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Mini-review

Recent development of therapeutics for chronic HCV infection

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Dedicated to Prof. Erik De Clercq on the occasion of reaching the status of Emeritus-Professor at the Katholieke Universiteit Leuven in September 2006.

Abstract

The global prevalence of hepatitis C virus (HCV) infection and serious health consequences associated with chronic state of the disease have become a significant health problem worldwide. Currently, there is no vaccine to prevent the disease and no specific antiviral drug directed against HCV infection. The current standard of care, interferon-based therapies, both alone or in combination with ribavirin, has demonstrated limited success and is associated with undesirable side effects. Thus, the treatment of the chronic HCV infection represents an unmet medical need. With advances in the understanding of HCV replication and the crystal structures of the virally encoded enzymes, the HCV NS3/4A serine protease and the NS5B RNA-dependent RNA polymerase have emerged as ideal targets toward the control of the disease and the development of new anti-HCV agents. In this review, we will summarize the current treatment options, and outline the approaches toward discovery of small molecule antivirals against the virally encoded enzymes. The current clinical studies of promising lead compounds are also reviewed.

Keywords: Hepatitis C virus (HCV); NS3 serine protease; NS5B RNA-dependent RNA polymerase; Target for antiviral therapy; Drug resistance

Contents

1. Introduction

Hepatitis C virus (HCV) infection is the leading cause of parenteral non-A, non-B viral hepatitis worldwide. Although the infection is often asymptomatic, approximately 80% of infected patients progress to chronic hepatitis, which may lead to liver cirrhosis and eventually development of hepatocellular carcinoma. It is estimated that 180 million people worldwide and over 4 million people in the United States are infected with HCV. No vaccine is currently available to prevent HCV infection. The gold standard therapy is pegylated interferon (IFN) in combination with ribavirin, which has yielded a sustained virological

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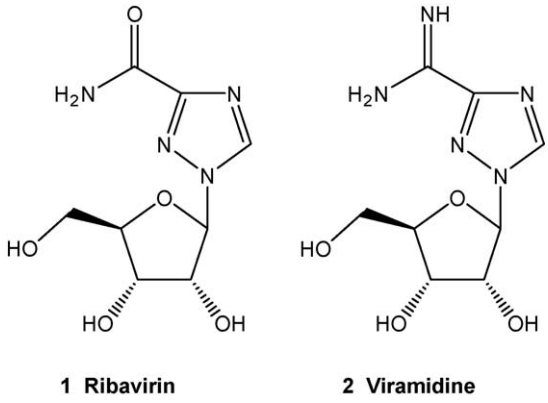
response rate of 40–50% in genotype 1-infected patients, the majority of the hepatitis C population in the United States and Japan, and of 80% in those infected with genotypes 2 and 3 (Cornberg et al., 2002). Though remarkable progress has been made in its effectiveness, the therapy is expensive and often associated with side effects that may lead to discontinuation (Cornberg et al., 2002). Given the high prevalence of the disease and the lack of specific anti-HCV drugs for treatment, novel and more efficacious therapies for HCV infection are urgently needed.

HCV is a small enveloped virus with a positive-sense, single-stranded RNA genome that encodes a large polyprotein of ~3010 amino acids. The polyprotein is co- and post-translationally processed by cellular and virally encoded proteases to produce the mature structural and non-structural (NS) proteins. Among the NS proteins, the NS3 serine-like protease and the RNA-dependent RNA polymerase (RdRp) are essential for viral maturation and replication, and therefore represent ideal targets for the development of small molecule anti-HCV compounds (De Francesco et al., 2003; Beaulieu and Tsantrizos, 2004). Other therapeutics directed at different mechanisms such as cell entry, virus assembly, maturation and prophylactic or therapeutic anti-HCV vaccines, have been explored. The HCV internal ribozyme entry site (IRES) possesses a unique functional structure and is required for viral translation and replication, and thus, represents another viral specific target for drug development efforts (Pisarev et al., 2005). Various strategies, such as Hepatozyme, anti-sense oligonucleotides (e.g. ISIS 14803), interfering RNA and compounds that bind RNA have been investigated. Though various novel therapies are under development, this review will focus on the development of small molecules against the virally encoded enzymes, mainly the NS3/4A serine-like protease and the NS5B RNA-dependent RNA polymerase.

2. Past and current therapies

For many years, the mainstream treatment for HCV infection was monotherapy using conventional IFN-α. However, this therapy is not satisfactory due to poor efficacy and the associated severe side effects. Moreover, the sustained virological response (SVR) rate was only 15–20% (Carithers and Emerson, 1997). The treatment of chronic HCV infection has improved since the introduction of ribavirin as combination therapy with IFN. Ribavirin (**1**, Fig. 1) is a broad-spectrum antiviral nucleoside analog of guanosine. Though little activity against HCV has been demonstrated as a monotherapy agent, ribavirin enhanced the SVR rate when used with IFN in combination therapy (Poynard et al., 1998). Subsequently, many antiviral adjuncts with immunomodulatory properties have also been evaluated along with IFN for their antiviral activity; however, none of these showed effects superior to combined therapy of IFN and ribavirin (Ni and Wagman, 2004).

Several strategies have been taken to improve the therapeutic efficacy of natural IFNs. The introduction of the introduction of pegylated IFNs represents a significant step forward in this regard. Clinical results suggest that compared to conventional IFN, the pegylated IFN achieved enhanced SVR both as monotherapy (Heathcote et al., 2000) and in combination therapy with ribavirin (Hadziyannis et al., 2004). Other modifications of IFN for better bioavailability and optimized pharmocokinetics have been explored. Albuferon-α, a fused form of IFN-α2b with human serum albumin, entered into a phase II clinical study. Preclinical and clinical results suggest that Albuferon-α is well tolerated with a half-life substantially longer than those for conventional and pegylated IFN, and exhibits robust antiviral activity (Nelson et al., 2005).



1 Ribavirin **2 Viramidine**

Fig. 1. The structures of ribavirin and viramidine.

