Recent Advances in Discovery and Development of Promising Therapeutics against Hepatitis C Virus NS5B RNA-Dependent RNA Polymerase

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Abstract: Lack of highly effective and safe therapeutics for hepatitis C virus (HCV) infection provides an opportunity as well as a challenge to discover novel and potent anti-HCV drugs. HCV NS5B RNA-dependent RNA polymerase (RdRp) is responsible for viral genome replication and thus constitutes a valid target for therapeutic intervention. To date, numerous HCV NS5B RdRp inhibitors have been discovered. This review focuses on the recent advances in discovery, mechanism of action studies and biological characterization of several distinct classes of potent inhibitors for NS5B RdRp. The clinical efficacy and developmental status of several promising compounds are also outlined.

Keywords: Hepatitis C virus, HCV, NS5B RNA-dependent RNA polymerase, polymerase inhibitor, structure-activity relationship, nucleoside analogue, non-nucleoside inhibitor, allosteric inhibitor, mechanism of action, drug resistance.

INTRODUCTION

As one of the leading causes of liver disease, HCV is responsible for the majority of sporadic and post-transfusion related non-A and non-B hepatitis. According to a World Health Organization report, there are estimated 170 million HCV infected individuals worldwide, of whom approximately 4 million reside in the United States [1]. Although often asymptomatic, around 20% of these chronic infected patients eventually progress onto the sequelae of fibrosis, cirrhosis and hepatocellular carcinoma during a period of 20 years or more [2]. HCV accounts for about one-third of hepatocellular carcinoma cases and ascribes to the majority of liver transplantation in the developed countries. Overall, nearly 10,000 annual deaths result from HCV infection in the US. Currently there is no vaccine available for prevention of HCV infection. With more infected patients diagnosed for medical attention over the next decade, the death toll attributed to HCV is projected to rise significantly. In the absence of a highly efficacious and safe anti-HCV therapy, current option of treatment is limited. The standard care of treatment is the combination therapy of subcutaneous pegylated interferon-α and oral nucleoside drug ribavirin. The endpoint of treatment is defined by the sustained viral response (SVR) or undetectable viral load for at least six months following cessation of therapy. The overall SVR for this combination therapy is around 54-56% [3]. Whereas the SVR for patients carrying HCV genotype 2 or 3 is as high as 88%, it is only 48% for genotype 1, the major genotype in the US, Japan and part of Europe. More importantly, this treatment suffers from many serious adverse effects, including severe flu-like symptoms derived from interferon-α and hemolytic anemia due to the accumulation of ribavirin in red blood cells [4]. These undesirable side effects can lead to dose reduction and discontinuation of treatment. Hence, attaining highly effective, well-tolerated and safer antiviral agents are urgently needed for the treatment of intractable HCV infection.

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HCV is a small enveloped single-stranded (+)-RNA virus that belongs to the *Hepacivirus* genus in the *Flaviviridae* family [5]. It comprises six major genotypes and more than sixty subtypes [6]. The 9.6-kb viral genome encodes a single polypeptide that is processed to generate core and envelope proteins for assembly of new viral particles and nonstructural proteins for viral replication. Among the nonstructural viral proteins, the bifunctional NS3 serine protease/helicase represents a prime target for therapeutic interference. Major progress has been made in the discovery of potent and specific NS3 protease inhibitors [7]. Notably, an orally bioavailable small molecule inhibitor of the NS3 protease, BILN 2061, has demonstrated good antiviral activity in an early phase of clinical trials [8]. NS5B RdRp is considered another attractive drug target. This membrane-associated viral RNA polymerase is the catalytic core of the viral genome replication machinery. Thus far, numerous nucleosides and non-nucleoside NS5B RdRp inhibitors have been discovered and revealed in the literature and patent applications. A number of compounds are currently undergoing the early phase human clinical trials.

Antiviral chemotherapy targeting viral polymerases has been highly successful for the treatment of human immunodeficiency virus (HIV), herpes simplex virus and hepatitis B virus (HBV) infections. More than 10 chemical entities that function as reverse transcriptase (RT) inhibitors have been approved for the treatment of HIV infection [9]. According to their chemical structures, these compounds can be divided into nucleoside RT inhibitors (NRTI) and non-nucleoside RT inhibitors (NNRTI). The NRTIs serve as substrates for HIV RT and can be incorporated into the elongating viral DNA. Incorporation of these nucleotide chain terminators inhibits further chain elongation and aborts viral DNA synthesis. These NRTIs can be utilized by RTs of various HIV strains as well as related viruses such as HBV, and thus possess a broad-spectrum antiviral activity. In contrast, the approved NNRTIs act as allosteric inhibitors by targeting an inhibitor-induced lipophilic pocket near the HIV-1 RT nucleotide substrate binding site. They are non-competitive to the native deoxynucleoside triphosphate (dNTP) substrates, and are more specific to HIV type 1. A

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similar scenario exists for the HCV NS5B RdRp inhibitors, the counterparts of HIV RT inhibitors. Most NS5B RdRp nucleoside inhibitors function as RNA chain terminators. These are not only active against HCV, but also against related flaviviruses including yellow fever virus, dengue virus, and West Nile virus. Conversely, non-nucleoside inhibitors are more specific to HCV. This review serves to supplement several recent reviews on NS5B RdRp inhibitors [7,10-12] by focusing on the mechanism of action (MOA), biological activity, resistance profile and developmental status of four distinct classes of NS5B RdRp inhibitors.

1. NUCLEOSIDE ANALOGUES

Nucleoside and nucleotide analogues are a fertile ground for antiviral drug discovery. Many nucleoside analogues targeting viral polymerases have been successfully exploited as DNA virus and retrovirus therapeutic agents. The substrate specificity of hepaciviral polymerase differs from the host counterpart, which makes it possible to develop potent and virus-specific nucleoside chain terminators or genomic mutagens. Since HCV is an RNA virus, most of the known nucleoside inhibitors active against HCV are ribonucleosides. Among them, NM283 is a promising drug candidate. NM283, a prodrug of 2'-*C*-methyl cytidine, is currently in phase I/II clinical trials.

