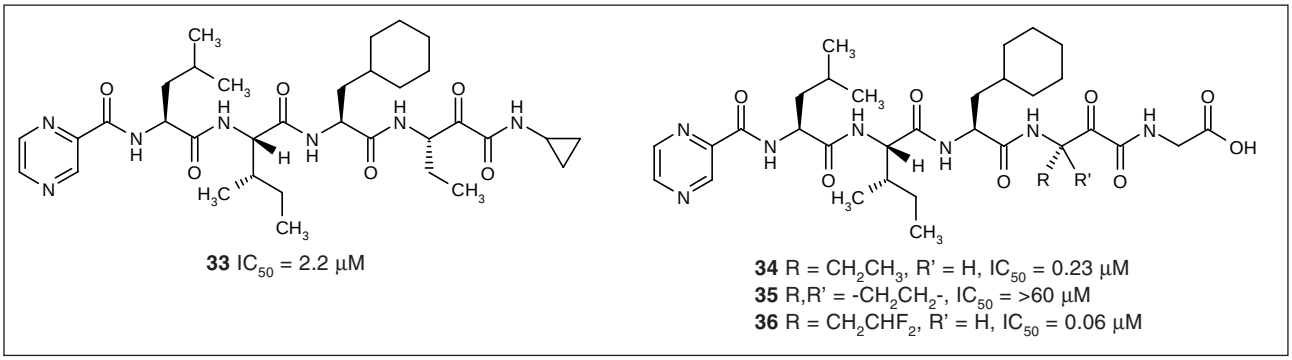


Fig. 9. α-Ketoamide HCV Pr inhibitors disclosed by DuPont Pharmaceuticals (Bristol-Myers Squibb).

and synergistic inhibiting effects were observed. A related series of compounds based on the NS4A/4B cleavage site have also been described by DuPont Pharmaceuticals (now Bristol-Myers Squibb). For example, the representative cyclopropyl analog **33** (Fig. 9) exhibits an IC_{50} of 2.2 μM, which is improved 10-fold when the C-terminal is capped with a glycine (**34**, IC_{50} = 0.23 μM) (149). The effect of P1 substituent variation on the inhibitory activity of this series was examined. Remarkably, a dramatic difference in the activity was observed when the P1 ethyl side chain of **34** was converted either to a cyclopropyl group (as in **35**) or a 1,1-difluoroethyl group (as in **36**), with **36** being >1000-fold more potent than **35**. This result indicates a very different binding mode between the NS4A/B and NS5A/B cleavage site derived N-terminal product inhibitors. Although to date the exact structure of ketoamide/HCV Pr complex has not been elucidated, a computer model of **34** bound to the enzyme suggests that the glycine moiety interacts with Lys136 and Arg109 in the S2' site, which is reminiscent to that of the phenethyl amide **21** (Fig. 5).

The C-terminal carboxylic acid is an important structural element in the development of product-based inhibitors (*e.g.*, tripeptides **10**, **11** and ketoacids **17** and **18**). This group confers activity to the inhibitors via binding in the oxyanion hole. The design of inhibitors in which the carboxylic acid in NS5A/B-based product inhibitors is replaced with boronic acid has been disclosed by DuPont