Consensus interferon (CIFN, interferon alfacon-1, Infergen) is a recombinant non-naturally occurring type-I IFN originally developed by scientists at Amgen. It was derived by comparing the amino acid sequences of natural IFN-α subtypes and assigning the most frequently observed amino acid in each corresponding position to generate a 166-amino acid consensus molecule. CIFN binds with high affinity to type-I IFN receptors and has more portent biological activity than naturally occurring IFN-α (Melian and Plosker, 2001). The utility of CIFN in the clinic has yet to be proven definitively. Data reported from several recent studies, although small, may lead to treatment alternatives for individuals that do not respond to the current standard of care. A limited number of Peginterferon/ribavirin non-responders have been shown to achieve a sustained virological response with the use of high dose CIFN. Results of a large international phase III study in non-responders is forthcoming.

Much effort has been devoted to developing ribavirin analogs to improve the safety profiles associated with hemolytic anemia. Among several ribavirin-like molecules, viramidine has produced promising data. A liver targeting ribavirin prodrug, viramidine (**2**, Fig. 1) is converted to ribavirin by adenosine deaminase, an enzyme abundant in the liver. Compared to its parent, viramidine has a favorable pharmacokinetic profile, which can lead to better antiviral activity and dosing schedules. Pharmacokinetic and safety studies have demonstrated that viramidine was safe and well tolerated (Lin et al., 2004). A phase II combination therapy of pegylated IFN-α2a with either viramidine or ribavirin for chronic hepatitis C demonstrated no significant difference between the treatment groups in terms of the early virological responses; however, significantly fewer patients developed anemia in the viramidine-treatment

groups (Gish, 2006). The phase III trail (VISER 1) confirmed that Viramidine resulted in a significant reduction in anemia compared to ribavirin (5% versus 24%; $p < 0.0001$). However, Viramidine did not meet the non-inferiority to ribavirin efficacy endpoint on an intent-to-treat (ITT) basis based on the analysis of 637 patients (Benhamou et al., 2006). Further analysis of the data based on weight (mg/kg) indicated that SVR was improved with increased mg/kg concentrations while preserving the safety benefit. A second phase III Viramidine trail, VISER 2, is currently underway.

Peginterferon plus ribavirin combination therapy is the current standard of care for HCV-infected individuals. While it is well known that greater than 50% of individuals infected with HCV genotype 1 treated with the standard of care, fail to achieve a sustained virological response, at this time, there are no FDA approved treatment options for these individuals.

3. HCV NS3/4A serine protease inhibitors

The HCV NS3 is a multifunctional protein in which the N-terminal third of the protein contains a serine-like protease whereas the C-terminus possesses helicase and NTPase activities. The coupled NTPase and helicase unwinds duplex RNA structures, and thus, plays a crucial role in viral replication. Despite extensive efforts directed toward screening inhibitors against HCV helicase, only a few small molecule compounds have been disclosed (Borowski et al., 2002). The NS3 protease requires the NS4A co-factor, or peptidyl derivatives thereof, for enzymatic activity (Bartenschlager et al., 1993). The NS4A is required to improve the anchoring and orientation of the highly conserved catalytic triad, which is composed of His57, Asp81 and Ser139 (Yao et al., 1999). Because of its essential role in the viral replication, the NS3 protease represents an attractive target for HCV antiviral intervention.

The structure of the NS3/4A complex was solved at 2.5 Å resolution by X-ray crystallography (Yao et al., 1999). The complex contains two β-barrel domains and four short α-helices. The active site of the NS3/4A protease lies in the shallow and solvent-exposed cleft between two β-barrels, which makes development of potent small molecule inhibitors challenging. Nevertheless, enormous progress has been made in a multidisciplinary effort. Innovative, structure-based rational approaches generated two broad classes of inhibitors against the NS3/4A protease: peptidomimetic and non-peptidic molecules.

3.1. Peptidomimetic protease inhibitors

Biochemical studies surprisingly demonstrated that the amino-terminal products derived from the cleavage are competitive inhibitor of the enzyme (Llinàs-Brunet et al., 1998; Steinkuhler et al., 1998). In most cases, the hexapeptide with residues P_6 to P_1 displayed a lower K_i value (Llinàs-Brunet et al., 1998). Therefore, efforts were focused on the natural substrates as the starting points for rational design and lead optimization of peptidomimetic inhibitors (De Francesco et al., 2003; Chen and Tan, 2005). Based on structure–activity relationship (SAR) studies, unnatural amino acids were introduced to increase the specific binding affinity and improve pharmacokinetic profiles of the compounds. At the same time, truncations in the size of the compounds were pursued to render the peptidomimetic inhibitors more suitable for drug development. Different medicinal chemistry strategies were taken to generate substrate-based inhibitors of either a covalent or non-covalent mode of action.

3.1.1. Non-covalent peptidomimetic inhibitors

Initial SAR studies revealed that the optimal binding of a hexapeptide inhibitor required an acidic anchor at the N-terminus and the carboxylic at the C-terminus (Ingallinella et al., 1998). This unique, free carboxylic acid present on the P_1 residue contributes considerably to potency and imparts selectivity with respect to other serine protease (Llinàs-Brunet et al., 1998). Starting from hexapeptide inhibitors derived from the amino-terminal NS3 cleavage product, researchers focused on alternating P_1 residue as the first step in lead optimization. To date, several classes of non-covalent, product-based inhibitors have been disclosed, with C-terminal moieties including carboxylic acid (Llinàs-Brunet et al., 2004), acyl sulphonamide (Campbell et al., 2004) and phenethyl amide (Colarusso et al., 2003). Among these, (1*R*,2*S*)-1-amino-2-vinylcyclopropane carboxylic acid represents one of the best available P_1 cysteine substituents.