1.1. 2'-*C*-Methyl Ribonucleoside

The chemistry of 2'-*C*-methylation was originally introduced in the 1960s to reduce the *in vivo* deamination of adenosine [13]. Recently, 2'-substituted ribonucleosides have been revisited and found to possess potent anti-HCV activity both *in vivo* and *in vitro*. They are a focal point of anti-HCV nucleoside chemistry efforts. Current MOA studies suggest that they function as RNA chain terminators.

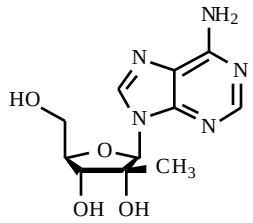
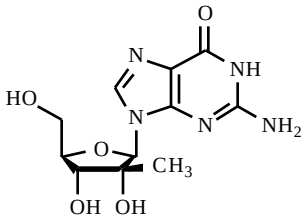
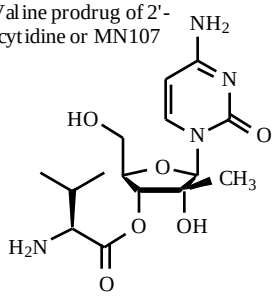
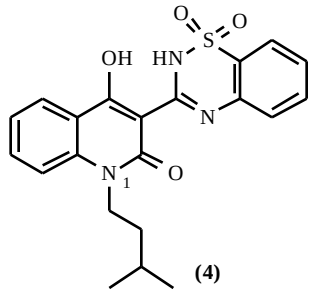
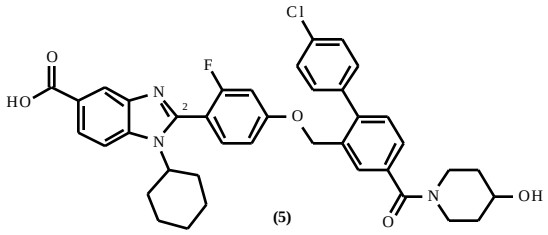
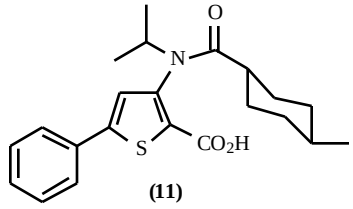
The structure-activity relationship (SAR) studies of nucleoside inhibitors have been performed by synthesizing both nucleoside analogues and cognate triphosphate derivatives. The nucleoside analogues are tested for anti-HCV activity in a cell-based HCV subgenomic replicon assay, whereas their triphosphates are directly evaluated in an NS5B RdRp enzymatic assay. The SAR on purine nucleoside scaffold has been reported in detail [14]. Initial modifications focus on the 2' ribose position. The hydrogen bond donor 2'-hydroxyl group is replaced with a methoxy or a fluoro group, and is inverted to the arabino orientation. Alternatively, a 2'-*C*-methyl group is added. Alteration of the 2'-hydroxyl group generally correlates with reduced activity; however, inclusion of a 2'-*C*-methyl group to either adenosine or guanosine yields compounds with potent activity in both the NS5B RdRp enzymatic assay and HCV replicon assay. Further modifications on the furanose moiety by increasing the size of 2'-methyl to ethyl substituent or changing the stereo or regional chemistry of the methyl group at 2' and/or 3' position lead to a complete loss of activity. Moreover, changes in the hydrogen bonding capacity of the adenine or guanine nucleobase are found detrimental to the activity of 2'-*C*-methyl adenosine or guanosine. In a separate study, an acyclic ATP has been reported to act neither a substrate nor an inhibitor for NS5B RdRp [15]. These results underscore the importance of preserving ribose and canonical nucleobase for activity. The chemical space available for investigation is narrow, highlighting the difficulty in performing lead optimization on a nucleoside scaffold to concurrently meet the requirements of maximizing antiviral activity while retaining the 5'-phosphorylation activity by nucleoside kinases in order to be efficiently converted to the active 5'-triphosphate *in vivo*.

Kinetic analyses with 2'-*C*-methyl adenosine and guanosine triphosphates reveal that these behave like competitive inhibitors with regard to the native nucleoside triphosphate (NTP) substrates in an NS5B RdRp enzymatic assay. The corresponding IC_{50} values (inhibitor concentration exhibiting 50% suppression of enzyme activity) are 1.9 and 0.13 μM, respectively (Table **1**) [16,17]. Nucleotide incorporation experiments demonstrate that these 2'-*C*-methyl purine nucleotides are substrates for the HCV NS5B RdRp and can be incorporated into nascent elongating nucleotide chains. However, following the nucleotide incorporation, further chain elongation is impaired, presumably due to the misorientation of the 3'-hydroxyl group required for subsequent nucleotide addition, and/or possibly out of alignment of the α-phosphorous of the incoming nucleotide substrate. Consistent with the *in vitro* activity, 2'-*C*-methyl adenosine exhibits good potency in a cell-based HCV subgenomic replication assay. The EC_{50} (effective concentration of an inhibitor with 50% inhibition of viral replication) is 0.25 μM at 24-hr in the replicon assay (Table **1**), and it is further reduced to 0.17 μM at 48 hours. By comparison, 2'-*C*-methyl guanosine is less active in the replicon assay (EC_{50} = 1.4 μM at 48-hrs). The inferior cellular activity may be explained by intracellular metabolism. Whereas 2'-*C*-methyl adenosine is efficiently phosphorylated, 5'-phosphorylation of 2'-*C*-methyl guanosine is extremely low with the intracellular NTP level of 0.2 pmole/10^6 cells [16]. Preparation of 5'-monophosphate prodrugs might remedy this problem by bypassing the first step in 5'-triphosphate production.

Both 2'-*C*-methyl purine nucleosides (adenosine and guanosine) display broad spectrum activity against a number of RNA viruses [16]. They are active against HCV and related RNA viruses, including bovine viral diarrhea virus (BVDV), yellow fever and West Nile virus. Interestingly, they exhibit little activity against other RNA or DNA viruses, suggesting that they are selective against flaviviruses. Further studies with the corresponding 5'-triphosphates reveal that they lack activity against human DNA polymerase α, β, or γ (IC_{50} > 50 μM) [17]. Consistent with these results, 2'-*C*-methyl guanosine does not exhibit discernible cytotoxicity in many cultured cell lines tested. However, cytotoxicity became apparent for 2'-*C*-methyl adenosine at high concentrations with longer incubation times [16].

