Approaches to hepatitis C treatment and cure using NS5A inhibitors

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Abstract: Recent progress in the understanding of hepatitis C virus (HCV) biology and the availability of in vitro models to study its replication have facilitated the development of direct-acting antiviral agents (DAAs) that target specific steps in the viral replication cycle. Currently, there are three major classes of DAA in clinical development: NS3/4A protease inhibitors, NS5B polymerase inhibitors, and NS5A directed inhibitors. Several compounds thought to bind directly with NS5A are now in various clinical trial phases, including the most advanced, daclatasvir (BMS-790052), ledipasvir (GS-5885), and ABT-267. While many NS5A-targeted compounds demonstrate picomolar potency, the exact mechanism(s) of their action is still unclear. In the clinic, NS5A HCV inhibitors show promise as important components in DAA regimens and have multifunctionality. In addition to inhibiting viral replication, they may synergize with other DAAs, possibly by modulating different viral proteins, to help suppress the emergence of resistant viruses. Structure-based models have identified target interaction domains and spatial interactions that explain drug resistance for mutations at specific positions (e.g., residues 93 and 31) within NS5A and potential binding partners. This review provides insights into the unique complexity of NS5A as a central platform for multiple viral/host protein interactions, and possible mechanism(s) for the NS5A inhibitors currently undergoing clinical trials that target this nonstructural viral protein.

Keywords: HCV replication complex, direct acting antivirals (DAAs), clinical trials

Introduction

Hepatitis C virus (HCV) is a global health burden, with approximately 170 million people (3% of the world’s population) estimated to be infected worldwide. More than three million people contract HCV each year, and while 15%–30% of all HCV infections clear spontaneously, the remaining 70%–85% (an estimated 120–130 million) of infections will develop into chronic hepatitis, which can lead to steatosis, cirrhosis, and hepatocellular carcinoma. Unfortunately, most are unaware of their infection – HCV-associated liver diseases may only manifest after decades in undiagnosed individuals – and can potentially transmit the virus to others, primarily through contaminated blood. In addition, HCV reinfection after treatment has been reported, making vaccine development desirable. Accordingly, the burden of HCV-associated disease is predicted to rise over the next 20 years. In fact, in the US, HCV has now superseded human immunodeficiency virus type 1 (HIV-1) as the leading cause of mortality due to an infectious agent.

As a member of the Flaviviridae, the general replication cycle of HCV is similar to that of other viruses of this family and replicates entirely within the cytoplasm.
Because it does not establish latency, HCV is curable, although the mechanism by which it mediates persistence remains unclear. Among all recognized positive-strand ribonucleic acid (RNA) viruses, HCV is unique in its ability to establish a chronic infection.\(^9\) The HCV genome consists of a 9.6 kb, positive-sense, single-stranded, enveloped RNA, which encodes three structural proteins (core, E1, and E2), the ion channel protein p7, and six nonstructural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B).\(^10\) Each of these proteins has a role in HCV entry, infection, replication, or maturation and is therefore a potential drug target.

HCV is highly heterogeneous, which can be an obstacle to the development of a universal treatment and a preventative vaccine. According to the World Health Organization, six major HCV genotypes and several subtypes have been identified throughout the world. Subtypes 1a/b account for approximately 70% of all infections in the US, Europe, China and Japan,\(^11\) and the remainder are generally genotype 2, 3, and 4.\(^12\) The HCV genotype strongly predicts the response to the currently approved HCV treatments.

Over the last decade, the standard of care comprised a dual-therapy regimen containing peginterferon alpha (PEG-IFN), given once per week as a subcutaneous injection, and ribavirin (RBV), a guanosine (ribonucleic) analog given orally twice daily. Individuals with HCV genotype 1 or 4 infection are less likely (40%–50%) to demonstrate a sustained viral response (SVR) with these treatments compared with individuals with genotype 2 or 3 disease (75%–85%).\(^13,14\) In 2011, the first NS3/4A HCV protease inhibitors (PIs), telaprevir and boceprevir, were approved. These direct-acting antiviral agents (DAAs) have now been licensed in several countries for use in combination with PEG-IFN and RBV, for the treatment of genotype 1 subjects. Unfortunately, many infected individuals, regardless of genotype, have been ineligible or unable to tolerate the standard of care regimen due to adverse effects and long treatment durations. Therefore, newer treatments with improved characteristics are needed to address the growing unmet medical needs.