Based on the SAR studies and computational chemistry techniques, the research group at Boehringer Ingelheim demonstrated that replacement of the natural P_1 residue with 1-aminocyclopropyl-carboxylic acid resulted in an inhibitor with equal potency as the parent counterpart. Introduction of large lipophilic aromatic bases to the P_2 proline ring significantly enhanced the potency and culminated in the discovery of the 2-phenyl-4-oxoquinoline as an improved replacement (De Francesco et al., 2003; Tsantrizos, 2004). Capitalizing on the discovery, subsequent SAR studies were conducted to truncate the P_5 and P_6 residues, which led to the discovery of tetrapeptide inhibitors with EC_{50} values in the submicromolar range (De Francesco et al., 2003; Tsantrizos, 2004). Much effort has been devoted to improving the poor biopharmaceutical profile of the compounds by using a shorter peptidic scaffold than that of the linear tetrapeptides. Further truncation on the tetrapeptide yielded a potent tripeptide inhibitor, which demonstrated potency in the cell-based HCV replicon system. To further improve the potency, a novel P_1 group attached through an alkyl bridge was introduced, which forms a rigidly bound macrocylic ring connecting the P_1 and P_3 residues (Llinàs-Brunet et al., 2004). This led to the discovery of the bioavailable tripeptide inhibitor, BILN-2061 (**3**, Fig. 2). It is thought that binding of BILN-2061 to the active site of the NS3/4A protease may result in the distortion of the catalytic triad and disruption of H-bonds (Lamarre et al., 2003). BILN-2061 displayed potent and competitive inhibition of HCV protease of genotypes 1a and 1b with a mean K_i of 0.3 and 0.66 nM, respectively. The compound exhibited a mean EC_{50} of 4 and 3 nM for the HCV replicon 1a and 1b, respectively (Lamarre et al., 2003). In a small proof-of-concept study, BILN-2061 (200 mg) was orally administered to patients infected with HCV genotype 1 twice a day for 2 days. BILN-2061 was highly effective, inducing a 2–3 $\log_{10}$ or greater

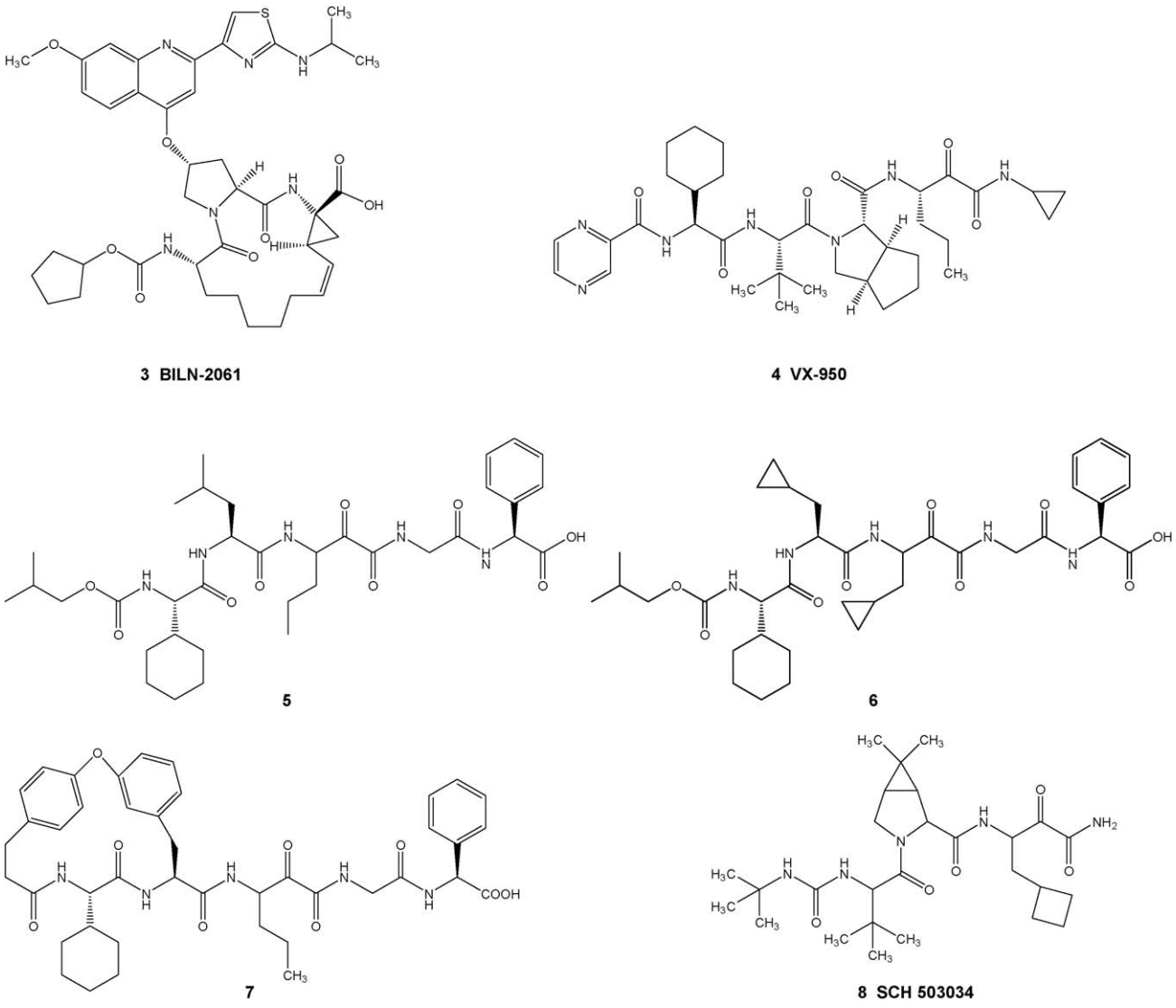


Fig. 2. Structures of NS3 protease inhibitors.

reduction in viral load within 24–28 h post-administration. The viral load was undetectable in most patients at 48 h after treatment at detection limit of 50 HCV RNA copies ml^{-1} (Lamarre et al., 2003). While this study established the first proof-of-concept in man for an HCV protease inhibitor, routine safety testing of supra-therapeutic doses in animals did, however, reveal relevant side effects. Further development of BILN-2061 was suspended.

