Bioorganic & Medicinal Chemistry 20 (2012) 3150–3161

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Review

Hepatitis C RNA-dependent RNA polymerase inhibitors: A review of structure–activity and resistance relationships; different scaffolds and mutations

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ARTICLE INFO

Article history:
Received 29 December 2011
Revised 28 February 2012
Accepted 21 March 2012
Available online 29 March 2012

Keywords:
HCV
NS5B
RdRp
Structure–activity-relationships
Viral resistance

ABSTRACT

Hepatitis C virus (HCV), like many other flaviviruses, is widely distributed worldwide with estimated chronically infected victims between 170 and 200 million. HCV inherent error-prone RNA-dependent RNA polymerase (RdRp) is an attractive target for medicinal chemists because of the conservative nature of NS5B nucleotide-binding site. In addition, the availability of several crystal structures for HCV RdRp paved the road for conducting rational-based drug design. At the same time, RdRp is responsible for high mutation rate and rapid development of resistance to the clinically-used therapeutics. To improve the viral response, combination therapy is regularly used. The success of co-therapy disciplines depends on targeting two different active sites. This review provides an overview about different scaffolds that target HCV RdPp with insights about their binding modes and possible induced mutant strains.

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1. Introduction

Hepatitis C virus (HCV) was first identified as non-A non-B around 1989.[1] There are estimated 170–200 million people worldwide with chronic HCV infection, of which approximately 5.2 million are in the United States.[2] The highest reported HCV prevalence rate is in Egypt, approximately 25% of population are positive to HCV.[3] Chronic HCV can arise to serious liver diseases such as liver cirrhosis and hepatocellular carcinoma (HCC).[4] Chronic HCV infection is responsible for 40% of end-stage cirrhosis, 60% of liver cancer and 30–40% of liver transplants.[5] HCV is transmitted mainly by blood direct percutaneous exposure,[6] and is the most common chronic blood-borne infection in the United States.[7] A protective vaccine against HCV does not exist yet, and the current therapeutic options are very limited.

Historically, hepatitis C therapy started with interferon alpha (INFα) as a broad antiviral agent.[8,9] Then the therapy was developed to pegylated interferon (peg-IFN) plus ribavirin (RBV) combination.[10,11] However, this combination produces viral response rates of approximately 40–50% in genotype 1-infected individuals, it does not show the same viral response rate with the other genotypes.[12,13] In addition, peg-IFN/RBV treatment is associated with serious adverse effects such as depression, anemia, fatigue, and 'flu-like' symptoms.[14–17] Some rare undesirable side effects such as alopecia universalis was also reported.[18,19] Current research focuses on three disciplines:

1 Enhance the efficacy of the IFNα formulas to improve the patient tolerance.
2 Finding alternative ribavirin-like molecules to reduce its toxicity.
3 Direct-acting antiviral agents (DAAs) that target specific key steps of the viral life cycle (Chart 1). Recently, two NS3 protease inhibitors *boceprevir*[20] and *telaprevir*[21] have been approved by FDA for treatment of naïve patients with chronic HCV.

1.1. HCV RNA-dependent RNA polymerase (RdRp)

HCV RNA-dependent RNA polymerase (RdRp) is part from NS5B protein,[22] and it is responsible for viral genome replication.[23] This viral biochemical activity is not present in mammalian host cells, hence NS5B is an excellent target for selective HCV inhibitors.[24] Structurally, NS5B, like others polymerases, structured like a right hand (Fig. 1), but it adopts a unique shape, in where the *fingers* and *thumb* subdomains interact with RNA and encircle the enzyme active site, which is called the *palm*.[25] Beside the active site, there are several other pockets that act as allosteric binding sites.

Abbreviations: ADA, adenosine deaminase; DAAs, direct-acting antivirals; F, bioavailability; HCC, hepatocellular carcinoma; HCV, Hepatitis C virus; INF, interferon alpha; NIs, nucleosides inhibitors; NNIs, non-nucleosides inhibitors; PEG-IFN, pegylated interferon; PNP, purine nucleoside phosphorylase; RBV, ribavirin; RdRp, RNA-dependent RNA polymerase.

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0968-0896/$ - see front matter Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.bmc.2012.03.049

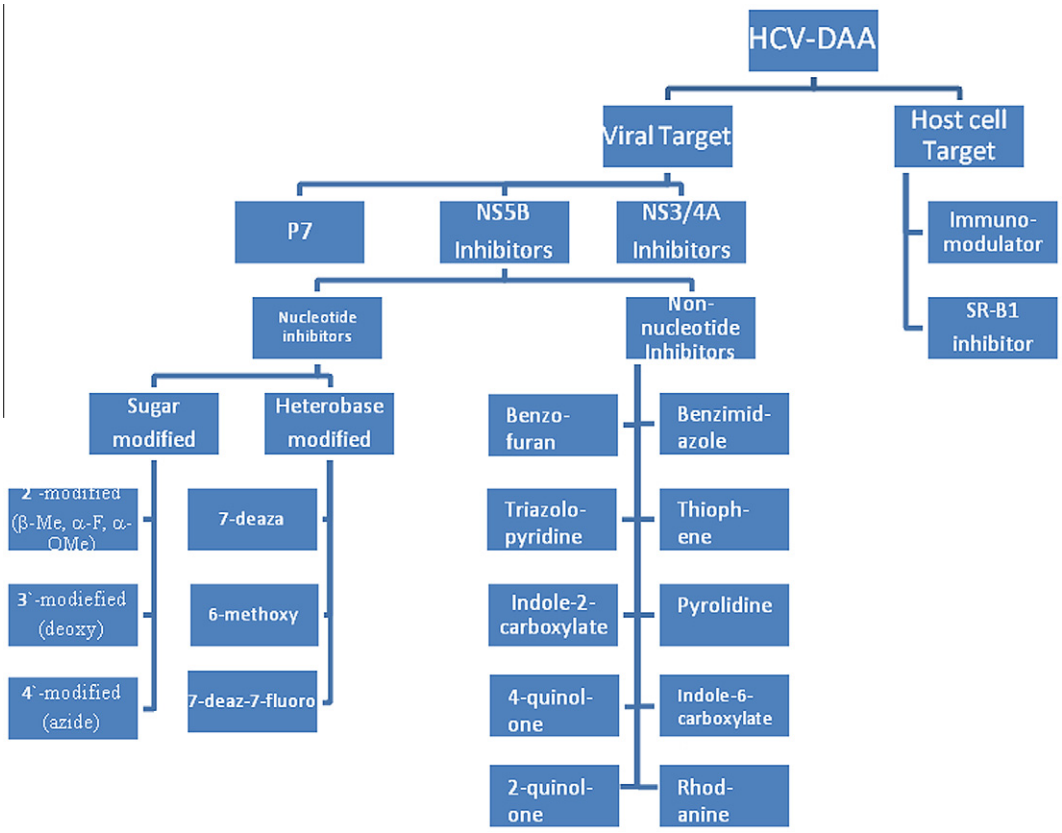


Chart 1. Diagrammatic chart for HCV–DAA categories.

