Journal of
Medicinal
Chemistry

Volume 48, Number 1 January 13, 2005

Perspective

Control of Hepatitis C: A Medicinal Chemistry Perspective

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Received January 16, 2004

Introduction

Hepatitis C virus (HCV) is a common pathogen with an estimated 170 million people infected worldwide in the year 2000.[1] The virus was unambiguously identified in 1989 as the major etiological agent responsible for post-transfusion non-A and non-B hepatitis.[2] HCV infection can cause a large variety of clinical symptoms; however, in most cases, clinical symptoms are benign or even subacute. Many patients do not realize the infection until chronic liver damage is manifested 1−3 decades after the initial contraction of the virus.[3]

At present, no vaccine or a broadly effective therapy for all genotypes of HCV is available. Until recently the development of such agents has been severely hampered by the lack of appropriate experimental systems.[4] The only animal that can reliably be infected with HCV is the chimpanzee, but ethical reasons, high costs, and the inherent difficulties in working with large animals severely limit their experimental utility. However, the greatest limitation for HCV research has been the lack of a cell culture system that supports the efficient and reliable propagation of the virus.[5]

Despite these obstacles, several promising opportunities for drug development have been discovered through molecular biology. The cloning of the HCV virus in 1989[2] has proven extremely useful in characterizing the virus and identifying potential targets for drug development. New antiviral agents in development include inhibitors of HCV replicative enzymes, such as protease, helicase, and polymerase, as well as several genetic approaches, such as ribozymes and antisense oligonucleotides.

Although a number of reviews outlining current and emerging therapeutic approaches to hepatitis C infection have been recently published,[6−12] little attention has been paid to the design and development of drugs targeting the virus. This article places the drug design and development of hepatitis C genotype 1 therapeuticals into perspective, outlining the available targets and the ligands that currently display activity against these targets.

Current Treatments

Currently, the only agents approved by the Food and Drug Administration demonstrating efficacy in the treatment of HCV are interferon-α (IFN-α), a cytokine with immunomodulatory and antiviral activity,[13] and ribavirin, a synthetic guanosine nucleoside analogue.[14] IFN-α possesses both immune-modifying and antiviral properties including increased expression of major histocompatibility complex (MHC) proteins, which facilitate recognition of viral antigens by the immune system, activation of natural killer cells and macrophages, and direct inhibition of viral replication.[15,16]

Unfortunately, combination therapy with IFN-α and ribavirin is relatively ineffective. Less than 41% of patients given this treatment have a durable benefit.[17] However, recent clinical studies have shown that a higher response rate may be achieved by increasing IFN-α dosage while concurrently reducing its clearance rate. Pegylated IFN-α, in which the cytokine is covalently bound to poly(ethylene glycol) (PEG-interferon), has resulted in improved therapeutic effectiveness compared with unmodified IFN, likely because of its sustained action resulting from a prolonged half-life.[14,18,19]

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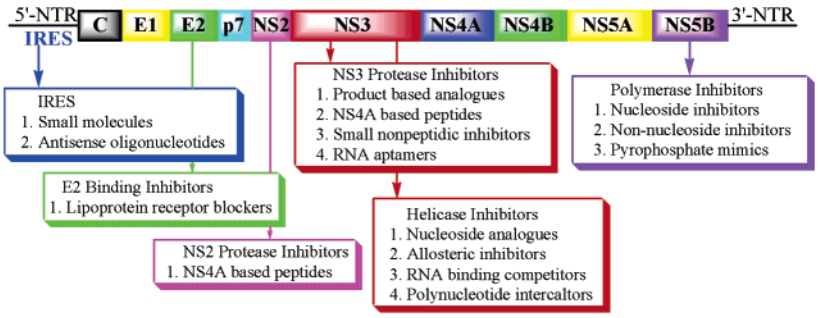
10.1021/jm0400101 CCC: $30.25
Published on Web 12/09/2004



Target	Size (kDa)	Function
C	21	Nucleocapsid
E1-E2	31,70	Envelope glycoprotein (heterodimer)
p7	7	Unknown function
NS2	23	Forms autoprotease with NS3
NS3	70	Serine protease, NTPase/helicase
NS4A	8	NS3-protease cofactor
NS4B	27	Unknown function
NS5A	56	Interferon sensitivity determining region
NS5B	68	RNA-dependent RNA polymerase

Figure 1. Structure of the hepatitis C virus genome and the targets of current antiviral agents. The HCV is a positive-stranded RNA virus possessing a genome of approximately 9000−9300 nucleotides in length and is classified as a separate genus of the *Flaviviridae* family. The genome contains a single open reading frame (ORF) coding for up to 10 proteins that are cleaved from the initially translated polyprotein by cellular and viral proteases.

Despite this progress and the forthcoming use of improved forms of ribavirin, it is unlikely that this treatment will succeed in a high percentage of cases. Current therapies based on pegylated IFN and ribavirin are limited in the treatment of the common HCV genotypes; moreover, combination therapy has significant side effects and is poorly tolerated, in particular by individuals affected by other diseases.[20] Consequently, many patients cannot be treated with current combination therapy and the overall chances of cure for patients are probably below 50%. Given the high prevalence of the disease, there is an urgent need to develop more effective and well tolerated therapies for chronic hepatitis C.

Emerging and Future Therapies

The development of antiviral agents that directly target the viral life cycle holds the greatest promise for effective HCV treatment. Unlike nonspecific antivirals such as IFN-α and ribavirin, target-specific antivirals would directly block viral replication and prevent ongoing infection of hepatocytes. As already mentioned, the development of such compounds has been severally hampered by the lack of efficient HCV replication in culture cells. Nevertheless, the cloning of the HCV genome in 1989[2] has proven to be useful in characterizing the virus and identifying several potential antiviral targets.

The HCV is a positive-stranded RNA virus possessing a genome of approximately 9000−9300 nucleotides (Figure 1) and is classified as a separate genus of the *Flaviviridae* family.[21] The genome consists of a single open reading frame (ORF) coding for up to 10 proteins that are cleaved from the initially translated polyprotein by cellular and viral proteases. These include a core protein C, two envelope proteins El and E2, and nonstructural proteins NS2−NS5 that are essential for viral replication, translation, and polyprotein processing.[22] In addition, the genome contains two nontranslated regions (NTRs); the first is the 5′-domain that functions as an internal ribosomal entry site (IRES), and the second site located at the 3′-end that probably plays a role in initiating replication of (−)-RNA.[22]

As is the case with HIV, efforts to develop anti-HCV agents have focused on the inhibition of key viral enzymes. In part, because of the proven success of protease inhibitors in the treatment of HIV, a large number of pharmaceutical companies have focused on the HCV NS3 proteolytic enzyme. HCV possesses two proteolytic enzymes: NS2/3 and NS3/NS4A. However, NS2/3 has received little attention as an antiviral viral target because of incomplete knowledge of the NS2/3 cleavage process. Furthermore, NS2/3 processing is partially mediated by host cell proteases, making it a less attractive target for drug development.[23,24]

In addition to the HCV protease, NS3 possesses an RNA helicase domain. HCV helicase represents a relatively new and unproven antiviral target. Although it is not yet known whether helicase inhibition would be effective in suppressing HCV replication, it has nonetheless been targeted in the search for novel therapies for HCV infection.

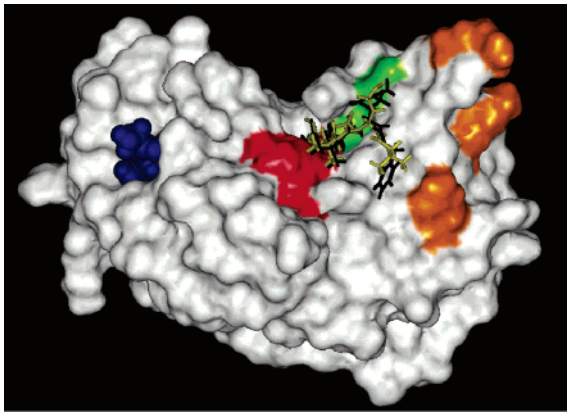


Figure 2. Superimposed X-ray crystal structures of the HCV NS3-4A protease complexed with the capped tripeptide α-ketoacids inhibitors Boc-Glu-Leu-L-(difluoro)aminobutyric acid (yellow stick representation) and Cbz-Ile-Leu-L-(difluoro)-aminobutyric acid (black stick representation). Both of these potent, reversible covalent inhibitors span S_1-S_4 residues of the protease active site. The NS4A cofactor is shown in blue, whereas residues His^{57}, Asp^{81}, and Ser^{139}, which together form the catalytic triad, are colored red. The side chains of the residues forming the S_1 specificity pocket of the enzyme, Val^{132}, Leu^{135}, and Phe^{154}, are shown in green, while Lys^{123}, Arg^{161} and Lys^{165}, which have been implicated in the binding of the P_5 and P_6 acidic pair of the substrate, are colored orange. The atomic coordinates were retrieved from the Protein Data Bank (codes 1DY8 and 1DY9).[35]

HCV NS5B RNA-dependent RNA polymerase (RdRp) is another attractive therapeutic target. It is a virus-specific enzyme with no functional homologues in the host and therefore may be less toxic to the host.[25] Weak inhibitors of HCV RdRp have been identified recently, but their exact role has not been investigated.

Several opportunities for targeted drug development also exist in the RNA genome of HCV. The 5′ nontranslated region of the RNA genome constitutes an internal ribosomal entry site (IRES) that binds to the ribosomes or ribosome-like structures of the infected cell allowing translation of the proviral DNA genome.[26] The IRES is highly conserved from isolate to isolate, making it an outstanding target for drug development.[27] Immune-based novel therapies also appear to hold great promise in treating chronic HCV infection. Examples of these immune-based therapies include anti-HCV immunoglobulins, interleukin-2, interleukin-10, and several T-cell vaccines that are at different stages of development.[28]

NS3 Protease

The HCV protease cleaves the viral polyprotein at four junctions: NS3/NS4A, NS4A/NS4B, NS4B/NS5A, and NS5A/NS5B. Cleavage at the NS3/NS4A site is an intramolecular event (cis cleavage), while the processing at the other three junctions occurs at the intermolecular level (trans).[29−31] In accordance with the Schechter and Berger nomenclature,[32] the cleavage sites are designated as $P_6-P_5-P_4-P_3-P_2-P_1\cdots P_1'-P_2'-P_3'-P_4'$, with the scissile bond between P_1 and P_1', while the relative binding pockets on the enzyme are termed $S_6-S_5-S_4-S_3-S_2-S_1\cdots S_1'-S_2'-S_3'-S_4'$, with cleavage occurring after cysteine or threonine.[33,34]

HCV protease (Figure 2)[35] is a heterodimeric enzyme comprising the N-terminal domain of the NS3 protein. This enzyme is a member of the chymotrypsin superfamily of serine proteases but is unique in requiring a peptide cofactor and a noncatalytic structural zinc atom.[36,37] The protease cofactor, NS4A, is a relatively small protein consisting of only 54 residues. The NS3/NS4A interaction crucially contributes to the proper folding of the protease, its stability, association with intracellular membranes, and catalytic activity.[38,39] An additional distinguishing feature of HCV protease is the absence of several surface loops connecting β-strands in the substrate-binding groove that typically form the pocket walls of the chymotrypsin family of enzymes. Consequently, the substrate-binding pocket is solvent-exposed and relatively featureless, rendering conventional chymotrypsin inhibitors inactive.[40,41] Although this indicates that specific nontoxic inhibitors could be developed, it has hampered the development of effective and selective inhibitors because binding relies on a series of weak and solvent-exposed interactions.[39]

Despite the challenges presented by the NS3 protease, inhibitors of this enzyme are beginning to emerge and can be tentatively classified into three classes: (1) product-based analogues competitive with substrate and larger peptide, (2) small-molecule inhibitors acting by noncompetitive mechanisms, and (3) RNA macromolecules operating by a variety of mechanisms.