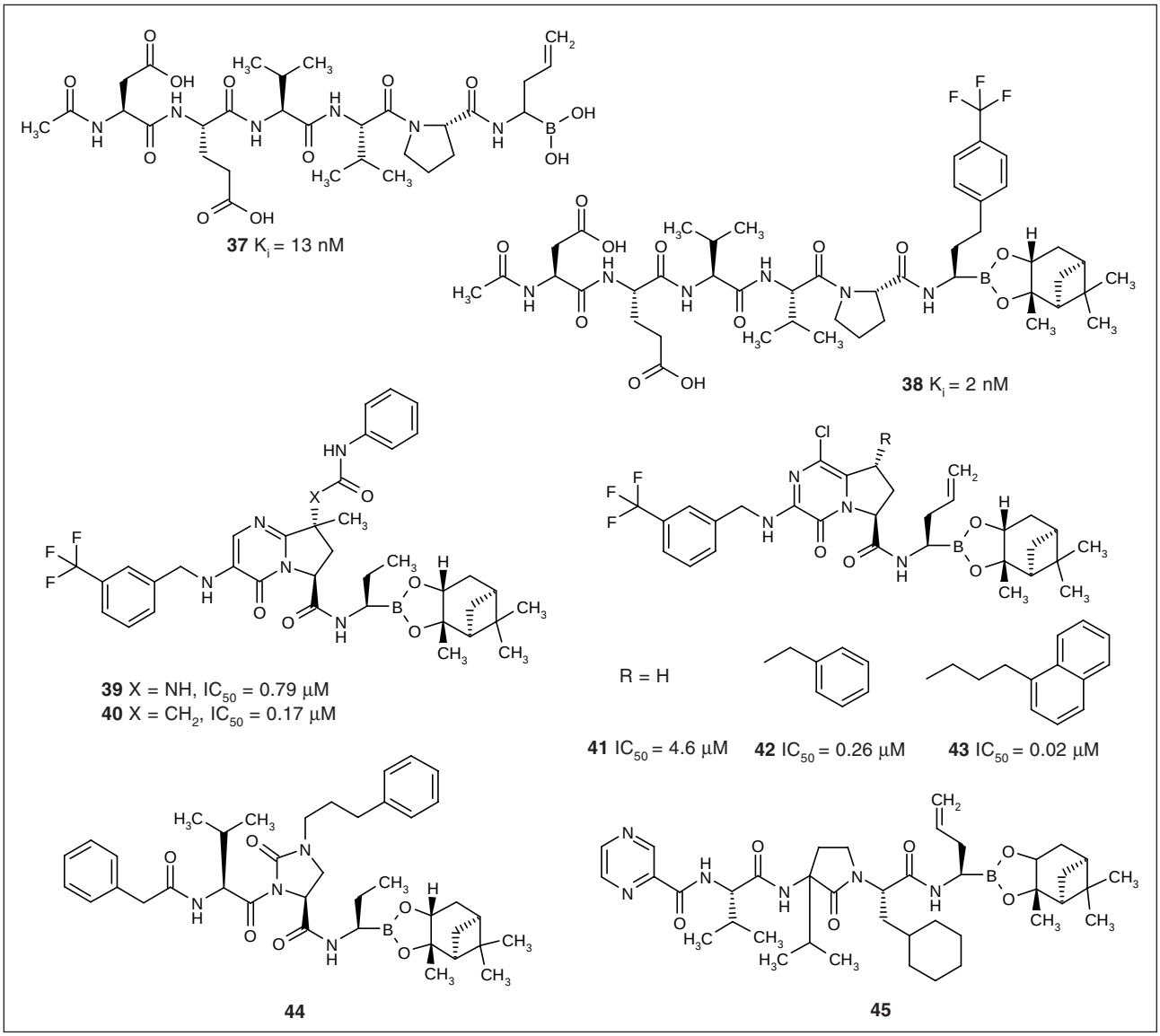


Fig. 10. Boronic acid HCV Pr inhibitors.

Pharmaceuticals (Fig. 10). These transition state analogs (**37-45**) feature different P2 proline variants and fused P2-P3 structures designed to reduce peptidic character. A solution structure of the boronic acid inhibitor **37** bound to HCV Pr in the presence and absence of NS4A cofactor (54) revealed that, in contrast to the α-ketoacids, the boronic acid binds in a fashion analogous to that observed with mammalian serine protease inhibitors. A tetrahedral adduct is formed between the boronic acid and the catalytic serine, in which one borate hydroxyl occupies the oxyanion hole and the other hydrogen bonds to the catalytic histidine. In the absence of NS4A cofactor, the inhibitor binds with 10-fold weaker affinity, consistent with the finding that a proper orientation of the catalytic triad is partly induced by NS4A cofactor binding.