HCV primarily infects liver parenchymal cells (hepatocytes). Because the liver is a highly specialized and complex organ, it is difficult to adequately model its biology in vitro. However, significant efforts have been directed at developing cell culture models to elucidate the viral replication in vitro.\(^15,16\) Specifically, the discovery of host cell receptor molecules that potentiate HCV infection has helped to overcome these obstacles, and the development of human hepatoma cell lines (eg, Huh-7 and Hep3B cells) has led to recent advances in the understanding of HCV structure and replication.\(^10,15\)

There is now a broad pipeline of drugs in clinical development for treatment of HCV that relies on DAAs alone. DAAs block viral production by directly inhibiting one or more steps of the HCV replication cycle and are in various stages of clinical development.\(^13,17–24\) DAAs currently include first-generation, second-wave, and second-generation NS3/4A PIs, nucleoside inhibitors and nonnucleoside inhibitors of the NS5B RNA polymerase, and NS5A complex inhibitors.\(^25–28\) Highly potent once-a-day, DAA-only combinations are now in Phase II and III of clinical development.\(^20\) This review will focus on the unique complexity of NS5A as a drug target, with its putative protein interactions and possible mechanism(s) of action. We will summarize the most recent mechanistic research on drugs targeting NS5A and the ongoing clinical trials with these agents.

**Structure and function of NS5A**

NS5A is a 447 amino acid (aa), zinc-binding phosphoprotein comprised of three domains separated by two linker regions (Figure 1).\(^29\) Structurally, the amino-terminus of NS5A comprises the amphipathic \(\alpha\)-helix, which is responsible for anchoring to the endoplasmic reticulum (ER) and ER-derived membranes, including lipid droplets (LDs).\(^30,31\)

In one study, the structure encoded in the first 31 aas of HCV genotype 1a domain I (D-I) was determined, by nuclear magnetic resonance spectrometry (NMR), to include an extended amphipathic helix (AH), which oscillates in plane with the cytosolic surfaces of the model membrane bilayers and which is thought to be conserved for all genotypes (Figure 1).\(^32\) The remaining subdomains were subsequently crystallized using different truncated sequences of genotype 1b (Figure 1).\(^33,34\) Both three-dimensional (3D) structures reveal a novel folded core between aa 36–100 designated as subdomain 1a (D1a), that coordinates a zinc atom via four cysteines, yet the two structures pack into two unique conformations of the homodimer. The remaining residues in D-I (aa 101–213), designated subdomain 1b (D1b), include a putative RNA-binding domain at the dimer interface of one of the structures.\(^35\) The structural predictions were recently confirmed by in vitro biochemical assays, where the D-I residues involved in dimerization and RNA binding were identified.\(^35\) Domains II (D-II) and III (D-III) of NS5A are intrinsically disordered and are therefore flexible. This may confer NS5A’s promiscuous ability to interact with numerous proteins.\(^36–40\) Specifically, D-II is involved with binding to cyclophilin A and, therefore, has the potential to antagonize the innate
The immune response to HCV.41 D-III seems to be important for the assembly of infectious viral particles.42,43

NS5A exists in two forms (designated p56 and p58), based on electrophoretic mobility.44 The p56 form is primarily unphosphorylated or is basally phosphorylated by several kinases in different regions,45–47 and data suggests that p58 hyperphosphorylation is performed by casein kinase I isoform alpha.48 More recently, the essential host factor, lipid kinase phosphatidylinositol 4-kinase III alpha (PI4KIIIα) has been shown to bind NS5A and regulate its phosphorylation state.49 Surprisingly, the upregulation of PI4KIIIα has been shown to result in lower levels of the hyperphosphorylated p58.50 While the exact role of NS5A phosphorylation in regulating HCV RNA replication is not completely understood, it may contribute to a switch between viral RNA replication versus RNA translation and/or packaging.50 Recent studies suggest that NS5A hyperphosphorylation appears to play different roles between in vitro and in vivo systems, and the degree and requirements for hyperphosphorylation may vary between different HCV genotypes and isolates.51,52