NS3 Protease Product Based Inhibitor Analogues

During substrate specificity studies of NS3-protease, the enzyme was observed to be susceptible to feedback inhibition by the N-terminal products **1** and **2** (Figure 3), released from the polyprotein substrate after enzymatic cleavage.[42,43]

Capitalizing on this seminal observation, two groups systematically modified the natural amino acids of the hexapeptide N-terminal products.[41,42] By use of traditional and combinatorial methods, optimal active site binding by these peptides was shown to require a dual anchor, a "P_1 anchor" and an P_5-P_6 "acidic anchor", and resulted in the synthesis of potent hexapeptide inhibitors, compounds **3** and **4** (Figure 3).[44,45]

The preference displayed by NS3-protease for a cysteine residue as the "P_1 anchor" results from the structure of the S_1 specificity pocket (Figure 2).[46,47] This small and lipophilic pocket, lined by the hydrophobic residues of Val^{132}, Leu^{135}, and Phe^{154}, is complementary to the small and lipophilic cysteine side chain. In addition, the sulfhydryl group can interact in a specific way with the aromatic ring of Phe^{154}. The second anchor of the hexapeptide inhibitors is the P_5-P_6 acidic pair, which has been implicated in electrostatic interactions with a region of the protein characterized by a cluster of basic amino acids, most prominently Lys^{165}, Arg^{161}, and Arg^{123} (Figure 2).[48,49]

Although highly potent inhibitors, the presence of a thiol-containing side chain on the preferred P_1 residue limits the potential use of the N-terminal hexapeptide products in a clinical setting.[50,51] Thus, a major effort has been devoted to designing suitable and chemically stable replacements for the P_1 sulfhydryl group.

Initially, amino acids with small hydrophobic side chains, such as alanine or α-aminobutyric acid, were investigated as a replacement of the sulfhydryl moiety. Although the enzyme tolerated these small hydrophobic

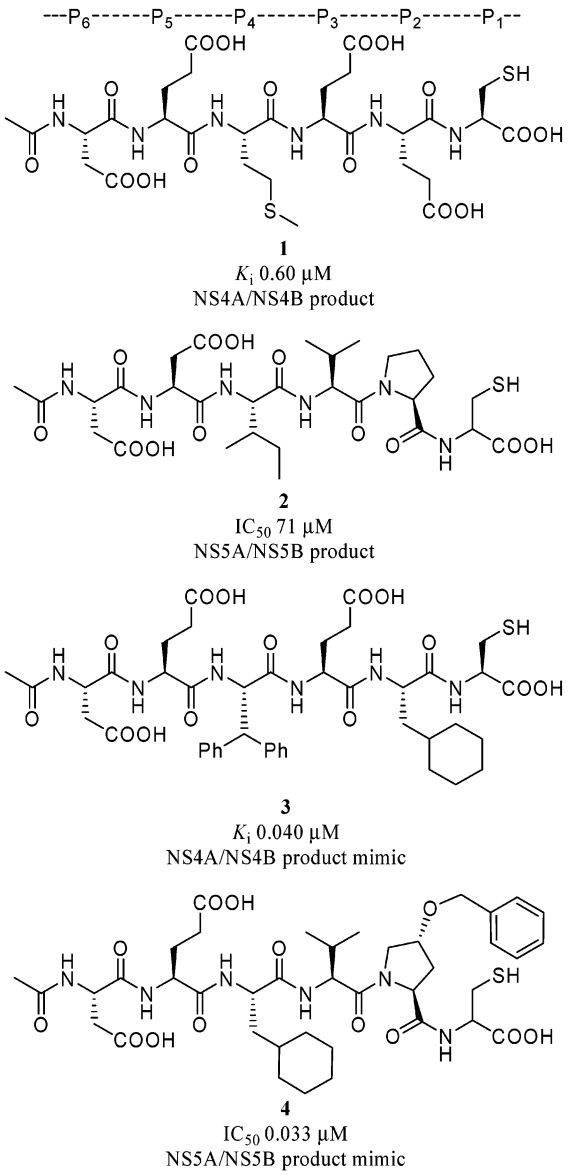


Figure 3. N-Terminal cleavage products of the peptide substrates corresponding to the NS4A/NS4B **1** and the NS5A/NS5B **2** cleavage products in addition to the first-generation hexapeptide protease inhibitors mimicking the NS4A/NS4B **3** and the NS5A/NS5B **4** cleavage products.

side chains, they resulted in loss of potency due to the reduction of the lipophilic contact surface area. Amino acids with larger side chains were also investigated; however, they also lad to a significant loss of activity possibly due to steric incompatibility.[41]

An analysis of the steric and electrostatic properties of the thiol group suggested a difluoromethyl group as a mimetic of the thiol.[52] This modification led to the development of competitive, noncovalent inhibitors, such as **5** (Figure 4), displaying higher potency than the initial hexapeptide **3** (Figure 3).

In addition to the difluoromethyl group, aminocyclopropylcarboxylic acid was proven to be an effective surrogate.[53] Replacement of the cysteine P_1 residue with this moiety produced an inhibitor, hexapeptide **6** (Figure 4), nearly equipotent to the parent compound.[45] Further improvements in activity were gained through traditional and combinatorial methods to optimize the P_2–P_6 amino acids producing a number of highly active

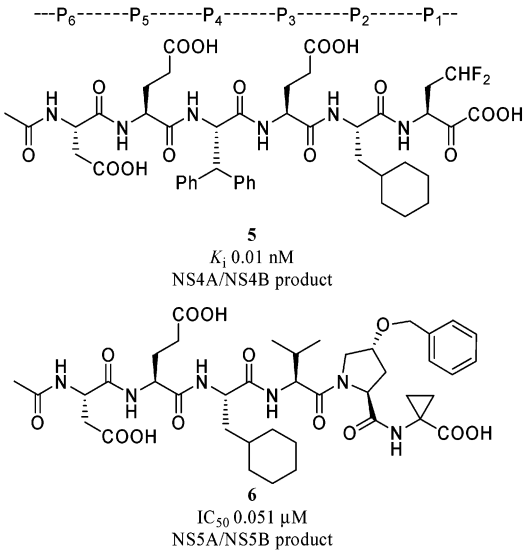


Figure 4. Structure of peptidomimetic inhibitors of NS3 protease activity.

Table 1. Hexapeptide Inhibitors of HCV Protease[55]

compd	inhibitor sequence[a] P_6–P_5–P_4–P_3–P_2–P_1	K_i (μM)
7	AcAsp-D-Glu-Leu-Glu-Cha-Cys	0.023
8	AcAsp-Glu-Leu-Glu-Cha-Cys	0.06
9	AcAsp-D-Gla-Leu-Ile-Cha-Cys	0.00075

[a] Abbreviations: Gla, γ-carboxyglutamic acid; Cha, β-cyclohexylalanine.

Table 2. Hexapeptide Inhibitors of HCV Protease Derived from Structure-Based Design[56]

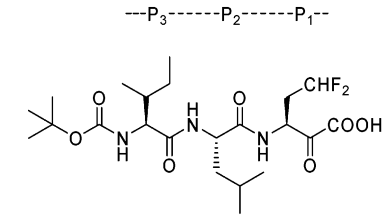
compd	inhibitor sequence[a] P_6–P_5–P_4–P_3–P_2–P_1	predicted K_i (nM)
10	AcAsp-D-Gla-Dif-Glu-Cha-Cyo	0.04
11	AcAsp-D-Gla-Dif-Glu-Cha-Fab	0.02
12	AcAsp-D-Gla-Dif-Glu-Trp-Cyo	0.03
13	AcAsp-D-Gla-Dif-Asp-Cha-Cyo	0.02
14	AcAsp-D-Gla-Trc-Glu-Cha-Cyo	0.02

[a] Abbreviations: Gla, γ-carboxyglutamic acid; Cha, β-cyclohexylalanine; Dif, β,β-diphenylalanine; Cyo, α-ketocysteine; Fab, δ,δ-difluoro-β-amino-α-ketopentanoic acid; Trc, 4-carboxytryptophan.

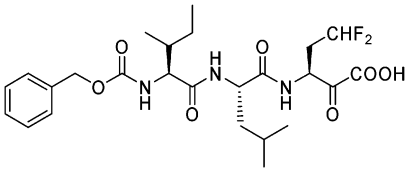
hexapeptides, the most active of which are illustrated in Table 1 (compounds **7**–**9**).[54]

Recently, a number of new potent pseudopeptidic inhibitors of NS3 protease were designed from information derived from both structure-based and traditional combinatorial optimizations.[55] Although this resulted in the identification of a number of promising drug candidates, such as those shown in Table 2 (compounds **10**–**14**), with predicted activities in the picomolar range, no significant increase in activity was obtained.[55]

A significant drawback of the polypeptides as therapeutics, especially those containing multiple carboxylates, is that they are unlikely to be bioavailable. Thus, to reduce the number of carboxylates, further truncation of the product-based peptides was conducted and resulted in the development of a number of potent tripeptides that clearly demonstrated that the P_5 and P_6 acids are not needed for activity.[35] A number of Cbz- and Boc-protected tripeptides, e.g., **15** and **16** (Figure 5) were also developed with the intention of partially mimicking the P_4 residue, which is integral to inhibitor–enzyme binding. The potency of these tripeptides is due to the slow dissociation rate of the covalent adduct,

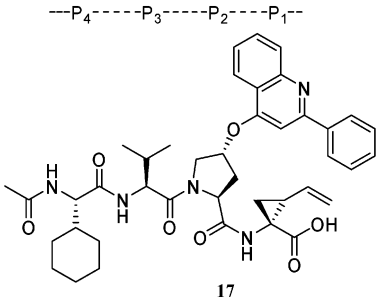


15
Boc-Ile-Leu-L-(difluoro)aminobutyric acid
IC_{50} 1.0 μM



16
Cbz-Ile-Leu-L-(difluoro)aminobutyric acid
IC_{50} 1.7 μM

Figure 5. Structure of two NS3 protease tripeptide inhibitors.



