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Perspective

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Nucleoside, Nucleotide, and Non-Nucleoside Inhibitors of Hepatitis C Virus NS5B RNA-Dependent RNA-Polymerase

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INTRODUCTION

Hepatitis C virus (HCV) infection is a global health problem that impacts approximately 180 million individuals. Of those initially infected with HCV approximately 80% will progress to develop chronic liver disease and a significant percentage will ultimately progress to liver cirrhosis and of these 75% will eventually develop hepatocellular carcinoma.[1] HCV infection is also the leading cause of liver transplants and consequently a major health economic burden. Until recently the current therapy for treating HCV infection has been regular injections of pegalated α-interferon (PEG-IFN) with daily oral administration of ribavirin (RBV). This standard of care (SOC) functions by boosting the host immune system and does not act directly on the virus. PEG-IFN/RBV treatment is moderately successful in treating infected patients, as only 40−50% of genotype 1 patients achieve a sustained virological response (SVR) indicative of a cure.[2,3] SVR rates can be as high as 75% for those infected with genotype 2 and 3 viruses. It has also been shown that subpopulations that include those of African descent respond less well to PEG-IFN/RBV treatment.[4] In addition, through genome wide association studies it was shown that a single nucleotide polymorphism 3kb upstream of the IL28B gene, which encodes the type III interferon IFN-λ3 known to be up-regulated by interferons and viral infections, correlates with a significant reduction in response to PEG-IFN/RBV treatment.[5] HCV patients who harbor a TT or TC allele in the IL28B gene respond less well to PEG-IFN/RBV treatment than those having the CC allele. Those patients undergoing PEG-IFN/RBV treatment also face the prospects of significant adverse effects that include fatigue, hemolytic anemia, depression, and flulike symptoms which ultimately lead to high rates of drug discontinuations. In attempts to improve on the overall HCV cure rate and reduce or eliminate the serious side effects associated with PEG-IFN/RBV therapy, the search for direct acting antivirals (DAAs) that are safe and effective has become an urgent endeavor.

HCV, a small, single-stranded positive-sense RNA virus, is a member of the *Flaviviridae* family of viruses that includes other flaviviruses such as bovine viral diarrhea virus, yellow fever virus, dengue virus, and West Nile virus and is the only member of the genus *Hepacivirus*. There is considerable nucleotide sequence variation distributed throughout the HCV genome, with as much as a 31−33% difference at the nucleotide level.[6] On the basis of the genomic variability in a small region of the gene that encodes for the viral NS5B polymerase, these naturally occurring variants are classified into six major genotypes (genotypes 1−6) and several subtypes.[6,7] The six major types of HCV may also be identified by comparing sequences in the 5′ noncoding region. Distribution of the six genotypes (GT) varies globally.[6] In North America and Europe, genotype 1 predominates followed by genotypes 2 and 3, whereas genotypes 4 and 5 are found almost exclusively in Africa. Genotype 6 is more prevalent in Asia than other parts of the world. Knowing the genotype is clinically important in determining the response to interferon-based therapy and the duration of interferon therapy.[8,9] Genotypes 1 and 4 are less responsive to interferon treatment than are the other genotypes, and therefore, the duration of interferon-based therapy for genotypes 1 and 4 is 48 weeks, whereas only 24 weeks of treatment is generally required for genotypes 2 and 3.

Development of first generation DAAs has focused on combinations with PEG-IFN/RBV with the desire to increase cure rates and reduce treatment duration from the standard 48-week PEG-IFN treatment regimen.[10−13] Within the past year, two NS3/4 protease inhibitors, telaprevir and boceprevir, in combination with PEG-IFN/RBV have been approved for treating HCV infection in genotype 1 patients. Both of these combination treatments have shown improved SVRs and reduced treatment duration. However, they are limited to treating genotype 1 patients, still require PEG-IFN/RBV adjunct therapy, and bring with them additional side effects that require further medical management. Therefore, the search continues for novel agents with complementary mechanisms of action that can be combined with existing or future DAA therapies and that have improved safety profiles over existing therapy. This Perspective focuses on inhibitors of the HCV NS5B RNA-dependent RNA-polymerase (RdRp) as therapies for treating HCV infection.

HCV VIRAL GENOME

The first step in the virus replication cycle involves the attachment of the virus to the surface of the target cell. This occurs through interactions between the virus and cell-surface receptors and is a multistep process. Cell-surface receptors required for the attachment and entry of HCV include HS glycosaminoglycans, LDL receptor, CD81, SR-BI, DC-SIGN, claudin-1, and occludin.[14−26] Cell entry involves clathrin-mediated endocytosis and release of the nucleocapsid and genomic RNA into the cytoplasm.[14,17,27,28] The 9.6 kb positive sense RNA genome is composed of 5′- and 3′-nontranslated regions that flank a long open reading frame (ORF) (Figure 1). The 3′-nontranslated region contains a poly U/UC tract and a highly conserved 98-nucleotide element that is required for viral RNA synthesis.[29,30] The 5′-nontranslated region is highly conserved among the different genotypes and contains the IRES (internal ribosomal entry site) element.[31,32] Translation of the viral genome requires the IRES element, which binds to the 40S ribosomal subunit near the start codon of the ORF.[33,34]

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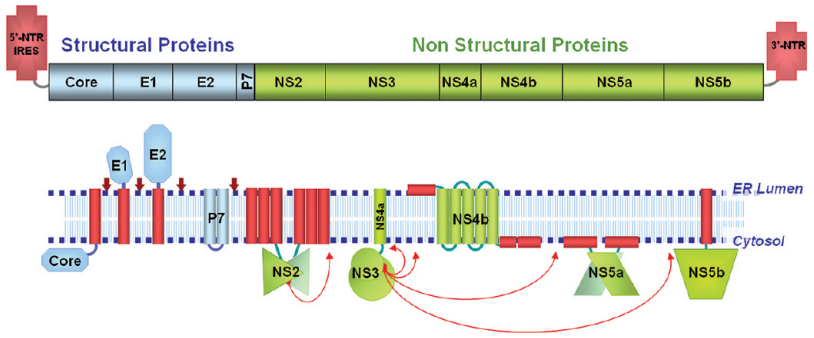

Figure 1. (a, top) Schematic of HCV genome organization. The 5′ nontranslated region (NTR) also incorporates the internal ribosome entry site (IRES). The long ORF that codes for a ∼3000 aa precursor polyprotein lies between the 5′ and 3′ NTRs. (b, bottom) Representation of HCV polyprotein processing and functional organization. Host ER signal peptidases clip the structural proteins (block arrows) required for viral capsid formation and release. The NS2-3 protease, a functional dimer, cleaves at the 2−3 juncture. The remaining cleavage sites downstream of NS3 are processed by NS3/4a (red arrows). NS3 also includes a larger helicase subunit. NS4b comprises four to six transmembrane helices forming a membranous web essential to viral replication. NS5a may function as a dimer based on crystallographic analysis. NS5b provides the essential RNA polymerase function.

Translation of the ORF results in the synthesis of a precursor polyprotein of approximately 3000 amino acids. The polyprotein precursor is processed into both structural and nonstructural proteins by host cell and viral proteases.[35−42] The N-terminal one-third of the precursor polyprotein is processed by cellular signal proteases and contains the structural proteins (Figure 1). These structural proteins include the core, a highly basic protein that is the major component of the nucleocapsid, E1 and E2 which are highly glycosylated transmembrane proteins that serve to encapsidate the viral genomic RNA and make up the viral particle, and p7 that is believed to form ion channels that are essential for assembly and release of infectious virions.[39,41]

The remainder of the precursor protein contains the nonstructural proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B (Figure 1). These proteins are processed by two viral proteases, NS2 and NS3. The first cleavage occurs at the NS2/NS3 junction.[35−38,40,42,43] This zinc-dependent autoproteolytic step involves the NS2 protease and the N-terminal one-third of NS3. NS3 is a bifunctional enzyme that contains a serine protease in the N-terminal 180 amino acid residues and is responsible for the downstream processing of the nonstructural region of the polyprotein, catalyzing cleavage at the NS3/NS4A, NS4A/NS4B, NS4B/NS5A, and NS5A/NS5B junctions to release NS3, NS4A, NS4B, NS5A, and NS5B. NS3 requires NS4A as a cofactor for efficient enzymatic function.[44,45] NS3 protein expressed in the absence of NS4A is relatively unstable suggesting that NS4A is important in maintaining the stability of the enzyme.[44−46] The C-terminal portion of NS3 displays an NTPase/helicase activity that is capable of separating duplex RNA and is essential for viral RNA synthesis.[29,47−49] In addition to functioning as a required cofactor for NS3 protease activity, NS4A also functions as a cofactor for the NS3 helicase, enhancing its ability to bind RNA in the presence of ATP.[50−52] The hydrolysis of ATP by the associated NTPase supplies the energy required to separate the strands of the RNA duplex. Other activities associated with NS4A include involvement along with NS3 and NS4B in the hyperphosphorylation of NS5A and interaction with other nonstructural proteins to form the replication complex.

HCV replicates its genome in the cytoplasm in association with specialized sites on the endoplasmic reticulum (ER) membrane.[53,54] NS4B, a highly hydrophobic protein, induces specific changes to intracellular membranes, forming what is referred to as the membranous web.[55,56] The membranous web is comprised of the nonstructural proteins and cellular proteins, which form a membrane-associated replication complex that provides the platform for viral replication.[55] NS4B, which contains at least four transmembrane domains and an N-terminal amphipathic helix, functions to affix the replication complex to membranes.[55−57]

The NS5A nonstructural protein is a phosphoprotein with no apparent enzymatic activity; however, it acts as a multifunctional regulator of cellular pathways, including host cell growth, immunity and innate immunity, and virus replication.[58−67] NS5A is associated with host cell membranes through its N-terminal amphipathic helix, where it is a part of the replication complex.[57,68] Recent studies suggest that NS5A is organized into three domains: the first 213 amino acids in the N-terminal domain constitute domain I and contain a zinc binding motif suggesting that the protein is a zinc metalloprotein, and domains II and III are in the C-terminal region of the protein.[69,70] NS5A exists in two phosphorylated forms: a basal form of 56 kDa and a hyperphosphorylated form of 58 kDa. The protein is phosphorylated at specific sites, primarily on serine residues within domains II and III, by host cell kinases.[71−76] Although no enzymatic activity has been attributed to NS5A, evidence suggests that NS5A is an essential component of the HCV replication complex and therefore is intimately involved in viral RNA replication. NS5A binds directly to the NS5B polymerase where it is believed to modulate the activity of NS5B.[66] A model has been proposed suggesting that the phosphorylation state of NS5A regulates HCV RNA replication.[59]

The catalytic subunit for replicating the viral genome within this complex is the NS5B RNA-dependent RNA polymerase (RdRp).[77] This viral specific enzyme is essential for HCV replication. Replication of the HCV genome begins with synthesis of full-length negative strand RNA. Positive strand RNA is produced from the negative strand RNA template which is used for additional translation and replication and packaging to yield progeny virions. On the basis of in vitro studies, it appears that the initiation of de novo HCV RNA synthesis in the infected cell makes use of a single nucleotide as primer. In vitro studies suggest that GTP is the preferred nucleotide and Mn^{2+} is the preferred cation for initiation, whereas

Mg^{2+} is the preferred cation for elongation.[78−81] However, the 3′-terminal residue for some isolates of HCV can be uridylate in which case ATP would be expected to be used to initiate RNA synthesis.[78,81] At high concentrations GTP binds at a low affinity binding site on the surface of the enzyme and can stimulate RNA synthesis.[82,83] As with other polymerases the elongation of HCV RNA by the RdRp likely involves a bireactant−biproduct mechanism in which binding of the substrates is ordered and incorporation of the bound nucleotide triphosphate (NTP) into the nascent RNA chain occurs via a S_N2 displacement of pyrophosphate by the 3′-hydroxyl at the primer terminus.[84−88]

Because of the genetic diversity in the HCV genome due to the high rate of replication of the virus, the error-prone nature of the polymerase, and lack of proofreading activity, it would be anticipated that preexisting drug resistant variants would arise naturally in untreated individuals.[89] It has been estimated that 10^{12} virus particles are produced each day in an untreated patient.[90] The mutation rate for HCV is estimated to be between 10^{-4} and 10^{-3} base substitutions per genome site per year, and based on the estimated number of virions produced per day, it is predicted that single and double mutants are generated several times a day.[91−93] Even with a very potent inhibitor that reduces viral load by as much as $-5 \log_{10}$ IU/mL, pre-existing resistant variants with two or more mutations are predicted to be present in untreated patients.[93] Indeed it is well documented that variants resistant to direct acting antiviral agents, including inhibitors of the NS5B polymerase, can be selected in vitro and in vivo.

STRUCTURE OF THE HCV RdRp

The NS5B RdRp is a 66 kDa protein of ∼590 amino acids found at the C-terminus of the virally encoded HCV polyprotein of ∼3000 amino acids (Figure 2).[94] The presence of several motifs common to RdRp's, in particular, the Gly317-Asp318-Asp319 (GDD) motif, an invariant element in RNA virus replicases, provided the first indication of the function of NS5B.[95,96] In 1999, three research teams independently reported the crystallographic determination of the NS5B structure that showed that the protein adopts a morphology similar to that of a right-hand maintaining the subdomains of "palm", "fingers", and "thumb" common to several classes of polymerases[97] as depicted in Figure 2 and has a secondary structural content of 47% helical and 15% β sheet,[98] on average. These first three crystal structures are all apo structures for the HCV genotype 1b BK strain NS5B protein deposited in the PDB as 1C2P,[99] 1QUV (Figure 2),[100] and 1CSJ[101] (in order of reference publication rather than deposition).

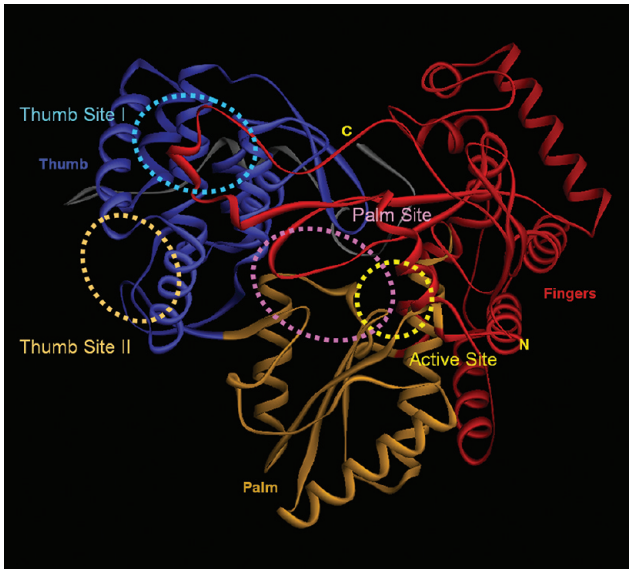

Figure 2. Ribbon diagram of the NS5b RNA dependent RNA polymerase (using 1C2P[99] data) oriented to show the NTP entrance to the active site in the palm subdomain (gold ribbon). The archway of the entrance is formed by the fingerloops with the finger domain to the right in red. The thumb domain is in blue on the left. It hosts two allosteric binding sites alone and provides key interactions on the interior to the palm allosteric binding site. The C-terminal loop connecting the thumb domain to the transmembrane anchoring helix (truncated) is in gray. These domain ribbon colors are maintained throughout the molecular graphics.

"Fingers" Subdomain. While having the "right hand" shape common to many polymerases, these initial HCV NS5B structures were also remarkably different (Figure 2). Whereas the other polymerase structures appear similar to an open hand as observed in the Klenow fragment from *E. coli* DNA polymerase 1 (1DPI),[102] for example, the HCV NS5B proteins have two loop elements that extend from the finger to the thumb subdomain as if the HCV RdRp "right hand" is making the "OK" gesture. The longest is ∼35 aa beginning near residue 10, and the shorter is ∼20 aa beginning near residue 140. Since then, it has become clear from examination of all of the HCV NS5B protein structures solved to date along with those solved for a variety of viral RdRps that these loops are a common structural feature; for example, see the RdRp structures for coxsackievirus 3D(pol) (2CDU),[103] foot-and-mouth disease virus (1U09),[104] or Norwalk virus (1SH0).[105] This observation had been anticipated by the designation of a portion of the shorter loop as structural motif F which encompasses residues Lys155−Ile160 (KPARLI) using the HCV NS5B 1b BK sequence.[99] While initially identified by sequence alignment of Dengue, BVDV, and HIV-RT to the structures of these loops in the HCV NS5B, this motif F has since been found to be universally conserved across viral RdRp's.[106] Interestingly, at the time of the disclosures of the first HCV NS5B structures, the only extant structure of the poliovirus RdRp had about a third of the protein disordered including this important loop region (1RDR) which precluded the identification of structural motif F at that time.[107] It would not be until 2004 that a complete structure of the poliovirus polymerase would be described showing that it too maintained the structural elements of motif F as anticipated (1RA7).[108]

The β-strand rich region of the fingers subdomain (β-fingers), of which these loops are a part, plays a critical role in providing access for the incoming NTPs (motif F) and the incoming template RNA strand to the catalytic site located in the palm subdomain. Similarly, the α-fingers, the α-helical rich region proximate to the palm subdomain, provide part of the exit route for the double stranded RNA product. Currently, no crystallographic complexes are available to indicate that the fingers subdomain functions as a binding site for allosteric inhibitors. However, the longer of the finger loops that reaches to the thumb subdomain forms a two-turn α-helix that packs within a groove created by two α-helices there. Two residues from that "fingertip", Ser29 and Arg32, form part of an allosteric binding site for a surface bound guanosine triphosphate (GTP) with the guanidinium moiety acting as a specificity determinant (1GX5).[109] Mutational analyses of the allosteric site residues show that it does not play a role in the RdRp function in vitro but is clearly important for HCV replication in vivo.[110]

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"Thumb" Subdomain. The "thumb" subdomain (Figure 2) appears to have the most variability among the various polymerases. In HCV NS5B, it comprises ~160 amino acids, which is significantly larger than seen in other polymerases, a hallmark of the *Flaviviridae* RdRp's.[111] This region contains seven α-helices and a relatively unique β-strand that descends into the palm domain partially blocking what is undoubtedly the exit path for the RNA product strand.

"Palm" Subdomain. The palm subdomain (Figure 2) is the most well conserved structural feature across all of the known polymerase structures comprising four β-strands and three α-helices.[111] RdRp sequence motifs A–E are found here including the GDD component of motif C.[95,99,112] The acidic side chains of these two aspartates in conjunction with that from Asp220 and the main chain O of Thr221 in motif A coordinate two divalent metal cations, either Mg^{2+} or Mn^{2+}, which catalyze the polymerization reaction. Most of the crystallographic complexes for the HCV NS5B were solved without these cations present; nine were found complexed with Mn^{2+} and only two complexed with Mg^{2+}. Without the metals present, the side chain of Asp319 rotates such that the carboxylate makes a good hydrogen bond with the main chain NH of Cys366 which is found in motif E of the palm (compare 1C2P[99] lacking Mn^{2+} to 1GX5,[109] for example).

Many of the structures of HCV NS5B are in what has been referred to as the "closed" conformation in reference to positioning of the fingers relative to the palm and their similarity to that in a ternary complex for HIV reverse transcriptase (1RTD),[113] suggesting that the crystal structures represent, for the most part, what one might expect to see in a ternary complex of HCV NS5B, template RNA, and incoming NTP. Superposition of NS5B and HIV-RT crystal structures provided some of the first models for the HCV elongation process. However, the presence of the β-strand from the thumb and the NS5B C-terminus in the Δ21 constructs blocks egress necessary for elongation. By recognition of the similarity to the blocked site observed in the bacteriophage ϕ-6 polymerase initiation complex (1HI0),[114] superposition with the HCV polymerase has been used to model an initiation complex. In 1HI0, two GTPs are positioned in the palm subdomain, forming base-pair interactions with a dCdCdTdT template with Tyr630 acting as a backstop for the initiation GTP. It has been suggested that Tyr448 at the β-turn of the thumb loop could function in a similar fashion during initiation of HCV replication.[109] Intriguingly, RdRp's for bovine viral diarrhea virus (1S48),[115] dengue virus (2J7U),[116] and West Nile virus (2HCN)[117] contain a similar thumb loop descending into the palm subdomain with an aromatic residue, tyrosine or histidine, in a position analogous to that of Tyr448 in HCV NS5B. Unfortunately, no crystallographic ternary complexes of HCV NS5B, RNA, and NTPs describing the initiation or elongation function are publicly available yet. However, RdRp elongation complexes are available for a variety of viral targets including the foot and mouth disease virus (1WNE),[104] Norwalk virus (3BSO),[118] and polio virus (3OL7)[119] which could provide additional structural insights into the HCV RdRp elongation process in lieu of crystallographic data.

NS5B crystallographic complexes are clearly useful in the screening, design, and confirmation of binding modes for a variety of non-nucleoside inhibitors (NNIs). However, these inherently provide an incomplete picture: all lack the C-terminus which contains an α-helical transmembrane anchor essential to viral RNA replication[120] in order to create protein suitable for enzymatic and crystallographic studies.[121] Similarly, once anchored in the endoplasmic reticulum, other HCV nonstructural proteins are part of the full replicase complex with the RdRp at its core; interactions between NS5B, perhaps in some homodimeric form,[122] and the NS3[123] and NS5A[66] proteins are important to replication.[124] Similarly, host proteins such as protein kinase C-related kinase 2 (PRK2) which regulates the polymerase function by phosphorylation of the fingers subdomain may also be associated with the complex.[125] A more complete structural picture of the replicase complex could prove useful in identifying additional molecular interactions as medicinal chemistry target options for HCV.[126]

■ IN VITRO AND IN VIVO TESTING MODELS

Insights into the life cycle of HCV previously had been hampered by the lack of cell culture systems that efficiently replicate the virus. With the establishment of the replicon system, tremendous progress has been made in our understanding of HCV RNA replication in cell culture and our ability to test compounds for anti-HCV activity. Typically, HCV replicons consist of a bicistronic RNA that contains the 5′- and 3′-untranslated region, a selectable marker, and the nonstructural genes NS3-NS5B.[127,128] The first cistron contains the 5′ nontranslated region and the HCV internal ribosomal entry site IRES that promotes the expression of the selectable marker. The second cistron downstream of the selectable marker contains the IRES element from the encephalomyocarditis virus, which directs the translation of the HCV nonstructural proteins, NS3-NS5B. The 3′ nontranslated region is required for the initiation of RNA synthesis. In the prototype replicon the structural genes are replaced by the gene that encodes for neomycin phosphotransferase that confers resistance to G418. The presence of this antibiotic selectable marker in the replicon allows cells that have high levels of sustained replication of the replicon to survive in the presence of G418. The NS3-NS5B region provides all of the enzymatic machinery necessary to replicate the replicon RNA. The first assays using the replicon system involved transfecting Huh-7 cells, a human hepatoma cell line, with replicon RNA generated by in vitro transcription. G418-resistant colonies of cells could be obtained in which the subgenomic replicon replicated and the number of surviving colonies could be counted.[128] This system was an important tool to begin studying HCV RNA replication, but as a tool for evaluating potential antiviral compounds the assay was not amenable for high throughput screening because of the lengthy selection procedure.

Following the development of the first HCV replicon genotype 1b replicon system in 1999, replicons and assays have been modified to make them more suitable for compound screening. A variety of different replicons and assays have been designed, including genotype 1a and 2a replicons and genomic length bicistronic replicons. Replication efficiency of replicons has been enhanced by the addition of adaptive mutations within the HCV sequences of the replicons.[58,59,129−131] One of the first replicon-based assays of relatively high throughput for identifying inhibitors of HCV RNA synthesis was a quantitative real-time PCR assay that directly quantified the amount of HCV replicon RNA synthesized.[132] Subsequently, transient transfection assays in which the neomycin resistance gene was replaced with a reporter gene such as luciferase were developed that could detect HCV RNA replication within 48–72 h post-transfection.[130,133] Other replicons, including monocistronic replicons that eliminate the need for the EMCV IRES for translation of the second cistron, that contain reporter genes

such as luciferase or green fluorescent protein have been generated.[134−137] Because the reporter gene in the monocistronic replicon is fused directly to the nonstructural proteins, the possibility of a false positive, due to interaction with the EMC IRES, is eliminated. These replicons provide reproducible high throughput assay systems for screening compound libraries. More recently an infectious assay system has been developed, which provides an opportunity to study the complete replication cycle of the virus and test compounds that may inhibit different steps in the virus life cycle, including attachment, entry, replication, and virus assembly and release.[138−140]

While there has been significant improvement in the development of in vitro models for studying HCV replication, there has been little progress in the development of robust animal models for the study of viral pathogenesis and drug discovery. Currently, the chimpanzee is the only well established animal model for HCV infection. This has been a valuable model because the clinical course of the disease and viral titers reached in the chimpanzee are similar to those observed in humans. Furthermore the chimpanzee model provides the opportunity to monitor and analyze the development of disease in the liver from the time of infection. However, there is reluctance to use the chimpanzee model because of the availability of these animals, expense, and ethical aspects to using chimpanzees as experimental models. Attempts have been made to develop other primate models including the use of tree shrews, marmosets/tamarins, and other primates.[141−145] The tree shrew currently is the only small animal that has been successfully infected with HCV.[144] For successful infection to occur, it is necessary to immunosuppress the animals. Even though the tree shrew can be infected with HCV, it is not possible to establish a persistent HCV infection. Other models include the use of tamarins and marmosets infected with GBV-B virus, a virus closely related to HCV, as a surrogate model.[146−149] A problem related to the development of a small animal model for HCV is the restricted host range of infection associated with the virus. Various attempts to circumvent this issue include the use of transgenic mice that express different parts of the genome; however, as yet a transgenic mouse has not been engineered with the full length HCV genome.[150−161] While translation of viral RNA takes place in these models, little or no replication occurs. Therefore, these models are unlikely to be of value in drug discovery. More recently, on the basis of the in vitro findings that CD81 and occludin allow mouse cells to be permissive to HCV infection, investigators demonstrated that expression of these two human genes in transgenic fully immunocompetent inbred mice was sufficient to allow HCV infection.[162] This particular model, while not useful in evaluating the in vivo activity of inhibitors of the HCV polymerase, may be useful for testing the activity of potential entry inhibitors. Xenograft mouse models using immunodeficient or transgenic mice have been established and used to evaluate novel anti-HCV compounds.[163−168] These models rely on transplantation of human hepatocytes into mice followed by infection with HCV. HCV replication has been demonstrated in these models, and replication in some cases continued for up to 35 weeks. Although this approach has been useful in demonstrating efficacy in vivo, the difficulties of establishing and working with chimeric mice make the model not practical for general use in drug discovery.

INHIBITORS OF HCV NS5B POLYMERASE

The search for inhibitors of HCV NS5B RdRp has led to the investigation of nucleoside and nucleotide inhibitors, which are alternative substrates for the polymerase, and NNIs, which bind to one of several allosteric sites on the enzyme. Through the intermediacy of their triphosphate anabolites, nucleos(t)ides become incorporated into the growing RNA chain, resulting in the termination of the replication elongation step. Non-nucleosides presumably function by inducing a conformation change in the RdRp, thus hindering the polymerase from functioning effectively and leading to inhibition of the initiation step in RNA replication. A number of HCV NS5B inhibitors have progressed into the clinical phases of development and have demonstrated proof of concept by reducing viral loads in HCV infected patients.[11,169] Several proof of concept studies have also shown that it may be possible to eliminate the use of PEG-IFN with combinations of DAAs that include NS5B inhibitors.[170] Therefore, inhibitors of HCV NS5B polymerase show great promise as future therapies for the treatment of patients infected with HCV.