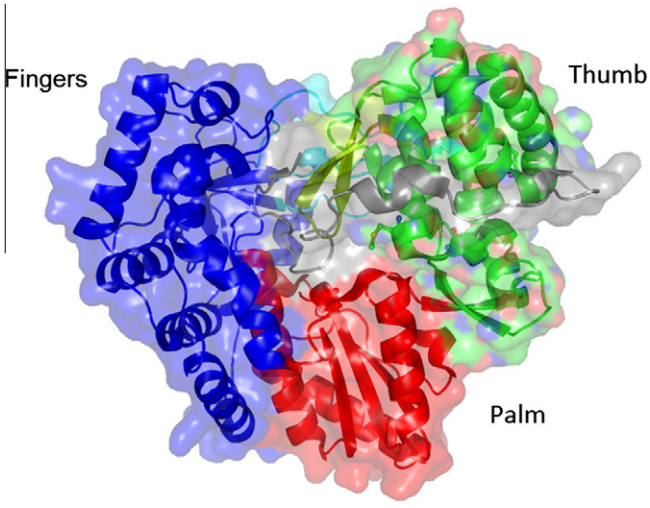


Figure 1. Right hand shape of NS5B, figure is generated using PyMol.

The availability of several crystal structures together with the conservative nature of NS5B nucleotide-binding[26] provides attractive features to medicinal chemists to conduct rational-based drug-design. As a result, large numbers of NS5B inhibitors have been recently reported and many of them are currently in clinical trials. However, treatment of chronic HCV infections is still challenging because of the inherent inaccuracy nature of the HCV RdRp; which leads to high error rates in the replication process of the viral genome. Consequently, resistant mutants arise rapidly.[27] To overcome the therapy-induced resistance and improve the viral response, a combination therapy is usually used. For instance, to overcome the resistance develops from using a thumb-pocket I inhibitor, another inhibitor with different binding mode has to be co-administrated.

This review focuses on classification of chemical scaffolds of HCV NS5B inhibitors that have published protein-complex crystal structures in the last 6 years. Analyzing the different binding modes will provide insights about the expected resistance types. Consequently, this will pave the road for choosing the proper therapeutic combinations to overcome the high mutation rate phenomenon.

2. Classification of NS5B inhibitors

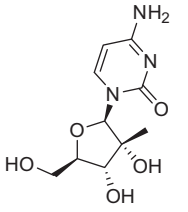
NS5B inhibitors can be classified according to their chemical nature into two major groups; nucleosides inhibitors (NIs) and non-nucleosides inhibitors (NNIs) as shown in Chart 1.

2.1. NS5B nucleosides inhibitors (NIs)

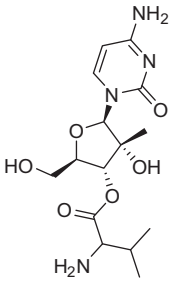
NIs are highly successful agents for the treatment of viral infections including HIV and herpes viruses. They compete with the natural substrates on the polymerase active site. Once they get incorporated into the viral genome, they stop the entire process. Hence, this group is also named '*Chain terminator inhibitors*'.[28] On the other hand, they also can interfere with the cellular proliferative machine causing undesirable side effects. Therefore, with such agents, the therapeutic index is critical. NIs can also be sub-classified into two main categories: sugar modified and heterobase modified subgroups (Chart 1). Antiviral modified nucleosides, in general, are prodrugs. They are activated into their corresponding triphosphates derivatives by the host cell kinases.[28]

2.1.1. Sugar modified NIs

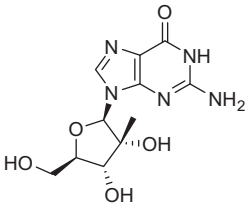
2.1.1.1. 2′-β-Methylated analogues. 2′-β-Methylated analogues of adenine and cytosine were tested for their NS5B inhibitory activity and they had IC_{50} values of 1.9 and 3.8 μM, respectively.[29,30] The 2′-*C*-methylcytidine (**1**) was reported as a potent HCV polymerase inhibitor (EC_{50} = 0.26 μM) with no activity against human immunodeficiency virus (HIV) or against DNA viruses.[31,32] The efficacy of 2′-*C*-methylcytidine (**1**) was hampered by its low oral bioavailability. Therefore, 3′-*O*-L-valinyl ester analogue (valopicitabine, **2**) was developed with a notable improvement in the pharmacokinetic profile (its oral bioavailability is close to 100%).[31,33–37] As a result of valopicitabine (**2**) promising in vitro results and its good pharmacokinetic profile,[38] several clinical studies have been launched to test its synergistic effect with INF.[39–45] Collectively, these studies addressed several clinical benefits for valopicitabine/peg-INF co-therapy, and peg-INF does not affect the pharmacokinetic properties of valopicitabine (**2**).[46] On the other hand, ribavirin antagonizes the anti HCV effect of valopicitabine (**2**).[47] This antagonistic effect might arise from restraining valopicitabine (**2**) phosphorylation process.[48]



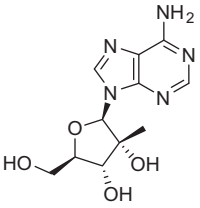
2'-*C*-Methylcytidine NM-107 (1)



valopicitabine NM283 (2)



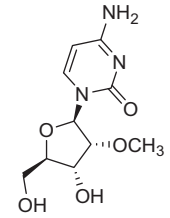
2'-*C*-Methylguanosine (3)



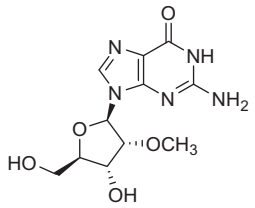
2'-*C*-Methyladenosine (4)

2′-*C*-Methylguanosine (**3**) is another nucleotide analogue that belongs to this class and it has moderate HCV RNA polymerase inhibitory activity (EC_{50} = 3.5 μM) in comparison with 2′-*C*-methylcytidine (**1**).[49] 2′-*C*-Methylguanosine (**3**) had the advantage of enhanced oral bioavailability over the other members in this class (*F* = 85%, and its 0% in case of the adenosine analogue **4**).[49] It is noteworthy to mention that the 2′-position with β-orientation is essential for the anti HCV activity. Moving the methyl group to 3′- or to 2′-α-position dramatically decrease the antiviral activity.[29] In addition, the 2′-β-C-methylated nucleotides cannot be utilized by human polymerase, which confers a higher degree of selectivity towards the viral protein.[30]

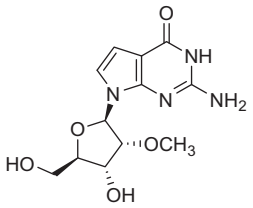
2.1.1.2. 2′-*O*-Methyl nucleotides. 2′-*O*-Methyl nucleotides represent the second class of HCV NIs. The triphosphate form of 2′-*O*-methylcytodine (**5**), guanosine **6** and 2′-*O*-methyl-7-deazaguanosine (**7**) derivatives had RdRp IC_{50} values of 1.2, 3.8 and 0.35 μM, respectively.[50] The nucleosides themselves have weak anti HCV in cell-based assay because of their poor cellular penetration or lower capacity of host cell phosphorylation, as indicated by their EC_{50} values in replicon assay.[50] To improve the efficacy in cell-based assay, bis(*t*Bu-*S*-acyl-2-thioethyl) nucleoside 5′-monophosphate esters were developed (e.g., compound **8**), the resultant trimesters demonstrated 40 to 155-fold more antiviral efficiency compared with the parent nucleosides.[50]