While NS5A is essential to HCV genome replication and is required for virion morphogenesis, its specific role in these processes has yet to be determined. What is certain is that HCV, like virtually all plus-strand RNA viruses, has the hallmark feature of forming a membrane-associated replication complex composed of virus proteins, replicating RNA and...
altered cellular membranes.\textsuperscript{53,54} All the HCV NS proteins, including NS5A, interact with host cell membranes, either directly as membrane-binding proteins, or in the case of NS3, via interaction with the membrane-anchoring protein NS4A.\textsuperscript{16} The interaction of these viral proteins with the membrane forms tight structures that accumulate as a “membranous web” to house the viral replication complexes.\textsuperscript{53}

Even though NS5A has no known enzymatic activity, its interaction with the membranous web is critical for viral replication, and modification/deletion of specific residues of the membrane-binding AH domain impairs both ER localization and RNA replication.\textsuperscript{30,32,55} NS5A interacts with a myriad of cellular and viral factors, including viral RNA, which likely enables it to perform multiple roles within the virus replication cycle. For example, NS5A is a novel structural class of RNA-binding protein with the ability to bind to HCV RNA.\textsuperscript{56–58} NS5A also colocalizes with other HCV-encoded and host proteins, which may facilitate virus particle production\textsuperscript{59} and mediate replication complex movement via an association with microtubules and actin filaments.\textsuperscript{60,61} A theoretical model of the membrane-bound NS5A D-I structure, with viral RNA as a platform for the assembly of replication complexes, is presented in Figure 2.

In addition to forming viral protein complexes within the membranous web, evidence suggests that NS5A interacts with diverse host proteins. For example, NS5A has attracted considerable interest because of its potential role in modulating the response to interferon (IFN)-\(\alpha\) therapy,\textsuperscript{62} although this remains controversial. NS5A also alters intracellular events (eg, calcium levels), which leads to oxidative stress and the activation of the nuclear factor kappa-light-chain enhancer of activated B cells (NF-\(\kappa\)B) and signal transducer and activator of transcription (STAT-3) transcription factors in the liver.\textsuperscript{63,64} As previously referenced, NS5A was found to directly interact with PI4KIII\(\alpha\) and stimulate its kinase activity, which reduces p58 hyperphosphorylation.\textsuperscript{49} Importantly, this leads to the formation of a phosphatidylinositol-4 phosphate (PI4P)-enriched membranous environment, which may be critical for the HCV replication cycle as the inhibition of PI4KIII\(\alpha\) abrogates HCV replication.\textsuperscript{65} Also, PI4P has been shown to rearrange during HCV infection and to colocalize with NS5A.\textsuperscript{66} Most recently, the cytoplasmic sorting factor tail-interacting protein of 47 kD (TIP47) was found to have a crucial role in the HCV replication cycle and to bind NS5A directly at the conserved AH residue tryptophan (W) \textsuperscript{9,57,68} In short, while the specifics of the HCV replication cycle have yet to be fully understood, it is likely that NS5A functions as a key modulator of this process.

**NS5A inhibitor mechanism(s) of action**

A recent clinical study of nine HCV-infected persons who were administered 10 or 100 mg single doses of the NS5A inhibitor daclatasvir revealed a multiphasic decline in serum HCV.\textsuperscript{69,70} An initial, rapid two-log\textsubscript{10} drop in HCV RNA levels over the first 6 hours and a half-life \((t_{1/2})\) of 48 minutes was observed, suggesting a direct inhibition of virus assembly or the secretion in serum. This was followed by a much slower phase of decline \((t_{1/2} = 6–9\text{ hours})\), which was indicative of an effect on viral RNA synthesis. These clinical findings suggest that NS5A inhibitors have mechanism(s) that affect both viral genome replication and the assembly/release of infectious HCV particles in humans, related to those previously seen in cell-based studies.