It appears that this chemotype can uniquely accommodate a more bulky P1 side chain moiety since the phenethyl derivative **38** is a potent inhibitor of HCV Pr and is selective against human leukocyte elastase and pancreatic chymotrypsin (150). As a strategy to reduce peptidic character, the P2 and P3 residues have been fused to form bicyclic systems, as exemplified by 3-aminopyrimidinone (**39** and **40**) and 3-aminopyrazinone (**41-43**, Fig. 10), which are used as β-sheet mimetics. Similar to SAR observations monitored in the BILN-2061 series (Fig. 3), 8*R*-subsitution on the ring (corresponds to 4*R* on the P2 poline ring) enhances the inhibitory potency via interactions made beyond the S2 site. For example, the phenyl urea **39** and the phenyl amide **40** are, respectively, 10- and 40-fold more potent than the 8-unsubstituted

parent pyrimidinone (IC_{50} = 7 μM) (151). Increasing the lipophilicity of the P2 substituent also increases activity in the pyrazinone series. For example, the 8-benzylpyrazinone derivative **42** is about 18-fold more potent than the parent **41**, while the naphthylpropyl **43** (IC_{50} = 0.02 μM) is 230-fold more potent (152). The SAR of the 3-amino substituent of **43** is flat which, as revealed by computer modeling studies, is a result of the fact that this group points away from the enzyme surface and into the solvent. The poor solubility of this pyrazinone series precludes it from further evaluation. Related series with different P2 proline variants, including imidazolidinone **44** (153) and lactam **45** (154), have also been disclosed.

While the emphasis of drug intervention efforts have focused mainly on peptide-based inhibitor approaches (66, 155), efforts directed toward the design of nonpeptide inhibitors for HCV Pr have also been reported. Several interesting approaches capitalize on the well-established, mechanism-based inhibitor design knowledge gained from the studies of mammalian serine protease. For example, Slater and colleagues (156) recently described application of a known mechanism-based serine protease inhibitor template, a pyrrolidine-5,5-*trans*-lactam, to HCV Pr. This template has been successfully developed to identify thrombin and elastase inhibitors (157) and, thus, provides a reasonable starting point for the design of nonpeptidic inhibitors of HCV Pr. The electrophilic carbonyl of the strained lactam is postulated to react with the catalytic serine hydroxyl group and form a covalent acyl-enzyme complex. Projection of functionality into the S1, S1' and S3-S4 enzyme pockets are possible by decorating the lactam ring, as shown in Figure 11. The prototype **46** displayed an IC_{50} of 30 μM after a 4-h preincubation (156). The crystal structure of **46** complexed with HCV Pr (158) suggests limited opportunities to add elements to this chemotype that can bind to the S2 pocket. This is based on the fact that substituents on the two methylene carbons of the pyrrolidine ring either point away from the enzyme surface or are sterically prevented from accessing the pocket. Crystallographic and modeling studies of these ethyl-substituted *trans*-lactams bound to HCV Pr also suggested that the S1 pocket could accommodate a 1,1-dimethyl or a spirocyclobutyl moiety. Indeed, the spirocyclobutyl derivative **47**, having a preferred cyclopropylcarbonyl at P1', gave a much improved IC_{50} of 0.51 μM, again with a 4-h preincubation. The specific inhibition of HCV Pr by the *trans*-lactam series in a cell-based environment has not been reported, although **47** shows activity in a replicon assay in the micromolar range (159). As in the example reported by Llinàs-Brunet *et al.*, structure-based design was utilized effectively to replace the reactive cysteine P1 residue with alkyl groups that have optimal binding interactions with the S1 pocket. The P1' cyclopropylamide group filled the S1' pocket more completely and also improved the plasma stability of the *trans*-lactam when compared to the electronically more activating methylsulfonamide group.

The 1,2-benzisothiazol-3-one-1,1-dioxide (saccharin) ring system is another mechanism-based template that