A drug's antiviral action mechanism in cell culture can be investigated by selecting drug resistant clones, mapping resistant mutations in the viral genome and finally, by applying reverse genetics to introduce putative resistant mutation(s) back into the wild-type virus for confirmation. This strategy allows the identification of potential inhibitor binding site(s) within viral proteins. It also provides crucial predicative information on drug resistance. The resistant

Table 1. Biological Properties of Selected NS5B RdRp Inhibitors under Development[a]

Compound Name/Structure	Company	Activity Profile and Status	Ref.
2'-*C*-Methyl adenosine 	Idenix/ Novartis Merck/ISIS	· RNA chain terminator · IC_{50} = 1.9 μM (triphosphate form) · EC_{50} = 0.17 μM (2 days) · NS5B resistant mutation: S282T · CC_{50} >100 μM · Efficient 5'-triphosphorylation · No bioavailability and/or rapid metabolism.	[16,17]
2'-*C*-Methyl guanosine 	Idenix/ Novartis Merck/ISIS	· RNA chain terminator · IC_{50} = 0.13 μM (5'-triphosphate) · EC_{50} = 1.4 μM (2 days) · NS5B resistant mutation: S282T · CC_{50} >100 μM · Poor 5'-triphosphorylation · 82% bioavailability in rat · C_{max} = 1.8 μM; T_{max} = 2.1 hr.	[16]
NM283 (Valine prodrug of 2'-C-methyl cytidine or MN107 	Idenix/ Novartis	· Active in HCV chimpanzee model · Phase I/II clinical trials for 2'-*C*-methyl cytidine · K_i = 160 nM for BVDV polymerase (5'-triphosphate) · IC_{50} = 0.2 μM (5'-triphosphate) · EC_{50} = 0.67 μM against BVDV[b] · EC_{50} = 7.5 μM in HCV replicon · CC_{50} > 100 μM[b] · $T_{1/2}$ = 13.8 h	[18,19]
 **(4)**	Glaxo-SmithKline	· Targets NS5B RdRp active site · IC_{50} = 80 nM · EC_{50} = 0.5 μM · CC_{50} > 50 μM · NS5B resistant mutation: M414T · Synergistic with interferon α and other nucleoside and NNIs.	[31,34,35]
 **(5)**	Japan Tobacco	· Allosteric inhibitor · IC_{50} = 0.28 μM · EC_{50} = 0.35 μM · NS5B resistant mutation: P495L · JTK-003 and JTK-109 from this series are in clinical trials.	[42]
 **(11)**	Shire Biochem	· Allosteric inhibitor · IC_{50} = 1.5 μM · EC_{50} = 0.6 μM · CC_{50} =123 μM	[48,49]

[a] If not specified, an IC_{50} is derived from an HCV NS5B RdRp enzymatic assay. Caution should be taken to compare IC_{50} for various inhibitors reported from different groups. An IC_{50} value could vary depending on the enzyme, RNA template and assay format used in an assay. EC_{50} is from an HCV subgenomic replicon assay. CC_{50} is from a MTS cytotoxicity assay in Huh7 cell.

[b] The assays were performed in MDBK cells.

HCV replicon colonies of 2'-*C*-methyl guanosine or adenosine have been selected [16]. Characterization of these colonies reveals a single mutation that confers resistance to both 2'-*C*-methyl nucleosides. The mutation, S282T, is located within the NS5B RdRp active site (Fig. (**1**)). This study confirms the MOA of 2'-*C*-methyl nucleosides by targeting the NS5B RdRp active site. Computer modeling studies suggest that this mutation could induce a steric hindrance to the binding of 2'-*C*-methyl group and prevent 2'-*C*-methyl nucleosides from being incorporated into the nascent chain. Consistent with this analysis, the S282T mutation reduces the binding affinity of 2'-*C*-methyl adenosine to NS5B by 100-fold [16]. Interestingly, the fitness of this mutant virus is somewhat reduced relative to the wild-type virus in terms of replication efficiency. Although the S282 residue is highly conserved among most HCV genotypes, HCV genotype 4 possesses a threonine at this position [12]. Perhaps certain HCV strains exhibit natural resistance to 2'-*C*-methyl nucleosides.

Pharmacokinetic (PK) properties of 2'-*C*-methyl adenosine and guanosine have been investigated in rats [16]. Oral dosing of 2'-*C*-methyl adenosine yields no detectable parental compound in plasma, suggesting little bioavailability or rapid degradation of the compound *in vivo*. On the other hand, 2'-*C*-methyl guanosine dosed at 2 mg/kg to rats has an excellent oral bioavailability (82%) with maximal plasma concentration (C_{max}) of 1.8 μM. The plasma clearance is slower as the half-life of elimination is around 2 h. The dose-normalized exposure is 3.7 μM·h·kg/mg. The poor rodent PK properties of 2'-*C*-methyl adenosine may preclude it from further clinical development. In spite of its favorable pharmacological profile, 2'-*C*-methyl guanosine must overcome its poor *in vivo* phosphorylation in order to advance to the next phase of development.

1.2. NM283

NM283 from Idenix Pharmaceuticals is a nucleoside analogue chain terminator that is currently in clinical trials. NM283 is a valine prodrug of 2'-*C*-methyl cytidine (NM107) (Table **1**) [18]. 2'-*C*-Methyl cytidine triphosphate has been reported to be a competitive inhibitor for NS5B RdRp of BVDB, a surrogate virus for HCV, with a K_i of 160 nM [19]. Although 2'-*C*-methyl cytidine exhibits good potency in a cell-based BVDV replication assay (EC_{50} = 0.7