NUCLEOSIDE AND NUCLEOSIDE PRODRUG INHIBITORS

The HCV polymerase catalytic site is located in the palm domain (Figure 3). It is where the conserved aspartic acid residue-containing GDD motif common to viral polymerases is located. As described above, at this site a ribonucleoside 5′-triphosphate binds through the coordination of a divalent metal (Mg^{2+} or Mn^{2+}) and is subsequently added to the 3′-end of the growing RNA chain through the formation of a

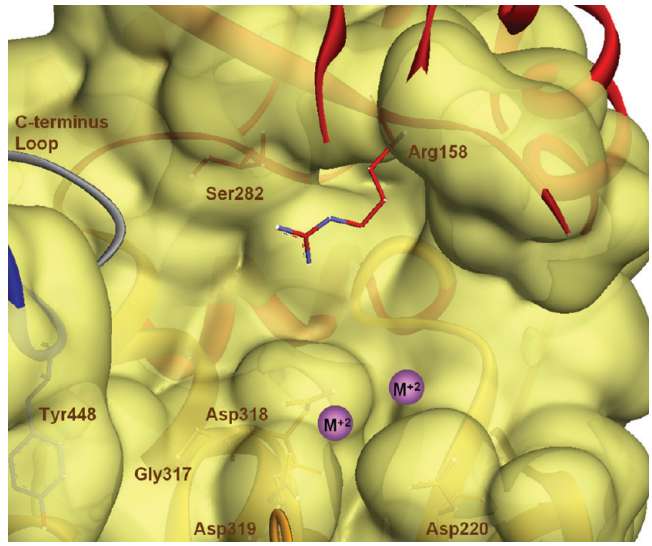

Figure 3. The active site of the polymerase is depicted with a smoothed vdW surface in light yellow (using 1C2P[99]/1GX5[109] data). The catalytic divalent metal cations are shown as purple spheres interacting with the side chains of Asp318, Asp319 (part of the GDD motif), and Asp220. Arg158 hangs from the fingerloop region and facilitates the formation of the phosphodiester link between the growing RNA strand and the incoming NTP. (Arg158 is shown without a surface for the sake of clarity.) Ser282, found to be important to the development of resistance to various 2′-MeNTP-based inhibitors, forms part of the surface against which the incoming NTP must fit when being incorporated into the RNA chain. Tyr448, found in the thumb loop, may help stabilize the formation of the initiation complex by interaction with the primer GTP during de novo initiation. The C-terminal loop (in gray) would necessarily be displaced upon initiation and both it and the thumb loop displaced during elongation.

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3′,5′-phosphodiester linkage. Consequently, the development of nucleoside inhibitors of HCV polymerase that function as chain terminators to block further replication events is a rational strategy in the search for anti-HCV agents. The discovery of nucleoside inhibitors of HCV polymerase has seen the exploration of both carbohydrate and base modifications to identify unique and potent agents. In addition, simple ester prodrugs have been developed to improve the pharmacokinetic properties of selected nucleosides.

For a nucleoside inhibitor of a viral polymerase, the NTP anabolite is the functional intracellular inhibitor. The NTP must be accepted as a substrate for the target polymerase and effect termination of the replication cycle after incorporation into the growing RNA chain. Intracellular kinases are required to convert the nucleoside to its active triphosphate. It is frequently observed in an isolated enzyme assay that a nucleoside triphosphate can inhibit a viral polymerase, and yet in a whole cell system the nucleoside itself is not an inhibitor primarily because phosphorylation is not allowed by one or more of the kinases in the phosphorylation cascade. Therefore, because of the multiple substrate requirements associated with nucleoside phosphorylation and the requirement that the polymerase recognize the nucleoside triphosphate as a substrate, the identification of an in vivo effective nucleoside polymerase inhibitor can be challenging.

Exploration of modifications to both the base and ribose sugar portions of a ribonucleoside identified structural features important to achieving anti-HCV activity both against the isolated NS5B polymerase and in the whole cell replicon assay. Structural modifications at the 3′ position of the nucleoside ribose core highlighted the importance of maintaining a 3′-OH in the α-orientation as necessary to achieving whole cell replicon activity without the occurrence of cytotoxicity.[171,172] Several monosubstitutions (α-F (**1**), α-OCH_3 (**2**), Figure 4) at

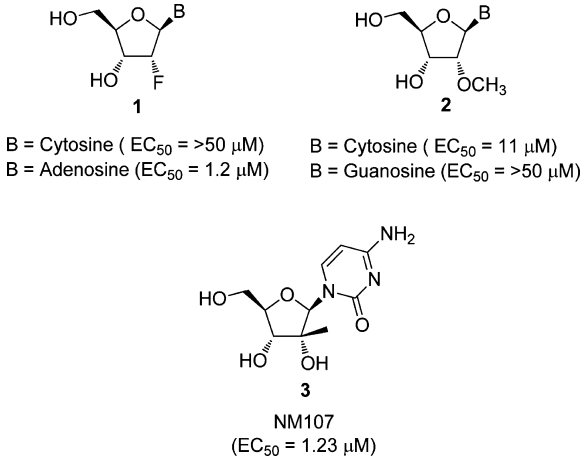

Figure 4. Nucleoside inhibitors of NS5B having 2′-modifications.

the 2′ position were tolerated in combination with an adenine, guanine, or cytosine base; however, for the α-F substitution, activity was only observed against the NS5B polymerase in an isolated enzyme assay and not in the replicon assay or substantial cytotoxicity or cytostatic activity was observed.[132,171,173,174] Uracil or modified uracil bases did not generally deliver active nucleoside inhibitors.[174,175] However, the combination of a 2′-β-*C*-methyl and a 2′-α-OH (**3**, Figure 4) resulted in the identification of a broad class of potent nucleoside inhibitors generally devoid of cytotoxicity.[171,176] In no instance has a 1′-α-nucleoside been reported to show activity as an inhibitor of HCV RNA replication.

Investigation of novel base moieties within the simple furanose class led to the identification of several active inhibitors of HCV (Figure 5). N^4-Hydroxycytidine **4** (EC_{90} =

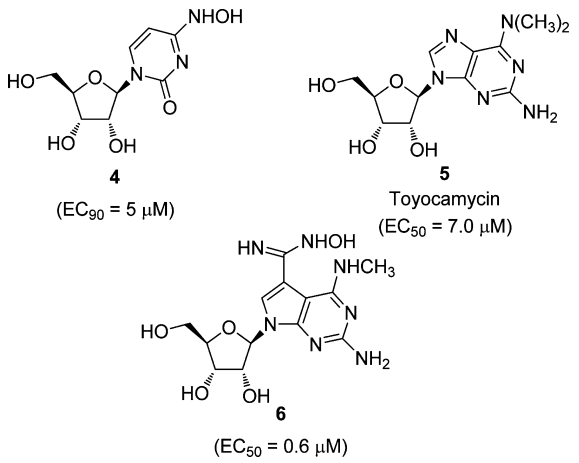

Figure 5. Riboside inhibitors of NS5B with base modifications.

5 μM) was shown to be an alternative substrate inhibitor.[132] A series of 7-deaza-6,7-disubstituted purines derived from **5** (toyocamycin) was identified as having potent activity.[177,178] In particular compound **6** showed submicromolar activity (EC_{50} = 0.6 μM) in the replicon assay. However, none of these compounds have progressed beyond the stage where their initial activity was identified.

Within the 2′-*C*-methyl ribonucleoside class, cytidine, adenosine, and guanosine derivatives have been shown to inhibit the HCV polymerase and HCV RNA replication in the whole cell replicon assay.[171,173,176,179] The 2′-*C*-methylcytidine derivative **3** (NM107, Figure 4) inhibited HCV RNA replication in the whole cell replicon assay (EC_{50} = 1.23 μM), and its triphosphate was shown to be an inhibitor of the HCV polymerase (IC_{50} = 0.09−0.18 μM).[176] Modifications to the pyrimidine base unit, however, did not lead to active analogues. Nucleoside **3** was identified as a nonobligate chain terminator with broad in vitro antiviral activity against flaviviruses initially against bovine virus diarrhea virus (BVDV), yellow fever virus, dengue virus, and West Nile virus and subsequently against HCV.[176,180] It was also shown that the S282T amino acid alteration in the HCV polymerase produced resistance to **3**.[181,182] In addition, animal pharmacokinetic studies raised concerns about poor oral bioavailability. Consequently, the 3′-*O*-valinyl ester prodrug **7** (NM283, valopicitabine) was developed with the hope of taking advantage of peptide transporter systems.[176,183] Prodrug **7** was shown to have an apparent oral bioavailability of 34% in rats and was subsequently progressed into human clinical trials.[176] In a monotherapy trial, **7** demonstrated proof of concept by delivering approximately $-1.2\ \log_{10}$ IU/mL reduction in HCV viral load when administered orally at 800 mg twice daily over 14 days. In a phase 2b study in HCV infected patients, when **7** was administered at 800 mg twice daily in combination with SOC over 28 days, a $>4\ \log_{10}$ IU/mL reduction in viral load was observed; however, clinical studies were eventually discontinued because of significant gastrointestinal toxicity.[169,184]

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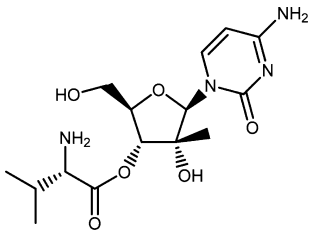
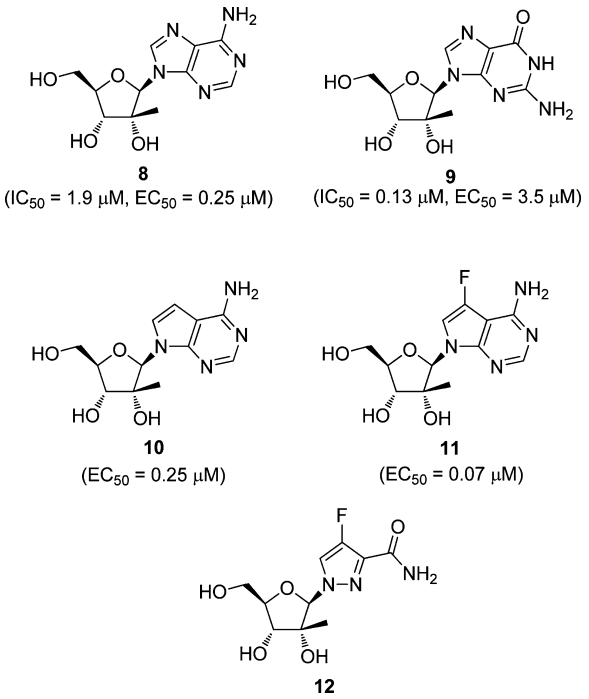


7

NM283

Further study of the 2′-*C*-methyl ribonucleosides showed that purine bases were compatible with the 2′-*C*-methylribose system (Figure 6).[171] Preparation of adenosine **8** (IC_{50} = 1.9 μM, EC_{50} = 0.26 μM) and guanosine **9** (IC_{50} = 0.13 μM, EC_{50} = 3.5 μM) derivatives produced inhibitors of the HCV polymerase and inhibitors of HCV RNA replication in the replicon assay; however, the activity of the guanosine derivative was limited by poor conversion to the active triphosphate.[171,179] For the adenosine derivative the observed poor oral bioavailability was attributed to metabolic deamination and purine nucleoside phosphorylase (PNP) conversion. To overcome these limitations, modifications to the purine base unit were investigated leading to the identification of a series of 7-deazaadenosine derivatives **10** showing potent activity in the HCV replicon assay with the most potent and least cytotoxic analogue being the 7-deaza-7-fluoroadenosine derivative **11** (EC_{50} = 0.07 μM).[179] Again, 2′-*C*-methyl nucleoside **10** suffered a significant loss of activity with the S282T amino acid alteration in the HCV polymerase. In vivo **11** was shown to have an oral bioavailability in the rat of 51%.[179] Several other 7-deazaadenosine derivatives with substitution at the C-6 and C-7 positions of the base were reported as inhibitors of HCV RNA replication.[179,185] By contrast the 7-deazaguanosine derivatives studied were not active in the replicon assay even though their triphosphates were shown to be inhibitors of the



8 (IC_{50} = 1.9 μM, EC_{50} = 0.25 μM)

9 (IC_{50} = 0.13 μM, EC_{50} = 3.5 μM)

10 (EC_{50} = 0.25 μM)

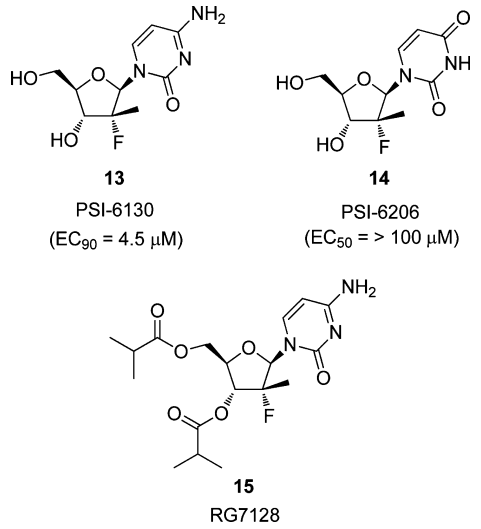
11 (EC_{50} = 0.07 μM)

12

Figure 6. 2′-*C*-Methylpurine riboside analogues.

NS5B polymerase.[186] Several 4-fluoropyrazole-3-carboxamide purine base mimetics **12** were also evaluated and were shown not to inhibit HCV RNA replication.[187] In vivo evaluation of the 2′-*C*-methyl-7-deazaadenosine derivative **10** (MK-0608) in a HCV infected chimpanzee animal model showed that the compound gave a robust antiviral effect after oral dosing.[188] In one chimp doses of 1 mg/kg produced a −4.6 $\log_{10}$ IU/mL reduction in viral load after 37 days of dosing, and another chimpanzee's viral load fell below the limit of quantitation at day 2 of dosing and remained there for 12 days after dosing ended. Subsequent in vivo chimpanzee studies intended to assess the effect of combining two direct acting antivirals, **10** and the HCV protease inhibitor (1′*R*, 21′*S*,24′*S*)-21′-*tert*-butyl-*N*-((1*R*,2*R*)-1-{[(cyclopropylsulfonyl)-amino]carbonyl}-2-ethylcyclopropyl)-3′,19′,22′-trioxo-2′,18′-dioxa-4′,20′,23′-triazaspiro[cyclpropane-1,16′-tetracyclo-[21.2.1.^{4,7}.0^{6,11}]-heptacosane]-6′,8′,10′-triene-24′carboxamide (MK-7009),[189] achieved an SVR in one of three treated animals with viral load levels below the limit of quantitation 3 years after study completion, thus demonstrating the possibility of achieving SVR in the absence of interferon treatment.[190] However, no further developments regarding **10** have been reported.

For 2′-*C*-methyl nucleosides, replacement of the α-hydroxyl group with a 2′-α-fluoro group resulted in identification of a class of nucleoside HCV inhibitors with unique in vitro and in vivo clinical characteristics (Figure 7).[191−193] Base variations on



13

PSI-6130

(EC_{90} = 4.5 μM)

14

PSI-6206

(EC_{50} = > 100 μM)

15

RG7128

Figure 7. 2′-α-*F*-2′-β-*C*-Methylpyimidine nucleoside inhibitors of NS5B.

the 2′-α-*F*-2′-β-*C*-methylribose sugar showed that the cytosine derivative **13** (Figure 7) (PSI-6130, EC_{90} = 4.5 μM) produced the most potent compound and the uridine, adenosine, and guanosine derivatives were either weakly active or inactive in the replicon assay.[191,194] These results contrast with those observed for the 2′-*C*-methylpurine derivatives **8** and **9**.[171,179] In addition, similar to the 2′-*C*-methylribonucleoside series, modifications at 2′ and 3′, stereochemical changes at 2′ and 3′, and carbocyclic analogues did not produce significantly active inhibitors of HCV RNA replication.[193] It was later shown that the weak activity for the uridine, adenosine, and guanosine derivatives was derived from poor phosphorylation in the first step toward triphosphate formation.[195−197] This was supported by the observation that the triphosphates of the uridine, adenosine, and guanosine derivatives were shown to be potent

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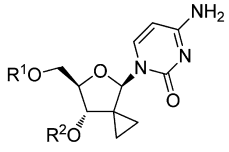
inhibitors of the NS5B polymerase in an isolated enzyme assay. Unlike the 2′-*C*-methycytidine **3**, the 2′-*α*-*F*-2′-*β*-*C*-methylcytidine nucleoside **13** demonstrated exquisite selectivity for HCV over other flaviviruses and other RNA viruses as well as for HIV and HBV. Nucleoside **13** also showed broad genotype coverage by inhibiting the HCV polymerase from genotypes 1−6.[192] The known 2′-*C*-methyl nucleoside S282T amino acid alteration in the HCV polymerase was shown to be only 3-fold resistant to **13**.[198] In clinical studies, **13** demonstrated modest oral bioavailability with a significant amount of the drug being metabolized to the inactive uridine metabolite **14**.[193] An ester prodrug approach was implemented to address these shortcomings, leading to the clinical candidate 3′,5′-di-*O*-isobutyrate ester prodrug **15** (RG7128, mericitabine).[193]

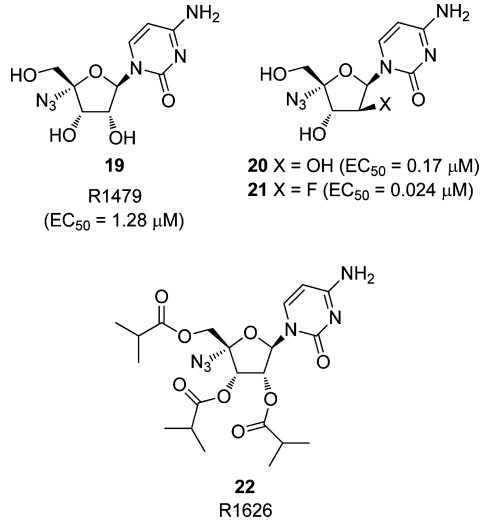
Nucleoside prodrug **15** demonstrated clinical proof of concept when administered as monotherapy or in combination with SOC.[169,184,193] A twice daily 1500 mg dose of **15** resulted in a maximal −2.7 $\log_{10}$ IU/mL viral load reduction after 14 days of therapy. Subsequent phase 2a studies were conducted in both genotype 1 and genotypes 2 and 3 patients in combination with SOC over 28 days. In these studies rapid virological response (RVR) rates of 85−90% were observed with viral load reductions of −5 $\log_{10}$ IU/mL and no reported significant adverse events. No viral breakthroughs were observed during the course of therapy nor were there any S282T viral variants identified in the treatment groups. These data supported the high barrier to resistance and the broad genotype coverage anticipated by preclinical results. Further phase 2b studies demonstrated SVR at 12 weeks after treatment.[199,200] In addition, a proof of concept PEG-IFN sparing study was undertaken in which the combination of **15** and an NS3 protease inhibitor (RG7227, danoprevir)[201] was administered to genotype 1 HCV patients.[170] Over 14 days in the absence of INF and RBV this DAA combination showed viral load declines of −4.9 to −5.1 $\log_{10}$ IU/mL, thus demonstrating for the first time the potential of an PEG-IFN free DAA combination for future HCV therapy in humans. The nucleoside prodrug **15** is expected to enter phase 3 studies in the near future.

Further exploration of 2′-substituted nucleosides resulted in the identification of 2′-deoxy-2′-spirocyclopropylcytidine **16** (TMC647078) as an inhibitor of HCV NS5B polymerase.[202,203] 2′-Deoxy-2′-spirocyclopropylcytidine **16** was determined to be a nonobligate chain terminator having EC_{50} = 7.3 μM with no cytotoxicity. Compound **16** exhibited broad genotype coverage against nongenotype 1 chimeric replicons and showed reduced potency against the S282T mutant replicon with a 39-fold increase in EC_{50}. In cell culture compound **16** was extensively metabolized to the uridine metabolite. Because **16** produced low plasma concentrations in a rat PK study, the 3′-isobutyrate ester (**17**) and 3′,5′-diisobutyrate ester (**18**) prodrugs were prepared. Prodrugs **17** and **18** were shown to have reduced activity in the whole cell replicon assay (EC_{50} of 42.8 and 20.3 μM, respectively) but produced a 10- and 24-fold increase in exposure, respectively.



16 R^1, R^2 = H, TMC647078 (EC_{50} = 7.3 μM)
17 R^1 = H, R^2 = C(O)CH(CH_3)$_2$ (EC_{50} = 42.8 μM)
18 R^1, R^2 = C(O)CH(CH_3)$_2$ (EC_{50} = 20.3 μM)

A series of 4′-substituted ribonucleosides having any one of four naturally occurring nucleic acid bases and azido, azidomethyl, ethynyl, allyl, vinyl, alkyl, and alkoxy 4′-substitutents were screened as inhibitors of HCV replication.[204−206] The 4′-azidocytidine derivative **19** (R1479, Figure 8) was identified



19
R1479
(EC_{50} = 1.28 μM)

20 X = OH (EC_{50} = 0.17 μM)
21 X = F (EC_{50} = 0.024 μM)

22
R1626

Figure 8. 4′-Azidocytidine nucleoside inhibitors of NS5B.

as the only active nucleoside (EC_{50} = 1.28 μM) that did not also exhibit cytotoxic or cytostatic behavior and was shown to be a chain terminator of RNA synthesis.[204,205,207] The S96T amino acid change in the NS5B polymerase produced a 4- to 5-fold resistance to the activity of **19**.[208] Several other 4′-substituted ribonucleosides having either a uridine base or a 4′-ethynyl group were shown to be inhibitors of the NS5B polymerase as their triphosphate derivatives in the isolated enzyme assay, indicating that a blocked phosphorylation path was contributing to their lack of whole cell replicon activity.[204] Subsequent investigation of 2′-*β*-substituted-4′-azidocytidine derivatives demonstrated that the 5′-triphosphates of both the 2′-*β*-hydroxyl **20** (IC_{50} = 0.24 μM) and 2′-*β*-fluoro **21** (IC_{50} = 0.51 μM) derivatives were potent inhibitors of the NS5B polymerase and the nucleosides themselves were also inhibitors of HCV RNA replication in the whole cell replicon assay (**20**, EC_{50} = 0.17 μM; **21**, EC_{50} = 0.024 μM).[205,209] Both **20** and **21** were shown to be potent inhibitors of replicon variants carrying the S96T or S282T point mutations in NS5B, thus showing no apparent cross-resistance with 4′-azidocytidine and 2′-*β*-methylcytidine. In a 2-week oral range finding rat toxicity study of the 2′-*β*-hydroxy derivative **20**, a no observed effect level (NOEL) of 2000 mg kg^{-1} day^{-1} was reported.

Further in vivo assessment of the 4′-azidocytidine derivative **19** revealed that its absorption was limited.[210] Consequently, a series of ester prodrugs was evaluated to improve the oral bioavailability of **19**. The physical chemical properties, Caco2 permeability, and PK in rats and monkeys of these prodrugs were assessed, and the 2′,3′,5′-triisobutyrate ester prodrug **22** (R1626, balapiravir) was selected for clinical development.[210,211] Preparation of this triisobutyrate ester prodrug increased oral bioavailability by >5-fold over **19**. The pharmacokinetics of **22** was evaluated in healthy male volunteers and showed that it was extensively converted to the nucleoside **19** as desired. Subsequent phase 1b studies in HCV genotype 1 patients receiving 500−4500 mg twice daily

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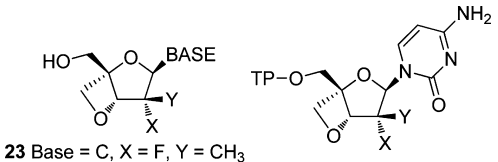
of **22** produced mean viral load reductions of −0.3 to −3.7 $\log_{10}$ IU/mL after 14 days.[184] Prodrug **22** was then taken into a phase 2a study in genotype 1 treatment naive patients at a dose of 1500 mg twice daily in combination with PEG-IFN/RBV. This combination with SOC demonstrated a −5.2 $\log_{10}$ IU/mL reduction in viral load and an 81% RVR after 28 days on therapy. Unfortunately, development of **22** was discontinued as a result of significant hematological adverse events observed in a phase 2b study.[212]

A series of 3′,4′-oxetane nucleosides was explored as inhibitors of HCV replication with the idea that preparing a conformationally constrained version of known 4′-substituted nucleosides might lead to active inhibitors (Figure 9).[213] In this



23 Base = C, X = F, Y = CH_3
24 Base = C, X = F, Y = H
25 Base = C, X = H, Y = F
26 Base = C, X = F, Y = F
27 Base = C, X = OCH_3, Y = H
28 Base = A, X = F, Y = H

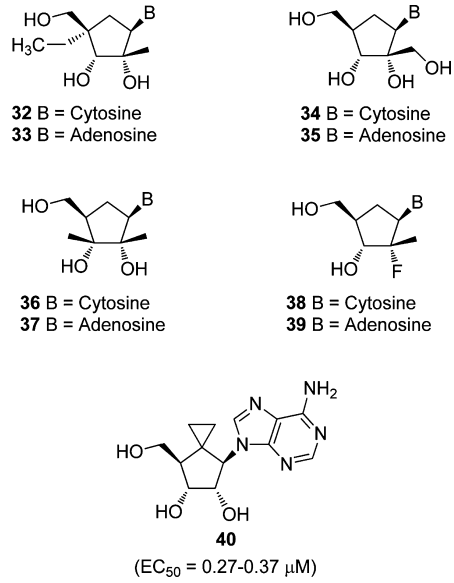
29 X = F, Y = H
30 X = H, Y = F
31 X = F, Y = F
TP = triphosphate

Figure 9. 3′,4′-Oxetane nucleoside NS5B inhibitors.

series a variety (**23**−**28**) of 2′-substituents (α- or β-F, di-F, α-OCH_3, α-F-β-CH_3) with cytosine and adenine bases was investigated. In no case did the 3′,4′-oxetane nucleosides exhibit whole cell inhibition of HCV replication; however, several of the corresponding cytidine triphosphate derivatives (**29**−**31**) did show modest inhibition of the HCV polymerase in the isolated enzyme assay. It was ultimately determined that although these compounds were substrates for deoxycytidine kinase (dCK) and formed the desired 5′-monophosphates, the 5′-monophosphates were not substrates for the uridine monophosphate−cytidine monophosphate kinase (UMP-CMP) needed to produce the required diphosphates on the way to the active triphosphates, thus highlighting the importance of a 3′-hydroxyl group in kinase recognition.

When the oxygen atom of the furanose ring is replaced by a methylene group, the glycosidic bond of a carbocyclic nucleoside is known to be resistant to both nucleoside phosphorylase and nucleoside hydrolase and therefore renders the nucleoside more metabolically stable. Therefore, to explore the potential of carbocyclic nucleosides as anti-HCV agents, a number of 2′- and 2′,3′- and 2′,4′-substituted carbocyclic nucleosides (**32**−**39**) were prepared (Figure 10).[214−216] However, none of these agents demonstrated anti-HCV activity in the replicon assay without the occurrence of substantial cytotoxicity. A novel cyclopropylspirocarbocyclic adenosine nucleoside **40** did show potent anti-HCV activity in a genotype-1 replicon assay (EC_{50} = 0.27−0.37 μM) with no observed cytotoxicity.[217] Compound **40** also showed some selectivity for inhibiting HCV over other viruses.

Incorporation of a heteroatom (O or S) in the place of the 3′-hydroxymethylene of a natural nucleoside was demonstrated to be a successful strategy in the development of potent antiviral agents. The 1,3-oxathiolane cytosine nucleoside lamivudine (3TC) is an efficacious anti-HIV and anti-HBV agent, and 1,3-dioxolanes have been shown to have broad antiviral activity. In an attempt to translate the favorable impact on anti-HCV



32 B = Cytosine
33 B = Adenosine

34 B = Cytosine
35 B = Adenosine

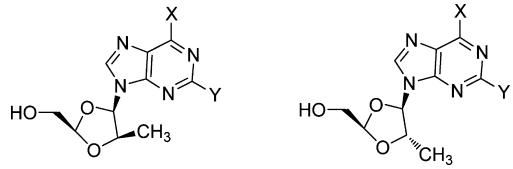
36 B = Cytosine
37 B = Adenosine

38 B = Cytosine
39 B = Adenosine

40
(EC_{50} = 0.27-0.37 μM)

Figure 10. Carbocyclic nucleoside inhibitors of NS5B.

activity of incorporating a 2′-methyl substitutent into ribonucleosides, a series of 5-methyl substituted 1,3-dioxolanes (**41**−**44**, Figure 11) having purine bases was prepared.[218]



41 X = OH, Y = NH_2
42 X = Y = NH_2

43 X = OH, Y = NH_2
44 X = Y = NH_2

Figure 11. Dioxolane nucleoside mimetic inhibitors of NS5B.