17
IC_{50} 0.013 μM

Figure 6. Structure of the tripeptide NS3 protease inhibitor **17**.

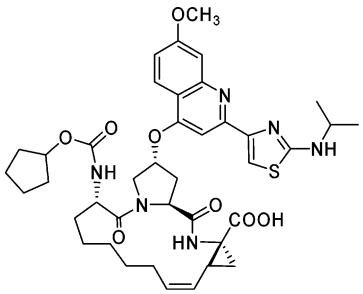
whereas the hexapeptide affinity is determined by high electrostatically driven association rates.[49]

In a further attempt to reduce both inhibitor size and peptidic character, an additional series of tetrapeptides were synthesized to bind within the S_1-S_4 region of the NS3 protease active site with a particular focus on optimizing the P_2 substituent.[56] It has previously been proposed that the P_2 residue plays a crucial role in the stabilization of the catalytic His−Asp hydrogen bond by shielding the region from solvent, earmarking the S_2 site as an attractive target.[57,58] This approach yielded a number of potent tetrapeptides. The most active of these contained a quinoline or biphenyl moiety that was attached to the P_2 proline residue via an ether linkage. The most potent of these compounds reported was compound **17** (Figure 6) with and IC_{50} of 0.013 μM.

Although the product-like inhibitors mediated potent inhibitory effects against HCV protease, the limitations of peptides as drug candidates are well documented.[59] Therefore, much effort has been devoted to reducing the peptidic nature of the peptidyl carboxylic acid inhibitors described above. Potent peptidyl carboxylic acids were thus obtained by designing a macrocyclic ring in order to connect the side chain of the P_1 and the P_3 residues.[60,61] One such compound, which has performed favorably in clinical trials, is BILN-2061 (**18**, Figure 7).[62]

NS3 Protease Noncovalent Substrate Mimetics and P′ Inhibitors

In contrast to the P region, the P′ region of the substrate has received considerably less attention.



18
IC_{50} 3.0 nM

Figure 7. Structure of the macrocyclic HCV NS3 inhibitor **18**.

However, the prime region of the substrate-binding groove possesses a number of pockets that significantly contribute to substrate recognition, which could possibly be exploited in the design of competitive inhibitors.

In a study of the mechanism of NS3 protease activation, the replacement of the P_1' residue of an NS5A/NS5B-decapeptide substrate resulted in poor enzyme turnover.[63−65] This was most noticeable for cyclic amino acids such as proline, pipecolinic acid (Pip), and tetrahydroisoquinoline-3-carboxylic acid (Tic). The pronounced affinity of the Tic-containing peptide for NS3 is consistent with previous models, indicating that the S_1' pocket can accommodate residues much larger than the natural serine residue. Further analysis showed that while the P-side residues contributed more to binding than the P′ side, a favorable interaction could be detected between the P_4'-leucine (Figure 8, decapeptides **19** and **20**) or the P_4'-tyrosine (Figure 8, decapeptides **21** and **22**) residues of the inhibitor and NS4A.[66]

Although a great deal of selectivity and potency can thus be obtained through the design of substrate analogues, their relatively large molecular weight limits cell membrane permeability and bioavailability, preventing their further development as clinical candidates. Therefore, a new class of NS3 inhibitors were developed that bind to the prime region in the absence of any contacts with the nonprime region of the enzyme.[67] The strategy combined key binding elements of the two previously described classes, namely, the optimized prime-site binding sequence and an N-terminal carboxylic acid suitably positioned in the active site to engage in interactions similar to those previously established for the C-terminal carboxylate of peptide product inhibitors. The success of this design required selecting the proper linkage between these two elements from a small combinatorial library. This effort has resulted in capped tripeptides that are low micromolar competitive inhibitors of the NS3 protease. The most potent compound obtained thus far in this series is **23** (Figure 9).

More recently, a novel series of reversible, competitive inhibitors have been reported that bind to the enzyme substrate cleft across the active site.[68] These inhibitors are characterized by the presence of a C-terminal phenethylamide group, which extends to make contacts into the S′ side of the enzyme, possibly with the side chain of lysine.[136] It was possible to evolve tripeptide phenethylamide inhibitors, e.g., **24** (Figure 9), that inhibit purified NS3/NS4A protease with potencies in the submicromolar concentration range. This finding

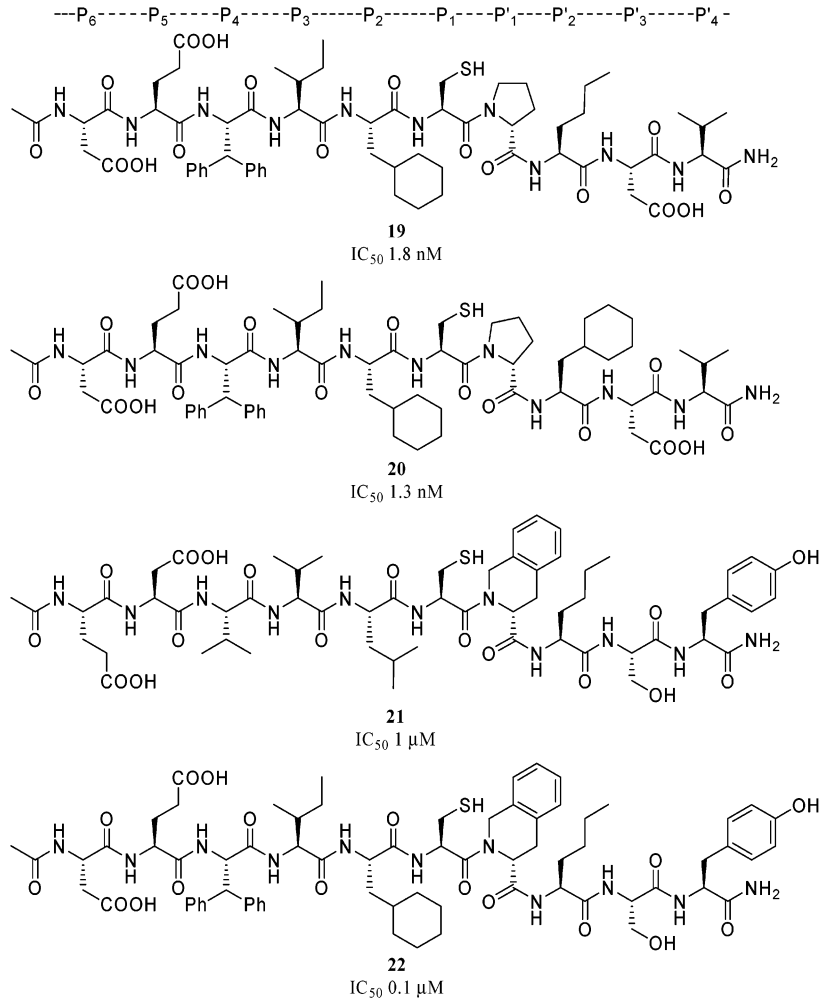


Figure 8. Structure of four decapeptide inhibitors that simultaneously bind to the S and S′ regions of the NS3 protease the active site.

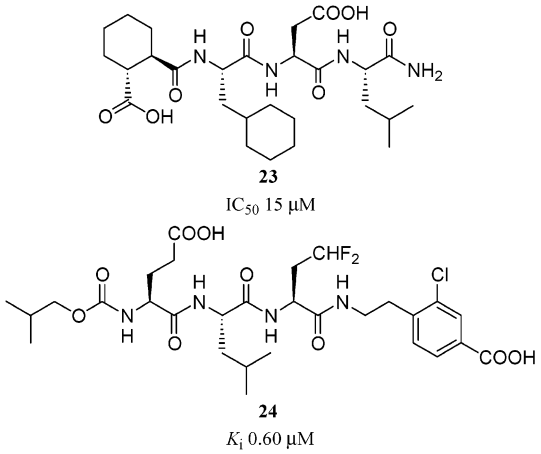


Figure 9. Structure of a capped tripeptide and a tripeptide phenethyl amide NS3 protease inhibitor.

suggests that the moiety might constitute a novel active site anchor and a potentially valid alternative to the C-terminal free carboxylic group present in the product analogue series.

Inhibitors of NS4A Binding

An interesting strategy has been developed using NS4A analogues alone or linked to an NS5A/NS5B peptide, yielding inhibitors capable of binding to both domains simultaneously.[8,65,69] The NS4A inhibitors were assembled from D-amino acid residues in standard order such as **25** or in reverse order such as compounds **26** and **27** (Table 3).

The mechanism through which these NS4A inhibitors repress NS3 protease is as yet unknown. Furthermore, the determinants, which lead to inhibition versus activation of the protease, are also yet to be elucidated. However, perhaps the most significant unanswered question of the NS4A binding inhibitors in terms of their potential as therapeutics is whether they will be able to effectively compete with viral NS4A during infection if the initial binding of NS4A to NS3 protease occurs cotranslationally.[69]

In addition to the NS4A binding inhibitors, several bivalent inhibitors (Table 4, compounds **28**−**32**) with two enzyme-binding domains have been described.[8,65] These inhibitors employ a P_6−P_1 element, either Glu-Asp-Val-Val-Cys-Cys or Asp-Glu-Val-Val-Cys-Cys, attached via a linker (L1−L3) to an NS4A inhibitor, similar to those discussed above.[8,65]

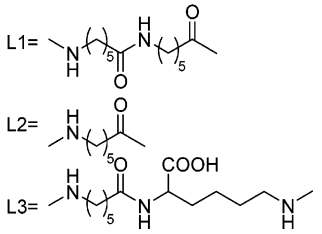
Small-Molecule Inhibitors of NS3 Protease

Because of the difficulties associated with the de novo design of small nontoxic inhibitors that bind to the NS3 protease substrate binding pocket, many groups have turned to high-throughput screening of chemical and

Table 3. Amino Acid Sequence and Inhibitory Constants of a Number of NS4A Binding Inhibitors[8,66,70]

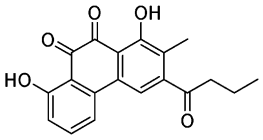
compd	inhibitor sequence	IC_{50} (μM)
25	Cys-Val-Val-Ile-Val-Gly-Arg-Ile-Val-Leu-Ser-Gly-Lys	0.2
26	Lys-Gly-Ser-Leu-Val-Ile-Arg-Gly-Val-Ile-Val-Val-Cys-Lys	0.2
27	Ser-Leu-Val-Ile-Arg-Gly-Val-Ile-Val	0.6

Table 4. Amino Acid Sequence of a Number of Dual Site Inhibitors That Simultaneously Bind to the Active Site and NS4A Binding Region of NS3 Protease[a]

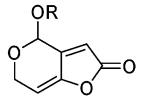


compd	inhibitor sequence	IC_{50} (μM)
28	Glu-Asp-Val-Val-Cys-Cys-L1-Cys-Val-Val-Ile-Val-Gly-Arg-Ile-Val-Leu-Ser-Gly-Lys	0.6
29	Glu-Asp-Val-Val-Cys-Cys-L2-Cys-Val-Val-Ile-Val-Gly-Arg-Ile-Val-Leu-Ser-Gly-Lys-Lys	3
30	Glu-Asp-Val-Val-Cys-Cys -L3-(Lys-Gly-Ser-Leu-Val-Ile-Arg-Gly-Val-Ile-Val-Val-Cys)[b]	3
31	Glu-Asp-Val-Val-Cys-Cys -L1-Lys-Gly-Ser-Leu-Val-Ile-Arg-Gly-Val-Ile-Val-Val-Cys[c]	0.2
32	Asp-Glu-Val-Val-Cys-Cys -L2-Lys-Gly-Ser-Leu-Val-Ile-Arg-Gly-Ile-Val-Val-Cys-Lys[c]	0.2

[a] The structure of each of the linker groups is illustrated at the top.[8,66] [b] Peptide sequence in parentheses is written from the C-terminus to the N-terminus. [c] Last 12 residues are D-amino acids.