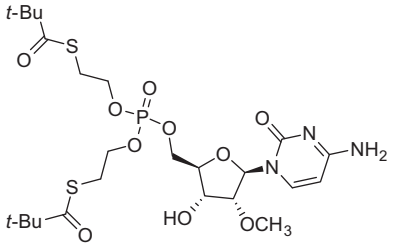
2'-*O*-Methylcytidine (5)



2'-*O*-Methylguanosine (6)



2'-*O*-Methyl-7-deazaguanosine (7)



Bis(*S*-pivaloyl-2-thioethyl)-2'-O-methylcytidine 5'-monophosphate (8)

The efficacy of 2′-modified nucleotide derivatives is highly terminated in the presence of the nearby active-site mutation S282T.[51] The antiviral termination is due to the reduced affinity of the mutant polymerase for the drug and the increased ability to extend the incorporated nucleoside analogue.[51] It is noteworthy to mention that the S282T mutation has slightly effect on utilization of GTP and the RNA substrate by the NS5B.[52] The active triphosphate form of 2′-*C*-methylcydine (**1**) demonstrated 36-fold lower affinity to S282T mutant NS5B genotype than the wild type, probably due to the steric hindrance by the methyl group of T287 during the elongation step (Fig. 2).[53] Surprisingly, this steric clash has not been observed in case of the corresponding 2′-*O*-methylcytosine analogue **5** due to relaxation of the T287 residue (Fig. 2), and no difference in its activity has been reported with S382T mutant one in comparison with the wild type NS5B.[53] Although, the activity of valopicitabine (**2**) against NS5B S282T mutant genotype has been reported, it had an excellent activity against some RNA protease mutant genotypes.[54]

2.1.1.3. 2'-Fluoro nucleotides. Another potent anti HCV deoxy nucleotide inhibitor is 2'-deoxy-2'-fluoro-2'-cytidine (**9**) that was evaluated as an effective inhibitor of the NS5B polymerase but, unfortunately, with high affinity towards some host cell polymerases.[55] This led to inhibition of some cellular growth functions.[55] To increase the selectivity towards viral polymerase,[31] β-*C*-methyl group has been introduced to the pervious compound, and

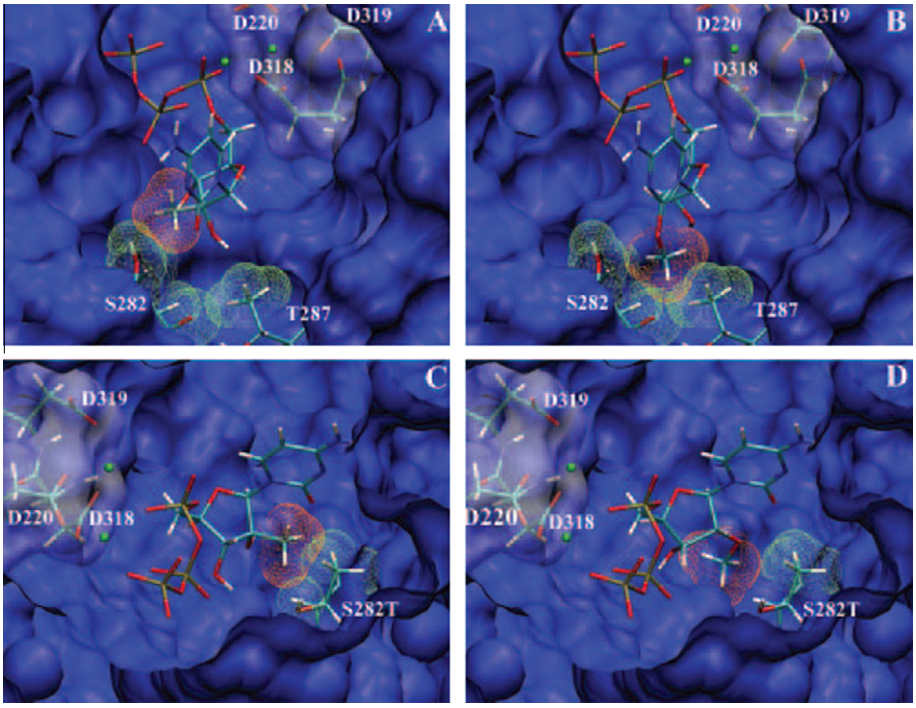
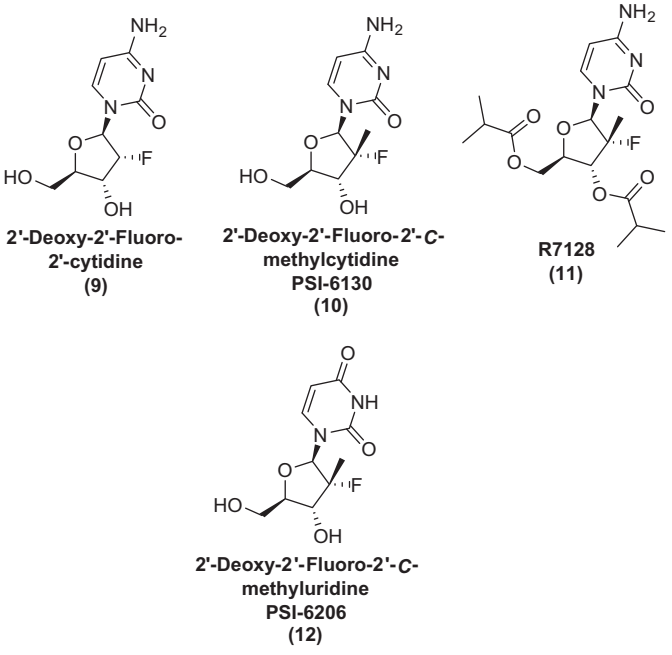


Figure 2. Modeling of 2′-*C*-methylcytosine triphosphate and 2′-*O*-methylcytosine triphosphate in the active site of the WT NS5B and S282T mutant polymerases. The GTP and ssRNA of initiation complex models are not shown. The active site of WT (A and B) or S282T (C and D) NS5B appears as a blue surface, with catalytic residues represented by sticks with a transparent surface and Mg^{2+} ions represented as green spheres. Residues 282 and 287 appear as sticks, with part of the surface shown as a green grid. Nucleotide analogues (2′-C-Me-CTP [A and C] and 2′-O-Me-CTP [B and D]) appear as sticks, with the surface of the methyl group shown as an orange grid. Fig. is reproduced with copyright permission from *Antimicrob. Agents Ch.* 2006, *50*, 4161.[53]