However, none of these compounds exhibited any anti-HCV activity in the replicon assay.

■ NUCLEOTIDE PRODRUG INHIBITORS

Since nucleosides must be converted intracellularly to their 5′-triphosphate derivatives to be active inhibitors of viral polymerases, many nucleosides fail to demonstrate activity in whole cell assays because they are poor substrates for one or more of the kinases in the phosphorylation cascade. To identify if phosphorylation is a potential problem, the 5′-triphosphate of a nucleoside is evaluated against the target polymerase in an isolated enzyme assay. In most cases it is the formation of the monophosphate by the first kinase in the cascade that is the problematic step. This is because this first kinase is the most discriminating of the three kinase enzymes. Therefore, in many cases bypassing the first phosphorylation step by delivering the 5′-monophosphate can result in achieving high intracellular levels of active triphosphate. However, nucleoside 5′-monophosphates are enzymatically dephosphorylated and negatively charged; consequently, they do not enter cells and are unstable making them undesirable as drug candidates. To overcome the problem of delivering a nucleoside monophosphate, nucleoside 5′-monophosphate prodrug strategies have been developed.[195,219−221] For the success of these prodrugs in HCV therapy, prodrugs of nucleoside 5′-monophosphates need to display several characteristics. They must have sufficient

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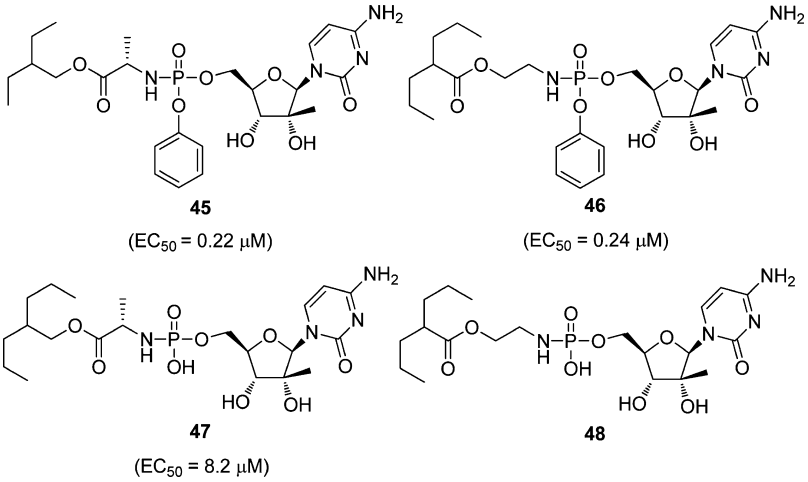

Figure 12. 5′-Phosphoramidate prodrugs of 2′-*C*-methylcytidine inhibitors of NS5B.

chemical stability to be formulated for oral administration and then be stable to conditions of the gastrointestinal tract in order to reach the site of absorption. The prodrug must be efficiently absorbed and be able to reach the liver intact after which intracellular hepatic enzymes unmask the 5′-monophosphate group, allowing further metabolism to the active triphosphate. Since HCV is a liver disease, several prodrug strategies have successfully leveraged first pass metabolism and liver metabolizing enzymes to achieve liver targeting. A number of these prodrugs have also demonstrated proof of concept in the clinic.

One prodrug construct that has been extensively employed to deliver nucleoside 5′-monophosphates to treat HCV infection is the 5′-phosphoramidate.[195] The phosphoramidate prodrug approach has been used to address several issues limiting the development of various nucleosides including poor phosphorylation and therefore lack of potency, unfavorable therapeutic index, and lack of desired liver to plasma ratio. First disclosed by McGuigan for delivering nucleotides to treat HIV and cancer, this phosphate prodrug construct masks the phosphate group by appending an aryloxy group and an amino acid ester.[222] In doing so, it increases lipophilicity of the nucleotide 5′-monophosphate, thus increasing cellular permeability. Unmasking of the phosphoramidate relies on a series of enzymatic and chemical steps. Phosphoramidate cleavage to the desired monophosphate is initiated by either carboxyesterase or cathepsin A to remove the ester of the amino acid moiety. After a chemical cyclization step followed by enzymatic removal of the amino acid unit by a phosphoramidase or histidine triad nucleotide-binding protein 1 (HINT1), the nucleoside 5′-monophosphate is fully revealed. These phosphoramidates contain a chiral center at phosphorus, and upon synthesis with existing methodology a diastereomeric mixture of compounds is generated, a potential issue in the development of any such prodrug.

Other prodrug strategies employed to deliver 5′-monophosphate nucleoside prodrugs for treating HCV include 3′,5′-cyclic phosphates and phosphoramidates, cyclic 1-aryl-1,3-propanyl phosphate esters (HepDirect),[223] and bis(*S*-acyl-2-thioethyl) phosphate esters (SATE).[219] 3,5-Cyclic phosphates and phosphoramidates have rarely been used to deliver nucleoside 5′-monophosphates; however, their application for the delivery of nucleotides for HCV has shown great promise.[195] Yet little has been reported about the cleavage mechanism to release the free 5′-monophosphate. HepDirect prodrugs were specifically developed to deliver agents to the liver.[223] These prodrugs are released by a cytochrome P450 mediated mechanism and produce an aryl vinyl ketone as a byproduct which clearly raises toxicity concerns. Bis(*S*-acyl-2-thioethyl) phosphate esters, also known as SATE prodrugs, have been extensively explored for the delivery of nucleoside 5′-monophosphates.[219] SATE prodrugs release the promoiety via an initial ester hydrolysis followed by expulsion of the free nucleoside 5′-monophosphate through liberation of episulfide, another byproduct with potential toxicity concerns. Each of these prodrug strategies has demonstrated success in delivering nucleoside 5′-monophosphates in the whole cell HCV replicon system and in vivo in animal studies. Applications of several of these prodrugs strategies also have shown human clinical proof of concept in delivering nucleoside 5′-monophosphates into hepatocytes.[195]

In an attempt to address several limitations associated with nucleoside **3** such as modest potency because of poor conversion of the nucleoside to its triphosphate and unfavorable therapeutic index that resulted in clinical adverse events, phosphoramidate prodrugs of the 5′-monophosphate were developed (Figure 12).[224,225] Phosphoramidate **45** showed significantly improved potency ($EC_{50} \leq 0.5$ μM) relative to simple 3′-valinate ester prodrug **7** and also showed increased levels of active triphosphate in human hepatocytes.[224] However, this improvement did not translate in vivo where only a 2-fold increase in liver triphosphate levels was observed in the hamster, attributed to low oral bioavailability or metabolic degradation.

Another attempt at addressing the issues with 2′-methycytidine **3** was the investigation of the acyloxyethylamino phosphoramidate promoiety.[225] The resulting phosphoramidate prodrug **46** did improve potency in the replicon assay by 30-fold and demonstrated increased levels of triphosphate in human hepatocytes; however, no improvement of liver triphosphate production in vivo was observed thus calling into question the oral bioavailability and stability of these prodrugs. The phosphoramidate monoesters of **45** and **46** were also explored. Surprisingly, although phosphoramidate monoesters **47** and **48** had inferior replicon potency (e.g., **47**, $EC_{50} = 8.2$ μM), they showed higher levels of nucleoside triphosphate in human hepatocytes and achieved liver levels of triphosphate when dosed subcutaneously but not after oral administration.

A major cellular metabolite of the 2′-α-F-2′-β-C-methylcytidine nucleoside **13** was identified as the uridine derivative **14** (PSI-6206).[196,226] This uridine derivative was inactive as an inhibitor of HCV replication; however, its triphosphate was a potent inhibitor of the NS5B polymerase (IC_{50} = 1.19 μM) and exhibited a long intracellular half-life of 36 h. A phosphoramidate prodrug approach was investigated to address the enzymatic blockade at the monophosphorylation step and to see if the long intracellular triphosphate half-life would translate into once a day dosing in the clinic (Figure 13).[227,228] SAR

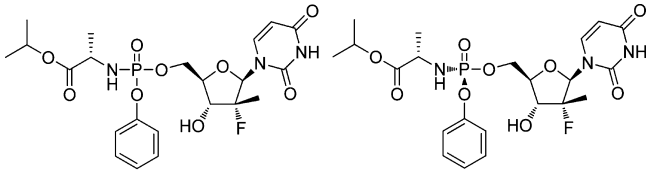

Figure 13. 2′-α-F-2′-β-C-Methyluridine 5′-phosphoramide inhibitors of NS5B.

around the uridine phosphoramidate showed that a small branched alkyl group on the terminal carboxylic acid ester moiety, a small alkyl group at the α position of the amino acid unit, and a simple phenoxy or halogenated phenoxy phosphate ester group were preferred to achieve submicromolar activity without cytotoxicity. Stability studies in simulated gastric fluid, simulated intestinal fluid, blood, and liver S9 fractions followed by evaluation of whole cell triphosphate levels in human hepatocytes and triphosphate liver levels in rats, dogs, and monkeys dosed orally showing high levels of desired nucleoside triphosphate ultimately led to the selection of **49** (PSI-7851, EC_{90} = 0.52 μM),[227] a 1:1 mixture of diastereomers, as a development candidate. When **49** was combined with PEG-IFN and/or RBV or other DAAs that included NS3/4 protease inhibitors, non-nucleoside polymerase inhibitors, and NS5A inhibitors, synergistic or additive effects were observed in the replicon assay.[229] Like other 2′-methyl nucleosides, the S282T mutant replicon was determined to be resistant (16-fold) to **49**.[229] In a phase 1 clinical study **49** was shown to be generally safe and well tolerated at doses up to 800 mg q.d. and PK results indicated low plasma exposure to prodrug **49** with a profile depicting rapid uptake of the drug by the liver.[193] In a 3-day multiple ascending dose monotherapy study in genotype 1 HCV infected patients, HCV RNA levels declined in a dose dependent manner with maximal mean change from baseline of −1.95 log_{10} IU/mL at the 400 mg dose.[193] No treatment emergent viral resistance was observed nor was there evidence of viral resistance after therapy. Further development of 2′-α-F-2′-β-C-methyuridine phosphoramidates proceeded with the single *Sp* diastereomer **50** (PSI-7977, EC_{90} = 0.42 μM)[227] which was obtained by selective crystallization from the mixture and was shown to be 10-fold more active than the *Rp* isomer.[227] The single diastereomer **50** was evaluated in a 14-day monotherapy study in genotype 1 patients and achieved a −5.0 log_{10} IU/mL reduction in viral load with 85% of patients achieving undetectability, thus showing a significant improvement over the diastereomeric mixture **49**.[230] A 28-day phase 2a study in combination with PEG-IFN/RBV produced a maximum RVR rate of 93% with no viral resistance and was followed by a phase 2b study in genotypes 1, 2, and 3 patients.[231,232] In the phase 2b study RVR rates of 98% at both the 200 and 400 mg q.d. doses were achieved at week 12 with no viral breakthrough.[233] For the genotypes 2 and 3 patients a 96% SVR rate was achieved 12 weeks after therapy. Studies assessing shorter treatment durations in addition to PEG-IFN free regimens that include combinations with the nucleotide inhibitor PSI-352938 (**80**),[234] the NS5A inhibitor ((*S*)-1-{(*S*)-2-[5-(4′-{2-[(*S*)-1-((*S*)-2-methoxycarbonylamino-3-methylbutyryl)pyrrolidin-2-yl]-3*H*-imidazol-4-yl}biphenyl-4-yl)-1*H*-imidazol-2-yl]pyrrolidine-1-carbonyl}-2-methylpropyl)-carbamic acid methyl ester (BMS-790052),[13] and the protease inhibitor cyclopropanesulfonic acid {(*Z*)-(1*R*,4*R*,6*S*,15*R*,17*R*)-17-[2-(4-isopropylthiazol-2-yl)-7-methoxy-8-methylquinolin-4-yloxy]-13-methyl-2,14-dioxo-3,13-diazatricyclo[13.3.0.$0^{4,6}$]-octadec-7-ene-4-carbonyl}amide (TMC-435)[235] are under investigation.[195]

In an attempt to circumvent some of the toxicity issues observed with the 4′-azidocytidine clinical candidate **22** (Figure 8), a phosphoramidate prodrug of the 5′-monophosphate of the parent nucleoside **19** was prepared.[236] The possibility of enhanced potency by increasing the level of intracellular triphosphate levels and liver targeting to reduce systemic exposure was envisioned to possibly ameliorate the liabilities observed with **22**. Extensive SAR analysis around the phosphoramidate moiety led to prodrugs such as **51**.[236] Unfortunately, these prodrugs did not show improved potency relative to **19**, thus supporting the conclusion that **19** was efficiently phosphorylated. However, preparation of a phosphoramidate prodrug of 4′-azidouridine 5′-monophosphate was shown to be successful in improving the activity of this uridine derivative (Figure 14).[237] Even though the 4′-azidouridine 5′-triphosphate inhibited the HCV NS5B polymerase (IC_{50} = 0.22 μM), its parent nucleoside **52** was not active at inhibiting HCV replication in the whole cell replicon assay. Preparation of phosphoramidate prodrugs ultimately led to active inhibitors of HCV replication with the most potent compound **53** (Figure 14)

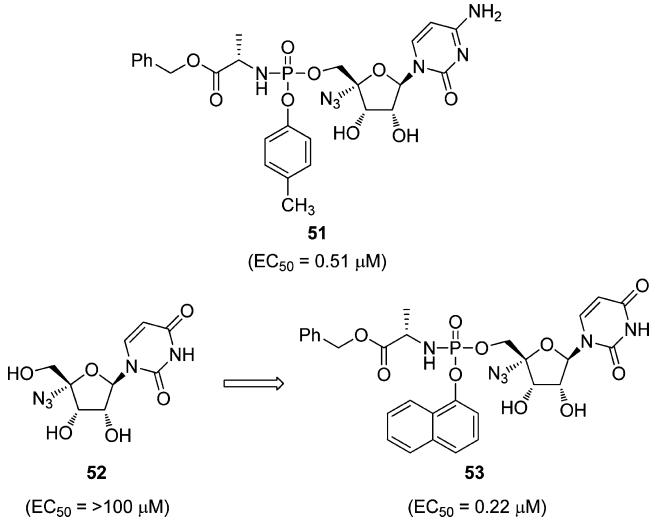

Figure 14. 4′-Azidouridine 5′-phosphoramidate prodrugs of NS5B.

having an EC_{50} of 0.22 μM.[237] Further development of these 4′-azidouridine 5′-phosphoramidates has not been reported.

In a continuing attempt to improve on the activity of 2′-C-methylcytidine **3**, preparation of a series of 3′,5′-cyclic

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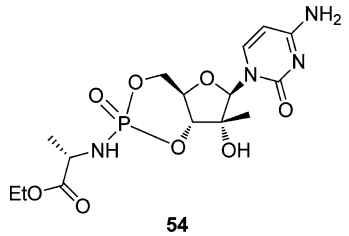
phosphoramidates was undertaken.[238] The expectation was that by reducing the rotational degrees of freedom, the 3′,5′-cyclic phosphoramidate would show enhanced cell penetration and therefore improved activity over the 5′-acyclic phosphoramidate. However, none of the cyclic phosphoramidates (e.g., **54**) demonstrated improved activity over the parent nucleoside **3** even though several of these cyclic prodrugs did produce improved levels of nucleoside triphosphate when incubated with primary human hepatocytes (2- to 7-fold). Oral bioavailability was also lacking for these agents as demonstrated by low liver levels of the nucleoside triphosphate.

HepDirect prodrug technology was investigated for the delivery of 2′-*C*-methylcytidine. To accomplish this, SAR around the aryl group of the prodrug moiety was studied (Figure 15).[239] Evaluation of both nucleoside triphosphate levels in rat hepatocytes as well as levels in rat liver after intraperitoneal and oral dosing showed that the 3,5-difluorophenyl substitution produced the highest triphosphate levels. After demonstrating that the *S*-diastereomer of the prodrug moiety was the more potent, compound **56** was taken into HCV-infected chimpanzees.[239] Oral administration at 10 mg/kg once daily produced approximately −1.3 and −1.5 $\log_{10}$ IU/mL reductions in viral load in two animals. Intravenous administration of **56** at 4 mg/kg over 6 days resulted in −4.8 and −3.6 $\log_{10}$ IU/mL decline in viral load with viral load in one animal reaching the lower limit of the assay on day 2. This result compares favorably to the approximately −1.0 $\log_{10}$ IU/mL reduction in viral load reported for the simple valinate ester prodrug **7** when administered to chimpanzees at 16 mg/kg.

In the case of 2′-*O*-methylcytidine derivatives or 3′-deoxycytidine, activity and intracellular nucleoside triphosphate production seemed to be limited by either poor phosphorylation or nucleoside degradation through either deamination of the base or demethylation of the 2′-*O*-methyl. Preparation of the 5′-SATE prodrug derivatives led to compounds **58** and **59** which demonstrated a 7- to 25-fold improvement in replicon potency and a 3- to 10-fold increase in intracellular triphosphate formation relative to the parent nucleosides.[172,173]

For purine nucleosides, 5′-monophosphate prodrugs of both adenosine and guanosine analogues were explored to overcome the lack of potency observed in the whole cell replicon assay and other metabolic deficiencies. Significant success was realized in the development of phosphate prodrugs of guanosine derivatives, several of which have progressed into

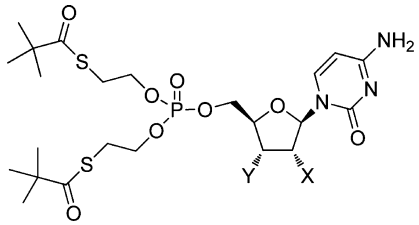


54

(EC_{50} = 4 μM)

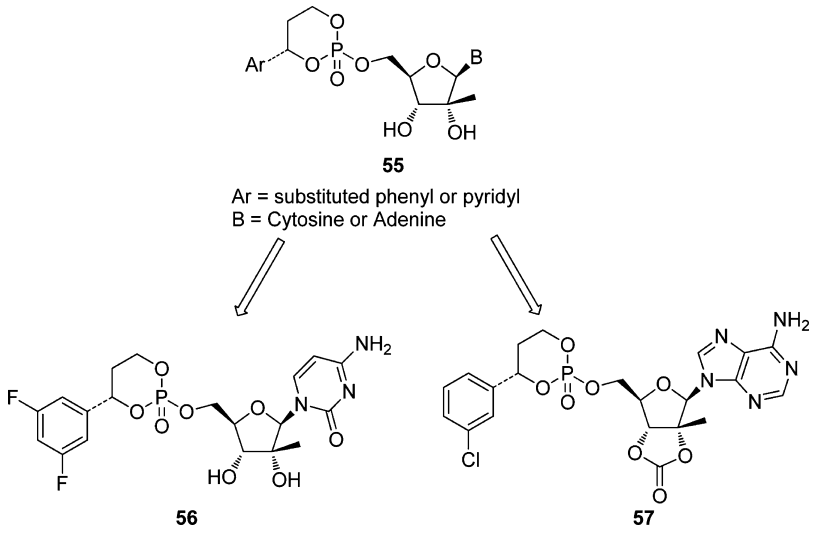
clinical development.[195,240,241] Unlike 5′-phosphate prodrugs of guanosine derivatives, no adensosine derivatives have progressed beyond the preclinical stage.

The development of a number of adenosine nucleoside analogue inhibitors of HCV has been hampered by several factors that include weak potency and deamination of the adenosine base by adenosine deaminase which limits their potential in vivo. Thus, it was hypothesized that a 5′-monophosphate prodrug could



58 X = OCH_3, Y = OH (EC_{50} = 3.2 μM)
59 X = OH, Y = H (EC_{50} = 1.4 μM)

increase potency by accomplishing kinase bypass, improve cell penetration, and block deamination. Preparation of the phosphoramidate prodrug **60** (EC_{50} = 0.25 μM) of 2′-*C*-methyladenosine did not deliver improved activity over the parent nucleoside **8**.[242] For 2′-*α*-*F*-2′-*β*-*C*-methyl-7-ethynyl-7-deazaadenosine **61** (Figure 16) reported to have low replicon activity (EC_{50} = 24 μM), a series of phosphoramidate prodrugs **62** were prepared and yet no antiviral activity was observed with these prodrugs.[185] However, use of the bis-SATE prodrug strategy to deliver 2′-*α*-*F*-2′-*β*-*C*-methyl-7-ethynyl-7-deazaadenosine



55

Ar = substituted phenyl or pyridyl
B = Cytosine or Adenine

56 **57**

Figure 15. HepDirect prodrugs of 2′-*C*-methylcytidine 5′-monophosphate and 2′-*C*-methyladenosine 5′-monophosphate.

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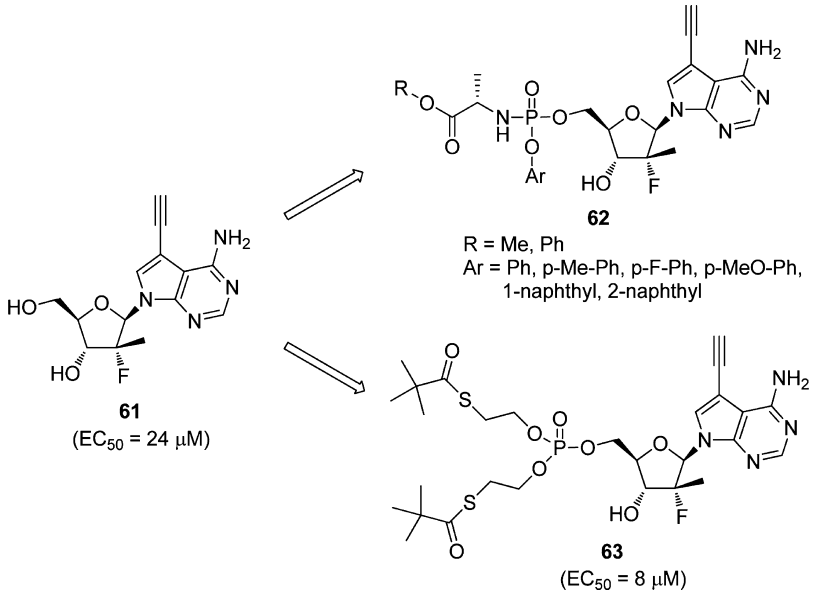
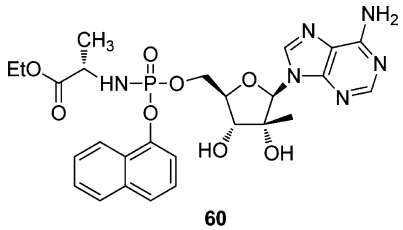


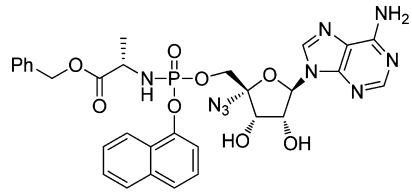
Figure 16. Phosphoramidate and SATE prodrugs of 2′-*α*-*F*-2′-*β*-*C*-methyl-7-ethynyl-7-deazaadenosine.

5′-phosphate led to **63** (EC_{50} = 8 μM) and did result in a 3-fold improvement in replicon activity relative to the parent nucleoside, but no additional data have emerged on this molecule.[185] In the case of 4′-*C*-azidoadenosine, where the nucleoside was shown to be inactive in the HCV replicon assay possibly because of poor monophosphorylation and deamination of the adenine base, a series of phosphoramidate prodrugs of 4′-*C*-azidoadenosine 5′-monophosphate led to improved activity (e.g., **64**, EC_{50} = 0.22−0.59 μM) with no cytotoxicity; however, no further development of these compounds was reported.[243]

3′,5′-Cyclic phosphate prodrugs **66** and **67** of a 2′-*C*-methyladenosine derivative **65** having a hydrazidosulfonamide at the C-6 position of the base were also investigated as inhibitors of HCV replication (Figure 17).[244] It is assumed that the C-6 hydrazidosulfonamide group would be metabolized to produce the natural adenine base. In this cyclic phosphate series the cyclic monophosphate **66** and SATE **67**, pivaloyloxymethyl (POM), and carbonate cyclic phosphate esters were prepared. It was speculated that cyclization of the monophosphate would reduce polarity and enhance cell penetration. Only weak inhibition was identified for the cyclic monophosphate. In each phosphate ester case active HCV inhibitors (EC_{50} = 0.039−0.46 μM) were produced with low cytotoxicity (CC_{50} > 50 μM), the most potent being the SATE derivative **67**; however, further development of these inhibitors has not been reported.



Another application of a 3′,5′-cyclic-SATE ester prodrug of 2′-*C*-methyladenosine entailed the application to a 4′-substituted carbocyclic nucleoside **68** and employed a phosphonate that would ultimately produce a stable form of



the nucleoside 5′-monophosphate (Figure 18).[245] The resulting 2′-*C*-methyladenosine 3′,5′-cyclic phosphonate SATE prodrug **69** (EC_{50} = 27.6 μM) was only modestly active in the HCV replicon assay, was <3-fold more potent than the phosphonate product it was intended to deliver, and was >14-fold less active than 2′-*C*-methyadenosine **8**.

Application of the HepDirect prodrug technology to deliver 2′-*C*-methyladenosine 5′-monophosphate had limited success.[246] SAR around the aryl group of the prodrug moiety favored halogenated aryl or pyridyl groups to achieve optimal activation to produce the nucleoside triphosphate in rat hepatocytes. However, oral bioavailability in the rat was only 5%, which prompted masking of the 2′- and 3′-hydroxyl groups to produce the 3′-monovalenate and 2′,3′-cyclic carbonate. The HepDirect cyclic carbonate double prodrug **57** (Figure 15) provided a 2- to 10-fold improvement in liver triphosphate production relative to the use of HepDirect alone, but no further developments have been reported on these compounds.

The development of guanosine derivatives for treating HCV infection has relied heavily on the use of phosphate prodrug technology to progress viable clinical candidates (Figures 19 and 21).[195] In several cases successful double prodrug approaches were implemented where in addition to the 5′-phosphate prodrug the C-6 position of the guanine base was masked to reduce overall polarity and improve cellular uptake.[197,240,241] The observations that 2′-*C*-methyguanosine **9** (Figure 6) inhibited the HCV NS5B polymerase with IC_{50} = 0.13 μM, EC_{50} = 3.5 μM in the replicon assay and exhibited low intracellular levels of the corresponding triphosphate suggested that phosphorylation was a problem. Implementation of a phosphoramidate prodrug development effort to address these

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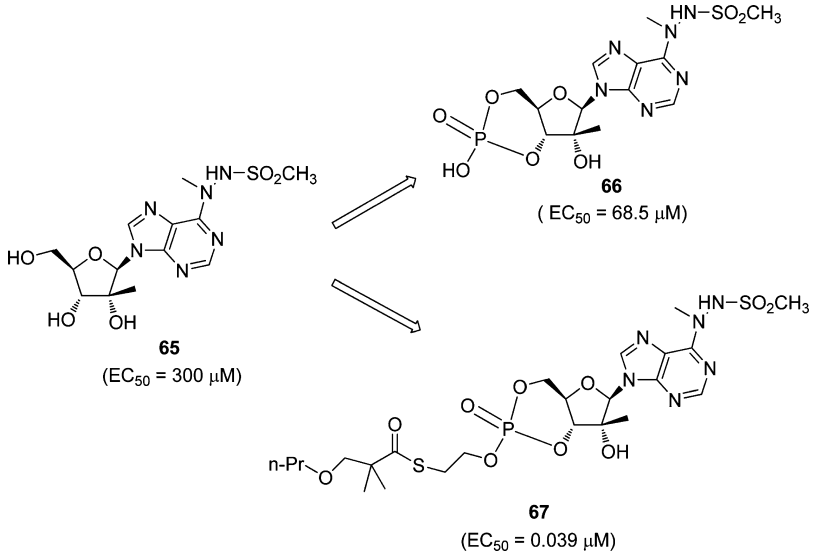

Figure 17. 3′,5′-Cyclic phosphate prodrug NS5B inhibitors of 2′-*C*-methyl-6-hydrazidosulfonamidoadenosine derivative.