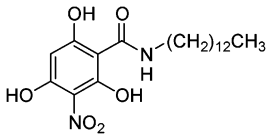


33
IC_{50} 7 μM



34; R = H, IC_{50} 3.8 μg/mL
35; R = Ac, IC_{50} 7.2 μg/mL
36; R = *m*-BrC_6H_4CO, IC_{50} 12.6 μg/mL

Figure 10. Structure of the small-molecule NS3 protease inhibitor murayaquinone (**33**) and structure of **34** along with its derivatives **35** and **36**.

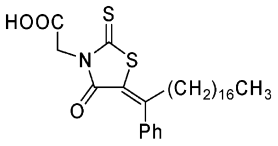


37
IC_{50} 5.8 μM

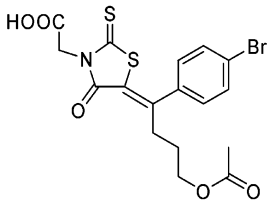
Figure 11. Structure of **37**, the most potent NS3 protease inhibitor from the 2,4,6-trihydroxy-3-nitrobenzamides series.

natural product libraries to search for novel lead molecules. Examples of such small-molecule inhibitors are illustrated in Figure 10 and include murayaquinone (Sch68631) (**33**), isolated from *Streptomyces*,[70] and Sch351633 (**34**) along with derivatives **35** and **36**, isolated from the fungus *Penicllium griseofulvum*.[71]

Additional compounds derived from random screening are the 2,4,6-trihydroxy-3-nitrobenzamides (THNBs).[72] As the most potent in a series of 12 derivatives, **37** (Figure 11) possessed an IC_{50} of 5.8 and 3 μM in the presence and absence of NS4A, respectively. Unfortunately, the majority of THNBs displayed low specificity toward NS3 protease, exhibiting strong inhibitory effects against human serine proteases such as chymotrypsin and elastase, making them inappropriate candidates for clinical use.



38
IC_{50} 2.3 μg/ml



39
IC_{50} 6.4 μg/ml

Figure 12. Structures of the NS3 protease inhibitors **38** and **39**.

A series of nine thiazolidine compounds were also described, some of which were claimed to inhibit HCV protease in a noncompetitive manner.[73] All nine derivatives inhibited the activity of NS3 protease in vitro by more than 50% at a concentration of 50 μg/mL, with the most potent being RD4-6250 (**38**, Figure 12), which elicited a 50% reduction in protease activity at a concentration of 2.3 μg/mL. However, the derivative with the greatest specificity toward HCV protease, RD4-6205 (**39**, Figure 12), only elicited a 50% reduction in enzyme activity at a concentration of 6.4 μg/mL.

Despite both the thiazolidine and THNB series of compounds eliciting a strong inhibitory effect against other human serine proteases such as chymotrypsin, trypsin, plasmin, and elastase, the results collected suggest that the skeleton of **39** is an important structure for the further development of small NS3 protease inhibitors.

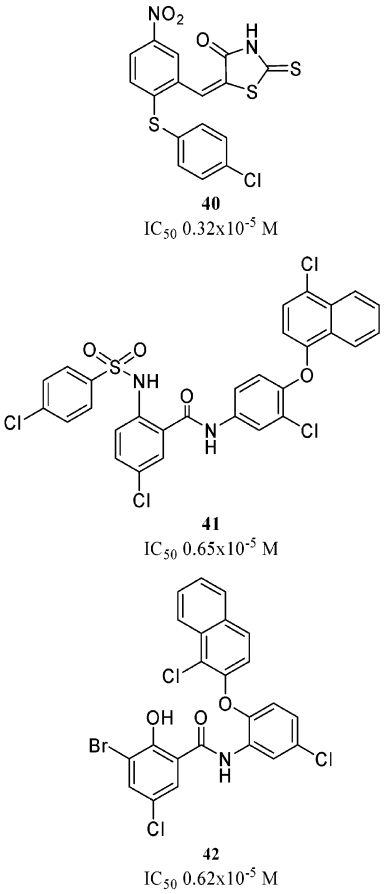


Figure 13. Structure of three potent NS3 protease inhibitors isolated from the screening of a 2000-compound library.

Three additional potent small inhibitors (Figure 13) of HCV serine protease were isolated from the screening a 2000-compound chemical library.[74] Although all three of these compounds exhibit comparable activities, **41** and **42** were found to be far more selective toward HCV protease than **40**, thus making them attractive candidates for further development.

To examine the inhibitory effects elicited by the position and chemical nature of **41** and **42** substituent patterns, a number of analogous compounds were examined. The results obtained from these studies indicated that the presence of a para and/or ortho halogen substituted phenol moiety along with a benzoyl moiety possessing a large hydrophobic group, such as a naphthyl para or ortho to a halogen atom, is required for inhibitory activity. Further, assays of **41** and **42** illustrated that inhibition occurred noncompetitively, suggesting that these compounds act a site removed from the substrate binding pocket.[74]

While conventional bioassay-based high-throughput screening has yielded a number of potent NS3 protease inhibitors, its limitations have driven the development of alternative and complementary tools. In this regard, novel NMR-based approaches, such as structure-based NMR screening, have emerged over the past few years and show great promise.[75−79] In general, structure-based NMR screening utilizes protein chemical shift perturbation data, generated in heteronuclear single quantum coherence (HSQC) based NMR screens, to rapidly provide an accurate structural representation of protein−ligand complexes under conditions unfavorable for

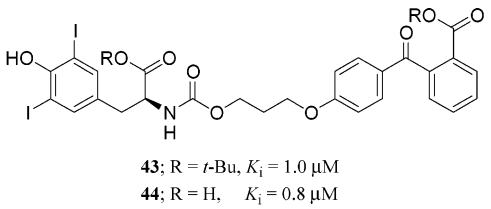


Figure 14. Structure of two nonpeptidic small-molecule inhibitors of NS3 protease **43** and **44** discovered through structure-based NMR screening.

traditional structural work using X-ray crystallography and/or solution NMR.[80,81] The NMR screening method uses protein chemical shift perturbation data in a more quantitative manner than the traditional "chemical shift perturbation mapping" widely used to qualitatively map molecular interfaces, and hence, they allow rapid determination of the ligand binding site with much higher precision.[77] By careful comparison of protein chemical shift perturbation data of closely related analogues, these methods also often enable an unambiguous determination of the binding mode of a small-molecule ligand.

In a recent study, 16 small-molecule hits that bind weakly ($K_D \approx 100$ μM to 10 mM) to the NS3 protease active site were identified.[80] Analogues for five classes of these NMR hits were evaluated by a combination of NMR and biochemical data yielding SAR and, in most cases, optimized hits with improved potencies ($K_I \approx 40$ μM to 1 mM). NMR chemical shift perturbation data were subsequently used to establish the binding location and orientation of the active site directed scaffolds in these five analogues. Two of these scaffolds, which were determined to bind at the proximal S_1-S_3 and S_2' substrate binding sites, were linked together yielding the competitive inhibitors **43** and **44** (Figure 14).

RNA Aptamers

Aptamers are single-stranded nucleic acids that directly inhibit a protein's function by folding into a specific three-dimensional structure that dictates high-affinity binding to the targeted protein.[81] The strategy involves isolation of nucleic acid molecules that have high affinity for a target molecule from a pool of random nucleic acids, with subsequent repeated rounds of selection and amplification. Through iterative in vitro selection techniques, aptamers can be generated that bind essentially any protein or small-molecule target.[82] A high-affinity, specific inhibitor can theoretically be made to order, provided that a small quantity of pure target is available. Because they inhibit the activity of existing proteins directly, aptamers are similar to monoclonal antibodies or small molecule drugs, and this property greatly increases the number of clinical indications that are potentially treatable by nucleic acid based compounds.[83]

By use of this genetic selection strategy, two aptamers that inhibit NS3 protease in vitro were isolated, designated G6−16 and G6−19.[84] Kinetic studies of the inhibition revealed that the aptamer G6−16 inhibited the NS3 protease with an inhibitory constant (K_i) of 3 μM. In addition, both G6−16 and G6−19 aptamers were found to inhibit the helicase activity of NS3. Since these aptamers inhibit two important functions of NS3, they could prove to be useful therapeutic agents.[85]

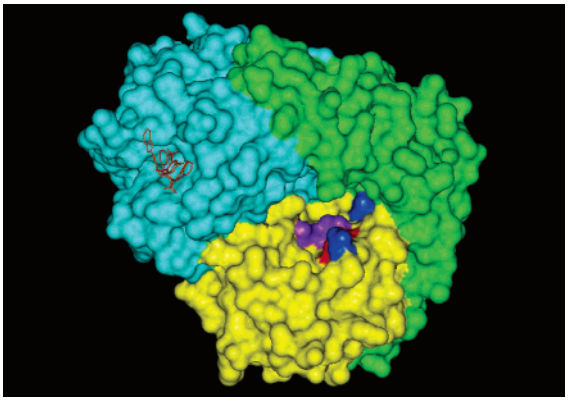


Figure 15. X-ray crystal structure of the HCV NS5B RNA-dependent RNA polymerase. The "thumb", "fingers", and "palm" subdomains are colored light-blue, green, and yellow, respectively. The residues essential for metal binding, Asp^{220} and the carbonyl of the Thr^{221} backbone, are colored red, while Asp^{318} and Asp^{319}, which are responsible for binding a second Mg^{2+} ion, are colored purple. Asp^{225} and Asp^{352}, which additionally contribute to the electrostatically negative surface surrounding the three essential aspartates, are colored dark-blue and partially obscure Asp^{220} and Thr^{221}. In addition, the X-ray structure shows two non-nucleoside inhibitors binding within the non-nucleoside-binding pocket. The atomic coordinates were retrieved from the Protein Data Bank (codes 1NHU and 1NHV).[89]