In vitro experiments indicate that NS5A is a “promiscuous” protein, interacting with multiple host and virus

**Figure 2** Structure-based theoretical model of membrane-bound NS5A D-I homodimer binding RNA duplex.

Notes: The ribbon representations of the dimeric subunits are colored gold and blue. The N-terminal AHs align in plane with the membrane and pack against residues Y93 and L31 at the dimer interfaces. This AH conformation suggests potential binding site(s) for endogenous cofactors and drugs at the Y93/AH/dimer interfaces that may help explain the activity of NS5A direct inhibitors at different stages of replication and inhibition, and the effects on membranous web morphology. An atom of Zn\textsuperscript{2+} (cyan) binds at the D1a site at the core of each monomer, stabilizing a novel fold. RNA is shown binding at the D1b interface of this dimeric form. The recently identified regions for PI4K-binding at the C-terminal are colored green (182–198), and critical TIP47-binding residues near the AH N-termini are colored magenta. (The templates used PDB ID IZH1, PDB ID IR7G, POPC phospholipid bilayer). Reproduced with permission of James H Nettles. Copyright © 2013.

Abbreviations: AH, amphipathic helix; D1, domain 1 subdomain 1; NS5A, nonstructural protein 5A; PI4K, phosphatidylinositol 4-kinase; PIP4, phosphatidylinositol phosphate 4; pdb, protein data bank; RNA, ribonucleic acid; TIP47.
components, most likely involved in the membranous web formation. For instance, NS5A has been shown to interact with core protein to enhance the formation of HCV particles and with NS3, NS4A, and NS5B to modulate its phosphorylation, in addition to its role in binding RNA. However, it is not precisely clear which complex(es) are the target of NS5A-directed inhibitor drugs. Attempts at monitoring inhibition, in a cell-free system with only purified NS5A, have not been successful to date, suggesting that targeting the NS5A complex may require additional host and viral factors. On the other hand, the HCV replicon system has provided a rapid and efficient way to monitor the effects of NS5A inhibitors on HCV RNA production within the membranous web complex. It is noteworthy that the effect(s) of NS5A inhibitors on viral assembly and release cannot be studied directly using the replicon system, as replicon cells do not contain the envelope gene.

Recent mechanistic studies using immunofluorescence to track NS5A localization in replicon-containing cells following treatment with NS5A inhibitors have suggested that these drugs reduce viral RNA production in new, rather than preformed, replication complexes, which may correlate with the observed slow rate of viral load decay seen in clinical trials. These studies also demonstrated that NS5A inhibitors significantly shift the distribution of NS5A from the ER to LDs and that such redistribution is ablated in cells with drug-resistant mutations (eg, Y93H and L31V). Since NS5A has previously been shown to colocalize with core proteins on LDs, drug binding at the LD stage may interfere with viral assembly and particle release at the cell membrane, thus accounting for the fast decay (as part of the multiphasic decline) seen in the clinic.

Additional mechanisms for the biphasic clinical response to NS5A-targeted inhibitors may include functional complexes with essential host factors, such as PI4KIIIα or TIP47. PI4KIIIα creates a PI4P-rich membranous environment supporting HCV replication as well as modulating NS5A phosphorylation. TIP47 is an LD-binding host protein that has previously been shown to regulate RNA replication via direct interaction with HCV NS5A and to have a key role in the viral assembly/release of the related dengue flavivirus.

While we have described at least two distinct possible modes of action for the drug response observed in infected persons, these modes of action are not likely to be exclusive. If NS5A inhibitors only affect the formation of new viral replication complexes, this mode of action would be limited to a relatively narrow time period in the HCV replication cycle; however, if NS5A inhibitors additionally affect the virus assembly complex, preventing the maturation of viral particles and the spread of infection, these mechanisms would occur at two different stages of the replication cycle and could account for the observed multiphasic kinetics.

Both mechanisms suggest that the ideal NS5A inhibitors should be administered at doses that maintain steady-state trough concentrations (C_{trough}) that will maintain an SVR at week 12 (SVR12). Because it is assumed that only the unbound fraction of drug is free to diffuse into hepatocytes, many studies have reported maintenance of C_{trough} > the in vitro determined 90% effective concentration (EC_{90}) against the targeted strain of HCV, corrected for binding to plasma proteins (protein-corrected potency), as a biomarker of therapeutic efficacy (Table 1). Once-a-day antiviral regimens generally demonstrate a greater degree of compliance. Once-a-day formulations are more easily achieved using drugs that have a long plasma t_{1/2} (eg, ledipasvir (t_{1/2} = 49.7 hours), ABT-267 (t_{1/2} = 28.1 hours), and daclatasvir (t_{1/2} = 12.8 hours). However, given the high potency of NS5A inhibitors, drugs with short t_{1/2} may also be considered as once-a-day regimens, provided a sufficiently high dose could be administered to maintain an effective steady-state trough without producing systemic toxicity. The pharmacokinetics of NS5A inhibitors in clinical development are summarized in Table 1.