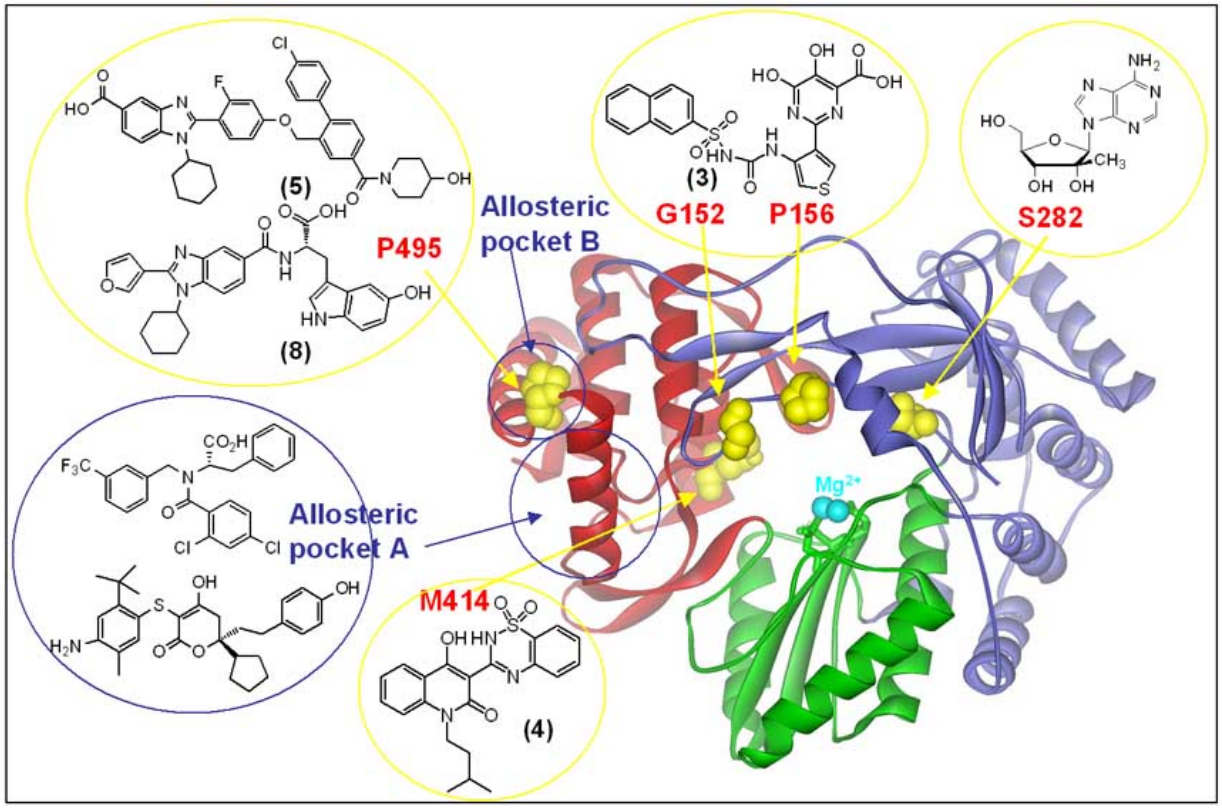


Fig. (1). HCV NS5B RdRp crystal structure highlighted with drug resistant mutation sites and allosteric inhibition pockets. The drug resistant mutation residues are denoted in yellow-colored balls with S282T for 2'-*C*-methyl adenosine (nucleoside chain terminator), G152L and P156E for dihydroxypyrimidine carboxylate (**3**) (pyrophosphate mimic), M414T for benzo-1,2,4-thiadiazine (**4**) (active site binder), and P495L for benzimidazole (**5**) and (**8**) resistance (allosteric inhibitors). Two known allosteric pockets A and B in the thumb subdomain are circled respectively. Please note the allosteric pocket A is located at the back the helix. The chemical structure of two allosteric inhibitors known to bind to the site A is shown. They are 2-[(2,4-dichloro-benzoyl)-(3-trifluoromethyl-benzyl)-amino]-3-phenyl-propionic acid (K_i = 2.2 μM) and 3-(4-amino-2-tert-butyl-5-methyl-phenylsulfanyl)-6-cyclopentyl-4-hydroxy-6-[2-(4-hydroxy-phenyl)-ethyl]-5,6-dihydro-pyran-2-one (IC_{50} = 0.93 μM). For clarity, the front view of NS5BΔ55 protein is shown here with the finger, palm and thumb subdomains in blue, green and red, respectively. Divalent Mg^{2+} ions in the catalytic site are displayed as cyan spheres.

μM), its activity in the HCV replicon assay is more discouraging (EC_{50} = 7.5 μM) [20]. NM107 is specific against HCV and related viruses with no activity against HIV and DNA viruses. PK analysis indicates that it is efficiently converted to 5'-triphosphate *in vivo* and has a long half-life of 13.8 hrs, which supports once daily dosing. Moreover, it is synergistic *in vitro* with interferon α. The valine prodrug of 2'-*C*-methyl cytidine, NM283, is adopted to improve oral bioavailability of NM107 (Table **1**). It is efficacious *in vivo* in HCV-1 chronically infected chimpanzees [19]. Once daily oral dosing of NM283 at 20 mg/kg for 7 days in chimpanzees reduces the HCV viral titer by one log10. Currently, NM283 is in a phase I/II clinical trial to evaluate safety, PK and antiviral activity [18]. The trial includes once daily dosing of 50, 100, 200, 400 or 800 mg for 15 days. NM283 is rapidly converted to 2'-*C*-methyl cytidine in plasma. It reaches C_{max} at 2 hours. Both AUC and C_{max} are proportional to the drug up to 400 mg. The C_{max} ranges from 500 to 3000 ng/ml. The drug is well tolerated with some transient GI toxicity. Antiviral activity becomes more obvious at once daily doses greater than 100 mg. The greatest antiviral effect has been observed in patients who received the highest cumulative dose of NM283 during the 15-day treatment period [21]. In this group, all patients demonstrated a viral load reduction from 79 to 99%. After stopping the therapy, viral load rebounded in all patients. A combination trial of NM283 with pegylated interferon-α is expected to start soon.