HCV Polymerase

The NS5B portion of the HCV genome encodes for the polymerase, an enzyme that is pivotal in viral replication and is thus a primary target for drug development. The enzyme facilitates the synthesis of both the negative stranded RNA intermediate, complementary to the viral genome, and the positive stranded RNA genome, complementary to the negative stranded intermediate.[86,87]

In accordance with virtually all known viral polymerases, the domain arrangement of HCV polymerase bears an anatomical resemblance to a right-hand with subdomains termed "fingers", "palm", and "thumb" (Figure 15).[88] However, different from many other viral polymerases, which adopt a "half-open right-hand" conformation, the HCV polymerase has a more compact architecture. This is primarily due to the presence of two extended loops protruding from the fingers subdomain that extend back against the thumb, which together with the palm completely encircle the polymerase active site forming a charged tunnel through which the RNA template and the NTP substrate access the active site.[89−92]

The palm subdomain possesses a two-metal-ion catalytic site, which is conserved in essentially all known viral polymerases.[93−95] The magnesium ions assist phosphodiester bond formation by polarizing the hydroxyl group at the 3′ terminus of the growing RNA chain, facilitating nucleophilic attack upon the dNTP substrate in addition to stabilizing the transition state in which the phosphorus of the dNTP is linked to five oxygens. The residues essential for metal binding are Asp^{220} and the carbonyl of the Thr^{221} peptide backbone, as well as Asp^{318} and Asp^{319}. Additionally Asp^{225} and Asp^{352} contribute to the electrostatically negative surface surrounding the three essential aspartates.[96]

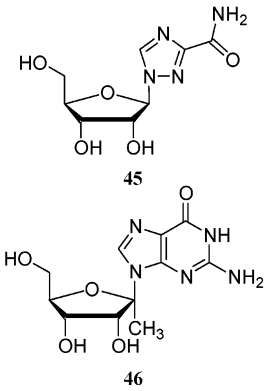


Figure 16. Structure of two nucleoside analogue inhibitors of HCV polymerase: ribavirin (**45**) and β-D-2′-methylribofuranosylguanosine (**46**).

Inhibitors of the NS5B Polymerase

As is the case with HIV-1 reverse transcriptase, the majority of currently known HCV polymerase inhibitors fall into two main categories according to their chemical structure and their mechanism of action, these being nucleoside analogue inhibitors (NI) and non-nucleoside inhibitors (NNI). However, in addition to the NIs and NNIs, a class of pyrophosphate mimics have also displayed an ability to inhibit HCV polymerase.

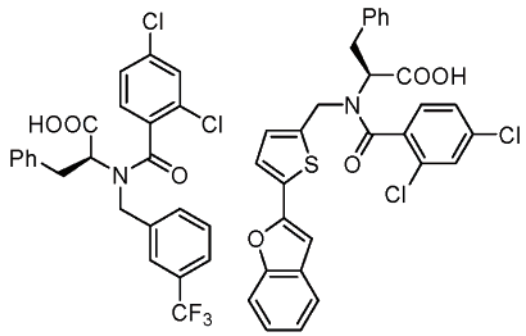
Nucleoside Inhibitors

NIs are all thought to inhibit polymerase activity in a similar fashion. That is, following the intracellular phosphorylation of the nucleoside analogue to the corresponding triphosphate, it is subsequently incorporated by the polymerase into the growing nucleic acid chain. This leads in turn to an increased error frequency of the polymerase and consequential early termination of the elongation reaction.[97−99]

Despite the success of nucleoside analogues such as AZT in the treatment of HIV, only a handful of NIs active against HCV polymerase have been identified. To date, the only nucleoside analogue shown to be therapeutically useful is the broad-spectrum antiviral agent ribavirin (**45**, Figure 16).[100,101] Recently, a number of dioxolane triphosphate and nucleoside triphosphate based inhibitors have been reported.[102,103] The most promising among these, β-D-2′-methylribofuranosylguanosine (compound **46**, Figure 16), is phosphorylated in cultured uninfected cells and is orally bioavailable in primates.

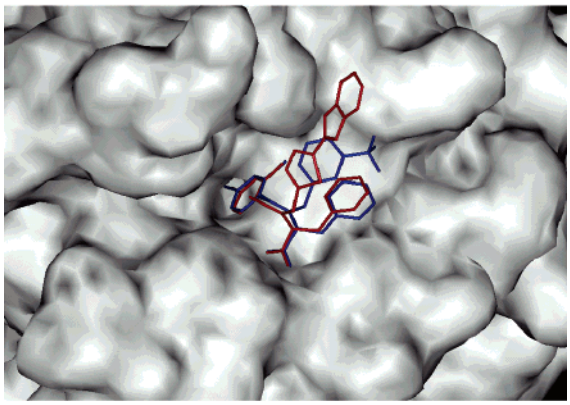
Non-Nucleoside Inhibitors

In contrast to the NIs, the NNIs are a structurally and chemically heterogeneous class and are not incorporated into the growing DNA strand. The NNIs indirectly inhibit the polymerase by binding to the enzyme in a reversible and noncompetitive manner. The recently reported X-ray crystal structures of two non-nucleoside inhibitors, (2*S*)-2-[(2,4-dichlorobenzoyl)-(3-trifluoromethylbenzyl)amino]-3-phenylpropionic acid (**47**, Figures 15 and 17) and (2*S*)-2-[(5-benzofuran-2-ylthiophen-2-ylmethyl)-(2,4-dichlorobenzoyl)amino]-3-phenylpropionic acid (**48**, Figures 15 and 17) bound to HCV polymerase, indicate that the binding site of the NNIs is located at the base of the thumb subdomain, approximately 35 Å away from the polymerase active



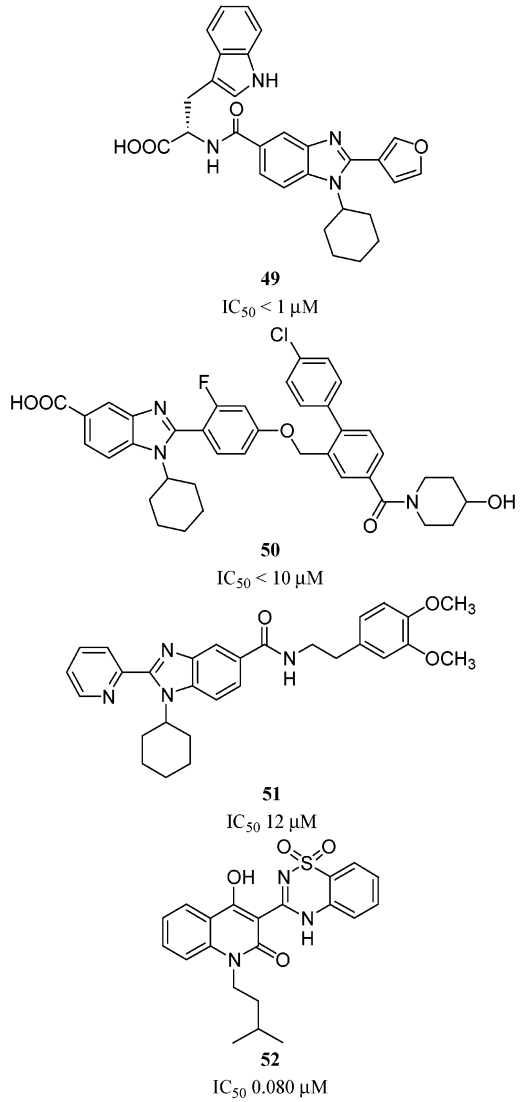
47 K_i 2.2 μM **48** K_i 2.2 μM

(a)



(b)

Figure 17. (a) Structures of the HCV polymerase inhibitors (2*S*)-2-[(2,4-dichlorobenzoyl)-(3-trifluoromethylbenzyl)amino]-3-phenylpropionic acid (**47**) and (2*S*)-2-[(5-benzofuran-2-yl-thiophen-2-ylmethyl)-(2,4-dichlorobenzoyl)amino]-3-phenylpropionic acid (**48**), (b) Superimposed X-ray crystal structures of the HCV polymerase non-nucleoside binding pocket with **47** (blue stick model) and **48** (red stick model) bound to the pocket. The atomic coordinates were retrieved form the Protein Data Bank (codes 1NHU and 1NHV).[105]



49 IC_{50} < 1 μM

50 IC_{50} < 10 μM

51 IC_{50} 12 μM

52 IC_{50} 0.080 μM

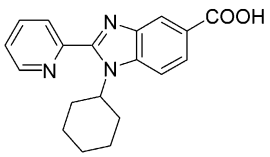
Figure 18. Structures of three benzimidazole HCV polymerase inhibitors **49**, **50**, and **51** as well as a benzothiadiazine **52** inhibitor of HCV polymerase.

site.[88] It is envisaged that the binding of inhibitors to this pocket may prevent the polymerase from undergoing a conformational change essential for enzymatic activity.

The inhibitor-binding site consists of two hydrophobic pockets and a pair of adjacent main chain amide groups. The predominate binding interaction is hydrophobic and exists between the primary binding pocket and the 2,4-dichlorophenyl group of both inhibitors. The side chain of Arg^{422} forms the bottom of the pocket, while the side chains of Trp^{528}, Met^{423}, Leu^{419}, and Tyr^{477} and the main chain atoms of His^{475} and Leu^{474} form the surrounding walls. Adjacent to the primary binding pocket are two main chain amide nitrogen groups belonging to Ser^{476} and Tyr^{477}, which form hydrogen bonds with the carboxyl group of both inhibitors. In addition, there is a second hydrophobic surface binding pocket that accommodates the phenyl ring of the region of the inhibitors. As indicated by the identical K_i of both inhibitors and illustrated in Figure 17b, the region occupied by the 3-trifluoromethylbenzyl moiety of **47** and the 5-benzofuran-2-yl-thiophen-2-ylmethyl of **48** makes only negligible contacts with the enzyme.[88]

In addition to the two propionic acid derivatives, several NNI HCV polymerase inhibitors have been reported, including three benzimidazole derivatives[104,105] **49**, **50**, and **51** as well as a benzothiadiazine derivative[106] **52** (Figure 18). Unlike the benzimidazole derivatives, which are suspected to bind in a fashion similar to the propionic acid derivatives, the benzothiadiazine compounds are thought to interact directly with the viral polymerase and to inhibit RNA synthesis noncompetitively with respect to GTP. It has been suggested that benzothiadiazine NNIs block the HCV RdRP prior to the formation of an elongation complex.[107] Similarly, the mechanism of action of the benzimidazole NNIs has been suggested to be antagonistic with respect to the formation of a productive enzyme−template complex.[108]

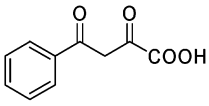
The structure−activity relationships (SAR) analysis of the benzimidazole derivatives indicated that a 1,2-disubstituted benzimidazole 5-carboxylic acid scaffold possessed by **53** (Figure 19) is the minimum core required for biological activity.[109] Exploration of the N^1-cyclohexane substituent revealed that increasing the ring size to seven, reducing it to a cyclopentyl ring, or even cleaving the ring to produce acyclic variants were



53

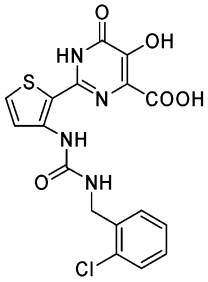
IC_{50} 4.3 μM

Figure 19. Structure of the HCV polymerase inhibitor **53**, which is composed of the 1,2-disubstituted benzimidazole-5-carboxylic acid scaffold, which has been shown to be the minimum core required for biological activity.