### Chemical space of NS5A-directed inhibitors

Daclatasvir was discovered using HCV replicon systems for high throughput screening (HTS), which identified a low micromolar hit that selected mutations in the NS5A coding region. Further screening of structurally similar compounds identified a low nanomolar lead that selected the same mutations. Investigation of the mechanism of the lead’s increased potency revealed a homodimeric metabolite to be the active component. Further medicinal chemistry optimization of the symmetric, dimeric scaffolds resulted in the picomolar-active structure of BMS-790052, also known as daclatasvir. A recent review mapped the chemical space of over 50 compounds from the patent literature. They were found to center around daclatasvir’s chemical structure. This large set of compounds can be grouped into two general chemotype classes (summarized in Figure 3). The class 1 compounds are dimers presenting a chemical motif of two peptidic caps joined by conjugated aromatic core linkers and include the known clinical compounds daclatasvir (BMS-790052), GSK-2336805, and ledipasvir (65-5885), with the structures shown in Figure 3. The most
Table 1 Pharmacokinetic parameters of NS5A inhibitors

<table>
<thead>
<tr>
<th>NS5A inhibitor</th>
<th>Dose (mg)</th>
<th>C_{max} (ng/mL)</th>
<th>T_{max} (hr)</th>
<th>AUC per dose (ng·hr/mL)</th>
<th>t_{1/2} (hr)</th>
<th>C_{trough} 90 (ng/mL)</th>
<th>PC EC_{50} (ng/mL)</th>
<th>Refs</th>
</tr>
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<tbody>
<tr>
<td>ABT-267†</td>
<td>25 QD</td>
<td>56.2 (38)</td>
<td>4.6 (16)</td>
<td>529 (36)</td>
<td>28.1 (22)</td>
<td>31.1*</td>
<td>NA</td>
<td>124,131</td>
</tr>
<tr>
<td>ACH-3102</td>
<td>300 QD</td>
<td>876.4 (28)</td>
<td>6.9 (18)</td>
<td>9,904.5 (31)</td>
<td>111.6 (24)</td>
<td>300</td>
<td>125</td>
<td></td>
</tr>
<tr>
<td>AZD-7295</td>
<td>90 tid</td>
<td>97.9</td>
<td>1.0–1.5</td>
<td>294</td>
<td>4.4**</td>
<td>29.5</td>
<td>NA</td>
<td>114,133</td>
</tr>
<tr>
<td></td>
<td>350 bid</td>
<td>304.4</td>
<td>1.0–1.5</td>
<td>1031</td>
<td>5.2**</td>
<td>145.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMS-790052‡</td>
<td>60 QD</td>
<td>1,726 (21)</td>
<td>1 (1–2)</td>
<td>15,120 (35)</td>
<td>12.8 (9.6)</td>
<td>254.6 (42)</td>
<td>99,126,134</td>
<td></td>
</tr>
<tr>
<td>GS-5816</td>
<td>150 QD</td>
<td>669 (48.1)</td>
<td>2–3</td>
<td>4,890 (45.4)</td>
<td>15.2 (12–15)</td>
<td>63.4 (42.8)</td>
<td>N/A</td>
<td>118</td>
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<tr>
<td>GS-5885</td>
<td>90 QD</td>
<td>247.7 (45.4)</td>
<td>4–6</td>
<td>1,592.4 (59.5)</td>
<td>49.7 (54.3)</td>
<td>115.9 (42.6)</td>
<td>104,127</td>
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</tr>
<tr>
<td>GSK-2336805§</td>
<td>75 QD</td>
<td>NA</td>
<td>NA</td>
<td>5,500 (90)</td>
<td>7–10</td>
<td>128§</td>
<td>116,132</td>
<td></td>
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<tr>
<td>IDX-719</td>
<td>50 QD</td>
<td>32.4 (25)</td>
<td>4 (2–4)</td>
<td>387 (30)</td>
<td>23 (17)</td>
<td>8.2 (34)</td>
<td>128,129</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 QD</td>
<td>65.3 (51)</td>
<td>3 (2–4)</td>
<td>728 (4.7)</td>
<td>20 (16)</td>
<td>15.6 (41)</td>
<td>128,129</td>
<td></td>
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<tr>
<td>PPI-668</td>
<td>320 QD</td>
<td>1,500–5,000</td>
<td>2</td>
<td>NA</td>
<td>6**</td>
<td>311</td>
<td>117</td>
<td></td>
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<tr>
<td>MK-8742</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>130</td>