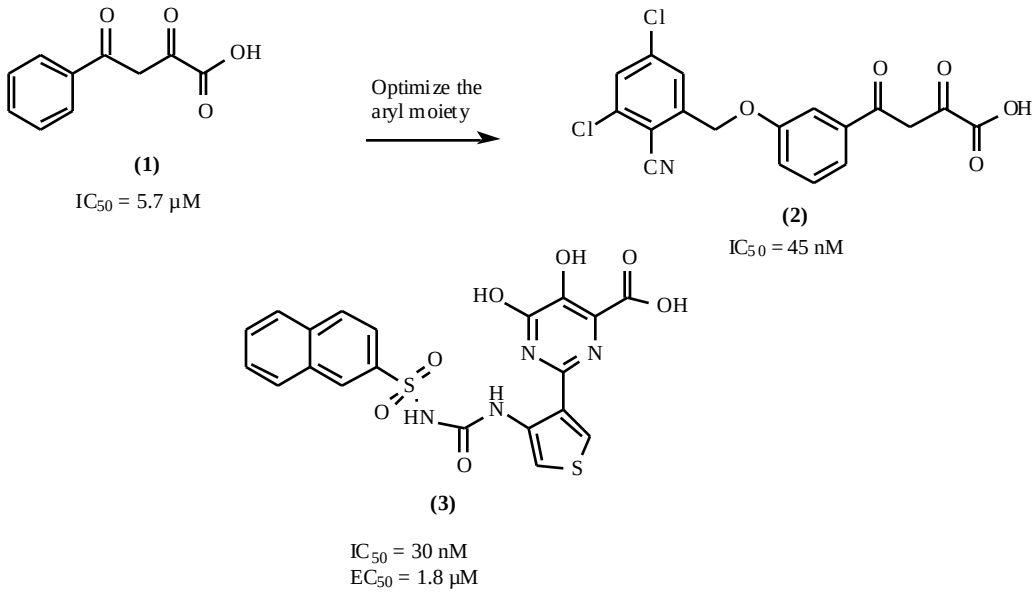
2. NON-NUCLEOSIDE INHIBITORS

Given the success of developing non-nucleoside inhibitors against HIV RT, intensive efforts have been made to identify non-nucleoside inhibitors for HCV NS5B RdRp. The NS5B RdRp enzymatic assay has been widely adapted for high-throughput screening of compound libraries, yielding numerous classes of non-nucleoside inhibitors. The inhibitory mechanism of most of these compounds is uncertain. However, available data indicate these inhibitors can be classified into three categories. The first class mimics the pyrophosphate motif of an NTP substrate and is believed to interact directly with the two catalytic Mg^{2+} ions in the NS5B RdRp active site. The second category binds directly to the NS5B RdRp active site and prohibits the enzyme's catalysis. The third recognizes allosteric surface pockets on the thumb subdomain of NS5B and inhibits NS5B RdRp by locking the enzyme in an inactive open conformation. The most potent inhibitors from each class are singled out and reviewed below. Notably, two allosteric inhibitors from Japan Tobacco are currently in early phase clinical trials.

2.1. Pyrophosphate Mimic

Screening against the HCV NS5B RdRp at the Instituto di Ricerche di Biologia Molecolare (IRBM) has identified a α, γ-diketo acid inhibitor (**1**), with a 5.7 μM IC_{50} (Scheme (**1**)) [22,23]. Further SAR studies show that the diketo acid moiety is essential for activity. Compound (**1**) likely mimics a pyrophosphate by directly chelating the two catalytic Mg^{2+} ions in the NS5B RdRp active site, blocking the active site from substrate binding and catalysis [7,24]. An inhibitor-NS5B binding model has been established to guide the lead optimization [24]. The aryl group from the γ-position of (**1**) is substituted and derived to build up the inhibitor's specificity and potency. The best compound from this series, (**2**), exhibits an IC_{50} of 45 nM in an enzymatic assay (Scheme (**1**)). It is highly selectively *in vitro* with no significant activity against human DNA polymerase α, β, γ, or HIV RT. Similar pyrophosphate mimics have been demonstrated to possess good antiviral activities. They include a broad-spectrum antiviral inhibitor phosphonoacetic acid, an approved antiherpetic drug foscarnet (phosphonoformic acid) [25], and several diketo acid inhibitors of HIV integrase [26].

IRBM has since disclosed another two series of pyrophosphate mimics. One series is based on meconic acid or 3-hydroxy-4-oxo-4*H*-pyran-2,6-dicarboxylic acid scaffold [27]. A representative from this series, a monoethyl ester of meconic acid, has an IC_{50} of 2.3 μM in an NS5B RdRp