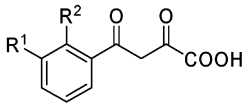
54

IC_{50} 5.7 μM



55

IC_{50} 0.100 μM



Compound	R^1	R^2	IC_{50} μMol
56	H	O-2-CN-5-BrPh	0.35
57	2-CN-5-BrPh	H	0.10
58	2-CN-3-ClPh	H	0.11
59	2-CN-3,5-Cl_2Ph	H	0.045

Figure 20. Structures of diketo acid inhibitors **54** and **56**−**59** and a dihydroxypyrimidine carboxylate inhibitor **55** of HCV polymerase.

all detrimental to activity, while removal of the N^1 substituent obliterated activity. Substitution of the cyclohexane ring at various positions as well as aromatization of the ring was also shown to elicit no beneficial effect.

A number of benzimidazole derivatives have displayed potent in cell antiviral activity and are consequently receiving a great deal of attention. Perhaps the most promising of these is **50** (Figure 18), of which an orally bioavailable analogue termed JTK-003 is currently being studied in phase I and II trials in Japan.[110]

Pyrophosphate Mimics

Like the nucleoside HCV polymerase inhibitors, the pyrophosphate mimics are a structurally and chemically homogeneous class with a majority of the compounds in the class possessing a diketo acid moiety (e.g., **54**, Figure 20).[111] However, novel classes of substituted dihydroxypyrimidine carboxylates, (e.g., **55**, Figure 20)[112] are also believed to act as pyrophosphate mimics. Binding of these inhibitors to the enzyme is mediated by active-site divalent cations such as Mn^{2+} or Mg^{2+}, thereby interfering with the binding of the phosphoryl groups of the nucleotide substrate and preventing the formation of phosphodiester bonds.

A recent study exploring structure−activity relationships of the diketo acid series of HCV polymerase inhibitors indicated that the structure of **54** (Figure 20) is the minimum scaffold capable of inhibiting the enzyme.[113] Exploration of the essential phenyl moiety meta to the diketo moiety revealed that a substituent at position 2 was required for activity as was demonstrated by an approximate 40-fold reduction in activity upon its removal. Replacement of the group with a neutral phenyl ring or with larger bicyclic substituents such quinolines and indoles was also shown to be significantly less potent; likewise, aliphatic cyclohexyl analogues and vinylic substituents were also shown to yield no beneficial effect. The greatest increase in activity was elicited with the addition of a butyronitrile ether chain ortho to the diketo acid (**56**, Figure 20) or with a benzyl ether moiety meta to the diketo acid possessing a nitrile group at the 2 position with a variation of halogens atoms at positions 3 and 5 (compounds **57**−**59**, Figure 20).[113]

Helicase

In order for genomic processing to proceed, the *Flaviviridae* family of viruses must synthesize negative-stranded RNA utilizing the parental positive-stranded RNA as a template. The resulting negative-stranded RNA is subsequently used as the template for synthesis of the positive-stranded progeny RNA that is assembled in viral particles. However, since the negative and positive orientated RNA strands are complementary, genome processing and thus viral replication cannot transpire if the helicase enzyme does not mediate RNA strand unwinding and subsequent separation.[114]

HCV helicase represents a relatively new and unproven target for antiviral intervention. Inhibition of the helicase action has the potential not only to terminate the proliferation of the virus but also to indirectly stimulate a cellular antiviral response because of the expected buildup of intracellular double-stranded RNA.[115] The necessity of unwinding activity mediated by the NTPase/helicase enzymes in the viral life cycle has recently been reported in "knock-out" experiments that demonstrated unambiguously that the switch-off of the helicase activity abolishes the virus propagation of bovine diarrhea virus (BVDV) and of dengue fever virus (DENV).[116,117]

The hepatitis C helicase enzyme exhibits a 3′-5′ directionality with respect to the template strand, and in contrast to the majority of the NTPase/helicases, it is capable of unwinding RNA/DNA heteroduplexes in addition to both DNA/DNA and RNA/RNA homoduplexes.[118] Two alternative mechanisms of the unwinding reaction have been postulated.[119−124] Both models predict that the enzyme binds and subsequently hydrolyzes NTPs in a well-defined NTP binding pocket. The energy released from the reaction is utilized in the translocation of the enzyme along the DNA or RNA structures, and the unwinding reaction results from capturing single strand regions that arise due to fluc-

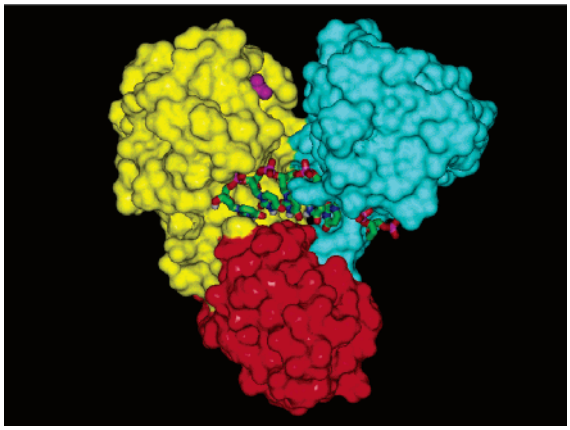


Figure 21. Surface diagram of HCV helicase complexed with single-stranded DNA (tube representation). The protein comprises three domains. Domains 1 (yellow) and 2 (blue) share a similar fold and make symmetrically equivalent contacts with the backbone of the bound single-stranded DNA. Domain 3 (red) is predominantly α-helical and has a carboxy-terminal 40 amino acid region that lacks any secondary-structure element. A sulfate ion (shown in purple) is bound within a phosphate-binding loop in the amino-terminal region of domain 1. The atomic coordinates were retrieved from the Protein Data Bank (code 1A1V).[125]

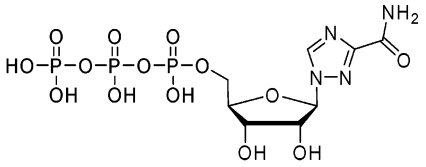
tuations at the fork.[119,120] Alternatively, the energy could be transferred to the fork and used for disruption of the hydrogen bonds that keep the strands together.[119,120]

The HCV helicase enzyme is composed of three subdomains, which adopt a Y-shaped conformation (Figure 21).[123] The enzyme possesses a number of conserved motifs common to other nucleoside triphosphate (NTP) consuming enzymes.[124−128] These include the Walker motif A located on the surface of domain 1, which binds the terminal phosphate group of the NTP, and the Walker motif B, responsible for the chelation of the Mg^{2+} belonging to the Mg−NTP complex.[122] Subdomain 2 possesses a highly conserved arginine-rich motif, which has been implicated in RNA binding; however, it has also been suggested that it may have a direct involvement in the binding of ATP. Unlike both domains 1 and 2, domain 3 is predominantly α-helical possessing no helicase-conserved motifs.[119,120,129]

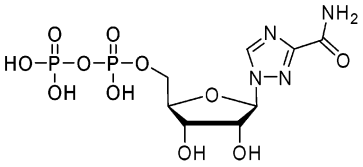
Thus far only a handful of inhibitors for the HCV helicase have been reported. This is surprising considering the quantity of structure information available. Nevertheless, existing groups of known inhibitors can be tentatively classified into three categories according to their chemical mechanism of action: (1) inhibition of NTPase activity by interference with NTP binding, (2) inhibition of NTPase activity by an allosteric mechanism, and (3) inhibition of the coupling of NTP hydrolysis at the unwinding reaction.

Inhibition of NTPase Activity by Interference with ATP Binding

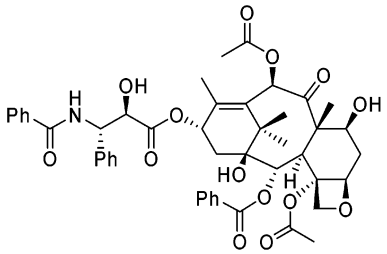
In general, the hydrolysis of ATP supplies the energy for the helicase unwinding reaction. The ATPase cycle allows the helicase to adopt various nucleotide ligation states that allosterically control the conformation of the nucleic acid binding site. Thus, it is believed that the ATPase cycle causes conformational changes in the nucleic acid binding site to drive the movement of the



60
IC_{50} 180 μM



61
IC_{50} 250 μM



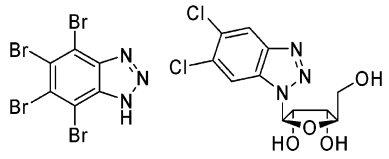
62
IC_{50} > 1 mM

Figure 22. Structure of three competitive HCV helicase inhibitors ribavirin 5′-triphosphate (**60**), ribavirin 5′-diphosphate (**61**), and paclitaxel (**62**).

helicase along the length of the nucleic acid chain.[130] Thus, a reduction of accessibility to the NTP-binding site may lead to decreased NTPase activity and therefore to a respective reduction of the unwinding rate.

A wide range of competitive NTPase inhibitors such as ribavirin 5′-triphosphate (RTP) (**60**, Figure 22) and ribavirin 5′-diphosphate (RDP) (**61**, Figure 22) have been tested as potential helicase inhibitors.[129] Surprisingly, although the IC_{50} values possessed by each of these compounds against NTPase activity lie in the low micromolar range, they demonstrate only moderate inhibitory action against the unwinding activity of HCV helicase. This phenomenon was also observed in assays utilizing paclitaxel (**62**, Figure 22), an antimitotic agent derived from the Western yew plant. Paclitaxel has been shown to compete with ATP at the ATP-binding domain, displaying an IC_{50} of 17 μM against the NTPase activity of the enzyme. However, when tested for helicase inhibitory activity, paclitaxel was not capable of inhibiting the enzyme below 1 mM.[131] This partial inhibition mediated by these competitive NTPase inhibitors is common to all members of the class, and the basis for the phenomenon remains unclear.