2'-deoxy-2'-fluoro-2'-*C*-methylcytidine (**PSI-6130**, 10) was designed.[56] **PSI-6130 (10)** revealed enhanced inhibitory activity in the HCV replicon assay compared with 2′-*C*-methylcytidine (**1**) and lower cellular toxicity.[56] Interestingly, **PSI-6130 (10)** is effective against NS5B with S282T mutation, which is known to confer resistance to 2′-*C*-methyladenosine (**4**), as the wild type.[57] Currently **PSI-6130 (10)** diisobutyryl prodrug (R-7128) is in phase IIb clinical trials.[58] The uridine analogue (**PSI-6206**, **12**) resulted in no inhibition of HCV RNA production due to the inability of **PSI-6206** (**12**) to be phosphorylated by cellular nucleoside kinases.[59]

2.1.1.4. 3′-Deoxy nucleotides. Similar to 2′-methoxy NIs **5**–**7**, the triphosphate forms of 3′-deoxy nucleotides **13**–**16** had RdRp IC_{50} values ranging from 1.2 to 0.08 μM, while their best EC_{50} value in replicon assay was 25 μM.[50] On the other hand, their bis(*t*Bu-*S*-acyl-2-thioethyl) nucleoside 5′-monophosphate derivatives (e.g., compound **17**) demonstrate EC_{50} as low as 1.4 μM value in the replicon assay.[50] To the best of my knowledge, there is no reported resistance for the 3′-deoxy nucleotides.



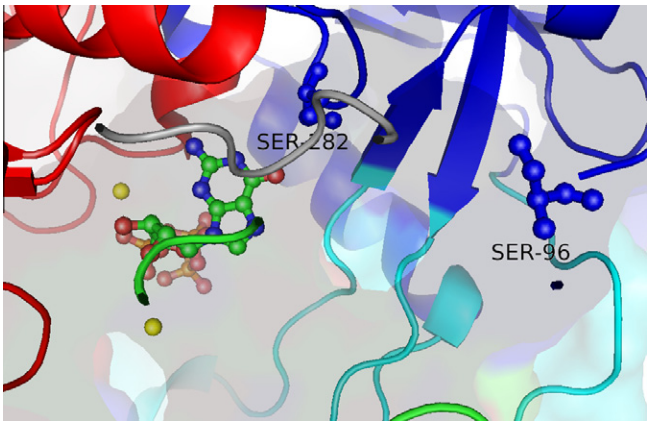
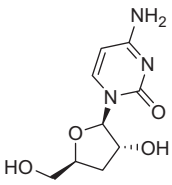
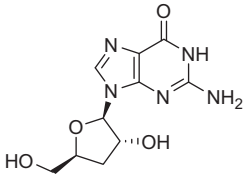


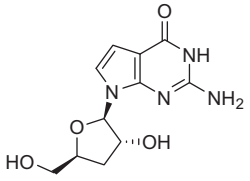
Figure 3. GTP in HCV NS5B active site (PDB ID 2XI3).[70] The following colors used; palm: red, fingers: blue, thumb: green, connecting loops: cyan, Mg ions: yellow. The active site was demonstrated by GTP, which is colored by elements and represented as balls and sticks. S282 and S96 residues are represented as balls and sticks too and colored blue (PyMol generated picture).



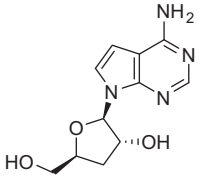
3'-Deoxycytidine
(13)



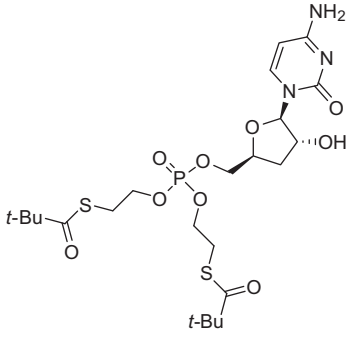
3'-Deoxyguanosine
(14)



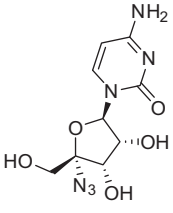
3'-Deoxy-7-deazaguanosine
(15)



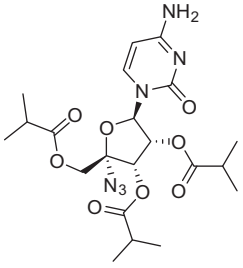
3'-Deoxy-7-deazaadinosine
(16)



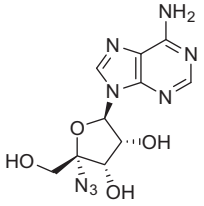
Bis(*S*-pivaloyl-2-thioethyl)-3'-deoxycytidine
5'-monophosphate (17)



R-1479 (18)



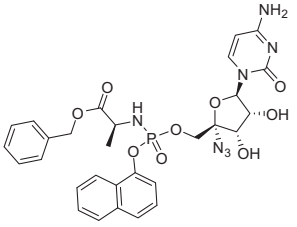
R-1626 (19)



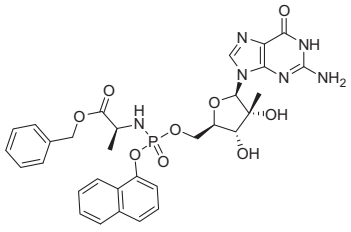
4'-Azidoadenosine
(20)

2.1.1.5. 4′-Azid nucleotides analogues. 4′-Azid nucleotides analogues **18**–**20** is another class of HCV nucleotide inhibitors. 4′-Azidecytidine (**R-1479**, **18**) is the first example in this class.[60] Compound **18** acts as a CTP-competitive inhibitor with K_i value of 40 nM.[60] The advantage of 4′-azid nucleotides is that they, unlike 2′-methyl nucleosides, are effective against S282T mutant genotype with good therapeutic indices.[60] Because of the azide group is long, it can reach S96 residue and therefore the anti HCV efficiency of 4′-azid nucleotides decreased significantly with S96T mutation (Fig. 3).[61] The disadvantage of **R-1479** (**18**) is its limited cellular permeability and oral bioavailability.[62] Therefore a more lipophilic tri-*iso*butyrate ester prodrug **R-1626** (**19**) was developed,[62] and it is in clinical trials now. Phase Ib and IIa results showed robust synergistic against HCV in combination with peg-INFα2a, with or without ribavirin.[63–69] A second example is 4′-azidoadinosine (**20**), which exhibited weak anti HCV activity.[28]

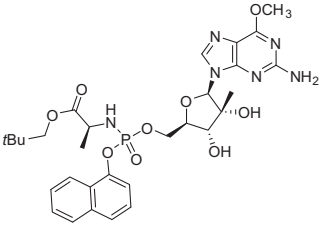
2.1.1.6. Phosphoramidate. As mentioned earlier, nucleotides analogues are not active by themselves and they have to be activated by some cellular kinases to their corresponding triphosphate active forms. To avoid low-rate or lacking of cellular phosphorylation, and taking in consideration that the phosphates are unstable, several nucleotide inhibitors have been derivatized as phosphoramidate analogues to bypass the first phosphorylation rate-limiting step.[28] The resultant phosphoramidate derivatives **21**–**25** showed significant HCV inhibitory activity improvement in both 2'-*C*-methyl and 4'-azide derivatives. For instance, 2'-*C*-methylguanosine phosphoramidates (e.g., compound **22**) are 10 to 30-fold more active than the parent drug **3**.[71] In terms of EC_{50} concept, 4′-azidoadinosine phosphoramidate analogues (e.g., compound **24**) had EC_{50} values as low as 0.22 μM.[28] The same strategy was applied also to 4′-azidecytidine (**18**), and the anti HCV potency of the corresponding phosphamidate **21** was improved more than 450-fold in the replicon assay.[72]