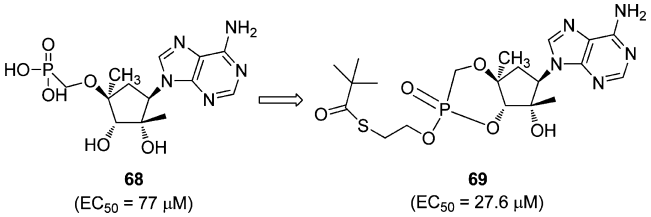

Figure 18. Cyclicphosphonate prodrug of a carbocyclic adenosine derivative.

limitations led to the identification of compounds **70** and **71** (Figure 19) with 10- to 30-fold improvement in HCV replicon potency and acceptable in vitro stability characteristics.[247] Substantial liver levels of the 2′-*C*-methylguanosine triphosphate were achieved after oral administration of either **70** or **71** to mice, but these levels were not substantially different from those generated on oral administration of the parent guanosine nucleoside itself. These results led to further modification of the guanine base by introduction of metabolically labile C-6 substituents, thus producing a double prodrug and ultimately the clinical candidate **72** (INX-08189, Figure 19).[241] Prodrug **72** was shown to be highly potent in the HCV replicon assay (EC_{50} = 0.01 μM, CC_{50} = 7 μM) and was active against genotype 1a and 2 replicons. In primary human hepatocytes, **72** produced substantial levels of the 2′-*C*-methylguanosine triphosphate after a 48 h incubation. Like other 2′-*C*-methyl nucleoside inhibitors of HCV, the replicon containing the polymerase with an S282T mutation was shown to be moderately resistant (3- to 10-fold) to **72**.[248] Since **72** was prepared as a mixture of phosphorus diastereomers, the individual diastereomers were separated and evaluated as inhibitors of HCV RNA replication. There was no difference in potency between the individual diastereomers; therefore, the mixture was advanced into clinical development.[241]

Results from a first in human phase 1a study with **72** reported that the drug was well tolerated and that PK supported once daily dosing. Further phase 1b studies in genotype 1b

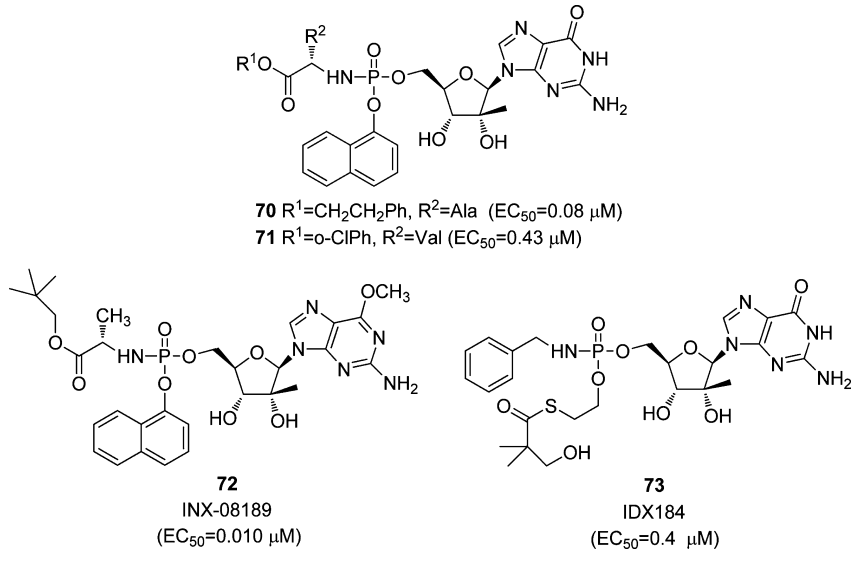

Figure 19. 5′-Phosphoramidate prodrugs of 2′-*C*-methylguanosine and *C*-6-guanosine derivatives.

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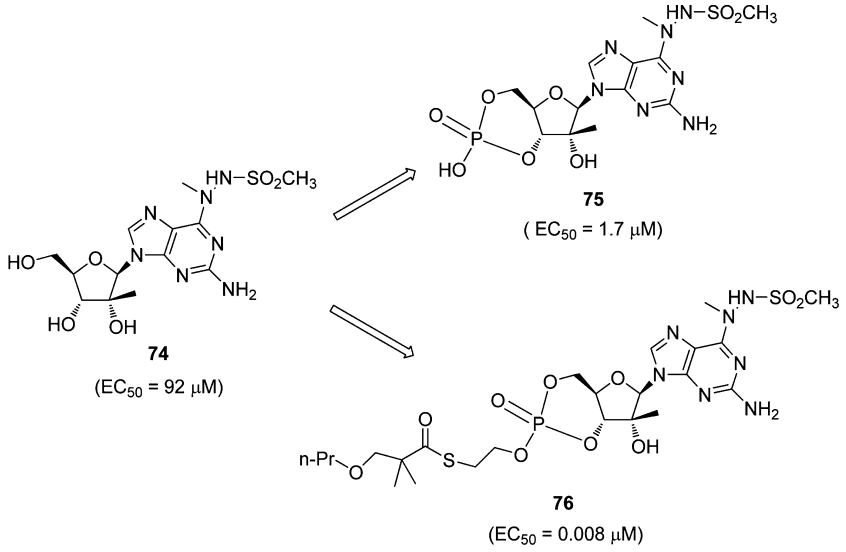

Figure 20. 3′,5′-Cyclic phosphate prodrug NS5B inhibitors of a 2′-*C*-methyl-6-hydrazidosulfonamidoguanosine derivative.

treatment naive patients demonstrated a mean reduction in HCV RNA of −0.71 and −1.3 $\log_{10}$ IU/mL at doses of 9 and 25 mg, respectively. Compound **72** has now progressed into phase 2 studies.[248,249]

Another phosphoramidate prodrug approach was also pursued to deliver the 2′-*C*-methylguanosine 5′-monophosphate. In this approach the phosphoramidate substituents included a benzylamine in place of the amino acid and employed the SATE moiety as the phosphorus ester, providing the clinical candidate **73** (IDX184, Figure 19).[250] Although a detailed mechanism for cleavage of the prodrug of **73** has not been revealed, it is believed to involve both CYP450-dependent and -independent processes. One can speculate that the SATE moiety is lost via the known SATE cleavage mechanism that releases an equivalent of episulfide as one step in the prodrug cleavage mechanism to obtain the free 5′-monophosphate. Nucleotide prodrug **73** was shown to be active as an inhibitor of HCV RNA replication (EC_{50} = 0.4 μM, CC_{50} > 100 μM) in a genotype 1b replicon and was also active in the genotype 2a JFH1 replicon (EC_{50} = 0.6−11 μM).[251] The replicon having the NS5B S282T mutation was also shown to be resistant to **73**, similar to other 2′-*C*-methyl nucleosides. Additive or synergistic effects were observed when **73** was combined with an NS3 protease inhibitor IDX320 (structure not disclosed), PEG-IFN, or RBV.[252] Oral administration of **73** to cynomolgus monkeys produced high liver triphosphate levels. Furthermore, oral administration of **73** to HCV-1 infected chimpanzees at 10 mg/kg resulted in a −2.3 $\log_{10}$ IU/mL decline in mean viral load over 3 days.[253] A phase 1b monotherapy study in genotype 1 infected patients administered **73** once a day at doses of 25−100 mg over 3 days produced a maximal viral load decline of −0.74 $\log_{10}$ IU/mL.[254] Subsequently, a 14-day phase 2a study of **73** in combination with PEG-IFN/RBV delivered viral load reductions of −2.7 to −4.1 $\log_{10}$ IU/mL for doses from 50 to 200 mg q.d. with no detection of resistant virus.[255] However, an attempt to study an interferon-free regimen of **73** with the protease inhibitor IDX320 resulted in three serious adverse events that ultimately led to institution of a clinical hold. A full clinical hold was eventually removed for **73** because evidence supported IDX320 as the cause for the observed toxicity.[195] Development of **73** is continuing in combination with PEG-IFN/RBV.

Preparation of 3′,5′-cyclic phosphate derivatives of base modified 2′-*C*-methylguanosine produced active inhibitors of HCV RNA replication (Figure 20).[244] Similar to the adenosine derivatives discussed above, this approach looked at both the 3′,5′-cyclic monophosphate **75** and the SATE ester derivative **76**. Surprisingly, the monphosphate derivative **75** achieved an EC_{50} of 1.7 μM despite the presence of an acidic phosphate group. The SATE ester derivative **76** was shown to be quite potent (EC_{50} = 0.008 μM), yet no further data are available for this compound.

SAR around the base unit of the 2′-α-*F*-2′-β-*C*-methyl nucleosides showed that the guanosine derivative **77** (Figure 21) was weakly active (EC_{90} = 69.2 μM) as an HCV inhibitor in the replicon assay but also showed that the 5′-triphosphate was an inhibitor of the NS5B polymerase (IC_{50} = 5.94 μM).[197,240] Further study showed that the triphosphate of **77** was equipotent as an inhibitor of both the wild type and S282T mutant NS5B polymerase.[197,240,256,257] This was the first example where a 2′-methyl nucleoside did not show differential activity between wild type and S282T mutant polymerases. Speculating that the monophosphorylation step was limiting activity of this 2′-α-fluoroguanosine nucleoside, 5′-monophosphate prodrug strategies were investigated (Figure 21).[197,240] Two strategies were reported: one strategy employed the phosphoramidate promoiety, and the other utilized a novel 3′,5′-cyclic phosphate ester approach coupled with C-6 substitutions on the guanine base to enhance lipophilicity and help cellular uptake. Both of these approaches proved successful at delivering clinical development candidates.

In the 2′-α-*F*-2′-β-*C*-methylguanosine phosphoramidate series, SAR focused on the alanate ester portion of the molecule and on the substiuent at the C-6 of the base while maintaining the simple phenolic phosphate ester.[197] Variation on the C-6 guanine substitutent had dramatic effects on potency with up to a 300-fold improvement over the corresponding natural guanine base. Maximum potency in the replicon assay, optimal stability characteristics in simulated gastric fluid, intestinal fluid, blood, and liver S9 fractions that supported liver targeting, and assessment of human hepatocyte triphosphate levels led to a series of low nanomolar inhibitors of HCV replication. A radiolabeled in vivo study showed that inhibitor **78** provided a (3.5−4.8)/1 liver to plasma ratio, supporting the concept of

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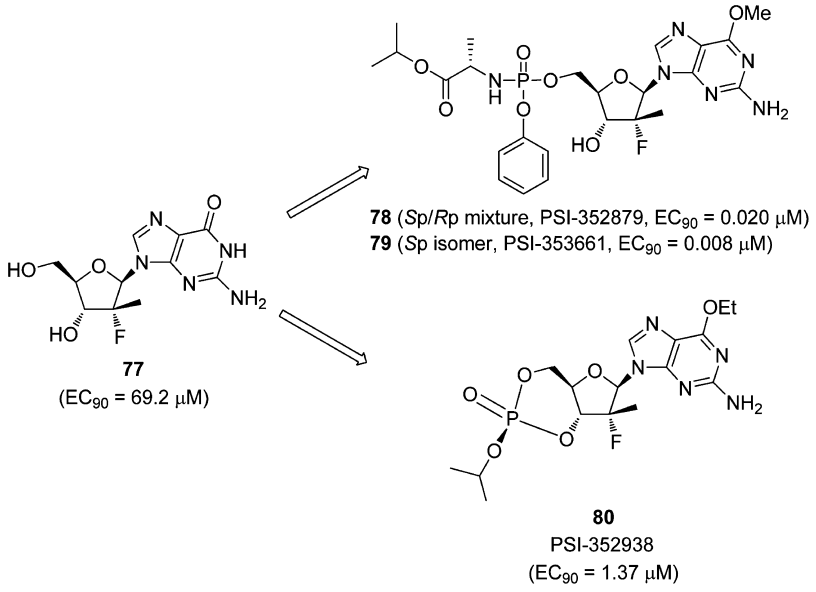


Figure 21. 5′-Phosphoramidate and 3′,5′-cyclic phosphate prodrugs of 2′-*α*-*F*-2′-*β*-*C*-methylguanosine as inhibitors of NS5B.

liver targeting. Crystallization of the single *Sp* diastereomer **79** (PSI-353661) from the diastereomeric mixture **78** showed that **79** was the more potent isomer. Further evaluation of **79** revealed that it was equipotent against both the wild type (EC_{90} = 0.008 μM) and NS5B S282T mutant replicon and was equipotent in the genotype 2a replicon.[257] The activity in the replicon containing the NS5B polymerase with the S282T mutation was unexpected for a 2′-*C*-methyl containing nucleoside and was consistent with the NS5B polymerase enzyme activity demonstrated for the corresponding 2′-*α*-*F*-2′-*β*-*C*-methylguanosine triphosphate. In addition, combination studies with PEG-IFN or RBV and other DAAs produced an additive or synergistic effect.[257] The 2′-*α*-*F*-2′-*β*-*C*-methyl-6-methoxyguanosine 5′-phosphoramidate **79** was selected as a clinical development candidate but has not started clinical trials at this time.

The second prodrug strategy investigated for delivering 2′-*α*-*F*-2′-*β*-*C*-methylguanosine monophosphate employed alkyl and aryl ester 3′,5′-cyclic phosphates (Figure 21).[240,258] As in the case of the corresponding phosphoramidate prodrugs, the cyclic phosphate esters were coupled with substitution at the C-6 position of the guanine base to maximize potency. However, potency of the cyclic phosphate ester prodrugs was generally >10-fold less than that of the corresponding phosphoramidate prodrug with the same C-6 substituted guanosine nucleoside. Although the 2′-*α*-*F*-2′-*β*-*C*-methyl-6-substituted guanosine 3′,5′-cyclic phosphate esters were shown to be metabolized to the corresponding 2′-*α*-*F*-2′-*β*-*C*-methylguanosine triphosphate, the mechanistic details of the metabolism have not yet been reported. Nucleotide cyclic phosphate **80** (EC_{90} = 1.37 μM, CC_{50} > 100 μM), a single (*Rp*) cyclic phosphate diastereomer, was selected as a lead development candidate. It showed broad genotype coverage, and in combination with PEG-IFN, RBV or other DAAs demonstrated additive or synergistic characteristics.[259] In combination with the nucleotide **50**, **80** effectively cleared both wild type and S282T mutant replicons in cell culture. In a phase 1 clinical study **80** was safe and well tolerated at doses up to 1600 mg once a day. Subsequently, in a 7-day multiple ascending dose monotherapy study in 40 genotype 1 HCV patients administered 100, 200, and 300 mg q.d. and 100 mg b.i.d., mean viral load declines of −4.31, −4.65, −3.94, and −4.59 $\log_{10}$ IU/mL, respectively, were observed.[260] In the 200 and 300 mg groups 11 of 16 patients achieved viral loads below the limit of detection. It was surprising that a compound with relatively modest replicon potency exhibited such dramatic viral load declines in human clinical studies. In addition, these results represent the first clinical proof of concept of the use of a 3′,5′-cyclic phosphate ester prodrug. Evaluation of an PEG-IFN-free dual nucleotide combination was undertaken. This 14-day proof of concept study showed that there were no PK interactions between the two drugs and that a −4.6 to −5.5 $\log_{10}$ IU/mL viral load decline was achieved with 94% of patient's viral loads below the limit of detection.[230] This DAA combination study was the first proof of concept that two nucleotides could be combined in an PEG-IFN-free treatment regimen and set the stage for the start of a dual nucleotide phase 2 study reported to begin in the near future.

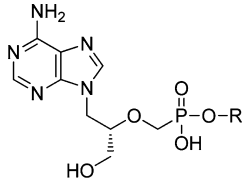
Recently, the in vitro selection of replicons that confer resistance to the nucleotide prodrugs **79** and **80** was reported.[258,261] Both compounds were metabolized to the same active 5′-triphosphate: 2′-*α*-*F*-2′-*β*-*C*-methylguanosine 5′-triphosphate. Unlike the other 2′-*C*-methyl analogues, including the 2′-*α*-*F*-2′-*β*-*C*-methylpyrimidine nucleos(t)ide analogues **13**, **49**, and **50**, which select for the S282T variant, **79** and **80** selected for a number of amino acid changes, including S15G, R222Q, C223Y, C223H, L320I, and V321I. Phenotypic analysis showed that these single amino acid changes were insufficient to significantly reduce the activity of **79** and **80**. Instead the combination of three amino acid changes, S15G/C223H/V321I, was required to confer a high level of resistance. Interestingly, replicons resistant to **79** and **80** remained fully susceptible to inhibition by **8**, **13**, **59**, **72**, and **73**. Resistant variants were only selected using a genotype 2a replicon, whereas selection studies using genotype 1a or 1b replicons failed to produce resistant replicons. Further studies showed that mutations that encode for the C223Y and C223H amino acid substitutions were lethal to genotype 1 replicons. Surprisingly, 2a replicons containing these amino acid substitutions remained sensitive to compounds containing the 2′-*C*-methyl modification, including the 2′-*C*-methylguanosine prodrugs **72**

and **73** and the 2′-*α*-*F*-2′-*β*-*C*-methylpyrimidine nucleos(t)ide analogues. Furthermore, genotype 1 replicons containing the S282T amino acid substitution remained fully sensitive to **79** and **80**.

The SATE prodrug approach was also applied to guanosine and 7-deazaguanosine derivatives of 2′-*O*-methyl nucleosides where the parent nucleosides were poor inhibitors in the cell based replicon system. However, none of these prodrugs showed activity.[172] The poor activity of the 2′-*O*-methylguanosine SATE prodrugs may be attributed to poor cellular penetration or lack of conversion of the delivered monophosphate to the downstream di- and triphosphates.

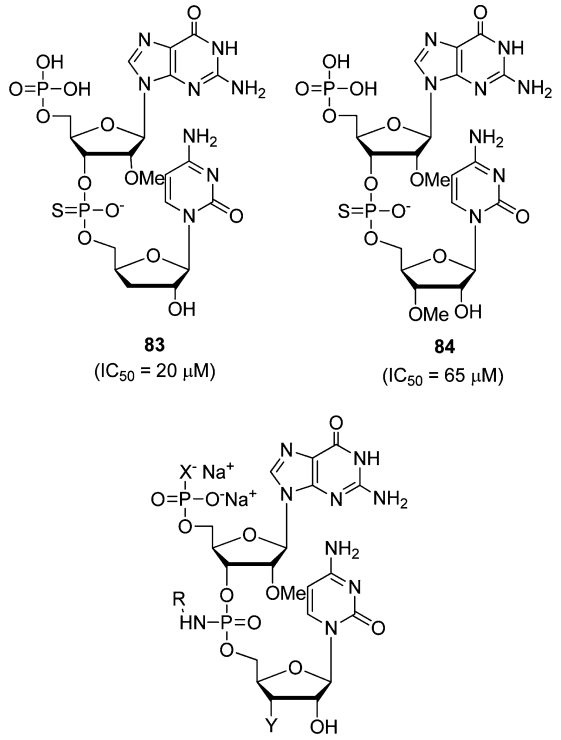
Acyclic nucleotides have played an important role in antiviral therapy. Drugs such as tenofovir and adefovir are important contributions to treatment regimens for HIV infection and hepatitis B virus infection, respectively. Attempts to identify acyclic nucleos(t)ide inhibitors of HCV studied the known herpes and orthopoxvirus inhibitor (*S*)-9-[3-hydroxy-2-(phosphonomethoxy)propyl]adenine ((*S*)-HPMPA) and prepared the octadecyloxyethyl (ODE) **81** and hexadecyloxypropyl (HDP) **82** esters.[262] HDP and ODE esters of acyclic nucleoside phosphonates were shown to greatly increase the cellular uptake and conversion to the acyclic nucleotide diphosphate in the study of acyclic nucleoside inhibitors of HIV-1 and cytomegalovirus (CMV). The ODE derivative **81** (for GT1b, EC_{50} = 1.31 μM and CC_{50} = 35.6 μM; for GT2a, EC_{50} = 0.69 μM and CC_{50} = 35.6 μM) was shown to be the most active inhibitor of HCV replication in the replicon assay. HDP-(*S*)-HPMPA **82** was also reported to provide excellent drug exposure in the liver.

Nucleosides and nucleotides are known to inhibit HCV during the elongation phase of viral RNA synthesis via a nonobligate chain termination mechanism. It has been proposed that dinucleotides have the potential to inhibit RNA replication at the initiation phase by behaving as primer molecules. Several reports have investigated dinucleotides as inhibitors of HCV replication.[263−265] The stable 5′GC dinucleotides **83** and **84** (Figure 22) employing phosphothioate linkage and 2′-*O*-methyl substitution were shown to inhibit initiation of RNA replication by HCV NS5B polymerase with IC_{50} of 20 and 65 μM, respectively.[263] An alternative approach looked at a phosphoramidate intranucleotide linkage with a phosphate, thiophosphate, or hydroxyl at the 5′-end.[264] In both the enzyme inhibition assay and in the HCV replicon whole cell assay the most potent compound **85** (IC_{50} = 25 μM, EC_{50} = 9 μM) was the *Sp* diastereomer containing a 5′-thioester and an intra-



81 (ODE) R = $(CH_2)_2O(CH_2)_{17}CH_3$, (EC_{50} = 1.31 μM)
82 (HDP) R = $(CH_2)_3O(CH_2)_{15}CH_3$, (EC_{50} = 2.02 μM)

nucleotide phosphoramidate substituted with a negatively charged hexanoate group. Further optimization of the phosphoramidate intranucleotide linked dinucleotides looked at the 3′-deoxycytidine moiety, thus removing the 3′-hydroxyl to exhibit a proper chain terminator effect. This led to the identification of several compounds with low micromolar EC_{50} in the replicon assay with the most potent being **86** (EC_{50} = 1.7 μM).[265]



83
(IC_{50} = 20 μM)

84
(IC_{50} = 65 μM)

85 X = S, R = -$(CH_2)_5CO_2^-Na^+$, Y = OH, Sp isomer (EC_{50} = 9 μM)
86 X = O, R = -$(CH_2)_5CO_2^-Na^+$, Y = H, (EC_{50} = 1.7 μM)

Figure 22. Dinucleotide inhibitors of NS5B.

Recently, data on a nucleotide prodrug GS-6620 (structure not disclosed) were presented.[266−268] GS-6620 was shown to have EC_{50} = 0.52 μM against a GT1b replicon. Selection of the S282T NS5B mutation in GT1b replicon cells and in a GT2a infectious virus (JFH-1) cell culture model suggests that this nucleotide prodrug is a member of the 2′-*C*-methyl class of nucleotides.[267] GS-6620 was demonstrated to have high intestinal absorption and high hepatic extraction in dogs. In addition, upon oral administration high liver triphosphate levels were observed in both hamsters and dogs.[268] GS-6620 is currently in phase 1 clinical development.

■ NON-NUCLEOSIDE INHIBITORS

Predominantly through the efforts of high throughput screening, three allosteric small molecule binding sites have been identified on the HCV NS5B polymerase. These allosteric binding sites reside in the palm and thumb domains. To date, no pure finger domain allosteric sites have been identified. The palm domain allosteric site is in essence a single long and deep hydrophobic pocket near the active site. However, the thumb domain has two well-defined allosteric binding sites. Structure based drug design methods have played an important role in lead optimization efforts of the more than 28 different chemotypes identified that bind to these allosteric sites. Two major challenges associated with development of allosteric non-nucleoside inhibitors (NNI) of NS5B have been resistance and genotype coverage.

Unlike the nomenclature used to address protease inhibitor binding sites in which subsites are named on the basis of cleavage of the peptide substrate, the various binding sites for allosteric inhibitors of the NS5B polymerase have been named using crystallographic and mutagenesis data over a period of 12

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years each eliciting a new name or number, sometimes contradictory and certainly confusing when reading across the literature. For this review, we have chosen to standardize our naming convention on the basis of the subdomains in which the allosteric site is found. There are two distinct and separate allosteric binding sites in the thumb domain that we identify as thumb site I, proximate to the GTP allosteric binding site, and thumb site II, a mainly hydrophobic cleft about 15 Å from thumb site I. The palm subdomain contains the largest allosteric binding site and the one most apparently able to change shape dependent upon the type of inhibitor bound. We refer to this as the palm site because the volume remains contiguous, and key residue interactions remain the same despite the change in shape or inhibitor presentation.

■ PALM DOMAIN INHIBITORS

The palm site allosteric binding pocket is at the interface of the thumb and palm domains near the polymerase active site. The hairpin loop that links the palm region to thumb comprising residues 363−369 is sometimes referred to as the "primer grip" site because of its intrusion into the catalytic region of the palm and its similarity to interactions observed in RNA-dependent DNA polymerases.[269] If the primer grip forms one wall of the palm binding site, the opposite wall is formed by the intrusion of another β-hairpin loop from the thumb ("thumb loop") descending into the palm domain (Figure 23). Both figure

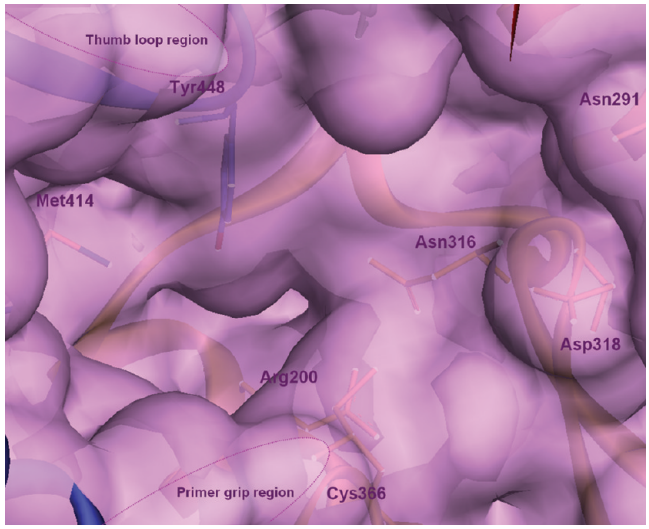

Figure 23. The palm allosteric binding site is large, 15 Å wide and 20 Å deep, encompasses part of the active site, and extends across the palm to the interior of the thumb domain with the thumb loop, particularly Tyr448, and the primer grip region providing critical interactions. From the vantage point of the finger loops, the molecular surface shown in purple also highlights a depression in the "floor" of the site into which some inhibitors descend in part or in whole, e.g., HCV-796 (**108**), dependent on presentation of Arg200. Some inhibitors appear to form covalent bonds to Cys366. Significant resistance develops with mutations at position 414, 316, or 447.

prominently in making stabilizing interactions with inhibitors as well as being regions in which resistant mutations can occur. Of the known allosteric sites, the size of the palm binding site, 15 Å wide and 20 Å deep,[99] permits the widest variety of chemotypes to act as inhibitors.