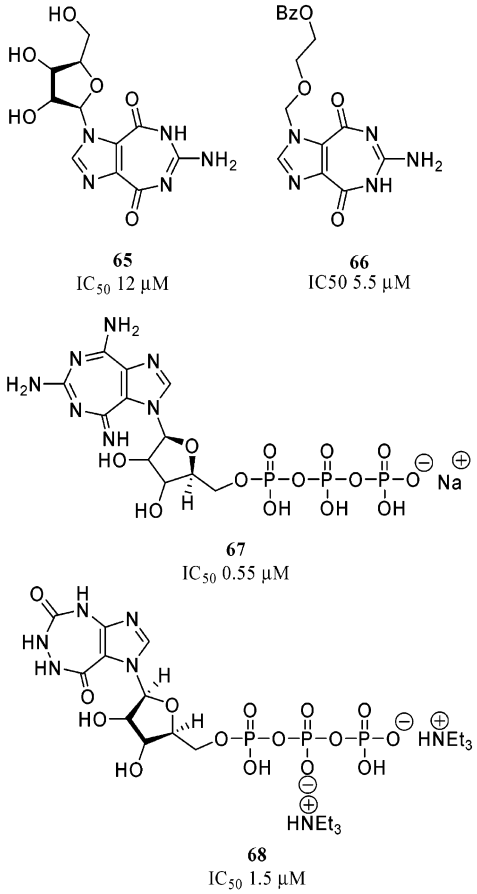
Recently a number of halogenated benzimidazoles and benzotriazoles were identified as inhibitors during the course of a random screening study.[132] Two of the most potent inhibitors identified were 4,5,6,7-tetrabromobenzotriazole (TBBT) (**63**, Figure 23,) and 5,6-dichloro-1-(β-D-ribofuranosyl)benzotriazole (DRBT) (**64**, Figure 23), displaying IC_{50} values of 20 and 1.5 μM, respectively. Although the two compounds have similar structures, the mode of action appears to differ. Preincubation of the enzyme with DRBT enhanced inhibitory potency



63
TBBT
IC_{50} 20 μM

64
DRBT
IC_{50} 1.5 μM

Figure 23. Structure of the halogenated benzotriazoles DRBT (**64**) and TBBT (**63**).



65
IC_{50} 12 μM

66
IC50 5.5 μM

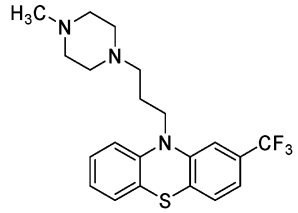
67
IC_{50} 0.55 μM

68
IC_{50} 1.5 μM

Figure 24. Structures of the ring-expanded nucleosides **65** and **66** in addition to the ring-expanded 5′-triphosphate nucleosides **67** and **68**.

from 1.5 to 0.1 μM. However, no effect of preincubation was noted with TBBT, suggesting that the mode of action does not involve the catalytic ATP-binding site but rather by occupation of an allosteric nucleoside/nucleotide binding site.[132]

In an extension of this study an additional class of nucleoside analogues known as ring-expanded (REN or "fat") nucleosides were reported to be active against the helicase unwinding reaction.[133] A number of RENs such as **65** and **66** (Figure 24) displayed IC_{50} values in the micromolar range. In view of the observed tight complex between some nucleosides and RNA and/or DNA substrates of a helicase, the mechanism of REN action might involve binding to the minor or major groove of the helical nucleic acid substrate.[133] The REN 5′-triphosphates such as **67** and **68** (Figure 24), on the other hand, did not influence the unwinding reaction but instead exerted their inhibitory effect on the ATPase activity of the enzyme.



69
IC_{50} 0.6 - 0.7 mM

Figure 25. Structure of the calmodulin antagonist trifluroperazine (**69**), which inhibits HCV helicase in a noncompetitive manner.

Inhibition of NTPase Activity by Allosteric Mechanisms

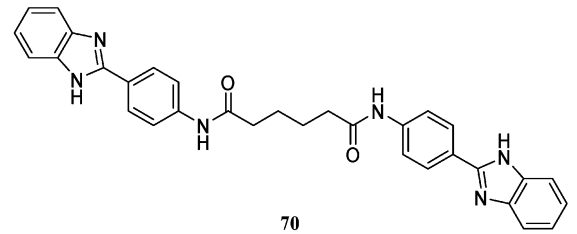
The partial inhibition mediated by the competitive NTPase inhibitors may be circumvented by utilizing compounds chemically unrelated to NTP, which reduce the accessibility to the NTP-binding site in a noncompetitive manner.[134] An example of such an inhibitor is the calmodulin antagonist trifluoroperazine (**69**, Figure 25). Although the molecule is known to interact with domain 1 of HCV helicase, it is uncertain if inhibition results from conformational changes or from blockage of the ATP-binding site.[131]

Competitive Inhibition of RNA Binding

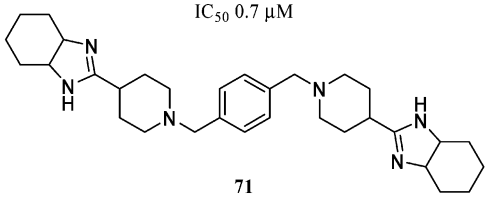
An important observation made during the initial studies of HCV helicase activity was that numerous polynucleotides elicited an inhibitory response on the enzyme. The inhibition is believed to result from the competition of the polynucleotides with DNA or RNA substrates, an effect that could possibly be mimicked by synthetic macromolecules.[131]

In an attempt to emulate the inhibitory response elicited on the enzyme by polynucleotides, two series of compounds containing aminophenylbenzimidazole and piperidinylbenzimidazole moieties such as those present in **70** and **71** (Figure 26) respectively attached to symmetrical linkers were synthesized.[135] Despite both **70** and **71** possessing submicromolar activities, the majority of corresponding benzoxazole (**72a**,**c**,**d(i−v)**, Figure 26) and benzothiazole (**72b**,**e(i−v)**, Figure 26) derivates were inactive. The aminobenzimidazole-derived diamides (**73a**,**b**, Figure 27) displayed 6−13% inhibition at 25 μg/mL, while the aminophenylbenzimidazole-derived ureas (**74a**,**b**, Figure 27) displayed 20% and 28% inhibitory activity at 25 μg/mL, respectively. Compounds **75** and **76** (Figure 27) displayed similar activity with a 14% and 10% inhibition, respectively, at a 25 μg/mL concentration.

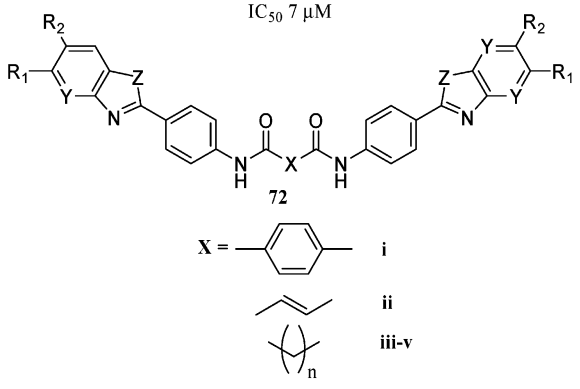
Preliminary SAR studies of these compounds demonstrated a dramatic decrease in potency with the replacement of the benzmidazole moiety present in **70** and **71** with the benzoxazole **72a**,**c**,**d(i−v)** and benzothiazole **72b**,**e(i−v)** moieties (Figure 26). Similarly, the linker was also implicated in inhibitor activity because replacement of the diamide linkage possessed by **70** with the diurea linkage, **74a** and **74b** (Figure 27), lad to diminished potency. Thus, the SAR data indicate that the benzimidazole ring, the benzene group at the C2 position of the benzimidazole moiety, and the nature of the linker are essential for inhibitory activity.[135]



70
IC_{50} 0.7 μM



71
IC_{50} 7 μM



72

X = i, ii, iii-v

n = 2, 4, 6

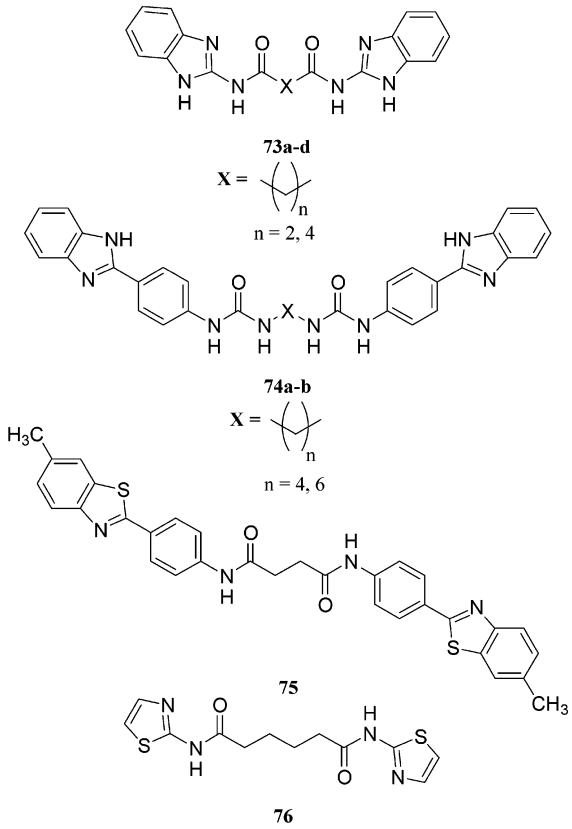
Compound	Z	Y	R_1	R_2
72a	O	CH	H	H
72b	S	CH	H	H
72c	O	N	H	H
72d	O	CH	CH_3	H
72e	S	CH	H	CH_3

Figure 26. Structures of a number of aminophenylbenzimidazole (**70**, **72a**−**d**) and benzathiozole derivatives **72b**,**e(i**−**v)** and a piperidinylbenzimidazole **71** competitive HCV helicase inhibitors.

Inhibition of the Unwinding through Intercalation of Polynucleotide Chain

There is increasing evidence that DNA or RNA duplexes, together with a bound/intercalated agent, are more stable than their unbound counterparts, increasing the energy required for duplex unwinding.[136−138] Therefore, DNA or RNA intercalating compounds are potential helicase inhibitors. Despite this, compounds that modulate the structure of the DNA or RNA substrates have received little attention as possible inhibitors.

Epirubicin (**77**, Figure 28) and nogalamycin (**78**, Figure 28), which are both members of the anthracycline family of anticancer/antibiotics, are effective inhibitors of the unwinding reaction catalyzed by the helicase enzyme.[115] Unfortunately the high cytotoxicity of such compounds and their weak penetration into the cell limit their application in the treatment of chronic viral infection. Thus, if intercalative modulation of the DNA or RNA substrates is to be considered as a possible antiviral therapy, less toxic and more selective derivatives must be identified.



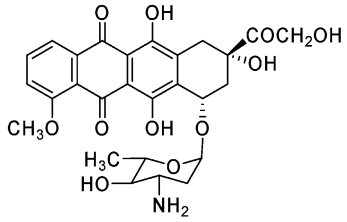
73a-d
X = n = 2, 4

74a-b
X = n = 4, 6

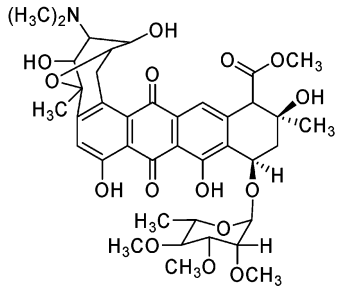
75

76

Figure 27. Structures of two aminobenzimidazole-derived diamides (**73a**,**b)**, two aminophenylbenzimidazole (**74a**−**d)**, an aminophenylbenothiazole **75**, and a thiazole **76** HCV helicase competitive inhibitors.