(1)
IC_{50} = 5.7 μM

(2)
IC_{50} = 45 nM

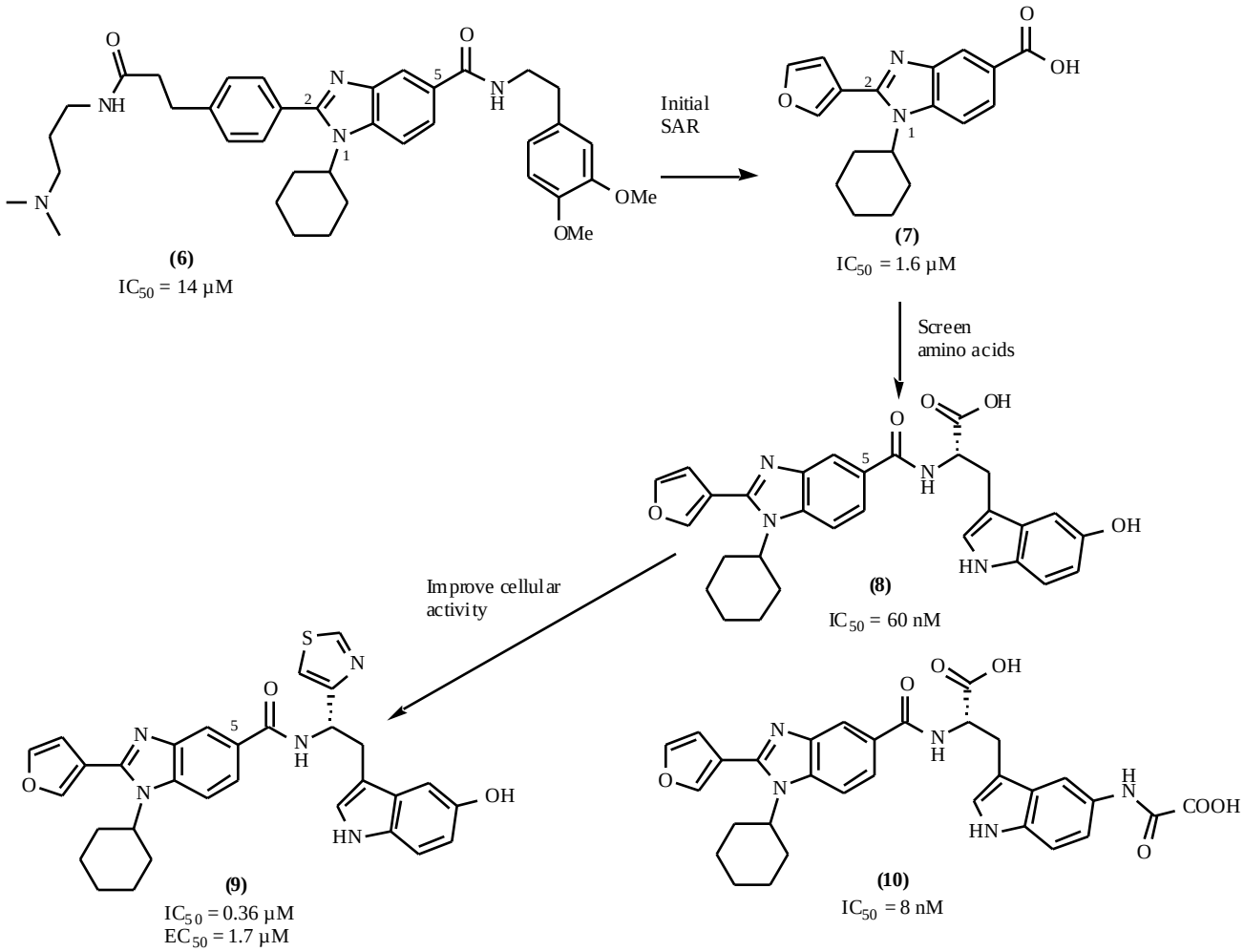
(3)
IC_{50} = 30 nM
EC_{50} = 1.8 μM

Scheme 1.

enzymatic assay. Due to the high ionic nature, none of the compounds in this series displays any significant activity in an HCV replicon assay. In the other pyrophosphate mimic series, the reactive diketo acid moiety is replaced by dihydroxypyrimidine carboxylate [7,28]. A representative compound (**3**) from this series exhibits an IC_{50} of 30 nM in an enzymatic assay and an EC_{50} of 1.8 μM in a replicon assay (Scheme (**1**)) [29]. Biochemical analysis has confirmed its MOA. It binds to the metal cofactors in the NS5B RdRp active site. Compound (**3**) is competitive with foscarnet and functions as an elongation inhibitor during the RNA synthesis. The cell-based HCV replicon system has been treated with (**3**), and drug resistant colonies selected, which are 10-fold less susceptible to (**3**). Resistant mutations have been mapped to P156L and G152E within the NS5B RdRp active site (Fig. (**1**)), supporting that this class of compounds directly targets the NS5B RdRp active site [29]. Reverse genetics have confirmed that either of these mutations can effectively confer resistance to (**3**). G152 is a residue that is conserved across all six major HCV genotypes. In contrast, P156, which is part of the conserved motif F in the NS5B RdRp, is an alanine in HCV genotype 2a [12]. Whether or not this HCV strain will be susceptible to (**3**) is of interest. To date, no *in vivo* activity for these pyrophosphate mimics is available. Their highly ionic nature and intrinsic affinity for a two-metal ion center could result in issues such as lower bioavailability and general toxicity upon clinical development.

2.2. Active Site Binders

Benzo-1,2,4-thiadiazine from GlaxoSmithKline is one of the few classes of non-nucleoside NS5B RdRp inhibitors that have been established as a potential binder of the highly conserved NS5B RdRp active site [30]. A representative compound (**4**) from this series has achieved good potency in both an NS5B RdRp enzymatic assay (IC_{50} = 80 nM) and an HCV replicon assay (EC_{50} = 0.5 μM) [31] (Table **1**). Compound (**4**) is non-competitive with respect to the NTP substrates. It inhibits the *de novo* initiation of RNA synthesis prior to elongation [32]. Biophysical binding experiments suggest that its binding to NS5B is mediated by an RNA template, forming a ternary complex of NS5B/template/inhibitor. Benzothiadiazine (**4**) is highly selective for HCV NS5B RdRp. It is not active against the related BVDV or GBV-B NS5B RdRp [33]. Moreover, a close analogue of (**4**) (with an isobutyl at position 1) has been tested with a Boehringer Ingelheim's benzimidazole allosteric inhibitor and is synergistic against the NS5B RdRp activity [34]. This study suggests that these two classes of non-nucleoside inhibitor target the differentregions of NS5B RdRp. A combination of multiple non-nucleoside



Scheme 2.

inhibitors could be an option for anti-HCV intervention. Compound (**4**) is highly synergistic with interferon-α in an HCV replicon assay. In the assay, viral load is reduced and replicon RNA synthesis is disrupted [33]. Unlike interferon-α that shows a 4-hr delay prior to inhibition of viral RNA synthesis, addition of compound (**4**) displays an immediate reduction of HCV replicon RNA synthesis.

Drug resistant studies with (**4**) and its close analogue with an isobutyl at position 1 have yielded HCV replicon clones with significantly reduced susceptibility [34,35]. The mutation that confers resistance has been mapped to residue M414T inside the NS5B RdRp active site (Fig. (**1**)). This mutation is located near the conserved motif E of NS5B RdRp, reinforcing the biophysical studies that compound (**4**) directly interacts with the NS5B RdRp active site. Computational analysis suggests that the M414T mutation may exert resistance by reducing the binding affinity of (**4**) to NS5B. Sequence analysis reveals that residue 414 is a methionine in 86% of HCV isolates [34]; however, it is a glutamine in HCV genotype 2 and a valine in genotype 4 [12]. Although the effect of the sequence variation on the inhibitory activity of (**4**) has not been determined, it does imply that this residue is highly variable and HCV could readily develop resistance to it. Although current evidences support that compound (**4**) directly targets the NS5B RdRp active site, the exact binding mode is elusive. Inhibition studies with its isobutyl analogue indicate that the inhibitory potency is at least one magnitude weaker against NS5B RdRp with C-terminal Δ55 truncation or with a β-loop deletion than against the enzyme with Δ21 truncation [34]. Thus the question remains whether benzothiadiazine inhibitors are pure active site binder or not. Potentially the C-terminal domain and the β loop of NS5B could also involve in the inhibitor's binding. Nevertheless, benzo-1,2,4-thiadiazine is the only non-nucleoside class except the pyrophosphate mimic that has been implicated to target the functionally critical NS5B RdRp active site. It deserves to be further explored at the atomic level to further define its binding mode for designing more potent and specific NS5B RdRp inhibitors.

A recent patent application from Abbott Laboratories has disclosed chemical synthesis of more than 100 compounds as HCV NS5B RdRp inhibitors [36]. These are based on scaffolds similar to benzo-1,2,4-thiadiazine. However, no biological activity of these compounds is currently available.