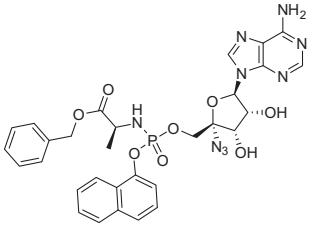
4'-Azidocytidine phosphamidate
derivative(21)



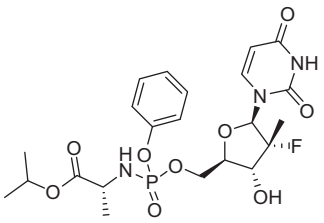
2'-*C*-Methylguanosine phosphamidate
derivative (22)



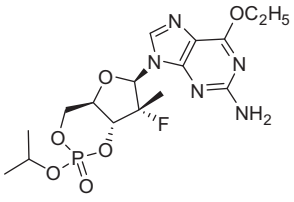
6-*O*-Mehyl-2'-*C*-methylguanosine
phosphamidate derivative (23)



4'-Azidoadenosine
phosphamidate derivative (24)



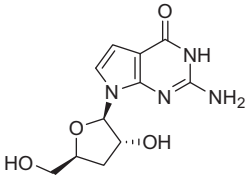
2'-Deoxy-2'-Fluoro-2'-*C*-methyluridine Phosphamidate PSI-7977 (25)



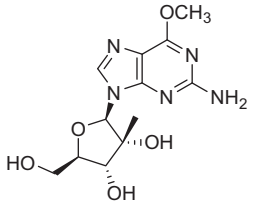
2'-Deoxy-2'-Fluoro-2'-*C*-Methyl-6-*O*-ethyl-guanosine PSI-352938 (26)

The pronucleotide **PSI-7977** (**25**), which is also known as **PSI-7851** is very active NI against HCV genotype 1b;[73] however, its uridine nucleotide **PSI-6206** (**12**), as mentioned earlier, is inactive due to its lower ability for phosphorylation.[59]

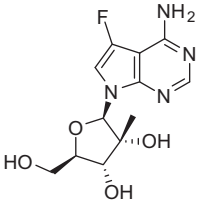
2.1.1.7. Cyclic phosphates. **PSI-352938** (**26**) is an example of cyclic phosphate pronucleotides.[74] **PSI-352938** triphosphate form has broad inhibitory activity against NS5B genotypes 1–4.[74] In contrast, it has no activity against HCB or HIV, and it does not affect human DNA or RNA polymerase.[74]



3'-Deoxy-7-deazaguanosine (27)



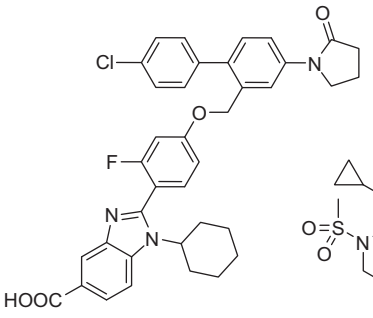
2'-Methyl-6-*O*-methyl-guanosine (28)



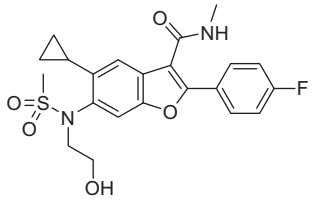
2'-methyl-7-deazadenosine derivative (29)

2.1.2. Nitrogenous-base modified NIs

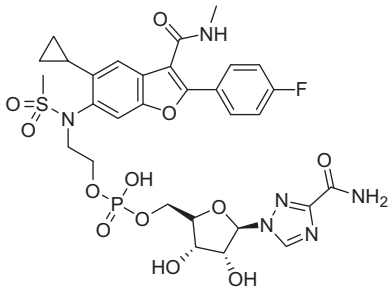
Nucleotides analogues with nitrogenous base modification include 7-deazaguanosines **7** and **15**, and 7-deazaadinosine **16** that mentioned earlier. Notably, incorporation of the 7-deaza-7-fluoro modification into the 2′-*C*-methyladenosine scaffold (**4**) results in an inhibitor identified as 2′-methylpyrolopyrimidine derivative **29**, which exhibited potent anti HCV activity in nanomolar range as measured in replicon assay (EC_{50} = 70 nM).[49] Furthermore, its triphosphate analogue revealed a 20-fold-increased potency in HCV RdRp assays and with reduced cellular toxicity.[75]



JTK-109 (30)



HCV-796 (31)



HCV-796-ribavirin prodrug (32)

Another example of hetero-modified nitrogenous base is 6-*O*-methylmodified-2'-*C*-methylguanosine (**28**), which is considered more lipophilic than 2'-*C*-methylguanosine (**3**) and consequently more potent in cell based assay.[76] Replacement of the 6-methoxy group with more lipophilic OEt or SMe moieties decrease the binding affinity to the polymerase enzyme.[76] Like other nucleotides, Compound **28** phosphoramidate derivatives (e.g., **INX-189**) are more potent and have better pharmacokinetic profile.[77] Several mutation types have been observed with **28** and/or its phosphoramide derivative **INX-189** including S282, I585T in case of HCV 1b genotype, and A540T in case of 1a genotype.[78,79] The anti HCV potency of **28** was reduced 13-fold only in case of S282T mutant replicons.[78] This reduction in potency might be due to increasing the bulkiness at this site clashed with guanidine 6-position as shown in Figure 3.

The advantage of heterobase modified nucleotide HCV inhibitors is that they are inert or, at least, more resistant to phosphorolysis by purine nucleoside phosphorylase (PNP) and deamination by adenosine deaminase (ADA), which deactivate others unmodified nucleotides inhibitors.[49] This results in higher oral bioavailability and better in vivo pharmacokinetic profiles in general.[75]

2.2. NS5B non-nucleosides inhibitors (NNIs)

'The search for hepatitis C virus polymerase inhibitors has resulted in identification of several non-nucleoside binding pockets. The shape and nature of these binding sites differ across and even

within diverse hepatitis C virus genotypes. These differences confront antiviral drug discovery with the challenge of finding compounds that are capable of inhibition in variable binding pockets'.[52] However the anti HCV activity of NNIs are more susceptible to be decreased or diminished than NIs by mutations,[80] there are more NNIs in clinical trials than NIs. In brief, nine NNIs entered clinical trials (**JTK-109**, **HCV-796**, **ABT-072**, **ABT-333**, **VCH-759**, **VCH-916**, **VCH-222**, **PF-868554**, **GSK-625433** and **ANA-598**). In this part, I will cover these drug candidates in brief because most of them are already covered in He 2011.[81] Then I will move to other related compounds that exhibited promising HCV NS5B inhibitory activities.