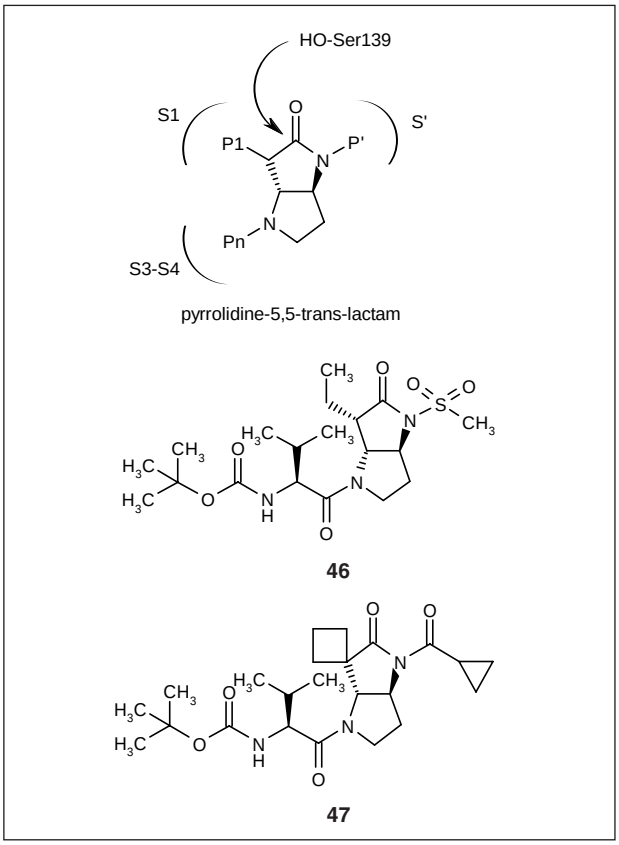


Fig. 11. Pyrrolidine-5,5-*trans*-lactam derived mechanism-based HCV Pr inhibitors.

has been successfully used in the development of orally bioavailable human neutrophil elastase inhibitors (160) and potent human mast cell tryptase inhibitors (161). This nucleus was examined as a vehicle to design inhibitors of the HCV Pr (162). Biochemical studies suggest the electrophilic carbonyl of the benzisothiazolone-1,1-dioxide reacts with the catalytic serine hydroxyl group to form a covalent acyl-enzyme complex. The leaving groups can be extruded in the next step to form a reactive iminium ion which then alkylates the nitrogen of the catalytic histidine imidazole ring. As a consequence, this class of inhibitors doubly covalently modifies the enzyme (**48**, Fig. 12). Binding to the S1, S2-S3 and S' enzyme pockets are possible by judicious substitutions of the saccharin ring and the leaving group, as shown in Figure 12. Diverse hydrophobic leaving groups were attached to the N of the heterocyclic ring to probe possible interactions with the prime site of HCV Pr. However, no significant inhibition of the protease was detected even for compounds with a thiomethyl substituent as the P1 element (*e.g.*, **49**) (163). Inhibitors with acidic leaving groups (*e.g.*, **50**), which take advantage of possible interactions with the basic residues surrounding the active site (164), were also inactive. These differences in saccharin-based inhibitor activity toward HCV Pr and human neutrophil elastase and

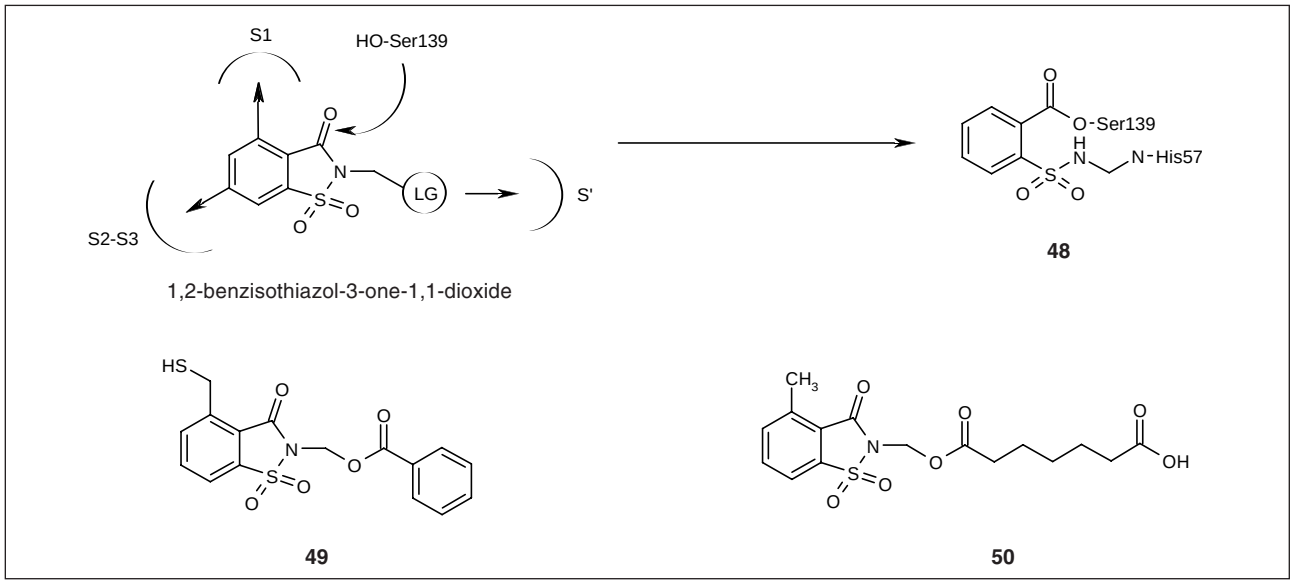


Fig. 12. 1,2-Benzisothiazol-3-one-1,1-dioxide derived mechanism-based HCV Pr inhibitors.

tryptase again highlight the unusual properties of the viral enzyme.