Most of the palm site inhibitors make two key interactions with Tyr448 which sits at the $i + 1$ position of the β-turn[270] on the thumb loop. The first is a hydrogen bond between the main chain NH and an acceptor atom on the inhibitor, and the second is what appears to be an edge to face $\pi-\pi$ aromatic stacking interaction between the tyrosine side chain (edge) and an aromatic moiety in the inhibitors (face) or at least a hydrophobic interaction. Some of the inhibitor classes form covalent bonds to the $S\gamma$ of Cys366. Guanosine triphosphate primer, important to de novo initiation of RNA synthesis, also binds in this palm region, forming key interactions with Arg386, Ser387, and Arg394.[109] Inhibitors bound at this site effectively block access by GTP and have been found to inhibit the polymerase in both the initiation and elongation stages.[269]

One of the most explored HCV non-nucleoside inhibitor classes is the benzothiadiazine class. The initial discovery of benzothiadiazines as palm binding site inhibitors arose from a high throughput screen of a proprietary library using an Rd/Rp scintillation-proximity assay[271] leading to benzothiadiazine **87** (IC_{50} = 0.200 μM, Figure 24).[272] Binding of the inhibitor interferes with RNA synthesis before the formation of the elongation complex.[273,274] A cocrystal X-ray structure (2FVC)[272] of the NS5B enzyme bound to an analogue of **87** with a C3 methyl on the alkyl side chain showed that the ABCD quinolinone-benzothiadiazine ring system fits tightly onto the hydrophobic surface of the binding pocket with the only apparent room for substitution on the C5 and C6 positions of the quinolinone AB rings and the C7 and C8 of the benzothiadiazine CD rings. The AB and CD ring planes were offset approximately 20°, whereas a single crystal X-ray of the drug molecule itself showed both ring systems in the same plane because of internal hydrogen bonding. There was an edge to face interaction between Phe193 and the benzo portion of the CD rings. Hydrogen bonding interactions with the sulfonamide came from Ser556 and Ser288 mediated through tightly bound water molecules. The quinolinone ketone was hydrogen-bonded to Tyr448. Met414 interacted with the A ring of the quinolinone and formed part of a lipophilic pocket that held the aliphatic side chain. In the presence of **87**, replicons can form five single mutations, but only Met414 leads to significant resistance, and thus this site must be crucial for binding of the inhibitor.[275] It was shown that there is also natural variation in the amino acids at this site.[276] Resistant strains were selected under benzothiadiazine drug pressure.

An initial SAR study on the lead structure **87** led to a series of compounds culminating in SB-711845 (**88**) with IC_{50} = 0.010 μM against GT1b, IC_{50} = 0.049 μM against GT1a, and IC_{50} = 0.011 μM against GT2a RdRp and far less activity against GT3a RdRp. In the 1b replicon it had EC_{50} = 0.038 μM and was selected as a clinical candidate. An X-ray cocrystal structure (2GIQ)[277] of **88** showed a binding pattern similar to that of the lead compound. Structure **88** showed good bioavailability in animal models and a high liver to plasma ratio. However, the presence of human serum albumin in the replicon assay led to a substantial decrease in potency, suggesting the potential for significant protein binding. Protein binding was reduced by adding an oxyacetamido side chain on C-7 of the benzothiadiazine ring (SB-750330, **89**), which led to improved replicon potency (EC_{50} = 0.002 μM) and its selection as a clinical candidate.[278] Although both of these compounds did not continue to advance in the clinic, this series did spawn a large medicinal chemistry exploration of this class by several research groups.[272] In general, the aromatic planar rings with numerous hydrogen bonding sites created a high polar surface area (PSA) and favored stable crystal packing which led to low solubility. The challenge for medicinal chemists was to find

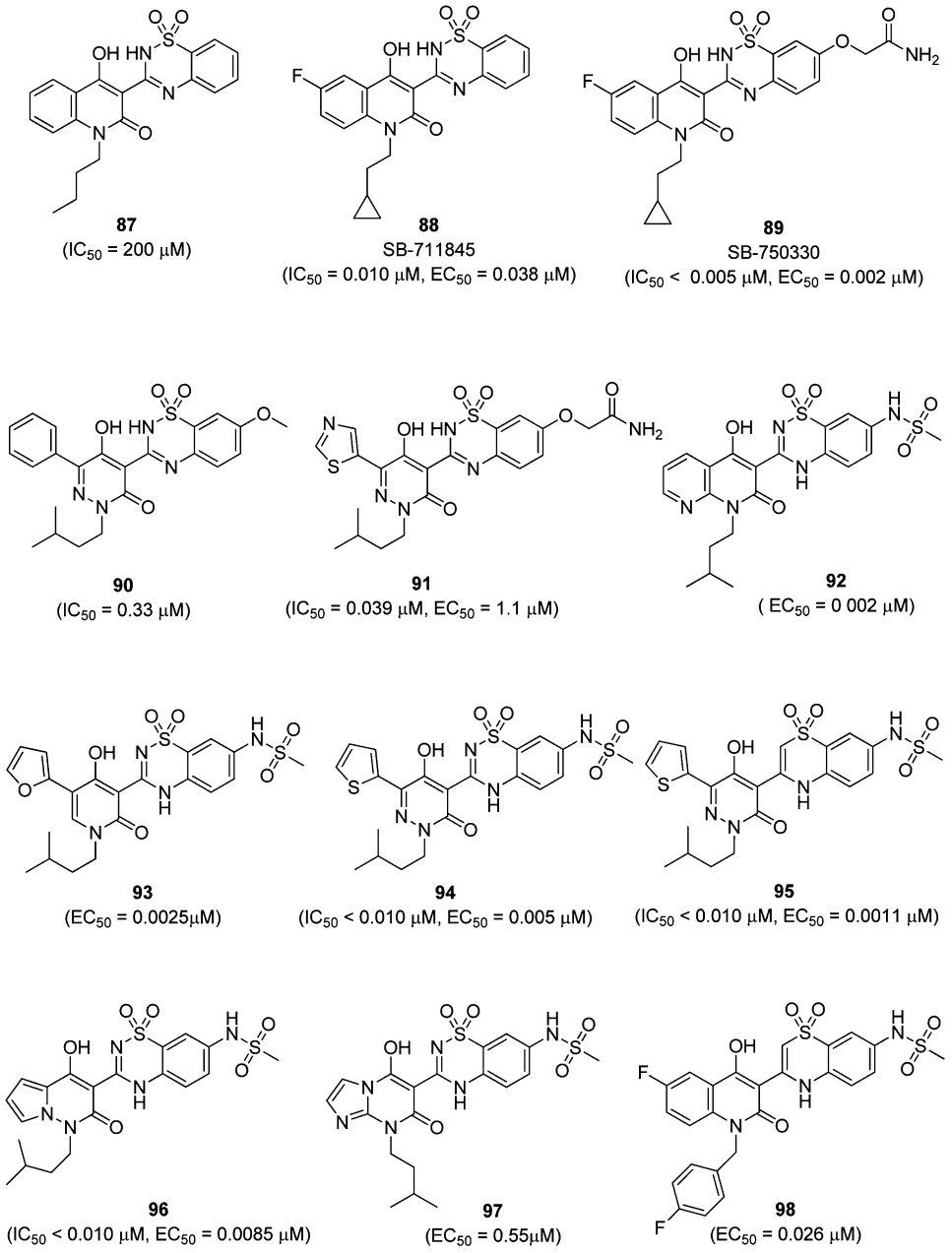

Figure 24. Benzothiadiazine palm site inhibitors (part 1).

strategies to reduce these interactions while maintaining or improving activity. This was accomplished by introducing saturated sites, branched aliphatic side chains, and various heterocyclic rings systems with a wide variety of substituents. Additional factors to optimize included moderating protein binding, reducing clearance, improving metabolic stability, increasing cell permeability, and overall increasing bioavailability.

Removal of the A ring led to a reduction of hydrophobic contact in the binding site.[279] Substitution of the remaining ring with hydrophobic groups led to improved activity. For example, replacing the bicyclic quinolinone with a dihydropyridazinone led to a new lead (**90**) with only modest GT1b activity against the RdRp (IC_{50} = 0.330 μM). Optimization included returning to the C-7 oxyacetamido side chain and an exploration of alkyl and heterocyclic substituents. This led to structure **91** with IC_{50} of 0.039 μM against GT1b RdRp and IC_{50} of 0.150 μM against GT1a RdRp and an EC_{50} value of 1.10 μM determined in a GT1b replicon assay and that had improved stability when incubated with human liver microsomes.[280] Shifting the heterocyclic substituent from a 5-thiazole to a 2-thiazole led to reduction in activity, whereas 2- or 3-thiophenes still maintained their potency. In the 2-thiophene series, substitution on the aliphatic side chain with a 3-F or using a more branched alkyl group improved stability toward microsomes. Introduction of a methanesulfonamide on C-7 of the benzothiadiazine ring (**92**) improved potency, EC_{50} = 0.002 μM, and proved to be a common substitution in most subsequent series.[281] Replacing the naphthyridinone of **92** with a furan substituted pyridinone (**93**) to gain back hydrophobicity maintained activity (EC_{50} = 0.0025 μM). In addition, in the presence of 40% human serum **93** retained more potency (EC_{50} = 0.060 μM) versus **92** (EC_{50} = 0.736 μM) because of lower protein binding.[279]

Substitution of benzothiadiazine in the dihydropyridazinone structure **94** with benzothiazine produced compound **95** which

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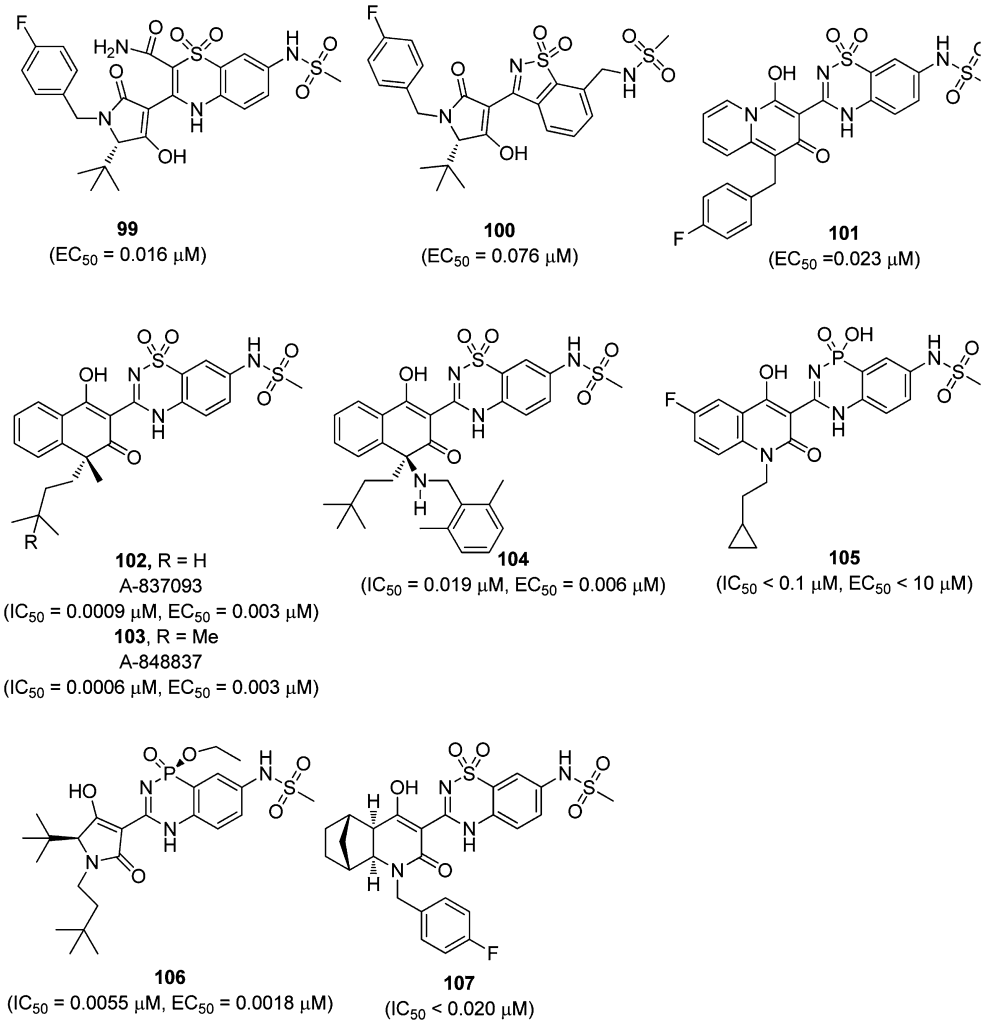

Figure 25. Benzothiadiazine palm site inhibitors (part 2).

maintained similar potency with IC_{50} < 0.010 μM against GT1b RdRp and EC_{50} = 0.0011 μM in a GT1b replicon assay as well as favorable stability in liver microsomes. However, Caco-2 permeability was not improved and bioavailability was limited to 4% in cynomolgus monkeys. In a similar series lacking the polar methanesulfonamide substituent, the analogue containing benzothiazine was significantly less bioavailable than the benzothiadiazine.[281] Another approach to improve cell permeability was replacement of the aromatic substituted dihydropyridazinone ring with a pyrrolopyridazinone (**96**) in an attempt to lower the PSA value. Activity was maintained with IC_{50} < 0.010 μM and EC_{50} = 0.0085 μM, but unfortunately, permeability was not improved.[282] Another variation of the AB ring system was the imidazolopyridinone **97** with EC_{50} = 0.55 μM. A 1.9 Å resolution X-ray cocrystal structure of the NS5B enzyme with **97** (3H98)[283] again showed similar interactions of the ring core with sulfonamide interactions mediated through bound water with Ser556 and Ser288 as well as with Gly449. The AB−CD planar offset was 22°. The sulfonamide side chain interacted with Asp318 and Asn291. The alkyl side chain protruded into the hydrophobic pocket defined by Pro197, Leu384, Met414, Tyr415, and Arg200.

Substitution of the quinolinone in the benzothiazine lead compound (**98**) which had EC_{50} = 0.026 μM against a GT1b replicon and a clearance in rat of 106 mL kg^{-1} min^{-1} with a nonplanar chiral tetramic acid led to **99** (Figure 25). Compound **99** had slightly improved potency (EC_{50} = 0.016 μM) and a substantially reduced clearance value of 5 mL kg^{-1} min^{-1}. The 4-F substitution on the benzyl substituent reduced inactivation by CYP 3A4, and the carboxamide substitution reduced clearance.[284] A related series (**100**) with a benzoisothiazole ring system and a methylene extension of the methanesulfonamide (3H5U)[284] had EC_{50} = 0.076 μM. Substitution of the quinolinone ring with a quinolizinone as shown in structure **101** resulted in EC_{50} = 0.023 μM.[285]

Introduction of a saturated carbon in the B ring of the quinolinone-benzothiadiazine series led to substitutions on the tetrahedral carbon to probe further into the palm binding site. A molecular modeling study on **102** (A-837093, EC_{50} = 0.003 μM)[285] showed overall that the ABCD ring structure occupied the normal site seen for other benzothiadiazines. The isoamyl side chain resided in the usual hydrophobic pocket. The methyl substituent on the same carbon was pointed toward an unexplored polar region with Arg386 and Ser367 residues. Substituting the isoamyl side chain with a neohexyl (**103**, A-888837, EC_{50} = 0.002 μM) blocked metabolic hydroxylation on the tertiary carbon and resulted in improved metabolic stability. Replacing the methyl with an amino substituent capable of interacting with these residues led to a new series resulting in **104** (EC_{50} = 0.006 μM).[286]

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There are four palm site inhibitors currently in clinical trials: ABT-072, ABT-333, IDX375, and ANA598 now known as setrobuvir (structures not disclosed).[287−289] ABT-072 and ABT-333 are in phase 2a trials. The structures of both have not been revealed, although CAS numbers have been assigned as 1214735-11-1 for ABT-072 and 1221573-79-0 for ABT-333. Perhaps the structures are related to **93** and **104** based on similar reported potencies. In preclinical studies, ABT-333 had IC_{50} = 0.002−0.004 μM for GT1a and IC_{50} = 0.002−0.010 μM for GT1b using various strains. It was not nearly as potent against other genotypes.[290] The EC_{50} values for cell culture replicons varied greatly if 40% human plasma were present with EC_{50} = 0.008 μM without plasma and 0.099 μM with plasma for GT1a and an EC_{50} = 0.002 μM without plasma and 0.021 μM with plasma for GT1b. By use of transient replicons isolated from infected patients, the EC_{50} values were 0.0008 and 0.0005 μM for GT1a and GT1b, respectively. The CYP metabolism and inhibition profile suggested a minimum potential for drug−drug interactions. Pharmacokinetic studies in dog, rat, and monkey showed an acceptable profile and significant accumulation in the liver in the last two species. The results supported further clinical development.

In a 12 week safety and efficacy study on ABT-072 and ABT-333, phenotypic and genotypic analyses were performed. Phenotypes with NS5B amino acid mutations at positions 316, 414, 448, 556, and 559 conferred resistance. At least one mutation was found in 12 of 13 patients that did not achieve undetectable RNA levels in clinical trials. In addition, 90% of those patients having the C/T or T/T IL28B allele did not achieve undetectable RNA levels. However, resistant viruses could be suppressed when ABT-072 or ABT-333 was administered in combination with SOC.[287]

In a phase 2a 12-week study with treatment naive patients, ABT-072 and ABT-333 were evaluated separately using 100, 300, and 600 mg q.d. and using 400 and 800 mg b.i.d., respectively, in combination with RBV and PEG-IFN. Test drug was administered as monotherapy for the initial 3 days of the trial. Preliminary results of the 12-week time point were reported. For the monotherapy period, at the respective highest doses, treatment with the test drug led to a decrease of viral titer of $-1.57\ \log_{10}$ IU/mL for ABT-072 and $-1.02\ \log_{10}$ IU/mL for ABT-333 compared to $-0.36\ \log_{10}$ IU/mL for the control. By the fourth week of combination therapy, the percentage of patients with undetectable levels of virus in each highest dose cohort was 42% for ABT-072 and 50% for ABT-333, compared to 9% in the RBV/PEG-IFN containing placebo control. At 12 weeks, the values were 86% and 63%, respectively, compared to 18% in the control. No serious adverse events were reported. The trial design included continuing RBV and PEG-IFN for a full 48 weeks, and those results have not yet been reported.[288]

The structure of palm inhibitor IDX375, currently in phase IIa trials, has also not been revealed. It was tested as both its free acid and choline salt in phase 1 studies, implying that there is an acidic group.[289] A series of novel isosteric phosphadiazine analogues have been revealed in the patent literature such as structure **105** (IC_{50} < 0.01 μM, EC_{50} < 10 μM).[291] Another possibility is **106** (IC_{50} = 0.0055 μM, EC_{50} = 0.0018 μM). Possibly one of these compounds or a similar analogue has progressed into clinical development as IDX375. IDX375 had IC_{50} = 0.030 μM for GT1a and IC_{50} = 0.018 μM for GT1b RdRp in enzyme assays and EC_{50} = 0.002 μM in a GT1b replicon assay. In the presence of 45% human serum, the EC_{50} increased 25-fold. IDX375 was not cytotoxic in test cell lines. The pharmacokinetic profile in rat and cynomolgus monkeys was favorable with drug concentrations observed only in the liver. Preclinical animal studies supported once or twice daily dosing. No adverse events including no clinical chemistry parameters or histological abnormalities were observed up to the highest dose of 100 mg kg^{-1} day^{-1} for 7 days in cynomolgus monkeys.[292]

IDX375 was evaluated in phase 1 single and multiple ascending dose studies in healthy volunteers and HCV patients. The choline salt form was dosed in the range 25−200 mg q.d. and 200 mg b.i.d. The more stable free acid form was tested in the range 200−1200 mg q.d. and 800 mg b.i.d. and at 200 and 400 mg b.i.d. for 3 days. The 800 mg b.i.d. arm showed an increase in indirect bilirubin levels not associated with increases in ALT, AST, or alkaline phosphatase levels. The levels returned to baseline upon completion of dosing. This was attributed to inhibition of UGT1A1 which was also observed during in vitro testing. The 200 mg single dose cohort of the free acid and choline salt forms had similar pharmacokinetic profiles. There were no discontinuations or serious adverse events.[293] IDX375 is reported to be currently in phase 2a studies.

The most clinically advanced benzothiadiazine is ANA598 (setrobuvir). The exact structure has not been revealed, but on the basis of a patent disclosure, setrobuvir appears to be in the family represented by structure **107** that constains a tricyclic octahydroquinolone moiety.[294] Currently, setrobuvir is in phase 2b clinical trials in combination with SOC. The first disclosure of its activity dates to 2007 with IC_{50} < 0.001 μM determined in an RdRp enzyme assay and EC_{50} = 0.052 μM (GT1a) and EC_{50} = 0.003 μM (GT1b) determined in replicon assays.[295] The difference in activity was later traced in clinical isolates to variation in position 415 in the genetic sequence which encoded for either a tyrosine or phenylalanine in the NS5B protein.[296] In preclinical studies, cynomolgus monkeys were treated with ANA598 up to 1000 mg kg^{-1} day^{-1} with no adverse events reported. It was also noted that there was a low potential for CYP-450 mediated drug−drug interactions.[297] In vitro synergism was observed with PEG-IFN, the nucleoside **13**, and the protease inhibitor telaprevir. Additive effects were noted with a cyclophilin inhibitor and an NS5B NNI.[298] A phase 1 single ascending dose 3-day monotherapy clinical study in GT1 patients of ANA598 using dosing of 200, 400, 800 mg b.i.d. reported day 4 viral load declines of −2.4, −2.3, $-2.9\ \log_{10}$ IU/mL, respectively.[299] No adverse events were reported after the 3 days of dosing. In a follow-up study, viral variant C316Y, Y448H, or G554D were observed in eight of nine patients. The EC_{50} values for these variants were 200- to 900-fold higher than that of wild type, but these variants were still susceptible to SOC which supported further exploration of combination studies.[300]

A phase 2a study was conducted using 200 and 400 mg b.i.d. ANA598 dosing arms plus SOC. The 200 and 400 mg arms resulted in 73% and 75% of patients with viral loads below the limit of detection, respectively. Unfortunately, the study observed a relatively high response rate for the SOC control arm that was attributed to a greater percentage of patients in the control group having the CC IL28B allele.[301] No adverse events were noted in the 200 mg dose group over those expected from the SOC, but in the 400 mg arm there was an increase in rash incidence and severity.[232]

Beyond the benzothiadiazines, a number of chemotypes have been identified that bind into the palm binding pocket allosteric site. One of these chemotypes is the benzofuran represented by

108 (HCV-796).[302,303] Benzofuran **108** was the first nonnucleoside inhibitor to demonstrate proof of concept in the clinic. It was shown to be a potent inhibitor (IC_{50} = 0.081 μM) of the HCV RdRp in an isolated enzyme assay with slow binding kinetics and active in the replicon assay (EC_{50} = 0.013 μM). Cocrystal structures of **108** bound to NS5B for both the HCV 1b Con1 (3FQL) and BK (3FQK) strains[304] showed that it functions as a palm site inhibitor binding near the polymerase active site into a deep cleft in the core of the palm domain.[302] Furthermore, **108** induced structural rearrangements in the protein that include a rotamer shift of Arg200 and movement of the primer-grip loop backbone. Several polymerase mutations arose on exposure to **108**, with those at C316N/Y and S365T exhibiting the strongest impact on potency (>200-fold) in the replicon assay.[302] In a phase 1 monotherapy clinical study in treatment naive genotype 1 patients receiving 50−1500 mg b.i.d. for 14 days, mean reductions in viral load of −1.2 to −1.5 $\log_{10}$ IU/mL at day 4 were observed.[303] However, 52% of patients on therapy who achieved a viral load decline experienced a viral breakthrough that was associated with the emergence of a viral variant expressing the C316Y amino acid substitution in NS5B. In a study evaluating the combination of **108** with PEG-IFN, a mean reduction in HCV RNA for genotype 1 patients of approximately −3.1 $\log_{10}$ IU/mL was observed after 14 days of treatment whereas for nongenotype 1 patients a reduction in HCV RNA of approximately −4.5 $\log_{10}$ IU/mL was reported. Unfortunately, the development of **108** was terminated when in a phase 2 study severe liver toxicity was observed in several patients after 8 weeks of treatment.[303]

The starting point for a series of proline sulfonamides came through high throughput screening (Figure 26).[305] The proline

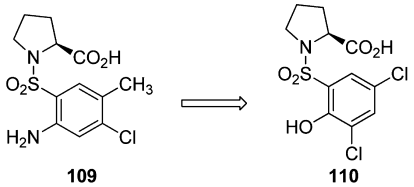
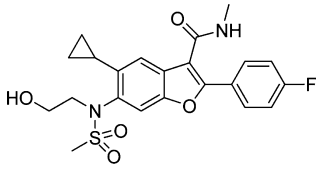


Figure 26. Proline sulfonamide palm site NS5B inhibitors.

sulfonamide **109** was identified as an active inhibitor of the HCV polymerase with IC_{50} = 3.1 μM and was shown to be highly selective for HCV polymerase over other polymerases. Subsequent SAR around the aryl group of the sulfonamide moiety and the proline moiety led to compounds optimized for HCV polymerase inhibition; however, these optimized compounds showed only modest cellular replicon activity



possibly due to poor permeability characteristics. A cocrystal complex of HCV polymerase with compound **110** (2GC8)[305] revealed that this class of inhibitors bound in the active site approximately 10 Å from the catalytic aspartic acids, making interactions with both the palm and thumb domains and particularly with the loop containing Tyr448.

Another high throughput screening campaign identified the acrylic acid **111** (IC_{50} = 6.7 μM) as an inhibitor of the NS5B polymerase enzyme (Figure 27).[269,306] Subsequent cocrystalli-

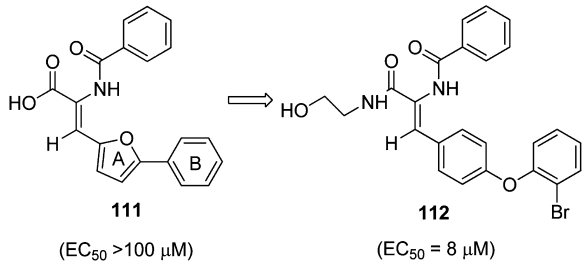


Figure 27. (2*Z*)-2-Benzoylamino-3-(4-phenoxyphenyl)acrylic acid palm site NS5B inhibitors.

zation studies using a more potent analogue of **111** (1YVF)[269] showed that this class bound to the polymerase in the palm binding domain sandwiched between the thumb hairpin loop with which the amide oxygen H-bonds with Tyr448 NH and the primer grip, there interacting primarily with the side chain of Cys366. Optimization of activity against the polymerase enzyme focused first on modifications at the A and B rings of **111** and then on the benzamide moiety.[269] This led to compounds exhibiting IC_{50} between 0.10 and 0.03 μM, though several of these compounds exhibited only modest activity in the cellular replicon assay. The lack of replicon activity was ascribed to either poor permeability or high levels of serum protein binding. Eventually, replacement of the carboxylic acid moiety with functionalized amide groups led to compounds with substantially improved replicon activity even though they only showed modest activity as inhibitors of the NS5B polymerase.[306] Although compound **112** was only a modest inhibitor of both the polymerase and of HCV RNA replication in the replicon assay, it did show substantially improved oral bioavailability (F = 76%) over its carboxylic acid derivative.

A series of rhodanine derivatives were explored as inhibitors of HCV polymerase starting from the high throughput screening hit **113** (IC_{50} = 1.5 μM).[307] In this series a lipophilic benzylidene moiety was required for enhanced potency as was a lipophilic phenylsulfonylamino group. Optimization led to compound **114**. X-ray crystal structures of inhibitor−NS5B complexes (2AWZ, 2AX1, 2AX0)[307] revealed that the inhibitors bound covalently to Cys366 in the palm domain approximately 8 Å from the Gly-Asp-Asp sequence at the active site, stabilizing the position of the β-loop and inhibiting a conformational change needed to bind template or form the initiation complex.[307] The formation of a covalent complex was of note because inhibitors were shown to be reversible inhibitors of HCV NS5B polymerase. Although these inhibitors were found to be covalently bound to the NS5B polymerase, studies showed that they were not nonspecific alkylators or broad spectrum inhibitors of proteins containing active cysteine residues. Unfortunately, no cellular replicon data were reported for this series even though a rat PK study indicated good exposure after oral dosing.