Early study revealed that the prime region also contributes to substrate recognition of the binding site, and that the substitutions in P'_1 with proline, tetrahydroisoquinoline-3-carboxylic acid or pipecolinic acid resulted in non-cleavable substrate analogs (Landro et al., 1997). Based upon the observations, by combining the N-terminal carboxylic acid at P_1 with the optimized prime-site binding sequence, scientists at Instituto di Ricerche di Biologia Molecolare (IRBM, a subsidiary of Merck) were the first to report a novel class of the protease inhibitors that bind to the prime site (Ingallinella et al., 2002). Taking advantage of the P'_1 binding, researchers at Boehringer Ingelheim reported a series of macrocyclic azapeptide inhibitors by extending BILN-2061 via a non-cleavable P_1/P'_1 amidation connecting a P'_1 sulfonamide (Tsantrizos, 2004). The azapeptides are metabolically more stable than their amino acid counterparts and SAR studies indicated that the methyl benzyl moiety was the most preferred group. Nuclear magnetic resonance studies demonstrated that the inhibitors bind in a non-covalent competitive mode to the protease active site (Tsantrizos, 2004).

Following the lead of others in the field, scientists at InterMune used a structure-based design approach in combination with protein crystallography to guide the rational design of a potent HCV protease inhibitor. Optimization of the P_2 moiety, the linker P'_1 and the contacts to the NS3/4A catalytic triad led to the discovery of ITMN 191, previously referred to as ITMN-B (Condroski et al., 2006). Preclinical characterization suggest that ITMN 191 is a potent, selective HCV protease inhibitor with the EC_{50} = 900 nM in a biochemical assay utilizing genotype-1b protease and an EC_{50} = 2.1 nM in a genotype-1b replicon system. The compound is active across different genotypes and retains activity against variants that exhibit reduced sensitivity to other

experimental HCV protease inhibitors in development (Seiwert et al., 2006). ITMN 191 is currently undergoing IND-enabling toxicologic evaluation.

3.1.2. Covalent substrate-based inhibitors

The NS3 protease–substrate interactions encompass hydrogen bonds with a substrate backbone and electrostatic contacts along the binding site. One approach is to design the peptidic inhibitors that interact with the catalytic serine residues, reversibly or irreversibly. These inhibitors are typically derived from known substrates by replacing the scissile amide bond with an electrophilic moiety, such as α-ketoamides, boronic acids, aldehydes, α-ketoacids, azapeptides or pyrrolidine-5,5-*trans* lactams. The lactams bind to the serine residue in the protease active site in a covalent irreversible fashion while the other groups can act as serine traps in a covalent reversible mode (Narjes et al., 2003). Among the reported moieties, the α-ketoamides have been extensively explored and disclosed, cumulating in the discovery of VX-950 (**4**, Fig. 2) (Narjes et al., 2003; Chen and Tan, 2005; Goudreau and Llinas-Brunet, 2005). The X-ray crystal structure revealed that the α-ketoamide moiety interacts with the catalytic serine residue forming a reversible covalent bond (Narjes et al., 2003). Moreover, the α-ketoamide-derivatives are able to penetrate cells, a favorable feature in drug development.

The SAR studies demonstrated that structure-based hexapeptides containing an α-ketoamide moiety in place of the scissile amide bond are potent protease inhibitors (Bennett et al., 2001). Based on this observation, scientists at Vertex Corporation synthesized a series of tetrapeptidyl α-ketoamide inhibitors with hydroxyproline as the P_2 units (Goudreau and Llinas-Brunet, 2005; Lin et al., 2006). Later, scientists at Eli Lilly discovered a novel series of HCV protease inhibitors with ketobiocycloproline incorporated as P_2 residue (Babine et al., 2002). The compounds exhibited excellent inhibitory effects in biochemical assays; however, they did not display any activity in the cell-based HCV replicon system. Subsequently, in a collaborative effort between Vertex and Lilly, systematic SAR optimization on P_2 and further work on both P_1 and P_3 culminated in the discovery of VX-950 (Goudreau and Llinas-Brunet, 2005; Lin et al., 2006; Perni et al., 2006). VX-950 demonstrated potent activity with an $EC_{50} = 354$ nM in the genotype-1b replicon system and an $EC_{50} = 280$ nM in human fetal hepatocytes infected with genotype-1a HCV positive patient sera (Perni et al., 2006). The available preclinical and clinical data suggested that VX-950 has the potential to shorten the duration of therapy and may yield an improved SVR with favorable pharmacokinetic profiles (Lin et al., 2006; Perni et al., 2006). Final results of a phase 1b, multiple-dose study demonstrated that VX-950 was well tolerated for 5–14 days in both healthy subjects and HCV-infected patients, and that the compound had sustained antiviral effects. All individuals in this limited study demonstrated at least a $2\log_{10}$ drop in viral load after treatment (Reesink et al., 2005). Late in 2005, the Food and Drug Administration (FDA) granted Fast Track designation to VX-950 for the treatment of HCV infection. A phase II clinical study of VX-950 is currently ongoing.

In research focusing on SAR studies surrounding the P'_2 position and building on their previous work with α-ketoamide-containing peptides, scientists from Schering-Plough discovered that improved potency can be found through manipulation of the amino acid at this site. Compounds, such as **5**, with a phenylglycine at the P'_2 location, were found to be quite potent. The carboxylic acid **5** has a K_i^* of 120 nM, and the corresponding carboxamide has a K_i^* of 66 nM. Structural data suggests that the P_1–P'_2 moiety forms a C-clamp around the lysine 136 side chain (Arasappan et al., 2005). Building even further on this structural improvement, it was found that altering the amino acids at the P_1 and P_2 sites to incorporate the more hydrophobic cyclopropylmethyl side chains provided an even further improvement, with compound **6** having a K_i^* of 50 nM. Structural studies indicate that this compound also forms a similar C-clamp, and also that the cyclopropylmethyl groups reside favorably in the S1 and S2 pockets (Bogen et al., 2005). Another interesting modification was made by linking the side chain residues that reside in the S2 and S4 pockets. This resulted in a series of macrocyclic inhibitors, of which compound **7** was the best, with a K_i^* of 22 nM (Venkatraman et al., 2005).