Unlike NIs that binds to the polymerase active site, NNIs bind to variable allosteric pockets leading to conformational changes of the polymerase-catalytic active site. At least 4 allosteric sites have been identified from the NS5B co-crystal structures.[81] Site I (Thumb I Site): lies between thumb and fingertips region close to GTP non-catalytic binding site, Site II (Thumb II Site): lies at the border between thumb and palm domains 35 Å away from the active site, Site III (Palm I Site): locates at the top border of palm domain site close to Thr418, Site IV (Palm II Site): is also part from the palm region close to Asp316 and it is the closest one from the active[24,82] (Fig. 4).

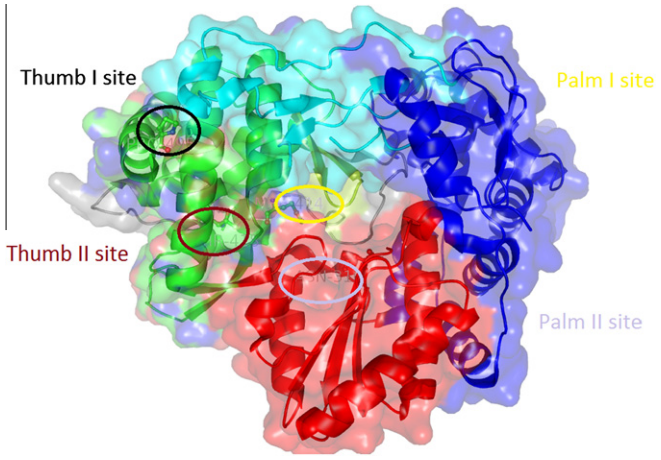
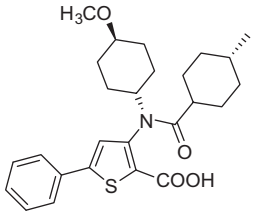


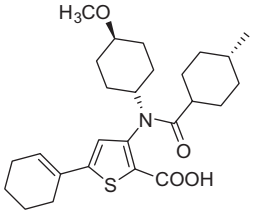
Figure 4. The allosteric binding sites of HCV NS5B. Binding sites are circled and colored differently.

2.2.1. Benzimidazole derivatives

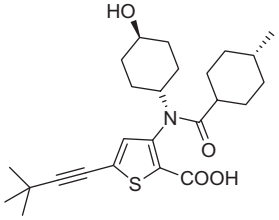
Benzimidazole derivatives bind to allosteric *Thumb Site I*. The first HCV NNI polymerase that entered clinical trial is the benzimidazole derivative **JTK-109** (**30**),[83] which inhibits the HCV polymerase in low nanomolar concentration (IC_{50} = 17 nM for genotype 1b). In addition, it demonstrated favorable pharmacokinetics, high HCV polymerase selectivity, and good therapeutic index.[83] Mutation of P495, a residue that locates on thumb domain away from the active site, into L or A confers resistant to this class of compounds.[84] The activity against P495 mutant decreased by a factor of 1000 as indicated by IC_{50} value that was 1.5 μM.[85]



VCH-759 (33)



VCH-916 (34)



VCH-222 (35)

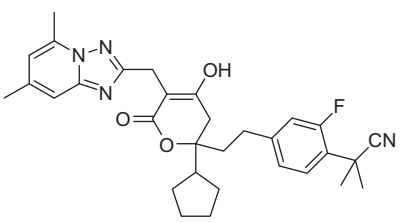
2.2.2. Benzofuran derivatives

HCV-796 (**31**) yielded IC_{50} values of 0.01–0.14 μM in biochemical assays for genotype 1, with EC_{50} values of 5 nM and 9 nM against genotype 1a and 1b replicons.[86] In addition, additive impact has been reported from **HCV-796** (**31**) in combination with INF in treatment of naïve patient with several chronic HCV genotypes.[86] Triple therapy of **HCV-796**/pegINF/ribavirin has also demonstrated high clinical values.[86] Recently, **HCV-796** (**31**) molecule has been connected with ribavirin via phosphate bond (compound **32**) as a new class of sequential HCV therapy.[87] The **HCV-796** (**31**) associated mutations include Leu314, Cys316, Ile363, Ser365, and Met414 of NS5B, which directly interact with **HCV-796** (**31**) leading to 10 to 1000-fold decrease in anti HCV efficacy.[88] In a particular example, the **HCV-796** (**31**) IC_{50} values of wild/C316 mutant types is 0.0008/2.16.[85]

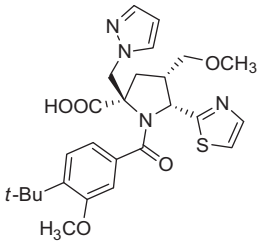
Both **ABT-072** and **ABT-333** are potent non-nucleoside genotype-1-selective HCV polymerase inhibitor. **ABT-072** has 5 to 9-fold greater potency than **ABT-333** in the presence of 40% plasma.[89] **ABT-333** has IC_{50} values vs. HCV 1a and 1b polymerase of 2.2 and 10.7 nM.[81] The reported mutation associated with this class of anti-HCV involve C316Y, S368T, M414T/I/V, Y448H/C, A443V and S556G.

2.2.3. Thiophene derivatives

VCH-759 (**33**) **VCH-916** (**34**) and **VCH-222** (**35**) are thiophene derivatives that are well tolerated as NNI of HCV polymerase with sub-micromolar IC_{50} values versus genotype 1a and 1b replicons.[90] Both bind to Thumb Site II; therefore with M423 mutant NS5B form, the IC_{50} value of **VCH-759** (**33**) increased from 17 nM into 450 nM.[85]



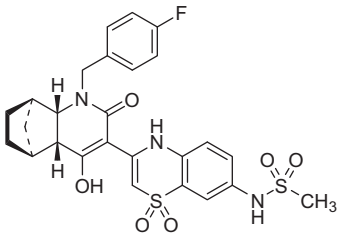
PF-868554 (36)



GSK-625433 (37)

2.2.4. Triazolopyridine derivatives

PF-868554 (filibuvir, **36**) is a triazolopyridine derivative that binds with Thumb II Site.[91] 'In phase I and a IIa clinical trial in treatment of naïve patients infected with genotype 1 HCV, filibuvir (**36**) monotherapy or in combination with pegylated IFNα2a/ribavirin for up to 4 weeks significantly reduced HCV RNA levels compared with placebo.'[92]



ANA-598 (38)

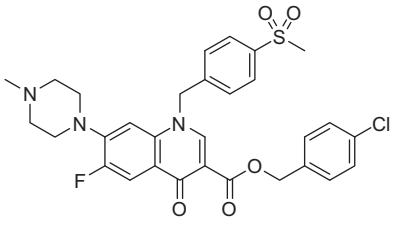
2.2.5. Pyrolidine carboxylate derivatives