2.3. Allosteric Inhibitors

Allosteric inhibition is an effective approach to suppress viral polymerase activity as evidenced by the multitude of HIV NNRTIs. Although different from the bi-subunit structure of HIV RT, allosteric binding pockets do exist in the HCV NS5B RdRp. A surface cleft in the thumb subdomain of NS5B has been discovered by x-ray crystallography as an allosteric binding pocket for non-nucleoside inhibitors [37,38]. This binding pocket (Fig. (**1**), allosteric pocket A) is one of the two allosteric binding pockets identified in the thumb subdomain. It is located about 30 Å from the enzyme's catalytic site and is highly conserved across the HCV genotypes described to date. Previously, a GTP molecule was reported to bind to a smaller and shallow hydrophobic surface pocket in the vicinity of the allosteric pocket A (Fig. (**1**), allosteric pocket B) [39]. Two series of inhibitors based on the N, N-disubstituted phenylalanine [37] and 5,6-dihydro-pyran-2-one [38] scaffolds have been co-crystallized with NS5B RdRp and both bind to the allosteric site A (Fig. (**1**)). Detailed structural analyses of the inhibitor-NS5B interactions indicate that the binding is mainly mediated by a large hydrophobic region and two specific hydrogen bonds from a pair of adjacent backbone amides of Ser476 and Tyr477. Both co-crystallized inhibitors are bound in a wedge-like fashion with a cyclic group occupying a hydrophobic cavity located near the C-terminal region of the thumb subdomain. These allosteric inhibitors are noncompetitive with respect to the NTP substrates. They are believed to act by locking NS5B RdRp in an inactive open form, thereby disrupting catalysis [37]. Structural analysis of NS5B indicates that other allosteric pockets could exist in the rugged NS5B protein surface and could be potentially targeted by non-nucleoside inhibitors. Through mapping drug resistant mutation and co-crystallization with NS5B, many non-nucleoside inhibitors have been found to bind to the region of the allosteric pocket A and B. Of the current allosteric inhibitors, three series stand out.

Japan Tobacco has filed patents on a series of benzimidazole carboxylic acid derivatives as NS5B RdRp inhibitors [40]. The covered compounds include substitutions extended from the *para* position of 2-phenyl group (Table **1**). Many compounds have been claimed to have IC_{50} in the nanomolar range, although some turn out to be less potent [41]. One representative compound (**5**) has been extensively characterized [42]. It exhibits an IC_{50} of 0.28 μM in an NS5B RdRp assay and an EC_{50} of 0.35 μM in a HCV replicon assay (Table **1**). The replicon system has been employed for drug resistance studies with (**5**), and revealed a single mutation P415L in NS5B that confers to resistance. The P495 residue is located on the surface of the NS5B thumb subdomain and is one of the key residues in the allosteric pocket B (Fig. (**1**)). Apparently, the allosteric pocket B is too small to accommodate compound (**5**). Further structural analysis is needed to elucidate the inhibitor's binding mode. Nevertheless, this result suggests that the activity of the benzimidazole series may derive from an allosteric mechanism. Consistent with this assessment, compound (**5**) behaves non-competitive in kinetic analysis and it blocks RNA synthesis prior to the elongation stage. Currently, two lead compounds from the series, JTK-003 and JTK-109, are in early phase clinical trials [43].

Boehringer Ingelheim has published another series of benzimidazole inhibitors based on a benzimidazole 5-carboxylic amide pharmacophore [44,45]. From high throughput screening, a benzimidazole 5-carboxylic amide hit, (**6**), has been discovered with an IC_{50} of 14 μM (Scheme (**2**)). Contrary to the Japan Tobacco's benzimidazole inhibitors, NMR binding studies with (**6**) indicate that the left portion of the compound is not involved in binding to NS5B. The NMR guided SAR studies further reveal 1,2-disubstituted benzimidazole 5-carboxylic acid, (**7**), as the core structure essential for activity with an IC_{50} of 1.6 μM. High-throughput parallel synthesis has been adapted to optimize the two aryl substituents on the left and 5-carboxyl amide moiety on the right of (**7**), which leads to the identification of compound (**8**) with an IC_{50} of 60 nM

(Scheme (**2**)) [44,46]. Modification of (**8**) on the indole ring exploits more interactions with the enzyme, resulting in compound (**10**) with an IC_{50} of 8 nM. Compound (**10**) is one of the most potent inhibitors reported for NS5B RdRp. Despite the potent enzymatic assay activity, this series of compounds displays marginal cellular activity in an HCV replicon assay. The inferior replicon activity could be the result of the highly ionic carboxylate group that prevents them from penetrating cellular membranes. To resolve this cellular permeability problem, prodrug approaches have attempted to mask the carboxylic acid with a simple ester or by displacing it with a bioisostere tetrazole or thioazole group. One such modified compound (**9**) has an IC_{50} of 0.36 μM in an enzymatic assay and an improved EC_{50} of 1.7 μM in a replicon assay (Scheme (**2**)).

Kinetic analysis and binding experiments suggest that compound (**8**) mainly acts as an allosteric inhibitor by blocking the NS5B RdRp catalyzed RNA synthesis prior to the elongation step [42]. Although efforts to elicit drug resistant mutant was unsuccessful due to the lack of cellular activity of (**8**) in replicon, compound (**8**) has a reduced activity (64-fold) to the NS5B RdRp P495L mutant, the same mutant that causes resistance to Japan Tobacco's benzimidazole (**5**). This result suggests that both benzimidazole compounds share similar MOA by binding to the same region of the thumb subdomain. Residue P495 is conserved in most HCV strains, highlighting the importance of this residue for HCV replication. This also helps explain the reduced replication efficiency of the P495L substituted HCV replicon [42]. Importantly, residue 495 is a leucine in a HCV genotype 6 strain, suggesting that certain HCV strains could possess natural resistance to both benzimidazole inhibitors. Further biological characterization and PK analysis are needed to fully evaluate the potential of these allosteric inhibitors.