SCH 503034 (**8**, Fig. 2), a peptidyl protease inhibitor with α-ketoamide moiety, is an orally active inhibitor developed by Schering-Plough (Malcolm et al., 2006). This investigational drug has a mechanism of action distinctly different from current therapies. Preclinical study demonstrated that SCH 503034 displayed a potent time-dependent inhibition of the single-chain NS3 protease in the enzymatic assay with an overall inhibition constant of 14 nM as well as potent *in vitro* activity ($EC_{50} = 200$ nM) in the HCV replicon system (Malcolm et al., 2006). Treatment with SCH 503034 at six times the EC_{90} effective concentration for 15 days resulted in a greater than $4\log_{10}$ reduction in replicon RNA. Moreover, the combination of SCH 503034 with IFN was more effective to suppress replicon replication than either compound alone (Malcolm et al., 2006). In a multi-dose, double-blind study of patients infected with HCV genotype 1 and that failed PEG-IFN-α treatment ($<2\log_{10}$ viral RNA reduction after 12 weeks), SCH 503034 exhibited potent dose-dependent anti-HCV activity with safety profiles similar to that in patients receiving the placebo. Mean maximum viral load reduction in the 400 mg TID group was $2.06\log_{10}$ from baseline (Zeuzem et al., 2005a). In another study with HCV genotype 1-infected, PEG-Intron ± ribavirin non-responders, SCH 503034 monotherapy for 7 days reduced the HCV RNA 0.4–1.77 and 0.5–2.5 $\log_{10}$ at 200 mg TID and at 400 mg TID, respectively. As expected, SCH 503034 plus PEG-Intron combination therapy demonstrated potent antiviral activity. Mean maximum HCV RNA levels of reduction ($\log_{10}$) were 2.4 and 2.9 for 200 and 400 mg SCH 503034 + PEG-Inron, respectively, versus 1.1 for PEG-Intron alone (Zeuzem et al., 2005b). The FDA granted Fast Track designation to SCH 503034 in January 2006, which is currently in phase II clinical development for the treatment of chronic HCV infection. The proposed first indication for SCH 503034 is for patients infected with HCV genotype 1 virus who have not responded to the standard combination therapy with pegylated IFN plus ribavirin.

3.2. Non-peptidic protease inhibitors

Enormous progress has been made in the search for peptidomimetic derivatives against the HCV protease. However, due to poor bioavailability and certain physicochemical properties, most of the inhibitors could not be further developed into promising drug candidates. An alternative strategy is to identify non-peptidic, small molecule inhibitors by the adapted protease assay. To date, several non-peptidic classes of compounds have been reported to demonstrate *in vitro* NS3 inhibitory activity, including thiazolidine derivatives, phenanthrenequinone compounds, aryl-alkylidene rhodanines and benzimidazole derivatives and isolate RNA aptamers (De Francesco et al., 2003; Ni and Wagman, 2004). None of these compounds are competitive inhibitors of the HCV protease and their mechanisms of action remain unclear. It remains to be seen whether these compounds warrant further development.

4. HCV NS5B RdRp inhibitors

The three-dimensional crystal structure of the HCV RdRp has been solved, revealing a classic right hand, 'thumb-palm-finger like' structure (Bressanelli et al., 1999). Extensive interactions exist between the fingertips and the thumb domain, forming a fully encircled active site. Subsequently, structural analysis of the polymerase in complex with ribonucleotides identified an rGTP pocket between the interface of the finger and thumb domains. The pocket acts as an allosteric site, providing alternative interactions between these domains during conformational changes for efficient initiation (Bressanelli et al., 2002).

The HCV RdRp is essential for viral replication and has no host equivalent, highlighting the enzyme as an attractive target for antiviral therapy. To date, the majority of approved antiviral drugs target viral polymerases as the primary mechanism of action (De Clercq, 2001). The available three-dimensional crystal structure of the HCV RdRp polymerase and the success achieved with the chemical agents targeting HIV reverse transcriptase fueled the search for the HCV polymerase inhibitors. Both biochemical and cell-based replicon assays have been employed in the identification and optimization of novel HCV RdRp inhibitors. Two structurally distinct classes of inhibitors with different modes of action have been reported: nucleoside analogs and non-nucleoside inhibitors. Whereas nucleoside analogs generally target the polymerase active site in a competitive manner and typically exhibit broader spectrum activity, non-nucleoside counterparts have much greater specificity, acting by either interfering directly with the active site or binding to the allosteric site and preventing the initiation process.

4.1. Nucleoside inhibitors

Nucleoside inhibitors, whether chain terminators or non-chain terminators, can be effective in inhibiting the virus replication. Upon entry into the cells, nucleoside analogs are first converted to nucleotide triphosphates (NTP). The unnatural nucleoside inhibitors can serve as competitive substrates for the polymerase and can be incorporated into the nascent chain by the viral polymerase. This incorporation can lead to premature termination of the elongation process. Alternatively, studies with poliovirus suggested that the decreased fitness of progeny viruses by ribavirin-caused mutagenesis is the primary mode of action (Crotty et al., 2001). The incorporated nucleotides may cause base-mismatch in subsequent rounds of replication, resulting in accumulated mutations in the viral genome and then the so-called error catastrophe. However, other studies indicate that in the case of HCV, ribavirin in combination with IFN helps to restore the immune response (Lau et al., 2002). It appears that ribavirin is a pleiotropic agent that can influence its overall antiviral properties. While the mechanism of action for ribavirin is still unclear, thus far ribavirin is the only FDA approved nucleoside analog used for the treatment of HCV infection.

Several sugar-modified and base-modified nucleoside analogs have been reported to inhibit the RdRp enzymatic activity and block HCV replication in the replicon systems (Carroll et al., 2003; Sarisky, 2004). Among these, the most promising derivatives are of 2′- and 3′-substituted ribonucleoside analogs. Merck Research Laboratory described 2′-*C*-methyladenosine (**9**, Fig. 3) and 2′-*O*-methylcytidine (**10**, Fig. 3), which inhibited the *in vitro* NS5B polymerase assay with EC_{50} values in the low micromolar range (Carroll et al., 2003). Mechanisms of action studies suggest that these nucleosides act as competitive substrates and are incorporated as chain terminators. The 2′-methyl group prevents subsequent incorporation via steric clashes with the incoming nucleotides. 2′-*C*-methylguanosine (**11**, Fig. 3) and other nucleoside analogs probably act in the mode similar to 2′-*C*-methyladenosine and 2′-*O*-methylcytidine. Another nucleoside analog with a *C*-2′ methyl group has been reported to have good anti-HCV activity. 2′-Deoxy-2′-fluoro-2′-*C*-methylcytidine (**12**) has been prepared and evaluated in several systems. In an HCV replicon assay it had an EC_{90} of 5.4 μM, comparable to that of 2′-deoxy-2′-fluorocytidine. Additionally, it was not cytotoxic up to the highest concentration evaluated (100 μM). Interestingly, this compound was inactive in assays measuring the replication of bovine viral diarrhea virus, an HCV-related viru (Clark et al., 2005). Further, a series of 3′-deoxyribonucleosides derivatives have been disclosed as chain terminators and one representative compound, 3′-deoxycytidine (**13**, Fig. 3), exhibited submicromolar activity in a biochemical assay (Ismaili et al., 2002).