GSK-625433 (**37**) has a pyrolidine-2-carboxylate core. It is highly potent and selective inhibitor of genotype 1 HCV RNA-dependent-RNA polymerase.[93] **GSK-625433** (**37**) binds to palm-pocket I, so mutation in M414 and I447 appear to be the key resistance mutations in vitro.[93] In the HCV replicon assay, the EC_{50} value of **GSK-625433** (**37**) is 2 nM versus 1b genotype and 290 nM versus 1a genotype.[93] However, the promising in vivo results of **GSK-625433** (**37**), its use in the clinical trials was terminated early after Phase I.[94]

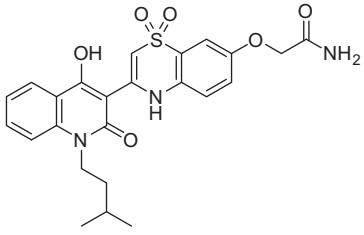
2.2.6. Benzothiadiazine derivatives

ANA-598 (setrobuvir, **38**) binds to palm region I close to Tyr448. Setrobuvir (**38**) is a promised NNI prodrug. It gave 75% SVR when administrated to patients.[95] '**ANA-598** (**38**) is orally bioavailable and potent inhibitor of HCV genotype 1 NS5B polymerase. **ANA598** (**38**) shows excellent in vitro antiviral potency with sub-nanomolar IC_{50} against sol. recombinant HCV NS5B 1a and 1b. **ANA-598** (**38**) is potent in Huh-7 cell-based HCV replicons [EC_{50}(1b) 3 nM and EC_{50}(1a) 52 nM] without appreciable cytotoxicity (CC_{50} ~100 μM). **ANA-598** (**38**) demonstrates good in vitro metabolic stability with a half-life of more than 60 min in human and monkey liver microsomes at 0.5 mg/mL microsomal protein'.[96] Setrobuvir (**38**) showed rapid and sustained reduction of HCV RNA at different doses level (200, 400 and 800 mg BID), and it is safe and well tolerated.[97] Setrobuvir (**38**) is already enrolled in phase IIb clinical trials.[98]

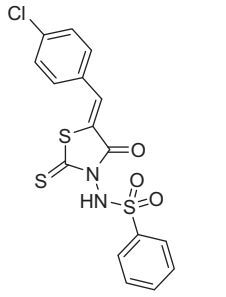
interactions with the protein backbone atoms of Ile447 and Tyr448, and the hydroxyl group of Ser367. C2-carobxylate interacts with Gly449 via a bridging water molecule.[99] However, there is no available mutation study on this particular class of HCV inhibitors; Y448H mutation, which arises in cases of benzothiadiazines (e.g., compound **38**), is expected to confer resistant to this class of compounds because Tyr448 is important for the protein binding.



4-Quinolone (43)



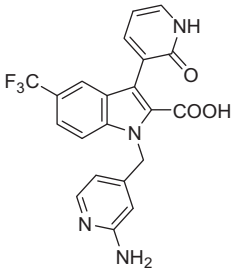
2-Quinolone (44)



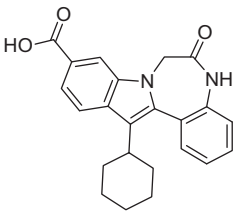
Rhodanine derivative (45)

2.2.8. Indole-6-carboxylate and bridged 2-arylindoles

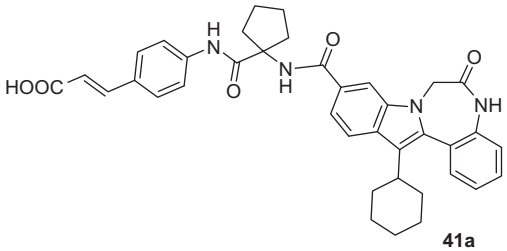
Indole-6-carboxylate & bridged 2-arylindoles, represented by compounds **40** and **41a**,**b**, bind to Thumb Site I close to Pro495. Incorporating the indole nitrogen with another heterocycle improved the replicon inhibitory activity.[100] However, the carboxylate moiety at indole position-6 plays a pivotal role in protein



Indole-2-carboxylate (39)



40

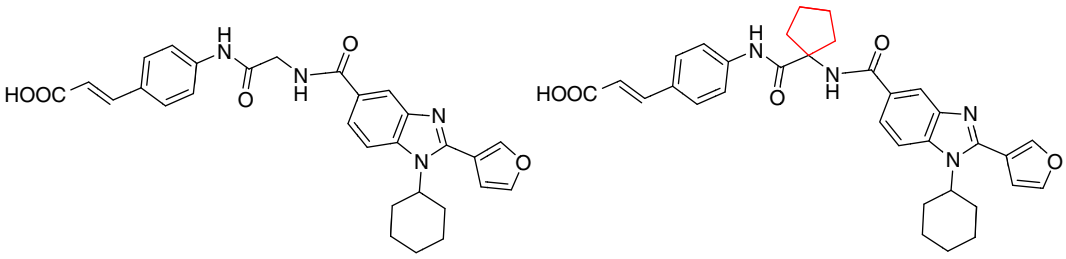


41a

Indole-6-carboxylate & Bridged indolobenzodiazepine

2.2.7. Indole-2-carboxylate

Unlike other clinically tested indole-related derivatives that bind to the palm region II (e.g., compound **31**), indole-2-carboxylates (e.g., compound **39**)[99] behave like benzothiadiazines (e.g., compound **38**) and bind to palm region I close to Tyr448. The reported NS3B inhibitory activity was as low as 17 nM and the replicon EC_{50} value was 0.3 μM. The crystal structures (PDB ID: 3ska, 3ske, 3skh) of indole-2-carboxylates exhibited intended binding at thumb-pocket 1, where it forms a salt bridge with Arg503 (PDB ID 2xwy),[101] introduction of *E*-3-[4-(1-aminocyclopentanecarboxamido)phenyl]acrylic acid moiety to the indole-6-carboxylate significantly improved the replicon EC_{50} values from 240 nM to 10 nM.[100] The acrylate carboxylate moiety form another salt bridge with Arg498 (PDB ID 3ska).[100] The role of the cyclopentyl moiety is to confer the biologically active *'L-shape'* configuration to ligand (Fig. 5).[102]



42
IC_{50} = 0.28 μM
EC_{50} = 41 μM

41b
IC_{50} = 0.095 μM
EC_{50} = 0.072 μM

Figure 5. Effect of the cyclopentyl moiety on HCV NS5B and replicon inhibitory activities.

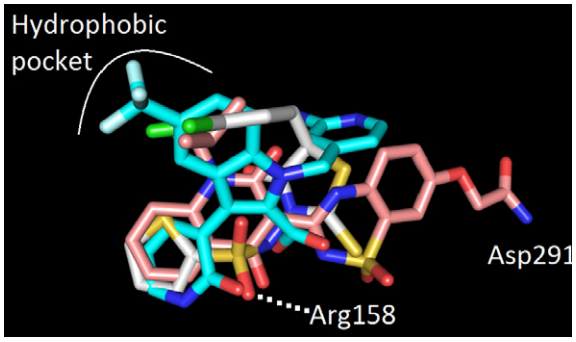


Figure 6. Overly of indole-2-carboxylate (cyan), 2-quinolonee derivative (salmon), and rhodamine (gray) extracted from crystal structures 3ska, 3hhk and 2ax1, respectively and aligned using PyMol.