Two series of HCV polymerase inhibitors which contain the α,γ-diketo acid pharmacophore believed to interact with divalent metal ions at the active site of the polymerase were identified through high throughput screening (Figure 28).[308]

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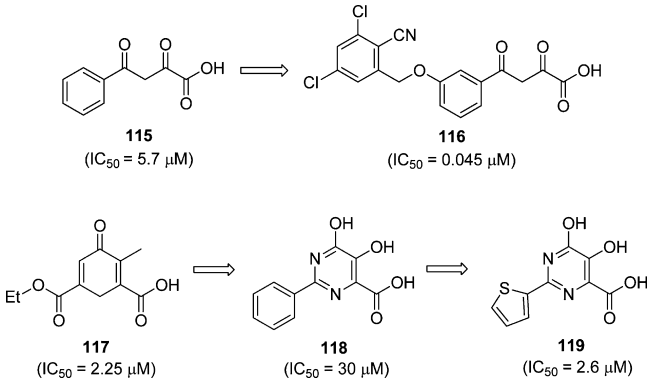
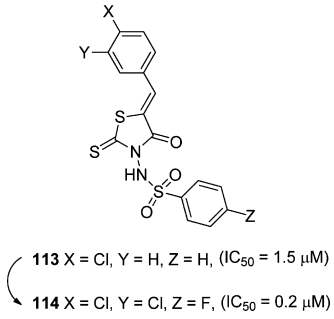


Figure 28. α,γ-Diketo acid and dihydroxypyridine palm site NS5B inhibitors.

The simple α,γ-diketo acid **115** (IC_{50} = 5.7 μM) was shown to be selective for HCV polymerase over DNA polymerase, poliovirus RdRp, and HIV reverse transcriptase (HIV-RT). SAR on the α,γ-diketo moiety showed that it was essential for activity. Modifications to the aryl substituent that included replacement with five- and six-member heterocycles and substituted phenyl groups led to the identification of



compound **116** (IC_{50} = 0.045 μM) as the most potent derivative when tested in the HCV polymerase isolated enzyme assay.[308] Compound **116** was also shown to be highly selective as an inhibitor of HCV polymerase over other human polymerases and HIV-RT. However, no data on inhibition of HCV RNA replication in the cellular replicon assay was reported. In a second screen the monoethyl ester of meconic acid **117** (IC_{50} = 2.25 μM) was identified as an inhibitor of the HCV NS5B polymerase, and kinetic competition experiments indicated that inhibitors **115** and **117** inhibited the HCV polymerase in a mutually exclusive manner, thus indicating a common mechanism of action.[309] However, since the chemotype **117** was known to be both chemically and biologically unstable, the dihydroxypyrimidinecarboxylic acid **118** (IC_{50} = 2.3−30 μM, dependent on divalent cation) was designed as a hybrid of both the diketo acid **115** and meconic acid **117**. Subsequent SAR around the aryl substituent led to the thiophene derivative **119** (IC_{50} = 2.6 μM).[310] However, none of these dihydroxypyrimidinecarboxylic acid derivatives were shown to be active in the cellular replicon assay presumably because of poor cellular permeability.

An *N*-acylpyrolidine 2,4-diacid **120** (IC_{50} = 0.3 μM, Figure 29) having the 2*S*,4*S*,5*R* configuration and obtained from screening a combinatorial library became the starting point for a medicinal chemistry effort not only to improve potency against the genotype 1 NS5B polymerase but also to improve cellular replicon activity, as the diacid analogues were not active in the cellular assay.[311] Structure based design played an important role in the potency optimization effort, as cocrystal structures (2JC0, 2JC1)[312] showed that this class of molecules bound in the palm binding domain close to the catalytic site of HCV polymerase. Intriguingly, one crystallographic complex, 2JC0, showed that this chemotype was capable of binding at both the palm allosteric site and the thumb site II. Improved cellular potency and in vivo PK profile was achieved by introducing substitution on the *N*-acylphenyl ring and converting the C-4 acid to a methylene ether. This led to compound **121** (IC_{50} = 0.44 μM, EC_{50} = 0.39 μM) which was further optimized to the clinical development candidate **122** (GSK625433, IC_{50} = 0.028 μM, EC_{50}(GT1b) = 0.003 μM, EC_{50}(GT1a) = 0.29 μM).[313] In vitro selection of resistant replicons in the presence of **122** identified M414T and I447F as mutations in the NS5B polymerase conferring 83- and 170-fold resistance, respectively. In vitro combination studies demonstrated synergy between **122** and PEG-IFN.[313]

Application of an affinity selection−mass spectrometry platform for label-free high throughput screening of mixture-based combinatorial libraries (ALIS: automated ligand identification screening) identified several classes of compounds as inhibitors of the NS5B(Δ21)-genotype 1b (Con1) enzyme.[314] One class was based on (*S*)-*N*-(2-cyclohexenylethyl)-2-fluoro-6-(2-(1-hydroxy-3-phenylpropan-2-ylamino)-2-oxoethoxy)-benzamide **123**. SAR on this series focused primarily on the amide moiety and resulted in compound **124**, where submicromolar inhibition (IC_{50} = 0.8 μM) of the HCV polymerase GT1b enzyme was achieved. However, these compounds lost significant or complete activity when evaluated against the GT1a enzyme or against GTs 2, 3, and 4 enzymes. In addition, none of these benzamide derivatives demonstrated activity in the cellular HCV replicon assay. Cocrystallization of

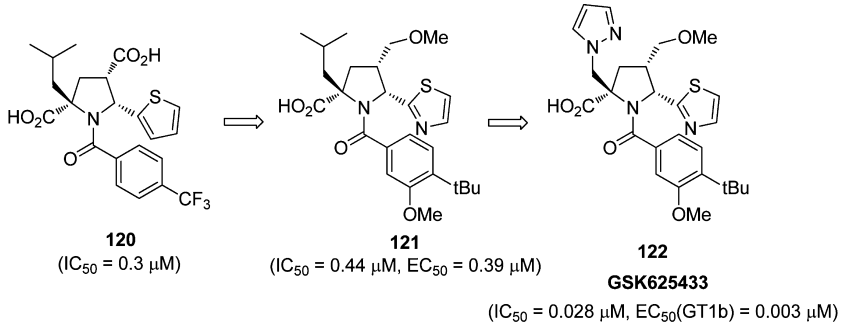


Figure 29. *N*-Acylpyrrolidine palm site NS5B inhibitors.

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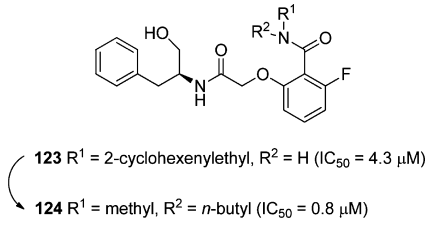
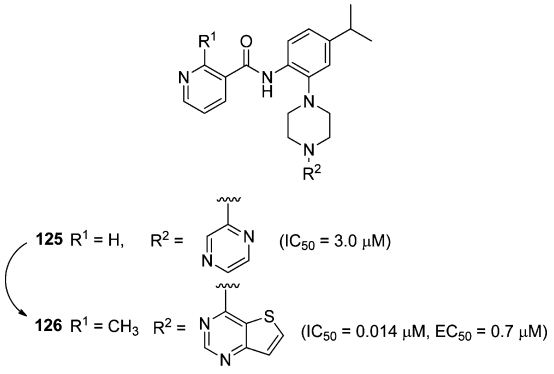
124 with the HCV polymerase (3LKH)[314] did show that this class of inhibitors bound near the top of the palm domain between the β-loop (residues 443−454) and the C-terminal tail region (residues 551−555) in what appeared to be a novel palm binding site.[314] Amino acid changes M414T, C316Y, and



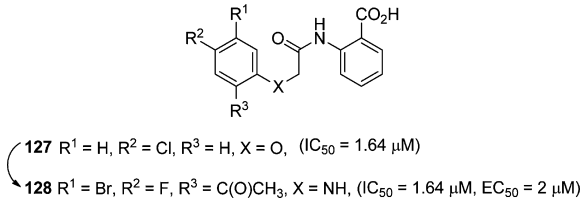
G554D in the NS5B enzyme resulted in significant loss of activity for this class.

A second inhibitor identified through ALIS was the *N*-(4-isopropyl-2-(4-(pyrazin-2-yl)piperazin-1-yl)phenyl)-nicotinamide **125** (IC_{50} = 3.0 μM).[315] 2D ^{15}N-HSQC NMR studies were undertaken to identify the binding site on the NS5B polymerase and concluded that these inhibitors share an overlapping palm binding site with the benzofuran **108**. It was also shown that C316N/Y, M414T, and G554D amino acid changes in the NS5B polymerase resulted in significant loss in activity for this class of inhibitors. Modifications of both the pyridine carboxamide moiety and the piperazine substituent led to the most active compound **126** (IC_{50} = 0.014 μM,



EC_{50} = 0.7 μM); however, genotype coverage beyond GT1b was not observed and cellular toxicity was clearly an issue.

A cocrystal structure of HCV NS5B polymerase with the anthranilic acid screening hit **127** (IC_{50} = 1.64 μM) demonstrated that this inhibitor bound between the thumb and palm domains approximately 7.5 Å away from the NTP binding site (2QE5).[316] SAR development explored the phenoxy ring substitutents, the anthranilic acid substitution pattern, and the linker region. Although inhibition of NS5B polymerase in the isolated enzyme assay (**128**, IC_{50} = 0.01 μM)



was achieved, this range of enzyme activity translated into only an EC_{50} of 2 μM in the cellular replicon system.

Like many of the other non-nucleoside palm site inhibitors of HCV NS5B polymerase, the benzodiazepine chemotype was identified through screening of a library of small molecules.[317,318] Additionally, a structure-based design approach played a key role in the lead optimization of this series (Figure 30). The

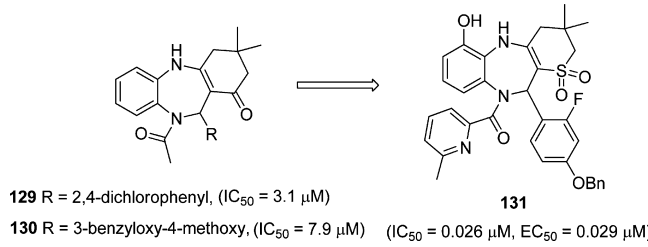


Figure 30. 1,5-Benzodiazepine palm site NS5B inhibitors.

initial screening hits **129** (IC_{50} = 3.1 μM, EC_{50} > 32 μM) and **130** (IC_{50} = 7.9 μM, EC_{50} = 12.3 μM) were observed to be modest inhibitors of the GT1b HCV polymerase and weak inhibitors of HCV RNA replication in the cellular replicon assay. An X-ray crystal structure of a benzodiazepine analogue bound to NS5B polymerase (3GOL)[318] demonstrated that this chemotype bound to the palm domain adjacent to the active site and did not require any conformational adjustment of the protein for binding. In addition, a critical H-bonding interaction with Tyr448 was observed, and it was this interaction that would become critical for the design of more potent derivatives. Further profiling of the benzodiazepines revealed that like other non-nucleosides they appeared to inhibit the initiation step in RNA replication and were only inhibitors of the genotypes 1a and 1b and not of genotypes 2−6 HCV polymerases. However, they did show good selectivity for the HCV polymerase over other viral and human polymerases as well as HIV-RT. The SAR of this series was extensively explored evaluating all regions of the template and also determined that the *R*-enantiomer was more active than the *S*. By leverage of crystal structure information obtained for the benzodiazepines and other palm site binders such as the proline sulfonamides (Figure 26), sulfone derivatives were developed to take advantage of the H-bonding interaction with Tyr448 and a potential H-bond interaction with Gly449. The sulfone derivative **131** did in fact show a substantial improvement in activity against the enzyme (IC_{50} = 0.026 μM) and in the replicon assay (EC_{50} = 0.029 μM) relative to its carbonyl parent (3GNW).[319]

Isothiazoles were identified as mechanistically unique direct active-site inhibitors of the HCV NS5B polymerase.[320] The initial lead isothiazole **132** was shown to be active as an inhibitor of the isolated NS5B polymerase (IC_{50} = 5.9 μM) and in the replicon assay (EC_{50} = 2.0 μM). The isothiazole core was shown to be critical to activity, and electronegative substituents on the phenyl ring were important in maintaining potency. The importance of the isothiazole core was revealed in the X-ray structure of the complex of **133** with the NS5B polymerase (2IJN).[320] The X-ray structure of the complex showed that **133** was ring-opened forming a disulfide bond with the Sγ of Cys366 and that the SAR could be explained on the basis of the ability of the compound to undergo ring-opening upon nucleophilic attack by the Sγ. The analogous isoxazoles which cannot undergo disulfide bond formation and concomitant ring-opening were essentially inactive.

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THUMB DOMAIN SITE I INHIBITORS

The thumb site I, also known as the finger-loop site,[321] is approximately 30 Å from the active site of the polymerase and is the juncture where the thumb interacts with part of the finger loop. In the thumb site I, it was initially believed that inhibitors might have an overlapping binding site with that of the allosteric GTP (Figure 31).[322] However, crystallographic

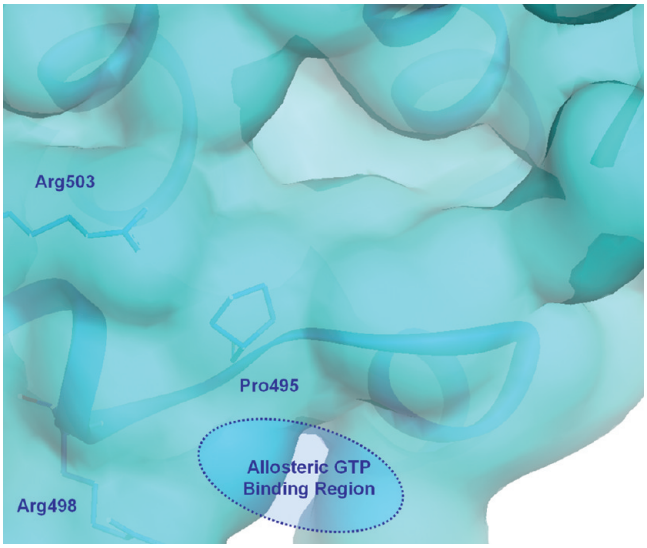

Figure 31. The allosteric binding pocket thumb domain I draped in a blue molecular surface is adjacent to the allosteric GTP binding site and is the pocket into which the side chains of Leu30/Leu31 of the finger loop would pack. With the indole class of inhibitors, these would be mimicked by substituents at the 3 and 2 positions, respectively. Arg503 provides stabilization to the acid/amide substitution typically found at the 6 position of the indole class. Arg499 has a similar role with some of the longer indole inhibitors. Pro495 plays a dominant role in development of resistance, since it provides part of the surface against which inhibitors pack. Both steric hindrance and increased flexibility with loss of the proline could lead to decreased inhibitor binding.

complexes of the site I inhibitors clearly show that they bind into pockets usually occupied by the side chains of Leu30 and Leu31. By preventing the "fingertip" α-helix residues from binding, this critical loop remains flexible and disordered in the crystallographic complexes studied. Since these two blocked residues fall between Ser29 and Arg32 which make critical interactions with the allosteric GTP,[109] the thumb site I NNIs indirectly prevent GTP binding. Mechanistic studies with inhibitors have shown that they are noncompetitive inhibitors that must act to inhibit the RdRp initiation process, as they are ineffectual once the RdRp−RNA complex has formed.[322]

Benzimidazole 5-carboxylic acids are the first class of molecules that were reported to bind to the thumb site 1 (Figure 32). Phenyl ether **134** (Figure 32), which inhibits genotype 1b NS5B polymerase with IC_{50} = 3.2 μM, was an initial lead compound discovered as a screening hit.[323] Further optimization mainly focused on extending the molecule from the C-2 position of the benzimidazole, providing compound **135** (JTK-109, EC_{50} = 0.32 μM) that exhibited inhibitory activity toward genotype 1a (IC_{50} = 0.062 μM), genotype 1b (IC_{50} = 0.017 μM), and genotype 3a (IC_{50} = 0.061 μM) HCV polymerase.[324] However, **135** demonstrated a >100-fold reduction in potency against genotype 2a (IC_{50} = 6.4 μM) and genotype 2b (IC_{50} = 2.0 μM) polymerases, which is a common feature among the thumb site I inhibitors. The rat PK profile of **135** distinguished itself from other compounds in the series by exhibiting a high plasma concentration (4.5 μM, 2 h after oral dosing of 30 mg/kg), acceptable bioavailability (F = 36%), and good liver/plasma ratio (>10-fold). Therefore, along with its predecessor JTK-003[303] (structure not disclosed), **135** advanced into phase 2 clinical trials in the U.S. and Japan. However, the trial was subsequently terminated for undisclosed reasons. Additional optimization efforts directed at investigating a non-biphenyl substructure afforded morpholine **136** (IC_{50} = 0.042 μM, EC_{50} = 0.27 μM), but the IC_{50}/EC_{50} ratio was high, signaling potential problems in membrane permeability, metabolism, and/or protein binding.[325] Efforts to address these issues provided novel tetracyclic indoles which became another important class of thumb site I inhibitors.

In separate efforts, 5-carboxylic acid **137** (IC_{50} = 1.6 μM, Figure 32) was identified by high throughput parallel synthetic techniques as the minimal core for inhibiting the NS5B polymerase.[326−328] Subsequent SAR efforts were concentrated on the elaboration of the C-5 position of the benzimidazole by extending the molecules through carboxamide derivatization. Tryptophan analogue **138** displayed enhanced potency (IC_{50} = 0.3 μM, EC_{50} = 1.7 μM), possessing the best subgenomic replicon activity in the series. From biochemical assays[329] and NMR experiments such as differential line broadening (DLB) and transferred NOESY,[330] the benzimidazole 5-carboxylic amides were confirmed to specifically interact with NS5B (not with RNA) by noncompetitively inhibiting the HCV polymerase activity at the initiation phase. In addition, amino acid substitutions (P495S/A/L, P496S/A, and V499A) as well as photoaffinity labeling experiments verified the interaction of the benzimidazoles at the top of the thumb domain binding pocket.[331] Further optimization on the amide chain[332] and the C-2 substitution[333] produced cyclopentyldiamide **139** with an EC_{50} of 0.55 μM and improved in vitro ADME profile. But this series of compounds showed poor rat PK characteristics due to low plasma exposure and rapid clearance.

In subsequent efforts to improve cellular permeability and anti-HCV potency in the cell-based replicon assay, exploration of the more lipophilic indole scaffold was favored over the bezimidazole core. The simple substitution of a nitrogen with a carbon in indole **140** (Figure 33) along with N-methylation produced more than a 20-fold enhancement of cell-based activity (EC_{50} = 0.05 μM) when compared to benzimidazole **138**.[334] Further optimization efforts were directed toward substitution on the N-1 position[335] as well as chain elongation on the 6-carboxamide of the indole scaffold.[336,337] By employment of combined efforts in X-ray crystallography (see 3QGF,[338] for example), NMR, computational modeling, as well as resistance mutations and photoaffinity labeling studies, it was discovered that the structural hinge derived from the gem-dialkyl/cycloalkyl moiety of the diamides such as **141** (BILB1941) induced the L-shape bioactive conformation that is required for effective inhibition.[339,340] In the replicon assay, **141** displayed submicromolar activity with EC_{50} = 0.084 μM against genotype 1b and EC_{50} = 0.153 μM against genotype 1a. Resistance mutations in NS5B were found to take place in the thumb site I (P495, P496, and V499), and animal PK studies showed high oral bioavailability (64−70%). In the phase 1 clinical trials, male HCV genotype 1 patients were treated with

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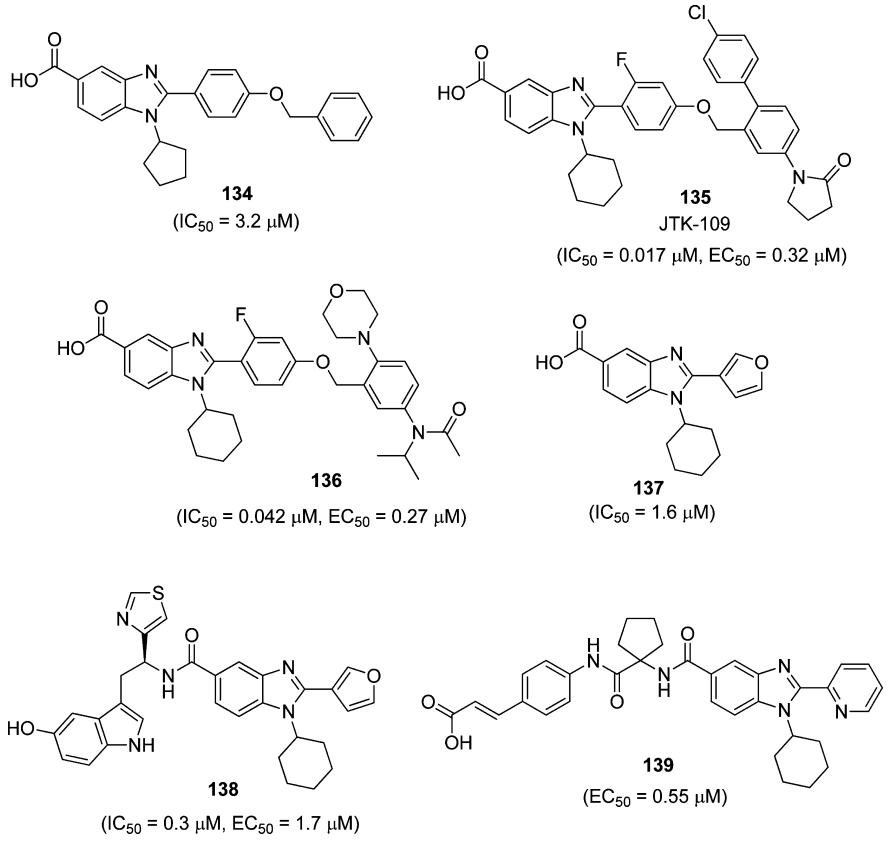

Figure 32. Benzimidazole-based NS5B thumb site I inhibitors.

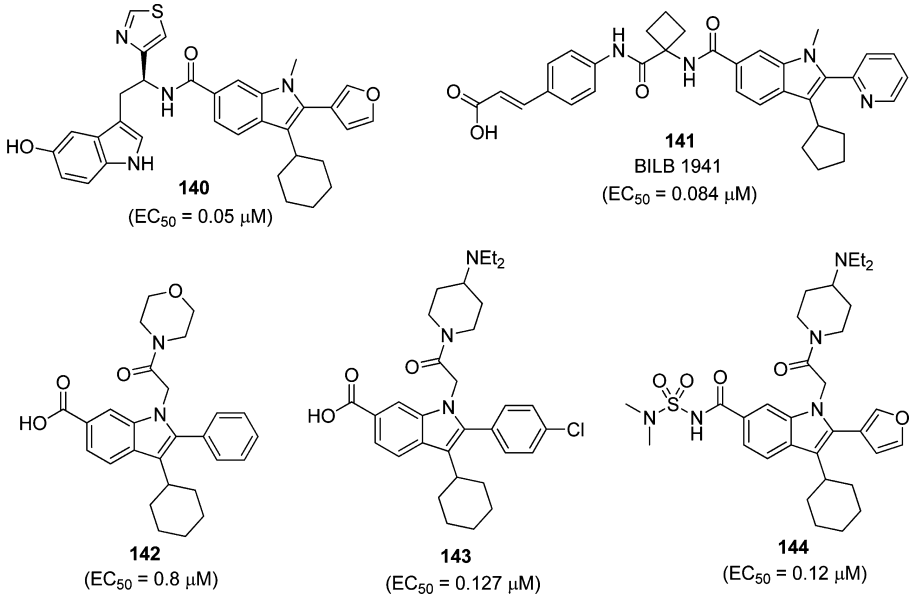

Figure 33. Indole-based NS5B thumb site I inhibitors.

10−450 mg of **141** q8h for 5 days.[341] Up to a 300 mg dose, **141** showed dose dependent increase in plasma drug levels, but only a small fraction of subjects (9 out of 47) showed more than −1 $\log_{10}$ IU/mL drop of viral load. Moreover, gastrointestinal intolerance prohibited testing of higher doses, and the study was discontinued.

The second generation NS5B thumb site I inhibitor, BI 207127[342] (structure not disclosed, GT1a EC_{50} = 0.043 μM, GT1b EC_{50} = 0.017 μM) also advanced to human clinical trials and produced improved results. In a 5-day monotherapy study, patients were treated with 100, 200, 400, 800, or 1200 mg of BI 207127 q8h via oral tablets and mean viral load declines of −0.4, −0.8, −1.3, −3.8, and −3.2 $\log_{10}$ IU/mL, respectively, were reported.[342,343] The tolerability was reported to be good in 41 out of 46 subjects, but moderate generalized erythema, mild localized rash, mild photosensitization, and moderate diarrhea were reported as adverse events among 800 and 1200 mg dose groups. From phenotypic analysis, P495L/Q/S

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resistant mutants were observed in 5 out of 46 patients on day 6.[344] These NS5B mutations conferred an 85-fold (P495S) to 380-fold (P495L) decrease in sensitivity to BI 207127. However, all of these resistant variants remained sensitive to PEG-IFN and a NS3/4A protease inhibitor, suggesting the feasibility of combination therapies with these drugs. Therefore, in later clinical studies, BI 207127 (400, 600, or 800 mg q8h) was dosed in combination with the SOC over 4 weeks.[345] In a treatment naive patient population (n = 27), the median reduction in HCV viral loads was −5.1, −5.6, and −5.4 $\log_{10}$ IU/mL in each dosing group, respectively. On the other hand, in a previously treated patient population (n = 30), the median reduction in HCV viral loads was only −2.9, −4.2, and −4.5 $\log_{10}$ IU/mL, respectively. In the SOUND-C1 trails, BI 207127 was dosed in an PEG-IFN-sparing regimen in combination with an NS3/4A protease inhibitor and RBV.[346] Over 4 weeks, 400 or 600 mg of BI 207127 q8h, 120 mg of the protease inhibitor q.d., and RBV were given to 32 treatment naive genotype 1 patients. All patients showed a rapid drop in viral load during the first 2 days of treatment, and 3 out of 15 patients in the 400 mg BI 207127 group and 12 out of 17 patients in the 600 mg BI 207127 group were found to have plasma HCV RNA levels below the limit of detection (<10 IU/mL) on day 29. The tolerability was rated as superior to the SOC, and no severe adverse event or early discontinuations were reported in this study. Phase 2 clinical trials using this triple therapy protocol that includes BI 207127 are under active investigation.