NM283 (**14**, Fig. 3), developed by Idenix, is a promising orally bioavailable pro-drug of 2′-*C*-methylcytidine, NM107 (**15**, Fig. 3). NM283 targets the HCV virus-encoded RNA polymerase in two ways. It inhibits the viral polymerase directly and it is incorporated into growing strands of viral RNA, which terminates RNA chain extension. NM283 is the first antiviral agent of this class to enter phase II clinical trials for the treatment of hepatitis C infection. Recently, partial four week data from an ongoing phase IIb clinical trial was released, which demonstrated that NM283 combined with pegylated IFN treatment exhibited improved efficacy and adequate tolerability compared to current therapies for chronic hepatitis C patients (O'Brien, 2005).

Recently, Roche announced interim results from a multiple ascending dose study of R1626, a novel nucleoside

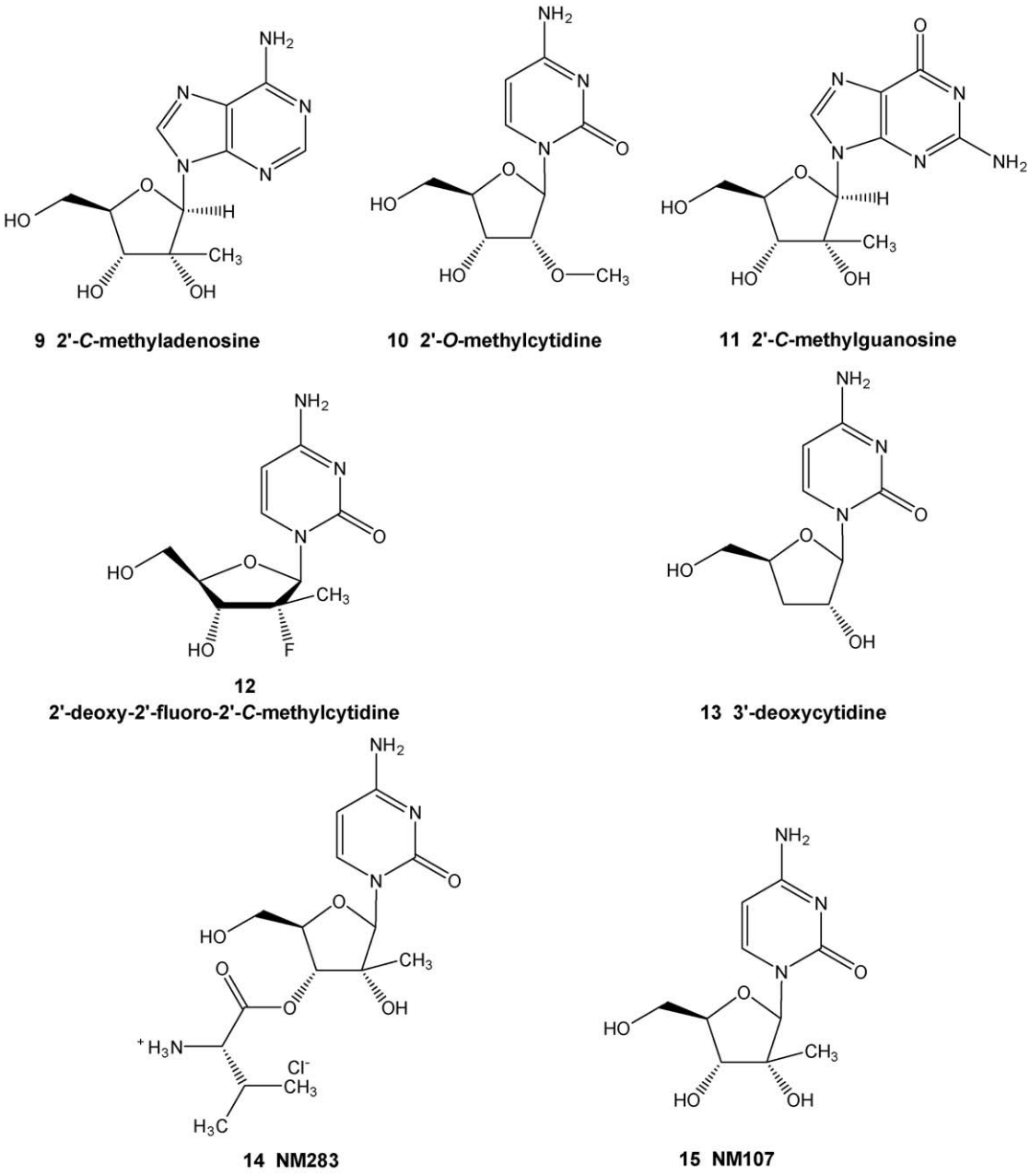


Fig. 3. Structures of nucleoside NS5B polymerase inhibitors.

analog targeting HCV polymerase in chronic HCV-infected individuals. R1626 is a pro-drug of R1479, which displayd potent anti-HCV activity *in vitro*. Following oral administration, R1626 was efficiently converted to R1479 and was well tolerated in naïve chronic patients. Moreover, dose-dependent antiviral activity was observed with a mean serum viral RNA reduction of 1.2 $\log_{10}$ from baseline after treatment with 1500 mg of R1626 for 14 days (Roberts et al., 2006).