In inhibitory studies of trypsin by a bisbenzimidazole derivative, it was determined that the bisbenzimidazole core occupied the active site of the enzyme. Together with the catalytic serine hydroxyl and histidine imidazole acting as the other two ligands, the core was found to form a tetrahedral coordination complex with a Zn^{2+} atom (165). The potential of this novel approach to serine protease inhibition was also explored in the context of HCV Pr, resulting in the identification of the phosphonoanaline derivative APC-6336 (CRA-6336) (Fig. 13). APC-6336 is a potent, active site-directed inhibitor of HCV protease that displays an IC_{50} of 0.20 μM in the presence of Zn^{2+}. In the absence of Zn^{2+}, APC-6336 is over 800-fold less active with an IC_{50} of 167 μM (166). APC-6336 also displays >100-fold inhibitory selectivity over other serine proteases including trypsin, thrombin and elastase. Extensive SAR studies around the core and the side chains on both sides of APC-6336 revealed a relatively flat SAR, leading to the suggestion that charge-charge interactions between inhibitor and enzyme contribute substantially to the potency observed (167).

Interesting lessons have been learned from the endeavor to identify a clinically useful HCV Pr inhibitor. The enzyme defies conventional wisdom and is recalcitrant to inhibition by a variety of well-known serine protease inhibition approaches. This is largely due to the unprecedented structural features of the enzyme, as discussed in the structure and function section above. This essential enzyme succumbs to potent inhibition by inhibitors derived from the N-terminal cleavage product of natural substrates. Ironically, the successful identification of the clinical candidate BILN-2061 relies, to a great extent, on the discovery of inhibitor/enzyme binding interactions that protrude beyond the S2 enzyme subsite. Such interactions have not been shown to be utilized by HCV Pr for processing of the HCV polyprotein. Although both the electrostatic and hydrophobic interactions with the labile Lys136 have been extensively exploited in the design of NS4A/4B derived product-based inhibitors, nanomolar inhibitors with minimal peptidic character and desirable physicochemical properties have, as yet, not been reported. Future peptide substrate-based inhibitor design may explore interactions at the prime sites (*e.g.*, at the S1') more extensively using rational design, as well as applying the concept of collected product inhibition (168). Knowledge gathered from the studies of peptidic inhibitors could possibly provide insight into the design of small-molecule nonpeptidic HCV Pr inhibitors. These different avenues will likely prove important for further improvement of the pharmacokinetic properties and resistance profiles of new generations of HCV Pr inhibitors.

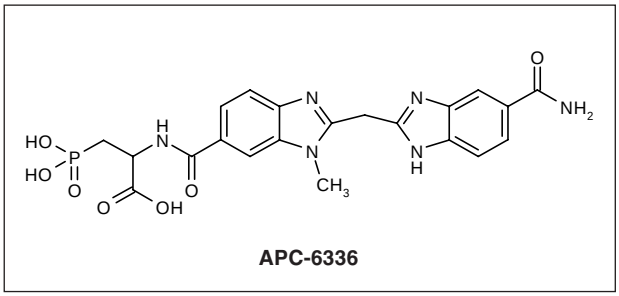


Fig. 13. Bisbenzimidazole-based Zn^{2+}-dependent HCV Pr inhibitor.