2.2.9. 4-Quinolones

4-Quinolones NNIs, represented by compound **43**, bind to thumb-pocket II close to leu419 (PDB ID 3phe) and it demonstrated IC_{50} value of 16 nM with low micromalar EC_{50} in replicon assay.[103] Carbonyl groups at position 3 and 4 are essential for the activity since they form two distinct hydrogen-bonds with amide NH of Ser476 and Tyr477, respectively.[103] Therefore, removal of the carbonyl group at position-3 or replacement with NH led to high increase in the IC_{50} values.[103] Both benzyl ester and methylsulfone moieties occupy hydrophobic regions in thumb-pocket II.[103] The methylpiperazine moiety was added to improve the water solubility and it has not apparent interaction with the protein.[103]

2.2.10. 2-Quinolones

Interestingly, 2-quinolones **44** bind differently than 4-quinolones **43**, they bind to palm-pocket I similar to indole-2-carboxylate **39** (Fig. 6). The amyl moiety occupies the same hydrophobic pocket that was occupied by the benzene ring of indole-2-carboxylate **39** close to Leu384 and Mse414. The role of the terminal amide moiety is to target Asp291 and that improve the replicon inhibitory activity more than 25 times.[104]

2.2.11. Rhodanines

Rhodanines based derivatives (e.g., compound **45**) represents a novel class of NS5B NNIs; however, they bind to palm-pocket I similar to 2-quinolones **44** and indole-2-carboxylate **39**.[105] Halogen

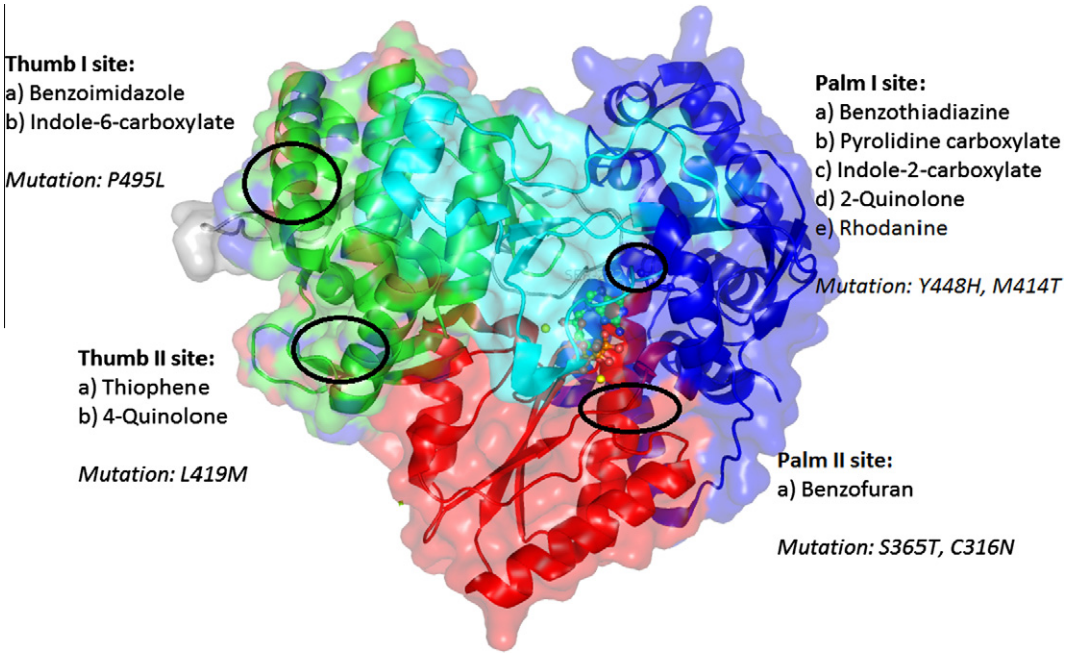


Figure 7. Summary of NNIs and their binding sites within NS5B.

substitution(s) on the benzylidine ring is favorable and the best IC_{50} values have been reported in cases of 3,4-di-Cl, 3,4-di-Br and 2,4,5-tri-F analogues.[105]

The co-crystal structures of indole-2-carboxylate, 2-quinolone, and rhodamine derivatives (PDB ID 3ska, 3hhk and 2ax1) revealed a hydrogen-bond with Arg158 as a common binding feature as well as a common aromatic ring lies within a hydrophobic region (Fig. 6).

3. Conclusion

In summary, NIs that target HCV NS5B can be classified into two major groups: sugar-modified and heterobase-modified derivatives. There are three reported positions for nucleotides sugar modification; namely 2′, 3′ and 4′ positions. 2′-β-Methylation is characterized by increasing the selectivity towards HCV polymerase vs. human or other non-flaviviral polymerases, which confers high therapeutic indices for this class of compounds. However, HCV S282T mutant confers resistant to all 2′-β-methylated derivatives. Moving methyl group to ribose 2′-α-position abolishes the anti HCV activity. 2′-α-Fluorinated derivatives have excellent anti HCV potency but with high tendency towards other mammalian polymerases. 2′-α-Methoxy NIs are, in general, less active than 2′-β-methylated derivatives but they have the advantage of the potency versus S282T mutant genotype. 3′ Modifications have been expected to be the best efficient chain terminators, since the 3′-position is directly involved in inter-nucleotides connection. Unexpectedly, most of the 3′-modified nucleotides had weak anti HCV activity. Only 3′-deoxy derivatives demonstrated descent HCV inhibitory activity as bis(*t*Bu-*S*-acyl-2-thioethyl) derivatives. The most important 4′-ribose modified HCV NIs are the azide nucleotides analogues. The advantage of 4′-azides is their inhibitory potency versus S282T mutants, but they loss most of their activity in the case of S96T mutation.

In general sugar-modified NIs have poor pharmacokinetic properties including weak cellular penetration and low oral bioavailability. Therefore, several approaches have been developed to improve their pharmacokinetics. These approaches include esterification with L-valine, isobutyrate, and *t*Bu-*S*-acyl-2-thioethyl derivatives, or phosphoramidation. *t*Bu-*S*-acyl-2-thioethyl derivatives and phosphoramidates not only provide the required enhanced pharmacokinetics, but also overcome the poor cellular phosphorylation.

Sugar-modified NIs are subjected to the action of purine nucleoside phosphorylase (PNP) and deamination by adenosine deaminase (ADA), leading to decreasing the oral bioavailability. Hence, the advantage of the second NIs class; that is, heterobase-modified NIs, which resist both deactivating enzymes, and show better pharmacokinetic characters and good oral bioavailability. Generally, 7-deaza modification improves the anti HCV of 2′-*C*-methyl NIs.

NNIs bind, at least, to four different allosteric sites based on the nature of their chemical scaffolds. They also induce different types of mutations. Different NNIs scaffolds, their binding sites within NS5B, and possible mutate residues are summarized in Figure 7.

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