4.2. Non-nucleoside inhibitors

In addition to the active site, the X-ray derived co-crystal structures of compounds bound to NS5B revealed distinct allosteric regulatory sites that are located distant to the active site and are targets for developing antiviral agents (Love et al., 2003). Accordingly, compounds that interact either with the RdRp active site or the allosteric site could potentially interfere with substrate binding and/or conformational change, thus effectively inhibiting initiation. Several classes of structurally distinct non-nucleoside inhibitors of the HCV RdRp have been identified and disclosed.

A series of α,γ-diketoacid compounds as inhibitors of HCV polymerase has been disclosed (Altamura et al., 2000). The SAR studies indicated that the diketoacid moiety proved essential for activity, and that the substitution of aryl to thienyl group on the γ-position was necessary for selectivity and potency (Summa et al., 2004). Further optimization led to the identification of a potent

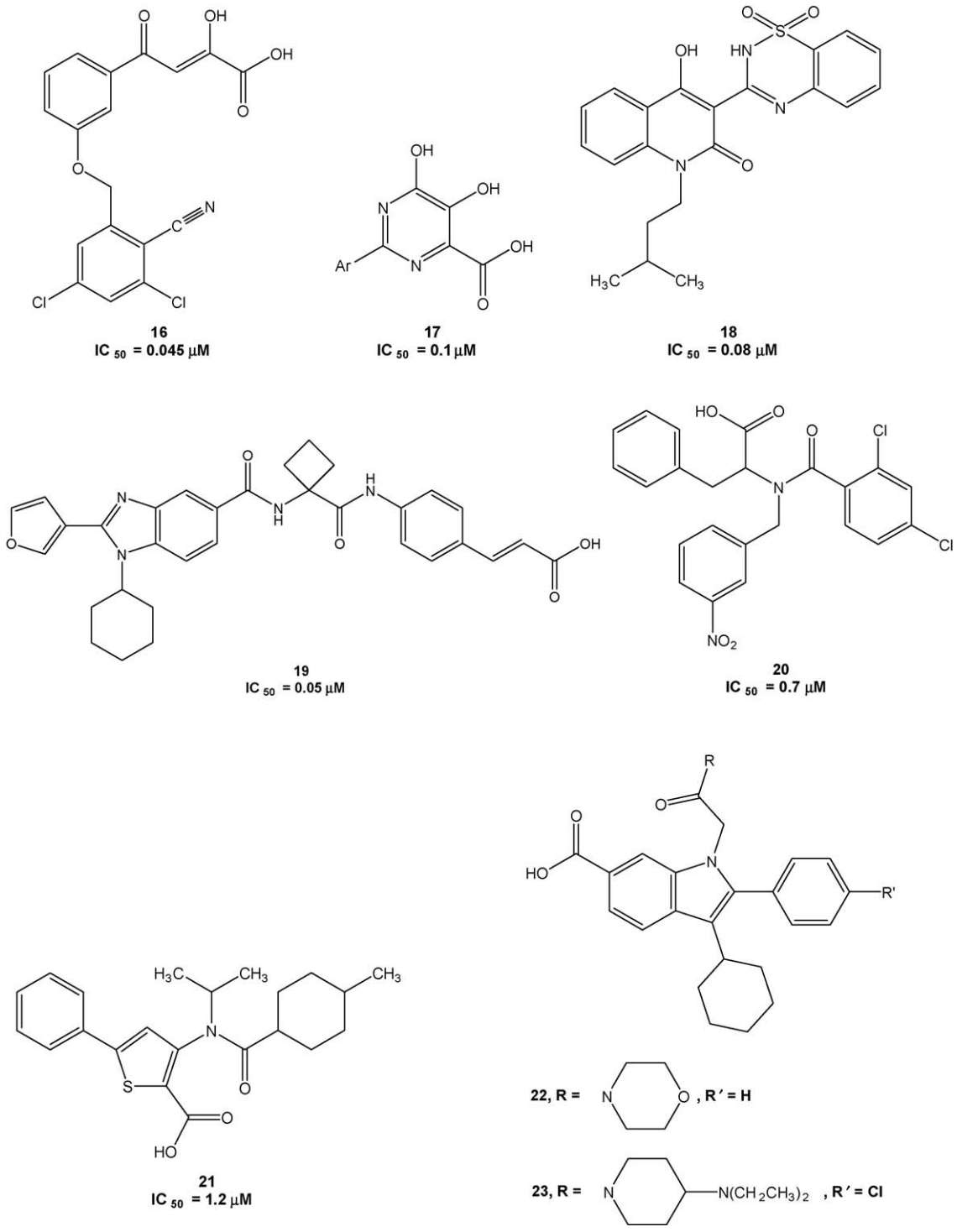


Fig. 4. Structures of non-nucleoside NS5B polymerase inhibitor.

HCV NS5b polymerase inhibitor with an EC_{50} of 45 nM (**16**, Fig. 4). Compounds of dihydroxypyrimidine carboxylic acid class, (e.g. compound **17**, Fig. 4), are believed to chelate the two catalytic Mg^{2+} ions in the active sites as diketoacid compound (Gardelli et al., 2002). However, no data of *in vivo* activity for the pyrophosphate mimics are available, and the high ionic nature of these compounds may raise concerns such as low bioavailability and toxicity.

GlaxoSmithKline (formerly SmithKline Beecham) disclosed a novel class of benzothiadiazine derivatives (Dhanak et al., 2001) from which a representative compound (**18**, Fig. 4) displayed good potency in both biochemical assay with an EC_{50} value of 80 nM and HCV replicon assay with an EC_{50} value of 500 nM (Dhanak et al., 2002). Moreover, the benzothiadiazine derivatives were shown to be highly selective for the HCV RdRp, failing to inhibit other viral and mammalian polymerases

(Dhanak et al., 2002). Treatment with the compound and IFN-α resulted in a highly synergistic effect in the replicon system (Gu et al., 2003). Further study demonstrated that the compounds are non-competitive for NTP incorporation and act to arrest the *de novo* initiation of RNA synthesis prior to the elongation phase, possibly through interacting with the functionally critical NS5B polymerase active site (Gu et al., 2003). Substitution of the quinolinone moiety with pyrrolone group led to compounds with greater potency (Dhanak et al., 2001).