In 2005, the X-ray cocrystal structures of HCV polymerase with indole-based thumb site I inhibitors (e.g., indole-6-carboxylic acid **142**, Figure 33) were reported (2BRK).[321] The binding site in the thumb domain was predominantly lipophilic with the aromatic ring of the indole scaffold stacked against the P495 side chain. The structure revealed that the inhibitor's binding to the surface at the top of the thumb domain prevented its intramolecular contacts with the α-helix A of the fingertip Λ1 loop, thus hampering the formation of a productive RNA−enzyme complex. This structural information guided the optimization efforts that led to the discovery of *N*-acetamide indoles such as **143** (Figure 33) which showed submicromolar cell-based efficacy (EC_{50} = 0.127 μM) and acceptable PK profile in rat and dog without any undesired off-target activity such as pregnane-X receptor activation.[347,348] Liability toward the formation of glucuronide conjugates with the carboxylic acid was an issue in the metabolism of this class. In vitro studies using rat liver microsomes showed that replacement of the acid functionality with oxadiazolone or acylsulfonylamide could reduce the problematic conjugation, and further optimization provided sulfonylurea **144** which maintained similar potency (EC_{50} = 0.12 μM) in the replicon assay.[349]

A number of variations of the indole scaffold have been explored as well (Figure 34). Among them, thieno[3,2-*b*] pyrrole **145** gave equivalent potency (IC_{50} = 0.058 μM, EC_{50} = 2.9 μM) in comparison to its indole congener (see 2WCX,[350] for example).[351] Further SAR studies produced the 3-methyl analogue **146** with submicromolar replicon potency (IC_{50} = 0.010 μM, EC_{50} = 0.13 μM), but its animal PK data were disappointing (no bioavailabilty in rats) possibly because of limited absorption in the GI tract. The thienopyrrole core with an extended C-5 chain (e.g., phenyl ether **147**) was also explored and showed submicromolar inhibitory activities (IC_{50} = 0.010 μM, EC_{50} = 0.052 μM).[325] Other scaffold modifications

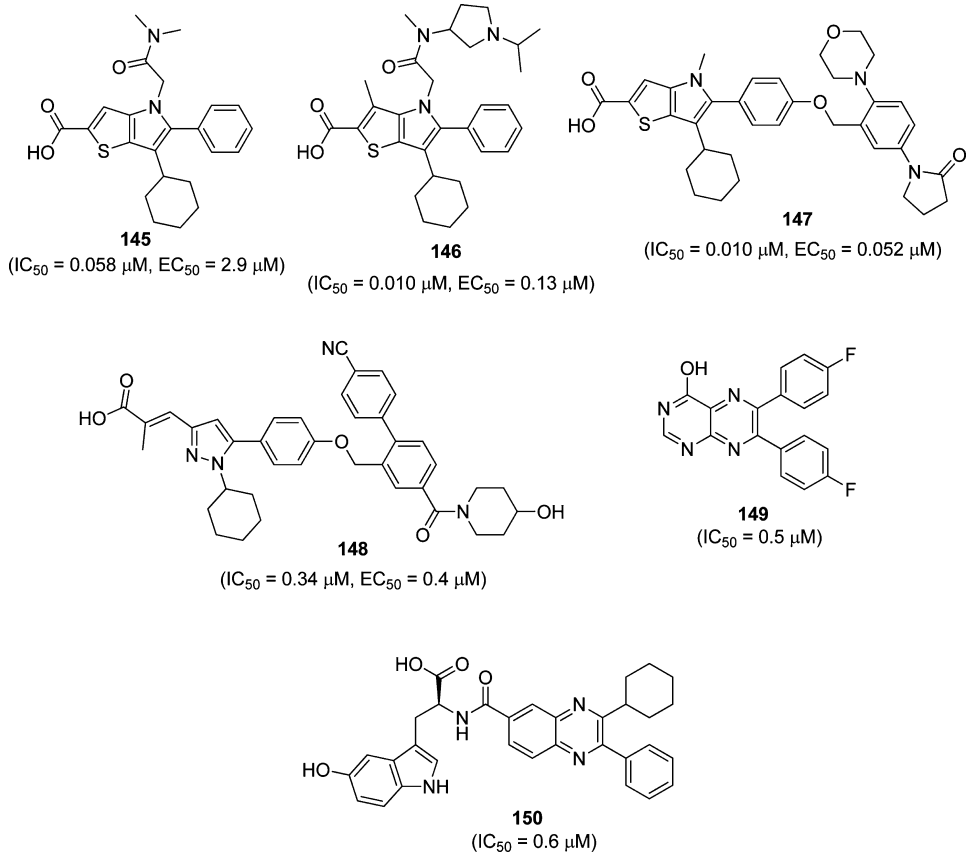

Figure 34. NS5B thumb site I inhibitors with indole derived scaffolds.

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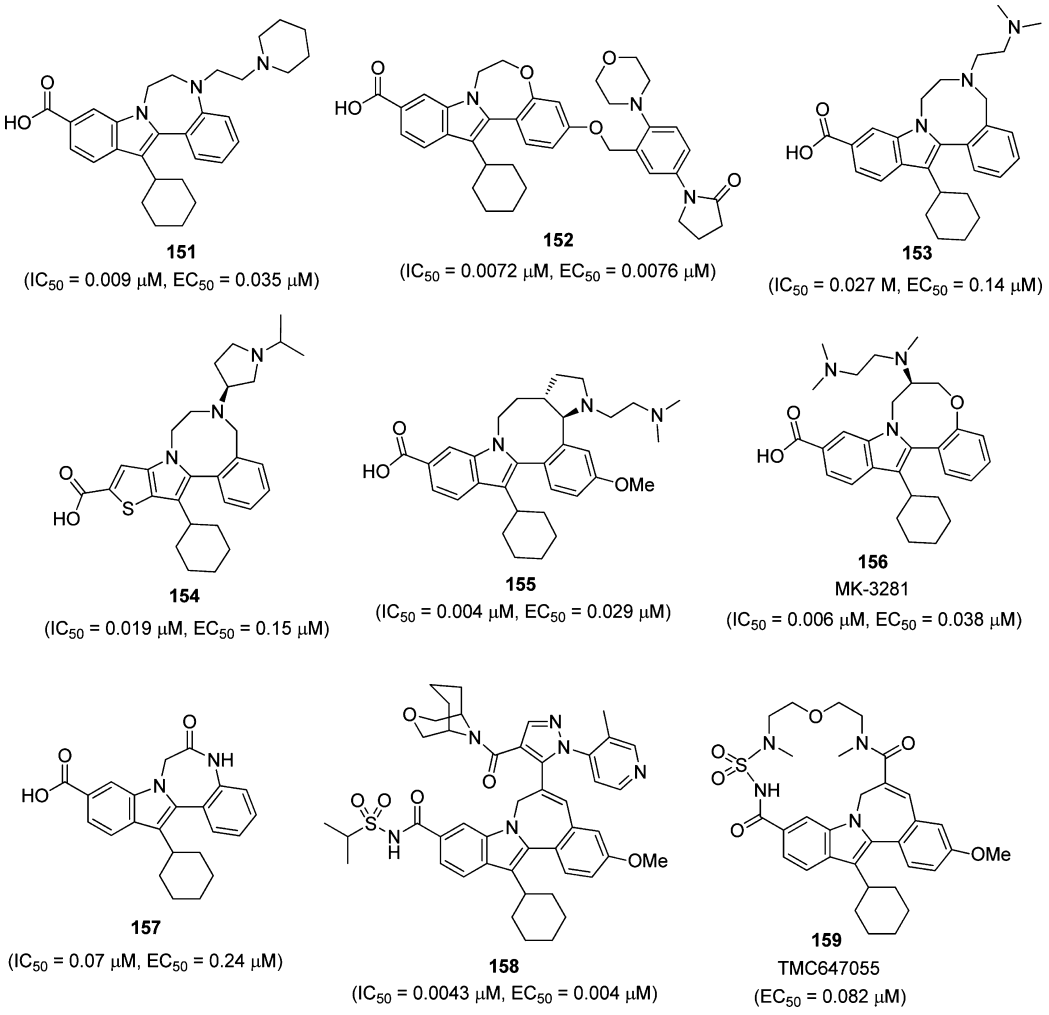

Figure 35. Conformationally restricted multicyclic NS5B thumb site I inhibitors.

reported in the literature include pyrazole **148** (IC_{50} = 0.34 μM; EC_{50} = 0.4 μM; resistant mutant, P495A),[352] pteridine **149** (IC_{50} = 0.5 μM),[353] and quinozaline **150** (IC_{50} = 0.6 μM).[354]

Introducing a conformational restriction by the use of a fused ring system has been demonstrated to be a successful approach in the development of potent thumb site I inhibitors, such as indole-based tetracycles.[355] The SAR studies on the dihedral angle (φ) between the benzimidazole and the C-2 phenyl group with varying size (five- to eight-membered) and atom composition (CH_2, NH, O, or S) of the ring, assisted by structural information obtained from ligand−enzyme cocrystallography, revealed that the best enzymatic and cell-based inhibitory activities were achieved when φ was around 46° in a seven-membered ring system. Benzodiazepinoindole **151** displayed remarkable potency with an IC_{50} of 0.009 μM and EC_{50} of 0.035 μM. Additional optimization on the benzyl ether side chain provided compound **152** (Figure 35), which was demonstrated to be equipotent in biochemical and cellular assays with single digit nanomolar inhibition (IC_{50} = 0.0072 μM, EC_{50} = 0.0076 μM).[325]

The strategy employing conformational constraint was quickly followed by several other research groups. In 2009, several articles describing the SAR on tetracyclic/pentacyclic thumb site I inhibitors were published.[350,356,357] The tetracyclic benzodiazocine **153** showed improved enzymatic and cell-based potency (IC_{50} = 0.027 μM, EC_{50} = 0.140 μM) and good animal PK profiles. Tetracycles based on thieno[3,2-*b*]pyrrole instead of indole, such as compound **154**, also gave similar in vitro activities (IC_{50} = 0.019 μM, EC_{50} = 0.150 μM) and good in vivo PK profiles. Benzoazocine **155** with a novel pentacyclic scaffold showed enhanced potency in both enzymatic and replicon assays (IC_{50} = 0.004 μM, EC_{50} = 0.029 μM), but this class of molecules was not orally bioavailable. Further optimization efforts were directed at the search for a novel scaffold with low nanomolar potency like **155** and good pharmacokinetic characteristics like **153**. As a result, benzooxazocine **156** (MK-3281, IC_{50} = 0.006 μM, EC_{50} = 0.038 μM) was discovered and later advanced into phase I human clinical trials.[358] The crystallographic complex with HCV polymerase was also solved (2XWY).[358] In preclinical studies, **156** exhibited comparable activities against genotype 1a (EC_{50} = 0.028 μM) and genotype 3a (EC_{50} = 0.037 μM) replicons but was only weakly active against genotype 2a (EC_{50} = 2.02 μM) and genotype 2b (EC_{50} = 1.83 μM) because of a difference in the shape of the binding pocket resulting from dissimilar amino acid composition.[359] Compound **156** displayed good oral bioavailability and moderate plasma clearance in animal PK experiments and demonstrated in vivo efficacy in HCV-infected (genotype 1b) humanized mice with a mean decrease of HCV plasma RNA levels of −3.13 $\log_{10}$ IU/mL after twice a day intraperitoneal injections of 50 mg/kg over 4 days. In phase 1 human clinical trials, **156** was generally well tolerated up to 800 mg b.i.d. over a 10 day

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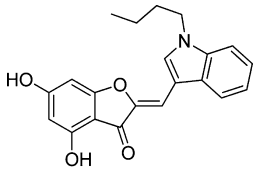
period.[360] In single ascending dose studies (25−2250 mg), **156** gave dose proportional increases in plasma drug levels with median T_{max} = 2.5−3.5 h, and the concentrations decreased in a biphasic manner with apparent $T_{1/2}$ = 14.3−18.6 h, supporting twice daily dosing to maintain effective drug concentrations in plasma. In a 7-day monotherapy study (800 mg b.i.d.), **156** demonstrated a significant anti-HCV response with mean max plasma HCV RNA changes of −1.28 $\log_{10}$ IU/mL for genotype 1a patients, −3.75 $\log_{10}$ IU/mL for genotype 1b patients, and −1.53 $\log_{10}$ IU/mL for genotype 3 patients.[361]

Benzodiazepinone **157** was found to show submicromolar biochemical and cell-based activities (IC_{50} = 0.07 μM, EC_{50} = 0.24 μM) and adequate iv PK properties in rat with increased exposure and bioavailabilty (F = 17.6%, CL = 35 mL min^{-1} kg^{-1} at 2 mg/kg dose). More optimized analogues such as benzoazepine **158** exhibited low nanomolar potency in vitro (IC_{50} = 0.0043 μM, EC_{50} = 0.004 μM).[362] The novel 17-membered macrocyclic indole **159** (TMC647055) was reported to demonstrate strong potency in a replicon assay (EC_{50} = 0.082 μM) and favorable PK characteristics in rat and dog (F = 70−80%).[363] The thumb site I inhibitor HCV polymerase inhibitor **159** is currently in phase 1 clinical trials.



160

(IC_{50} = 2.2 μM)

Recently, a new aureusidine analogue **160** was reported as an inhibitor of HCV RdRp (IC_{50} = 2.2 μM).[364] On the basis of the activity shift toward the P495L mutant polymerase (6-fold) and molecular modeling results, the authors postulated that the benzofuran-3(2*H*)-ones were a novel class of molecules that bound to the thumb site I of NS5B polymerase.

THUMB DOMAIN SITE II INHIBITORS

The thumb site II, also known as NNI site II, is a narrow hydrophobic cavity near the base of the thumb domain, approximately 35 Å apart from the active site (Figure 36). Sequence analysis showed that the primary structure of this binding site was relatively well conserved among the known HCV genotypes.[365] The binding site traverses the thumb from front to back of the RdRp "right hand" but is relatively shallow and narrow (30 Å × 10 Å × 10 Å). It is bounded by three of the α-helices in the thumb subdomain separated by the link between two of these from the fingertip α-helix/site I region that is 15 Å away and is proximate to the C-terminus of the thumb. The crystallographic complexes for the thumb site II NNIs are very similar to those for the free protein. The mechanism of action for these inhibitors is not known. It has been suggested that inhibition is due to lack of dynamism[366,367] and/or blocking association with other proteins/RNA important for replication.[365]

Several classes of structurally distinct molecules have been shown to bind to the thumb site II allosteric site. Its existence was first reported in 2002 in a patent application describing the X-ray cocrystal structure (1OS5)[365,368] of genotype 1b NS5B polymerase in complex with dihydropyrone **161** (IC_{50} = 0.93 μM, Figure 37). The cyclopentane moiety of **161** fit into the hydrophobic pocket surrounded by M423, W528, L419, Y477,

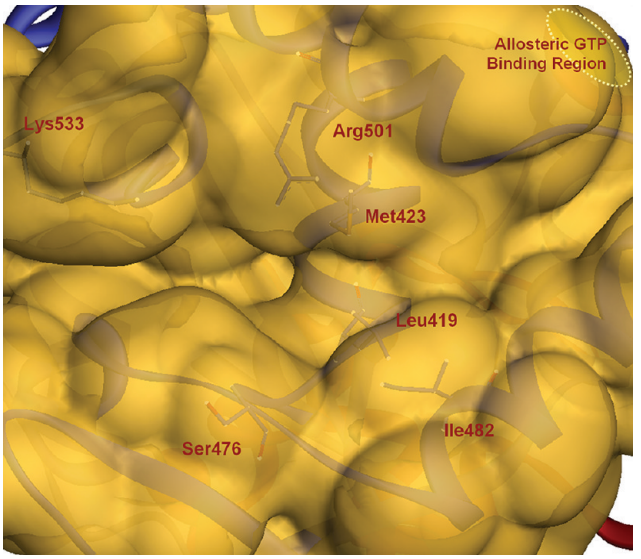


Figure 36. On the other face of the thumb domain and further removed from the allosteric GTP binding surface, a variety of inhibitor types can fit into the relatively shallow and narrow (30 Å × 10 Å × 10 Å). Thumb site II is indicated with the orange surface. Many of the bound inhibitors form stabilizing interactions with the NH of Ser476 and hydrophobic residues including Met423, Leu419, and Ile482. Some of the inhibitor classes contain acidic functionality which is stabilized by interaction wth Arg501 and Lys533. Unfortunately, resistance has been reported to occur with mutation of some of these hydrophobic residues.

and R422, while several polar functional groups made hydrogen bonds. These hydrogen bonds include the phenol oxygen with the main chain amide of L497 in a water-mediated way, the enol oxygen of the dihydropyran-2-one with the amide S476 directly and also with the amide of Y477 in a water-mediated way, and the carbonyl group of the dihydropyran-2-one with the guanidinium moiety of R501 in a water-mediated way. Separately, other X-ray crystal structures of the HCV NS5B RdRp were reported where phenylalanine analogues (e.g., **162**, IC_{50} = 1.7 μM) bound to the same binding pocket of the NS5B polymerase (1NHU).[367,369] The key interactions of the inhibitor and the enzyme include extensive van der Waals and hydrophobic interactions between the three aromatic rings of **162** and the side chains of L419, M423, W528, and Y477, and two hydrogen bonds between the carboxylate oxygens and the backbone amide nitrogens of S476 and Y477.

SAR studies following the discovery of compound **161** from a high throughput screen revealed that a substantial gain in potency could be obtained by halo substitution at the ortho position of the phenol ring and by replacement of the phenyl ring attached to the sulfur with a heterocycle ring (e.g., dihydropyrone **163**, IC_{50} = 0.038 μM).[370] However, none of the compounds in the series showed significant antiviral activity in the cell-based replicon assay. Later, in further optimizations, it was revealed that switching the sulfur linker to a methylene unit reduced the acidity of the dihydropyrone by 10- to 100-fold and therefore greatly improved cell permeation of the inhibitors.[371] An enantiomer of **164** (absolute stereochemistry unknown), which was separated from a racemic mixture by chiral chromatography, exhibited low nanomolar potency in enzymatic and cell-based assays (IC_{50} = 0.003 μM against GT1b, IC_{50} = 0.070 μM against GT1a, EC_{50} = 0.015 μM) and a favorable rat pharmacokinetic profile including low plasma

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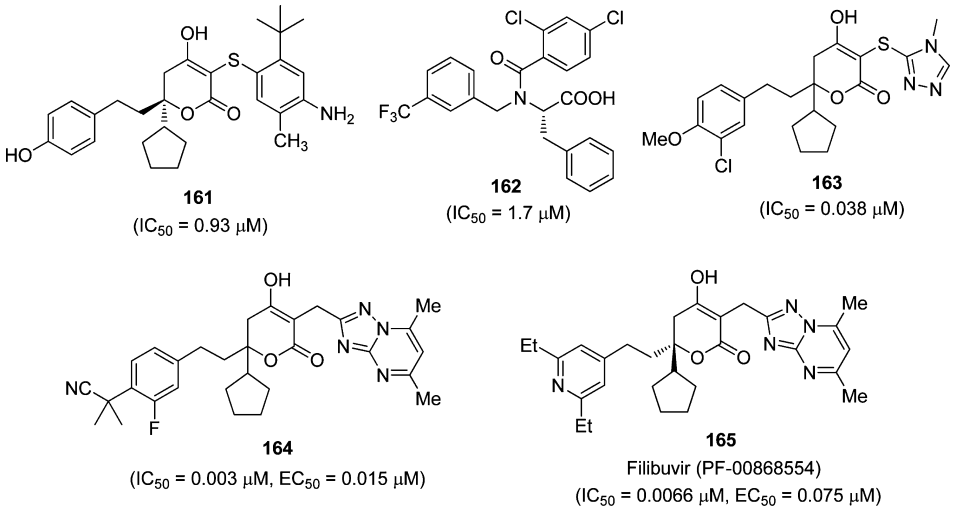

Figure 37. NS5B thumb site II inhibitors.

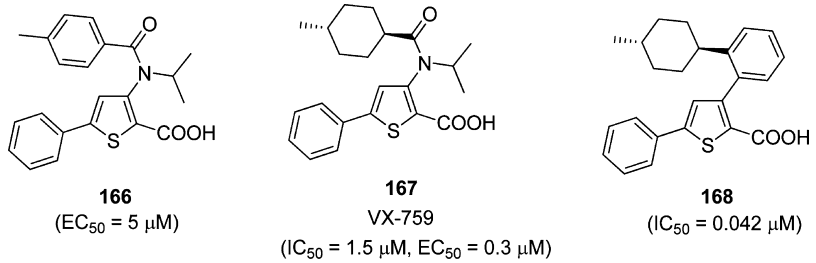

Figure 38. Thiazolone-based NS5B thumb site II inhibitors.

clearance and good bioavailability (CL = 3.7 mL min^{-1} kg^{-1} at 10 mg/kg po dose, F = 31%, effective $T_{1/2}$ = 0.88 h).

Further efforts to diminish CYP2D6 inhibition led to the introduction of a pyridine ring in the position of the phenyl group, resulting in the discovery of compound **165** (PF-00868554, filibuvir) (3FRZ).[372] Inhibitor **165** was selected to advance to human clinical trials based on its outstanding characteristics including strong inhibitory potency against genotype 1 HCV in biochemical and replicon assays (GT1b, IC_{50} = 0.015 μM; GT1a, IC_{50} = 0.021 μM and EC_{50} = 0.035 μM), no major CYP inhibition, no cytotoxicity in a panel of human cell lines, and enhanced animal PK profiles such as low plasma clearance and volume of distribution in dog, and high bioavailability supporting twice-daily dosing.[373] However, the inhibitory activity of **165** was significantly reduced against other genotype NS5B polymerases (GT2b, IC_{50} = 1.1 μM; GT3a, IC_{50} = 1.0 μM; GT4a, IC_{50} = 1.7 μM). The major resistant mutant was found as M423T, which gave a 761-fold activity shift (EC_{50} = 27 μM) but remained susceptible to PEG-IFN and a bezimidazole-based thumb site I inhibitor. In an 8-day monotherapy clinical trial, 24 treatment naive patients were treated with 100, 300, or 450 mg b.i.d. or 300 mg t.i.d. of **165** and exhibited mean reduction of HCV RNA levels of −0.97, −1.84, −1.74, and −2.13 $\log_{10}$ IU/mL, respectively, without severe adverse events.[374] On day 8, resistant mutations at M423 were observed in 11 out of 24 subjects. Six of them showed virologic breakthrough (>0.5 $\log_{10}$ IU/mL increase in HCV RNA from NADIR) during the treatment period.[375] In subsequent phase 2a studies, 200, 300, or 500 mg of **165** b.i.d. in combination with SOC were administered to HCV genotype 1 patients.[376] After 4 weeks, the mean reductions in HCV RNA were −4.29, −4.43, and −3.44 $\log_{10}$ IU/mL, respectively, and 6/10, 6/8, and 5/8 patients at the respective dose groups were found to be without any detectable plasma HCV RNA levels. Virologic breakthrough was observed among 1 out of 10, 1 out of 8, and 3 out of 8 patients, respectively. Viral resistant mutants M423V/T/I were detected from patients' plasma samples, and these mutations were shown to be a dominant pathway for **165** resistance in both genotype 1a and 1b patients.[377] But these variants did not cause cross-resistance to other anti-HCV drugs including NS5B nucleoside and palm domain inhibitors in vitro. As of 2011, a phase 2b clinical trial has been initiated where 300 or 600 mg of **165** b.i.d. in combination with SOC is administered over a 24-week period.

The early efforts on phenylalanine derivatives (e.g., **162**) quickly extended to the discovery of another new HCV polymerase inhibitor chemotype, thiophene-2-carboxylic acids such as **166** (Figure 38). Unlike its phenylalanine based predecessors, the thiophene-2-carboxylic acids **166** showed inhibition of HCV RNA replication in the subgenomic replicon assay in Huh-7 cells (EC_{50} = 5 μM).[378,379] The X-ray cocrystal structure confirmed that thiophene **166** fit into the same thumb site II binding site as other chemotypes (1YVX).[366] The main binding features of the thiophene-2-carboxylic acids were similar to those of the phenylalanine inhibitors: extensive van der Waals interactions with residues R422, M423, L474, H475, Y477, K501, and W528 and two hydrogen bonds between the carboxylic oxygens and the amide nitrogens of T476 and Y477 backbone. The presence of the thiophene inhibitors induced a change in the relative orientation of the thumb domain by 7.5°,

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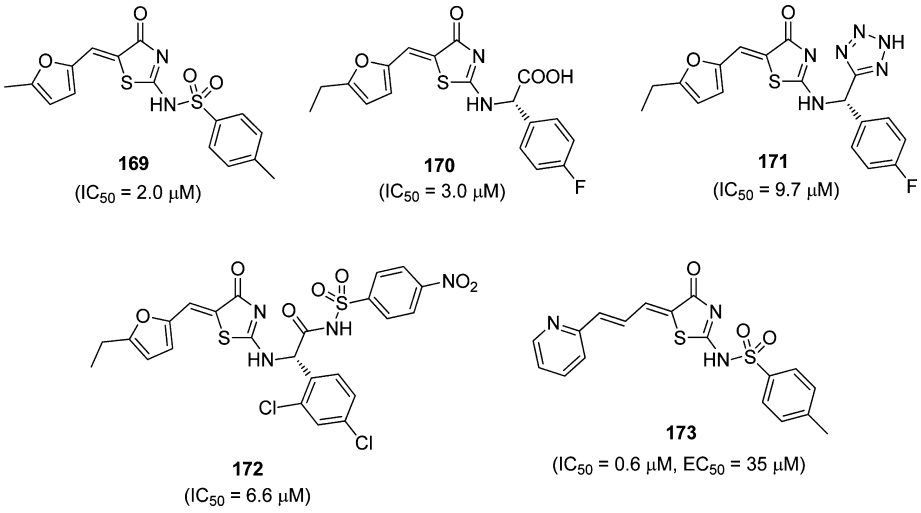

Figure 39. Thiazolone-based NS5B thumb site II inhibitors.

giving rise to the "open" conformation which was an inactive form of NS5B polymerase.

In subsequent efforts, thiophene analogue **167** (VX-759, formerly VCH-759) (2GIR)[277] was discovered to exhibit submicromolar replicon activity (IC_{50} = 1.5 μM, EC_{50} = 0.3 μM) and advanced into phase 1 human clinical trials. In a 10-day monotherapy study, patients were treated with 400 or 800 mg three times daily or 800 mg twice daily of **167** and exhibited mean maximal decreases in HCV RNA levels of −1.97, −2.30, and −2.46 $\log_{10}$ IU/mL, respectively.[380] Genotypic analysis of HCV NS5B variants revealed that the major resistance amino acid substitutions were M423T/V/I and L419M, which produced an 18- to 23-fold increase in EC_{50} compared to the wild type. Another thiophene-2-carboxylic acid derivative, VCH-916 (structure not disclosed, EC_{50} = 0.10 μM),[381] also has advanced into phase 1 clinical trials. Preclinical characterization of VCH-916 demonstrated good stability in human microsomes and hepatocytes, limited human CYP inhibition or induction, and excellent animal (rat, dog) PK profiles such as low total body clearance and superb oral bioavailabilities (>40%) as well as high liver to plasma ratio (5-fold).[381] In a 3-day monotherapy human clinical study using doses of 100 or 200 mg three times daily or 300 or 400 mg twice daily, VCH-916 produced HCV viral load declines of −0.6, −1.5, −1.5, and −1.5 $\log_{10}$ IU/mL, respectively.[382] Resistant mutants quickly emerged during the period of therapy, and the pattern was similar to that observed for VX-759: L419S/M, M423T/V/I, and I482L. Therefore, it was concluded that a drug combination therapeutic regimen was necessary with these drugs to prevent the selection of HCV polymerase resistant mutations.

VX-222 (formerly VCH-222, structure not disclosed) exhibited much enhanced in vitro potency in genotype 1 replicon assays (EC_{50} = 0.023 μM for GT1a, 0.012 μM for GT1b, and 4.6 μM for GT2a, CC_{50} = 45 μM).[383] VX-222 displayed a liver to plasma ratio of 5, good metabolic stability in human microsomes and hepatocytes, little potential to evoke CYP induction and inhibition, low body clearance, and good oral bioavailability in rat and dog PK studies (44% and 28%, respectively).[384] VX-222 was well tolerated at 750 mg b.i.d. treatment over 3 days and produced a reduction of −3.7 $\log_{10}$ IU/mL in HCV RNA levels among HCV genotype 1 patients without virologic rebound during treatment.[385,386] As of 2011, phase 2 clinical trials are in progress, where genotype 1 chronic HCV patients are treated with 100 or 400 mg b.i.d. of VX-222 in combination with protease inhibitor telaprevir (1125 mg b.i.d.) with/without PEG-IFN and RBV over 12 or 24 weeks. The dual all-oral therapy (VX-222 + telaprevir) was discontinued because of viral breakthrough. However, in the quadruple therapy (VX-222 + telaprevir + SOC), 83% (24 out of 29, 100 mg VX-222 b.i.d. regimen) or 90% (27 out of 30, 400 mg VX-222 b.i.d. regimen) of patients reached undetectable plasma levels of HCV RNA at the end of 24 weeks. Studies for another all-oral regimen (VX-222 + telaprevir + RBV) are also underway as a part of the phase 2 clinical program.