Shire Biochem has previously demonstrated that N, N-disubstituted phenylalanine derivatives inhibit NS5B RdRp by binding to the allosteric pocket A [37,41,47]. Recently, another series of allosteric inhibitors based on the thiophene-2-carboxylic acid scaffold has been reported with good *in vitro* activity [48,49]. Like the phenylalanine series, the thiophene carboxylate also binds to the same NS5B allosteric pocket. One representative compound (**11**) has an IC_{50} of 1.5 μM in an enzymatic assay and an EC_{50} of 0.6 μM in a replicon assay (Table **1**) [48]. Compound (**11**) has good selectivity in Huh7 cells with CC_{50} > 123 μM. In addition, it does not inhibit human DNA polymerase α, β or γ (IC_{50} > 100 μM) [48].

Although some of the NS5B RdRp allosteric inhibitors exhibit a very good potency in the NS5B RdRp enzymatic assay, in many cases, the activity fails to translate into the replicon activity in the cell-based HCV replicon assay [45,48]. Distinct factors may contribute to the suboptimal cellular efficacy. A recent study with the Boehringer's benzimidazole inhibitors reveals that an IC_{50} determined from an NS5B RdRp enzymatic assay is highly dependent on the type of NS5B construct used [50]. The enzyme construct with the higher K_m value for the temple/primer tends to give a lower IC_{50} for an allosteric inhibitor. This result indicates that the allosteric binding pocket is flexible and it changes in different NS5B molecules. In addition, NS5B RdRp constitutes the catalytic core of the HCV replication complex. It interacts with many other components in the complex including NS3, NS4B and NS5A [51,52]. Comparing to the monomeric NS5B RdRp in an enzymatic assay, we do not know how an NS5B's allosteric pocket is affected in the intact replication complex and whether the pocket is still accessible by an inhibitor *in vivo.* The poor correlation of IC_{50} from an enzymatic assay and EC_{50} from a replicon assay may reflect a possibility that accessibility and binding to an allosteric pocket is not the same between the two assays. Moreover, the ionic nature of many allosteric inhibitors may prohibit them from permeating cells efficiently, thereby affecting the cellular inhibitory activity. Thus, it is important to evaluate allosteric inhibitors in the assays that can truly reflect their biological activity *in vivo*. In this regard, HCV replicase assay can serve as another means in addition to the enzyme and replicon assays for evaluation of *in vitro* potency of allosteric inhibitors [51,52]. In this assay, the intact HCV replication complex is isolated from HCV subgenomic replicon cells, and utilized as the NS5B RdRp source for *in vitro* enzymatic assay. Likely, many features of the native *in vivo* HCV replication environment retain in the assay. The very nature of an allosteric inhibitor targeting a surface pocket of NS5B may also present a potential issue of inhibition specificity, as some host proteins could contort similar shaped surface pockets. This may explain some of general toxicity that some inhibitors encounter in cytotoxicity assays. Compared to the enzyme's active site, an allosteric binding pocket may have the disadvantage of being mutated without any functional consequence. It would be interesting to find out whether drug resistance is easier to develop against allosteric inhibitors than the active site binders.

3. OTHER PROMISING NON-NUCLEOSIDE INHIBITORS

Rigel Pharmaceuticals has initiated a multi-dose phase I/II clinical trial for a small molecule non-nucleoside NS5B RdRp inhibitor, R803, for the treatment of chronic HCV infection [53]. The chemical structure of R803 has not been released. The compound is orally bioavailable and is potent to suppress viral replication with an EC_{50} in the low nanomolar range in both a replicon assay and a live virus assay [53]. To date, no significant adverse effects have been reported for R803 at the clinical doses tested. A United Kingdom phase I escalating dose trial has found no difference between the treatment and placebo groups across a range of safety tests [53].

Viropharma has disclosed the results of a phase 1 clinical trial of a small molecular NS5B RdRp inhibitor, HCV-371, for the treatment of HCV infection. HCV-371, co-developed with Wyeth, is safe and well-tolerated at the doses evaluated. However, it has no impact on HCV viral load, which resulted in its termination in the clinical trial. Another oral NS5B RdRp small molecular inhibitor from Viropharma, HCV-086, is currently in clinical trials to assess safety, tolerability, and pharmacokinetic properties [54].

FUTURE DIRECTIONS

HCV NS5B RdRp constitutes a valid target for anti-HCV drug discovery. The rapid pace of drug discovery on

the NS5B RdRp inhibitors has revealed four distinct classes of small molecules including nucleoside analogues, pyrophosphate mimics, active site binders and allosteric inhibitors. Some of them possess both potent *in vitro* and *in vivo* activities, and have since progressed to the early phases of clinical trials for drug safety and efficacy evaluation. Because most of these inhibitors have been developed using enzyme assays and replicon system based on HCV genotype 1 backbone, their inhibitory profile against other five major HCV genotypes is lacking. It is important to determine the activity profile of these inhibitors against all HCV genome types. As demonstrated in the cell-based HCV replicon assay, drug resistance develops readily even for the most potent NS5B RdRp inhibitors. Unfortunately, many of these resistant mutations exist naturally in certain HCV isolates not used during drug discovery. Therefore, it is critical to assess the genetic fitness of these HCV mutations and help predict clinical resistance profiles. Monotherapy using direct anti-HCV agents would likely result in serious drug resistant issues in the clinical setting and limit their utility. Like HIV chemotherapy, combination therapy will continue as the trend for hepatitis C treatment. It is foreseeable that direct anti-HCV agents, like NS3 protease and NS5B RdRp inhibitors, coupled with the current interferon-α and ribavirin therapy will provide the backbone for future treatment of the chronic hepatitis C infection.

ABBREVIATIONS

BVDV = Bovine viral diarrhea virus

dNTP = Deoxynucleoside triphosphate

EC_{50} = Effective concentration of an inhibitor with 50% inhibition of viral replication

GBV-B = GB virus B

GTP = Guanosine triphosphate

HBV = Hepatitis B virus

HCV = Hepatitis C virus

HIV = Human immunodeficiency virus

IC_{50} = Inhibitor concentration exhibiting 50% suppression of enzyme activity

MOA = Mechanism of action

NRTI = Nucleoside reverse transcriptase inhibitor

NNRTI = Non-nucleoside reverse transcriptase inhibitor

NS5B = Non-structural protein 5B

NTP = Nucleoside triphosphate

PK = Pharmacokinetics

RdRp = RNA-dependent RNA polymerase

RT = Reverse transcriptase

SAR = Structure-activity relationship

SVR = Sustained viral response

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