Benzimidazole derivatives were the first non-nucleoside inhibitors disclosed to be active against the HCV polymerase by Japan Tobacco (Hashimoto et al., 2001). One representative compound (**19**, Fig. 4) showed an EC_{50} value in the submicromolar range. Investigation of the mechanism of action study indicated that the compound did not compete with incorporation of NTP (Tomei et al., 2003). Moreover, mutations conferring resistance to these compounds were mapped to proline residue 495, which is located on the surface of the polymerase thumb domain and away from the active site, suggesting that the compounds acted as allosteric inhibitors by blocking the activity of the polymerase prior to the elongation step (Tomei et al., 2003). Cross-resistance studies and synergistic inhibition of HCV polymerase by combinations of a benzimidazole and a benzothiadiazine further confirmed that these two structurally distinct classes of inhibitors had non-overlapping binding sites and thus acted with different mode of action (Tomei et al., 2004). Boehringer Ingelheim reported a series of benzimidazole-containing heterocycles by extending the original derivatives to topologically related scaffolds and incorporating an amide moiety inside the molecule (Beaulieu et al., 2004). Interestingly, subsequent substitution of the benzimidazole with a pyrazolopyrimidine moiety led to active compounds with an EC_{50} of less than 1 μM (Shipps et al., 2003).

Researchers at Shire Biochem disclosed two classes of HCV polymerase inhibitors including phenylalanine and thiophene carboxylate derivatives. Representative compounds **20** and **21** are shown in Fig. 4, respectively. Further studies demonstrated that the inhibitors bound to a distinct allosteric site in the thumb domain, (Beaulieu and Tsantrizos, 2004; Sarisky, 2004; Wu et al., 2005). In addition, other promising non-nucleoside inhibitors are in different phases of clinical trails. R803, a small molecule HCV RdRp inhibitor developed by Rigel Pharmaceuticals, was found to be active in the replicon system with EC_{50} below 10 μM. R803 entered into a multi-dose phase I/II clinical study for chronic HCV infection and recently the study has been cancelled. HCV-086, another orally available small molecule inhibitor of NS5B polymerase was co-developed by Viropharma and Wyeth. Results from a phase 1b study demonstrated that HCV-086 possessed favorable pharmacokinetics and was generally safe and well tolerated. However, the overall antiviral activity of HCV-086 did not warrant further development. The follow-on compound HCV-796, a novel non-nucleoside HCV polymerase inhibitor, is being evaluated in ongoing clinical trials in combination with PEG-IFN by ViroPharma and Wyeth. The safety and pharmacokinetics profiles from a randomized, double-blind, placebo-controlled, ascending single-dose study in healthy subjects, demonstrated that HCV-796 was generally well tolerated with no serious treatment-emergent adverse events (Chandra et al., 2006). Recently ViroPharma announced that oral administration of HCV-796 as monotherapy for 14 days to naïve patients was well tolerated with no dose limiting toxicities, and that it displayed dose-dependent antiviral activity across multiple genotypes, with peak mean HCV viral load reductions of 1.4 to 1.5 $\log_{10}$ on day four. The most significant and sustained reduction in viral load from baseline (>3.5 $\log_{10}$ at day 14 in the 1000 mg dose group) was observed on patient infected with genotype-1b HCV (ViroPharma News).

Recently, a novel class of allosteric inhibitor of NS5B has been report (Harper et al., 2005). A representative compound **22** showed potent affinity for the HCV RdRp and effective inhibition of subgenomic HCV RNA replication (EC_{50} of 300 nM). Based on the crystal structure of **22** with NS5B, an improved indole-*N*-acetamide (**23**) was found that had an EC_{50} of 127 nM in a replicon assay as well as the additional attractive property of not activating the pregnane X receptor (Harper et al., 2005). Further structural work with **23** bound to the enzyme has indicated that these molecules act as allosteric inhibitors by interference with contacts between two domains on the enzyme (Di Marco et al., 2005).

5. Conclusions

The lack of specific anti-HCV drugs highlights the necessity of developing novel, more efficacious and safe therapies for treating HCV infection. With advances in the understanding of virus replication and crystal structure information of the virally encoded enzyme, the NS3/4A protease and the NS5B RNA-dependent RNA polymerase, have emerged as ideal targets toward the control of HCV infection. The last few years have witnessed intensive efforts in developing potent protease and polymerase inhibitors as HCV therapeutics. Different classes of novel small molecule inhibitors against these enzymes have been reported. Medicinal chemistry focused on the SAR studies further enhanced the potency profiles and improved the pharmacokinetic properties of these compounds. Among these, some proof-of-concept investigational drugs are entering into clinical studies with promising potency and good pharmacokinetic profiles.

Recently, infectious HCV cell culture systems have been established that will promote a better understanding of virus tropism, virus–host interactions and virus life cycle (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). The availability and future improvement of the cell culture systems will also boost the search for HCV therapeutics. Fueled by more detailed elucidation of the crystal structures of the critical enzymes, it is reasonable to predict that effective small molecule antiviral therapeutics for HCV infection will emerge in the near future.

Given that HCV is an RNA virus with no known proofreading function during RNA replication and that the virus exists as a quasispecies in nature with a high replication rate *in vivo* and long duration of the disease, drug-resistant mutants may already preexist in HCV-infected patients. Moreover, it is apparent that new inhibitors directly targeting the enzymes involved

in HCV replication are likely to prompt the emergence of drug-resistant strains (Lin et al., 2005; Mo et al., 2005). Therefore, monotherapy with small molecule enzyme inhibitors will be unlikely to eradicate HCV infection. However, preclinical and clinical studies indicated it is possible to attack the virus on several fronts. It has been demonstrated that combination of small molecule anti-HCV inhibitors of different mode of actions or the small molecule inhibitor plus IFN-α synergistically inhibited viral RNA replication and facilitated viral clearance (Lin et al., 2005; Lamarre et al., 2003; O'Brien, 2005; Malcolm et al., 2006; Mo et al., 2005). Based on experience with the treatment of HIV/AIDS, cocktail therapies combining several agents that are directed against different viral targets with different mechanisms of action, alone or in combination with the current approaches, will most likely provide better treatment options for people with chronic HCV infection.

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