77
IC_{50} 0.75 μM

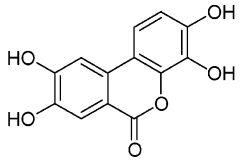


78
IC_{50} 0.1 μM

Figure 28. Structures of two DNA/RNA intercalators epirubicin (**77**) and nogalamycin (**78**) that have displayed inhibition of the unwinding reaction catalyzed by HCV helicase.

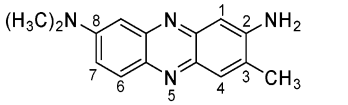
Internal Ribosomal Entry Site (IRES)

The 5′ nontranslated region of the RNA genome constitutes an internal ribosomal entry site (IRES) that binds to the ribosomes of the infected cell initiating the translation of the proviral DNA genome.[139] The IRES



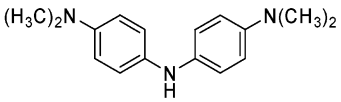
79

Figure 29. Small-molecule inhibitor of hepatitis C internal ribosomal entry site.



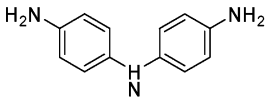
80

IC_{50} 0.02 μg/mL



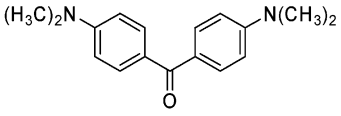
81

IC_{50} 2.19 μg/mL



82

IC_{50} >10 μg/mL



83

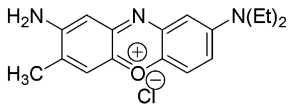
IC_{50} >10 μg/mL

Figure 30. Anti-IRES activity of compounds with truncated central ring.

allows HCV to bypass the normal cellular pathway for the initiation of protein synthesis by facilitating attachment of the viral RNA to the ribosome, hence enabling cap-independent translation. In addition to its unique function, the RNA sequence of the HCV 5′-NTR is highly conserved with a greater than 85% sequence identity among the various genotypes, thus making it an attractive target for antiviral therapy.[140,141]

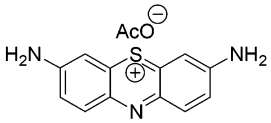
The IRES folds in a highly ordered way, forming a complex structure that possesses numerous pockets suitable for the binding of small molecules that may potentially inhibit the association of the IRES to ribosomes or other transitional cofactors.[142−144] Despite this potential, only a few reports have emerged describing small-molecule-based inhibitors of HCV translation. In one of these reports, analogues of the ellagic acid derivative (**79**, Figure 29) were reported to display an inhibitory action against HCV translation ranging from 30% to 70%.[145] It is believed that these compounds bind to the HCV IRES and not to the cellular components of the translation complex and should therefore selectively inhibit IRES-dependent translation over cap-dependent translations.

An additional class of small molecules reported to act as IRES inhibitors are known as the phenazines, and phenazine-like molecules have been reported (Figure 30).[146] The class was developed from the lead compound **80**, also known as neutral red, which was isolated from a screen of 132 000 fungal extracts.



84

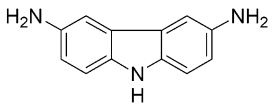
IC_{50} 1.0 μg/mL



85

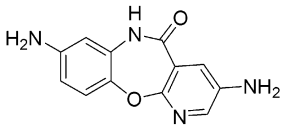
IC_{50} 2.86 μg/mL

Figure 31. Structures of phenazine **84** and phenazine-like **85** inhibitors of the HCV IRES.



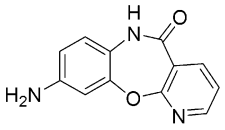
86

IC_{50} > 10 μg/mL



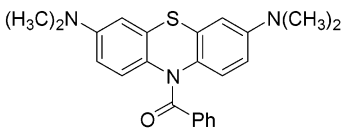
87

IC_{50} >> 10 μg/mL



88

IC_{50} >> 10 μg/mL



89

IC_{50} >> 10 μg/mL

Figure 32. Structures of a carbazole **86**, two benzoxazepione (**87** and **88**), and a thiomorpholine **89**, which have all demonstrated anti HCV IRES activity.

Preliminary structure−activity studies established that the phenazine core and polar substituents such as amines at positions 2 and 8 are crucial for potent activity, possibly because of a unique hydrogen-bonding array. The reduced activities observed with the "open" analogues (**81**−**83**, Figure 30) were indicative of change in the array due to geometry changes, at least in part. The results also suggested that a pyrazine moiety is important but not essential for activity because phenazine-like molecules (**84** and **85**, Figure 31) lacking a pyrazine arrangement were also comparatively active although more toxic.[146]

In an attempt to adjust the ring size, a number of compounds containing carbazole (**86**, Figure 32) and benzoxazepione (**87** and **88**, Figure 32) templates were also synthesized; however, these modifications lad to a complete loss in activity. The loss in activity appeared to result from the interruption of conjugation as opposed to the variation in ring size because compound **89** (Figure 32), containing the standard 6−6−6 ring scaffold but lacking in conjugation, was also inactive.[146]

Table 5. NS4A-Based Peptides and Their NS2/3 Cleavage Inhibitory Constants[159]

compd	inhibitor sequence	IC_{50} (μM)
90	Lys-Gly-Ser-Val-Val-Ile-Val-Gly-Arg-Ile-Ile-Leu-Ser-Gly-Arg-Lys	5.7
91	Ac-Arg-Gly-Gly-Ser-Val-Val-Ile-Val-Gly-Arg-Ile-Ile-Leu-Ser-Gly-Arg-Lys	3.4

Being highly conserved, the IRES is also an attractive target for ribozyme-based[147] and antisense RNA antiviral agents. Conventional antisense RNAs and a number of aptamers[148−150] in addition to ribozymes have been designed to target the HCV IRES and have given promising results in vitro, prompting the initiation of clinical trials. Two such antiviral compounds that have entered phase II clinical trials are the antisense oligonucleotide ISIS 14803[151] and the ribozyme Heptazyme.[152] However, the development of Heptazyme was stopped because of high toxicity in primates. Despite these trials, both types of molecules have limitations in terms of pharmacokinetics and uptake in mammalian cells.

Other Targets

In addition to the viral targets already discussed, information is emerging concerning the function of the remaining structural and nonstructural proteins of HCV such as the NS2 protease.[153] The NS2/3 protease of hepatitis C virus is responsible for a single cleavage in the viral polyprotein between the nonstructural proteins NS2 and NS3. The minimal protein region necessary to catalyze this cleavage includes most of NS2 and the N-terminal one-third of NS3. The NS2−NS3 protease is highly unusual in that it does not share significant sequence homology with any known protease, leading to conflicting opinions as to whether it is a metalloprotease or a zinc-dependent cysteine protease.[154] This protease has received little attention as an antiviral target probably because the protease acts co-translationally.[155]

Unlike its NS3 counterpart, the NS2 protease peptides representing the cleaved sequence have no effect on enzyme activity, suggesting that the NS2/3 cleavage is an intramolecular reaction. Surprisingly, peptides containing the 12-amino acid region of NS4A, such as **90** and **91** (Table 5), were shown to possess IC_{50} values as low as 3.4 μM against the NS2 protease.[156]

Inhibition of NS2/3 by NS4A peptides can be rationalized from the organizing effect of NS4A on the N-terminus of NS3 (the NS2/3 cleavage point) as suggested by the known three-dimensional structure of the NS3 protease domain. The X-ray crystallographic structures of NS3 protease alone and in an NS4A-bound form indicate that NS4A binding brings the N-terminus of NS3 into a stable β-sheet structure that is an integral part of the NS3 domain. The overall structure of NS2 may be affected similarly by NS4A binding, with residues critical for cleavage not positioned for the reaction in the NS4A-bound state. Specifically, the cleavage site may be rendered inaccessible by NS4A binding.[157,158]

A number of receptors also pose as potential candidates for hepatitis C therapy. Recent data indicate that the HCV envelope protein E2 binds specifically to CD81, indicating that CD81 acts as the putative receptor for viral attachment.[159] CD81 is located on the surfaces of many cell types, including hepatocytes.[160] HCV E2 binds to the large extracellular loop of CD81, but CD81 by itself does not appear to be sufficient to mediate virus entry,[161] and E2-CD81 binding correlates only in part with species permissiveness to HCV infection.[162] However, interaction between E2 and CD81 may modulate host B, T, and NK cell functions and may play a role in the pathogenesis of hepatitis C1.[163−165] The low-density lipoprotein receptor (LDLR) is also thought to be a HCV receptor because infectious HCV has been reported to be associated with low-density lipoprotein (LDL) or very-low-density lipoprotein (VLDL).[166] Presently, however, it is unclear whether interaction of HCV with the LDLR can lead to productive infection, and the search for additional receptor candidates is ongoing.

Conclusion

Despite recent progress in the molecular virology of hepatitis C, current medicinal chemistry successes have been hard-won, and numerous challenges remain. The development of effective long-term therapies will undoubtedly be hindered by the emergence of drug-resistant strains because HCV polymerase, like its HIV counterpart, lacks proofreading ability and therefore is susceptible to high levels of mutational change. For this reason, combination therapy with a number of agents will need to be developed using agents designed to bind to highly conserved essential regions of the HCV functional proteins. Moreover, because of the high genetic diversity and the geographic distribution of HCV genotypes, any new antiviral agents need to be active against all HCV genotypes.

Fortunately, there are numerous opportunities for inhibiting HCV and the clinical pipeline is showing promise for safer and more effective therapies. Inhibitors in phase II clinical development include those that target the IRES, NS3 protease, and NS5B polymerase. Thus, much progress has been made in developing antiviral agents in the relatively short time that the genome of hepatitis C has been available. Further opportunities are expected to arise as more is discovered about the virus and its life cycle.

Biographies

Christopher P. Gordon completed his BMedChem(Hons) degree at the University of Wollongong in 2003 investigating the design and synthesis of new therapeutics targeting the HIV-1 reverse transcriptase enzyme. Since then, he has continued under the direction of Paul Keller with his Ph.D. work, developing structurally novel and diverse entities to inhibit new anti-HIV targets. He continues his interest in developing synthetic and computational design methods in medicinal chemistry, with a particular emphasis on antivirals.

Paul A. Keller completed his BSc(Hons) (1985) and Ph.D. at the University of New South Wales, Australia, before undertaking an Alexander von Humboldt funded postdoctoral fellowship at the University of Wuerzburg, Germany, working in collaboration with Gerhard Bringmann. Since 1994, he has worked at the University of Wollongong, Australia, and is currently Assoc. Professor in Organic Medicinal Chemistry. His interests lie in the drug design and development of new generation anti-infectives.

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JM0400101