Summary and perspectives

Based on past success at delivering clinical candidates for protease targets, the pharmaceutical industry has invested considerable effort towards HCV Pr as a focus for drug intervention. Differences in structure relative to other serine proteases initially presented significant challenges to the research community, and has necessitated the development of a deeper understanding of substrate-protein and inhibitor-protein interactions. During this time, academic and patent literature have surfaced suggesting improved methodologies for reliably generating stable recombinant forms of the protein for both crystallographic and NMR studies to support rational design approaches. Furthermore, numerous systems have been established to efficiently evaluate inhibitor potencies and mechanism of action in both a recombinant enzyme and cell-based environment. Recently, murine models have also been described, potentially providing a more accurate gauge of the efficacy of possible clinical candidates. While INF-α-based therapies have improved, high expense, incidence and severity of side effects, and limited genotype coverage highlight the need for alternative therapies. Encouraging data has recently been disclosed for the orally administered and specific tripeptidic HCV NS3 inhibitor BILN-2061 (14, 169). This drug is reported to exhibit excellent potency against HCV, both at the enzyme level and against viral replication in a stable HCV cell-based assay. Despite being a tripeptide-based inhibitor, the drug displayed acceptable PK properties in several species (169) and, more importantly, was reported to be efficacious in patients infected with HCV genotype 1 (14). After 48 h of dosing, a drop in plasma viral load of greater than 2- to 3-fold was observed, with no adverse effects reported. This response is at least as effective as INF-α if not better (0.5- to > 2-fold log drop in viral load after 24 h). Should efficacy in patients infected with other genotypes prove similarly impressive, it may abrogate the requirement for INF-α as a first-line therapy.

The advantage of a protease inhibitor as a therapeutic target is the high homology in sequence and structure usually observed between strains. In the case of the rhinovirus protease 3C, excellent coverage has been reported against a broader spectrum of strains compared to capsid-binding agents (170). Efficacy with respect to efficient reduction of viral load is well documented for HIV protease inhibitors. The daunting ability of the virus to rapidly generate resistant mutants (171), however, suggests there is an ongoing need for new HIV protease inhibitors with superior pharmacokinetic and efficacy profiles. It should be noted that the serum half-life is estimated to be twice that of HCV; conversely, viral production is 2 logs less and the half-life of HIV-infected cells is only 2 days compared with 3-100 days for HCV. One could thus speculate that drug resistance is also going to be an issue for HCV-infected patients. There is an important difference, however, between HCV- and HIV-infected patients in that up to 15% of HCV patients can clear the virus and up to 50% of patients treated respond to INF-α therapy. HCV is thus potentially curable, unlike HIV (172). Response to therapy is less effective for immunocompromised individuals. Examination of the HIV-infected population suggests that approximately 30% of these individuals are coinfected with HCV (173). Hepatotoxicity associated with highly active antiretroviral therapy (HAART) is an issue for HIV-infected patients (174) that is significantly elevated in the HIV/HCV coinfected individual (175). In INF-α treatment trials, described at a meeting on therapies for viral hepatitis (176), rates of treatment discontinuation and severe adverse events were higher in the HCV/HIV patients than those without HIV. Much information is available on the potential hepatotoxicity of peptidic HIV Pr inhibitors (177) and this will have to be carefully examined for HCV Pr peptidomimetics, especially considering that HCV viral replication and subsequent damage occurs in the liver.

An understanding of the dynamics and life cycle of HCV (178, 179) can dramatically improve treatment strategies and overall success in lowering viral levels. With current INF-α therapies, rapid, dose-dependent exponential declines in viral RNA levels within 24 h of the first dose have been observed (30, 180, 181). This rapid decline is believed to be a consequence of the short serum half-life (~3 h) of HCV. After this rapid decline, there is a slower phase of viral decline that varies widely among patients and is attributed to the death rate of infected liver cells. The rate of the second phase is a good indicator of a patient's response to treatment (182) and is influenced by the infecting genotype (183). These INF-α viral kinetic studies will be useful for guiding drug administration decisions of HCV Pr clinical candidates.

HCV drug discovery is entering an exciting period. With Boehringer Ingelheim in phase II studies with its protease inhibitor and other companies such as Vertex and Schering-Plough disclosing preclinical candidates, distinct classes of protease inhibitors could be entering clinical trials in 2003. We may therefore be approaching an era where more efficacious, tolerable drugs with a broad spectrum of genotype coverage are identified. As more compounds enter the clinic, analysis of viral kinetics may suggest that these HCV Pr candidates provide a cure for the disease. Alternatively, we may find ourselves in a similar situation to HIV where drug resistance is a major issue which can only be resolved by multiple protease drugs and/or combination therapies with other HCV viral drug targets to achieve sustained viral responses.

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