Recently, an approach toward the bioisosteric replacement of the 3-amide of the thiophene core with various aryl/heterocyclic rings was reported.[387] A cocrystal complex of substituted benzene analogue **168** showed that it bound to the thumb site II region of NS5B polymerase (3MF5).[387] Benzene analogue **168** displayed similar enzymatic inhibitory activity (IC_{50} = 0.042 μM) as its parent amide.

Another class of thumb site II inhibitor, thiazolones (e.g., **169**, IC_{50} = 2.0 μM), was discovered via high throughput screening (Figure 39). Molecular design based on computational virtual screening guided by the structural information from X-ray crystallography (2HWI)[388] provided a new amino acid derivative such as thiazolone **170** (IC_{50} = 3.0 μM) which gave a comparable potency in a biochemical assay. The two oxygens of the carboxylic acid in **170** formed hydrogen bonds with the guanidium group of R501 and the amino group of K533.[389] Further optimization efforts focused on the acid functionality introducing heterocyclic (e.g., tetrazole **171**, IC_{50} = 9.7 μM (2I1R))[389] or acylsulfonamide (e.g., thiazolone **172**, IC_{50} = 6.6 μM)[390] acid bioisosters; however, none of these replacements led to significantly improved potency. Extension to the alkylidene chain afforded submicromolar NS5B polymerase inhibitor **173**, but its cell-based activity was shown to be rather weak (IC_{50} = 0.6 μM, EC_{50} = 35 μM).

In 2004, pyrano[3,4-*b*]indoles (e.g., **174**, IC_{50} = 3.0 μM) were reported as potent and selective inhibitors of HCV NS5B (Figure 40).[391] SAR studies revealed that the carboxylic acid functionality was essential for inhibitory potency, and only the *R*-isomer at the tertiary center possessed activity (see 2WRM, for example). Elaboration on the substitution of the indole core provided compound **175** (HCV-371), which displayed inhibition toward genotype 1a, 1b, 3a, and 4 NS5B polymerases

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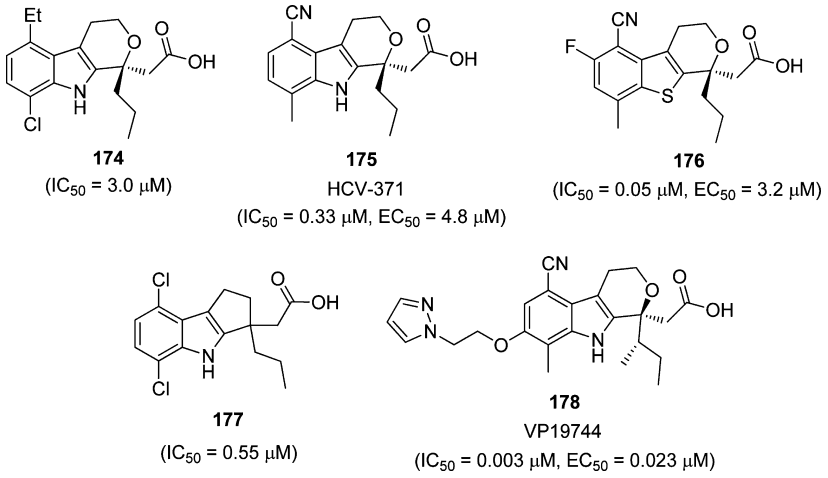

Figure 40. Pyranoindole and related NS5B site II inhibitors.

with IC_{50} of 0.3, 0.33, 1.4, and 17.8 μM, respectively.[392] Its cell-based activity was modest (EC_{50} = 4.8 μM), but multiple treatment with **175** over a 16-day period reduced replicon viral RNA levels more than $-3\ \log_{10}$ IU/mL, which was better than PEG-IFN treatment ($-2\ \log_{10}$ IU/mL reduction). Viral resistant studies showed that variations in the NS5B thumb site II region, such as L419M and M423V, resulted in 8- to 10-fold activity loss. In human clinical studies, **175** was well tolerated but did not demonstrate significant anti-HCV activity. A similar result was also obtained in a chimeric mouse model (scid-Alb/uPA) system.[167] Additional optimization efforts directed toward core modifications provided benzothieno[2,3-*c*]pyran analogues such as **176** (IC_{50} = 0.05 μM, EC_{50} = 3.2 μM)[393] and cyclopenta[*b*]indole analogues such as **177** (IC_{50} = 0.55 μM),[394] but the inhibitory potencies were not significantly improved. Enhanced cellular activity was obtained by substitution of the propyl moiety with an (*S*)-*sec*-butyl group and incorporation of an ethoxypyrazole chain into the indole scaffold.[395,396] Compound **178** (VP19744) exhibited low nanomolar potency in both enzymatic and cell-based assays (IC_{50} = 0.003 μM, EC_{50} = 0.023 μM for GT1b, EC_{50} = 0.0045 μM for GT1a). Replicon resistance studies in the presence of **178** showed that resistance was conferred by a single amino acid mutation, L419M, located in the dimple region of the thumb site II (EC_{50} = 0.49 μM). In an HCV-infected chimeric mouse experiment, **178** gave considerable reduction in plasma HCV RNA levels during the first 3 days of dosing, but the viral load rebounded in days 4–7, eventually leading to only an average $-1.2\ \log_{10}$ IU/mL reduction in HCV RNA levels when compared to untreated mice on day 8. The second clinical candidate in the series, HCV-086[397] (structure not disclosed), produced only a $-0.32\ \log_{10}$ IU/mL decline in plasma HCV RNA levels in a 14-day phase 1 study; consequently, its development was discontinued.

In 2009, a novel class of the NS5B thumb site II inhibitors represented by the benzo[*de*]isoquinoline-1,3(2*H*)-dione **179** (IC_{50} = 0.064 μM for GT1a, IC_{50} = 0.020 μM for GT1b) were reported to display nanomolar inhibition to NS5B polymerase genotypes 1a and 1b (Figure 41).[398] Dione **179** has been cocrystallized with the HCV polymerase for genotypes 1b BK and 2b HC-J8, 2WHO and 3HVO,[398] respectively. However, replicon activity was in the micromolar range (**179**, EC_{50} = 4.7 μM for GT1a, EC_{50} = 4.6 μM for GT1b) and most of the tetracycles in the series were somewhat cytotoxic (e.g., **179**, HB110A CC_{50} = 68 μM). SAR studies were primarily focused on the reduction of the cytotoxicity which potentially originated from DNA intercalation of the benzoisoquinolinediones. The carboxylic acid functionalized analogues such as **180** gave decreased cytotoxicity (CC_{50} > 100 μM), but replicon potency was moderated (EC_{50} = 13 μM for GT1b, EC_{50} = 17 μM for GT2b).[399] Recently, a novel quinolone-based thumb site II inhibitor **181** (IC_{50} = 1.15 μM) was reported as a screen hit, which gave a resistant mutation, M423F, in the NS5B thumb domain (>200-fold) (see 3PHE,[400] for example). Optimization efforts significantly improved the enzyme-based and replicon potencies of the series (e.g., compound **182**, IC_{50} = 0.008 μM, EC_{50} = 0.23 μM) as well as the safety window (~100-fold).

■ ALLOSTERIC INHIBITORS WITH UNDISCLOSED BINDING SITES

A series of imidazo[4,5-*c*]pyridines was discovered as a potent NS5B polymerase inhibitor class (Figure 42).[401] Initially, the imidazopyridine core was discovered in compound **183** (BPIP), an anti-pestivirus lead (BVDV EC_{50} = 0.070 μM).[402] BPIP **183** itself did not show any significant anti-HCV activity in a cell-based assay (EC_{50} > 50 μM). However, introduction of an ortho-fluorine to the 2-phenyl group (e.g., **184**) resulted in significant enhancement in HCV replicon inhibition (EC_{50} = 1.0 μM). Further optimization including additional halo substitutions and N-5 chain elongation provided imidazopyridine **185** (GS-327073),[401] which displayed nanomolar potency against HCV genotype 1b replicon (EC_{50} = 0.004 μM, Huh 5-2) but much reduced activity against BVDV (EC_{50} = 1.4 μM). Its activity against the HCV subgenomic genotype 2a replicon was approximately 100-fold weaker in comparison to its activity against the genotype 1b replicon. Compound **185** retained activity against various drug resistant replicons including the ones resistant to protease inhibitors such as telaprevir, nonnucleoside polymerase palm site inhibitor (benzothiadiazines), and nucleoside polymerase inhibitors (**3** and **19**).[401] *N*5-Phenylpyridazinylmethyl substituted analogue **186** (GS-9190, tegobuvir) was identified as the first clinical candidate in this class.[403] The NS5B NNI polymerase inhibitor **186** demonstrated strong in vitro potency against genotype 1 HCV replicons (EC_{50} = 0.0007 μM for GT1b, EC_{50} = 0.0025 μM for GT1a), and resistant mutations were shown to be in the HCV polymerase region (C316Y, C445F, Y448H, and C452H).

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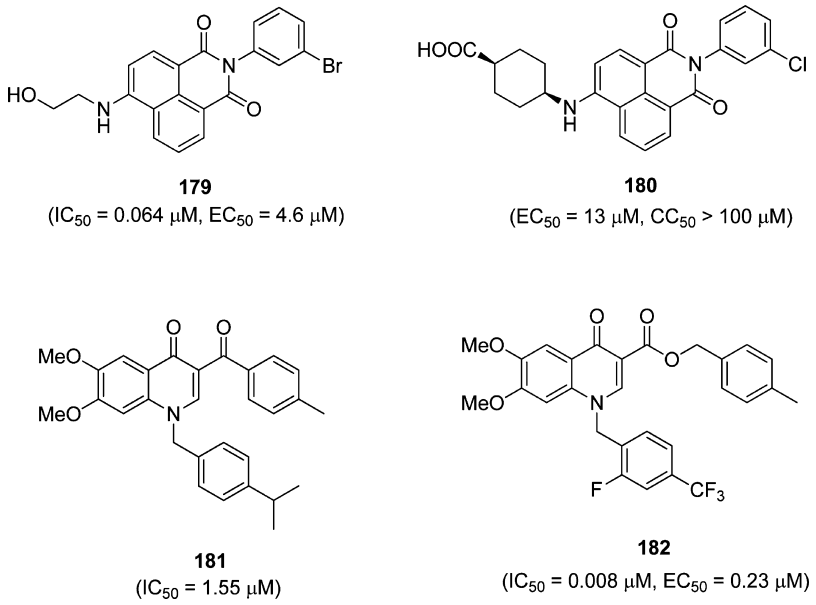

Figure 41. NS5B thumb site II inhibitors.

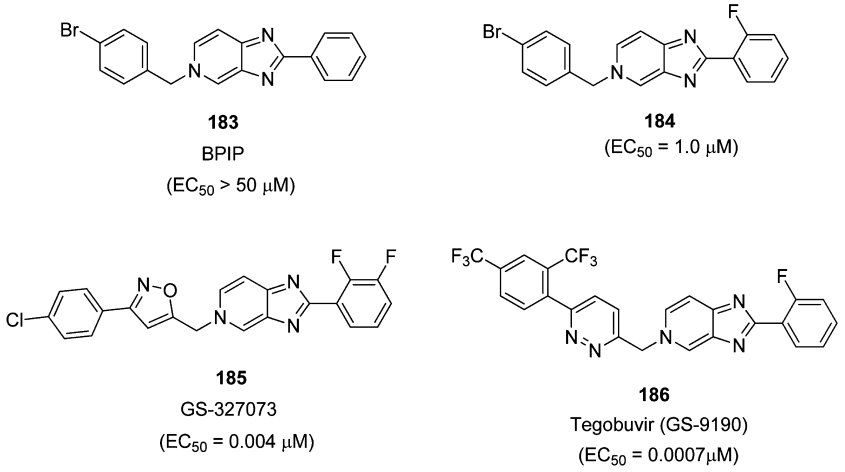

Figure 42. Imidazolopyridine NS5B thumb site II inhibitors.

In a phase 1 study, HCV genotype 1 patients were treated with 40 or 120 mg b.i.d. of **186** over 8 days, resulting in a −1.61 or −1.95 $\log_{10}$ IU/mL reduction in viral load, respectively.[404] Compound **186** was generally devoid of any characteristics involving drug interaction (CYP inhibition/induction); however, a concerning QT prolongation was observed especially at the higher dose. After an extensive evaluation of QT effects versus dose, further development of **186** was limited to the lower 40 mg dose. Phenotype analysis showed the Y448H mutation (27-fold increase in EC_{50}) in 58% of subjects and the double mutation Y448H + Y452H (78-fold increase in EC_{50}) in 5% of subjects on day 8. In vitro experiments showed that protease inhibitors retained wild type potency toward these NS5B mutations, and **186** did not show any activity shift against protease resistant mutants.[405] Therefore, the combination of these two DAAs with/without RBV and PEG-IFN was studied in a phase 2 clinical trial.[406] In the DAA only combination therapy, **186** (40 mg b.i.d.) and a protease inhibitor (75 mg b.i.d.) were administered to HCV genotype 1 patients over 4 weeks. The combination produced a median change in HCV RNA levels from a baseline of −4.1 $\log_{10}$ IU/mL; however, only 2 out of 15 subjects reached undetectable plasma HCV RNA levels (13% RVR) and significant viral breakthrough due to resistant virus was observed. Addition of RBV (1000−1200 mg/day) to the dual therapy regimen provided a greater viral load reduction (−5.1 $\log_{10}$ IU/mL) but only a 62% RVR. Finally, when **186** and a protease inhibitor were administered in combination with SOC over 4 weeks, 14 out of 14 patients (100%) reached RVR with a median HCV viral load decline of −5.7 $\log_{10}$ IU/mL.

In separate studies, patients were treated with 40 mg b.i.d. of **186** in combination with SOC over 24 or 48 weeks.[407] Patients treated with **186** showed faster declines in viral RNA levels than patients treated with SOC alone. However, similar SVR rates (56%) were obtained from both **186**-treated and SOC-treated groups. The viral rebound/relapse among the patients who were treated with the triple therapy was found to be mainly associated with resistant mutation Y448H, which was observed in 63% of genotype 1a patients and 57% of genotype 1b patients.[408] In a recent press release, two serious adverse events

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from patients receiving quadruple therapy (**186** + protease inhibitor + SOC) were reported. Consequently, all studies that included both **186** and PEG-IFN were discontinued. Currently, phase 2 clinical trials evaluating an all-oral quadruple therapy combining **186** + protease inhibitor + NS5A inhibitor and RBV over a 12- or 24-week period are still under active investigation.

DISCUSSION

Hepatitis C virus infection is a worldwide health problem for which new treatment regimens are desperately needed. HCV NS5B RNA dependent RNA polymerase is an essential enzyme in HCV viral replication and has become an important target in the quest to develop DAAs that can be used either in combination with PEG-IFN and RBV or in combination with other DAAs in interferon-free regimens. As with the evolution of HIV highly active antiretroviral therapy (HAART), HCV therapy is expected to require combinations of anti-HCV agents both to rapidly drive viral loads to undetectable limits and to inhibit the emergence of resistance virus. This effort to identify HCV NS5B polymerase inhibitors has involved the search for both nucleoside-based and non-nucleoside inhibitors. The nucleoside approaches have led to the identification of several new classes of nucleosides, particularly the 2′-C-methyl and 4′-azido classes, that have demonstrated proof of concept in human clinical trials. The development of nucleotide prodrugs to deliver nucleoside 5′-monophosphates into hepatocytes has added an exciting dimension to the nucleoside development story through the introduction of agents that not only are potent inhibitors but also have liver targeting characteristics. A number of these nucleoside 5′-monophosphate prodrugs have entered clinical development and have shown exceptional efficacy.

On the basis of their overall profile, nucleoside-based HCV inhibitors (nucleosides and nucleotide prodrugs) have the potential to become the backbone of HCV therapeutic regimens. These nucleoside-based agents have demonstrated pan-genotype activity and a high barrier to resistance. No other small molecule DAA developed to date has been able to mirror these characteristics. This high barrier to resistance suggests that there is a high degree of amino acid conservation in the active site of NS5B and that only subtle amino acid changes in the active site, which do not impair the replication fitness of the virus, are tolerated.[198,409,410] The first mutation to be described that is associated with nucleoside analogues resulted in a serine to threonine amino acid change at position 282.[181] This mutation confers resistance to 2′-*C*-methyl substituted nucleos(t)ide analogues (e.g., **3**, **73**, **72**) and certain 2′-*α*-*F*-2′-*β*-*C*-methyl nucleos(t)ide analogues (e.g., **13**, **49**, **50**) but not to the monophosphate prodrugs of 2′-*α*-*F*-2′-*β*-*C*-methylguanosine, **79** and **80**.[181,227,229,257,259,411] A second mutation, which causes a serine to threonine substitution at position 96, confers resistance to 4′-azidocytidine (**19**).[208] It has been shown previously that S96 and S282 are highly conserved amino acids and that replicons containing either the S96T or S282T amino acid change are severely impaired for replication.[181,277,412] Unlike the NNIs, selection of resistant variants in vitro requires long-term passage. Furthermore, detecting the mutations that give rise to the S282T and S96T amino acid changes in treatment naive patients has not been possible. This is likely because the replication fitness of these variants is impaired, and therefore, the level of these mutant viruses is below the limit of detection. This is supported by the fact that the emergence of resistance associated with nucleos(t)ide inhibitors in clinical studies has been uncommon. In a phase 2b trial only one patient receiving **7** (a prodrug of 2′-*C*-methylcytidine) and PEG-IFN and RBV developed viral breakthrough that was associated with S282T after 14 weeks of treatment.[169] However, no resistance variants have emerged in patients after 12 weeks of treatment with **15** or **50** in combination with PEG-IFN and RBV.

Interestingly, unlike for the 2′-*α*-*F*-2′-*β*-*C*-methylpyrimidine derivatives, the 2′-*α*-*F*-2′-*β*-*C*-methylpurine nucleotide prodrugs **79** and **80** were shown to be equipotent against both the wild type and S282T mutant replicon known to be resistant to 2′-*C*-methyl containing nucleosides. Instead a triple mutation, S15G/C223H/V321I, was required to confer resistance to **79** and **80** and only in a genotype 2a replicon because this triple mutation was shown to be lethal in genotype 1 replicon cells. These differences between genotypes in sensitivity to inhibition by the various nucleos(t)ide analogues suggest that conformational differences may exist between genotype 1 and genotype 2 NS5B polymerase. Additionally, the subtle interplay between the base unit of the nucleos(t)ide inhibitor and the 2′-substituents appears to have a major impact on resistance profile and viral selectivity. In the 2′-*β*-methyl substituted nucleos(t)ides, the 2′-*α*-F substitution appears to provide a significantly higher degree of viral selectivity and more favorable resistance profile relative to its 2′-*α*-hydroxy cousin.

Several proof of concept clinical studies have shown that the combination of a nucleoside (**15**) with a NS3 protease inhibitor and the combination of two nucleotide prodrugs (**50** + **80**) have the potential to deliver an PEG-IFN-free combination therapy. These combinations demonstrated dramatic viral load reductions and no emergence of resistant virus. Several additional proof of concept clinical trials investigating combining a nucleoside or nucleotide prodrug with a protease inhibitor, a NS5A inhibitor, or another nucleotide NS5B inhibitor are exploring further the concept of combination therapies and are designed to answer the ultimate question of whether an SVR can be achieved. It is expected that results from these trials will begin to appear in 2012. If such studies prove to be successful, a major paradigm shift in HCV treatment will result.

The non-nucleoside inhibitors comprise a large group of chemotypes (28) that inhibit the NS5B polymerase through an allosteric mechanism. These inhibitors belong to one of three broad classes of compounds: thumb domain site I and II inhibitors and palm site inhibitors. The identification of these small molecule allosteric inhibitors has relied heavily on the use of high throughput screening to identify new chemotypes and the use of structure-based methods to further optimize their intrinsic potency. In fact more than 80 crystal complexes of HCV NS5B with small molecule allosteric inhibitors have been reported. Although the exact mechanism of action of this class of compounds needs to be elucidated, it is likely that they induce or prevent a conformational change that adversely affects enzymatic activity. This would suggest that there is sufficient structural flexibility in the polymerase and at the NNI binding site to impact the catalytic site. Most NNIs lack broad genotype coverage.[409,413] This difference in the spectrum of activity of the NNIs is believed to be the result of the genetic diversity between genotypes at the three sites. Variants resistant to NNIs have been shown to preexist in treatment naive patients and have been attributed to the high replication rate of the virus and the error-prone nature of the polymerase.[89,414] Therefore, selection of viral variants displaying resistance to NNIs occurs readily in vitro and in vivo and has plagued the clinical

development of NNIs.[89,277,414] However, little or no cross-resistance occurs between inhibitors that interact at different sites. While many of the mutations associated with resistance to the different NNIs show reduced replication fitness or no effect on fitness, several NNI-associated mutations have resulted in a slight or significant increase in replicon replication fitness.[277,415−417] The C445F amino acid change was recently shown to cause a significant increase in fitness.[415] The selection of replicons containing the C445F amino acid change occurred with three different NNIs. Clonal sequencing showed that this mutation was present at low frequency in the wild-type replicon. An even greater increase in fitness was noted when the C445F amino acid change was combined with the C316Y amino acid change. On the basis of these data, it was suggested that the C445F change may serve as a compensatory mutation.[415]

Human clinical studies evaluating HCV NNIs as monotherapy have all shown viral breakthrough resulting from rapid emergence of resistant virus. Triple therapy combining HCV NNIs with PEG-IFN and RBV have had decidedly mixed results. Most NNIs have failed because of rapid emergence of resistance resulting in viral breakthrough prior to completion of a course of therapy. Others have failed because of unacceptable safety concerns. However, several NNIs have demonstrated good RVRs after 28 days of treatment and continue down the development path into late phase 2 clinical evaluation.

The quest to develop interferon-free DAA combination therapies by combining non-nucleoside inhibitors of NS5B with other small molecule DAAs of different mechanisms of action has yet to show signs of success. Several two-drug DAA combination studies that included an NNI and a NS3 protease inhibitor were plagued by early viral breakthrough as the result of emergence of resistant virus and are no longer under active investigation. In attempts to further address the low barrier to resistance seen for two-drug combinations containing NS5B NNIs, three- and four-drug DAA combination clinical studies were initiated. The three-drug combinations included an NNI combined with an NS3 protease inhibitor and RBV. The four-drug combinations married an NNI with an NS3 protease inhibitor, a NS5A inhibitor, and RBV. Although early data appear promising, it is yet to be determined if these combinations will provide the necessary long-term efficacy, safety, and ability to suppress viral breakthrough to be viable treatment options.

With numerous clinical studies currently underway that employ HCV NS5B inhibitors in a myriad of different drug combinations, the question of whether this target will eventually deliver a useful therapy will be answered in the very near future. Ultimately, the most successful candidates will be chosen based on efficacy (i.e., SVR rate), safety, resistance profile, and the ability to support viable HCV antiviral combination therapies. However, for those patients infected with HCV the future is bright with the expectation that a safe and effective cure with a tolerable treatment regimen is on the horizon.

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Biographies

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Wonsuk Chang graduated from Seoul National University, South Korea (B.S. and M.S. degrees). After receiving a Ph.D. in Organic Chemistry from the State University of New York at Stony Brook for the total synthesis of natural products under the guidance of Professor Kathlyn A. Parker, he completed his postdoctoral studies on antimalarial endoperoxides with Professor Gary H. Posner at Johns Hopkins University, MD. Since 2008, he has worked as a medicinal chemist at Pharmasset, Inc. and is currently a Senior Research Scientist. Wonsuk's research is focused on the discovery of small molecule therapeutics for infectious diseases, particularly hepatitis C.

Phillip A. Furman received his Ph.D. in Microbiology from Tulane University, LA, and completed his postdoctoral research at Duke University, NC, with Professor Wolfgang Joklik. In 1975 he joined Burroughs Wellcome as a Senior Research Scientist and later served as the Director of the Division of Virology at Burroughs Wellcome. Dr. Furman was named co-inventor of Retrovir (AZT) and Epivir (3TC) for HBV and was involved in the development of Zovirax (acyclovir), Vatrex (valacyclovir), Retrovir (AZT), and Emtriva (FTC). He was a cofounder and Chief Scientific Officer at Triangle Pharmaceuticals. He joined Pharmasset in 2004 and serves as the Vice President of Biological Sciences. He has over 90 publications and 20 patents. In 2012 he will serve as President of the International Society for Antiviral Research.

Ralph T. Mosley received his B.Sc. in Chemistry from the University of Missouri, Columbia, in 1983 and his M.S. in Organic Chemistry from Purdue University, West Lafayette, IN, in 1989. He then spent 18 years at Merck (Rahway, NJ), providing molecular modeling support for a variety of medicinal chemistry targets including enzymes, GPCRs, and nuclear hormone receptors. In 2007, Ralph joined Pharmasset, Inc. as the Head, Computational Chemistry, where he provides modeling and cheminformatics support for the discovery of viral enzymatic inhibitors. His scientific interests include structure-based drug design and methods for visual property analysis. He has authored over 55 publications and is a co-inventor on six U.S. patents.

Bruce S. Ross received his B.S. in Medicinal Chemistry from the University of Michigan in 1979 and his Ph.D. in Medicinal Chemistry from the University of Kansas in 1984. Following a postdoctoral appointment with Dr. Sydney Archer at Rensselaer Polytechnic Institute, he took a 1-year appointment as Research Assistant Professor in Medicinal Chemistry at the University of North Carolina. In 1987 he began his industrial career at Ash Stevens. In 1992, he joined Isis Pharmaceuticals and rose to become Executive Director of Process Research Chemistry. In 2005, he joined Pharmasset and is currently Senior Director of Process Research focusing on the process synthesis of nucleotide antiviral agents. He has authored over 35 publications and is a co-inventor on 18 patents.

ABBREVIATIONS USED

HCV, hepatitis C virus; PEG-INF, pegylated interferon; RBV, ribavirin; SOC, standard of care; SVR, sustained virological

response; INF-λ, interferon λ; GT, genotype; DAA, direct acting antiviral; LDL, low density lipoprotein; ORF, open reading frame; IRES, internal ribosomal entry site; E1, envelop protein 1; E2, envelop protein 2; NS2, nonstructural protein 2; NS3, nonstructural protein 3; NS4A, nonstructural protein 4A; NS4B, nonstructural protein 4B; NS5A, nonstructural protein 5A; NS5B, nonstructural protein 5B; ER, endoplasmic reticulum; RdRp, RNA-dependent RNA-polymerase; GBV-B, George Baker virus, flavivirus that infects tamarins; NNI, non-nucleoside inhibitor; NTP, nucleoside triphosphate; BVDV, bovine viral diarrhea virus; PNP, purine nucleotide phosphorylase; q.d., quaque die (once daily); b.i.d., bis in die (twice daily); q8h, quaque octa hora (every 8 h); po, per os (orally); RVR, rapid virological response; PK, pharmacokinetic; NOEL, no observed effect level; dCK, deoxycytidine kinase; UMP-CMP, uridine monophosphate−cytidine monophosphate kinase; 3TC, lamivudine; HINT1, histidine triad nucleotide binding protein; HepDirect, cyclic 1-aryl-1,2-propanyl phosphate ester prodrug; SATE, bis(*S*-acyl-2-thioethyl)phosphate ester prodrug; SAR, structure−activity relationship; POM, pivaloyloxymethyl; (*S*)-HPMPA, (*S*)-9-[3-hydroxy-2-(phosphonomethoxy)propyl]adenine; ODE, octadecyloxyethyl; HDP, hexadecyloxypropyl; PSA, polar surface area; CMV, cytomegalovirus; ALIS, automated ligand identification screening; ADME, absorption, distribution, metabolism, and excretion; NOESY, nuclear Overhauser effect spectroscopy; CYP, cytochrome P450; BPIP, 5-(4-bromobenzyl)-2-phenyl-5*H*-imidazo[4,5-*c*]pyridine; HAART, highly active antiretroviral therapy

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