<td></td>
</tr>
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Notes: BMS-790052 = daclatasvir; GS-5885 = ledipasvir; IDX719 = samarinavir. The C_{max}, C_{trough}, and terminal t_{1/2} were reported with %CV or ranges (in parentheses), when available. Clinical doses may not be finalized; some dosages displayed were inferred from early conference presentation or press release. The PC EC_{50} were calculated using the replicon EC_{50} molecular weight, and/or serum protein binding. The C_{max} was calculated by extrapolation using C_{trough} and t_{1/2}. The t_{1/2} was estimated using the C_{max}, C_{trough}, and dose interval (t). Coadministration of ABT-267 with ritonavir increased the mean C_{max} and AUC by 68% and 62%, respectively, and by 88% and 56%, respectively, when administered with food compared with administration under fasting conditions. The dose adjustments of BMS-790052 of 30 mg QD with atazanavir (300 mg QD) plus ritonavir (100 mg QD), and of 90 mg QD with elafaviren (600 mg QD) is expected to generate a daclatasvir exposure similar to that for 60 mg of daclatasvir administered alone. GSK-2336805 demonstrated a two- to fourfold greater C_{max} and AUC in chronic HCV-infected than in healthy persons. Clinical doses may not be finalized; some dosages displayed were inferred from early conference presentation or press release. The PC EC_{50} were calculated using the replicon EC_{50} molecular weight, and/or serum protein binding. *The C_{trough} was calculated by extrapolation using C_{max} and t_{1/2}. **The t_{1/2} was estimated using the C_{max}, C_{trough}, and dose interval (t). †Coadministration of ABT-267 with ritonavir increased the mean C_{max} and AUC by 68% and 62%, respectively, and by 88% and 56%, respectively, when administered with food compared with administration under fasting conditions. ‡Coadministration of ABT-267 with rifampin increased the mean C_{max} and AUC by 68% and 62%, respectively, and by 88% and 56%, respectively, when administered with food compared with administration under fasting conditions. §Coadministration of ABT-267 with ritonavir increased the mean C_{max} and AUC by 68% and 62%, respectively, and by 88% and 56%, respectively, when administered with food compared with administration under fasting conditions. **The C_{trough} was calculated by extrapolation using C_{max} and t_{1/2}. The t_{1/2} was estimated using the C_{max}, C_{trough}, and dose interval (t). ††Coadministration of ABT-267 with ritonavir increased the mean C_{max} and AUC by 68% and 62%, respectively, and by 88% and 56%, respectively, when administered with food compared with administration under fasting conditions.

Notes:

- **A** phenyl rings with shorter, nonaromatic linkers, but some other class 2 members clearly point to alternative mechanism(s) of action for NS5A-targeted compounds. For example, 4-aminoquinoline (AL-9) from Arrow Therapeutics Limited (London, UK) was first thought to target NS5A, based on selected mutations that emerged in NS5A. However, AL-9 was recently shown to directly inhibit purified PI4KIIIα and to reduce PI4P levels in the plasma membrane, which are known to regulate viral propagation.

- Systematic explorations of the structure–activity relationships (SARs) for compounds from both class 1 and class 2 were recently reported by our group, in collaboration with RFS Pharma, LLC (Tucker, GA, USA). Briefly, analog libraries of symmetric dimer compound 1, sharing a proline-valine-carbamate cap, were synthesized in three series (Figure 4). Series 1 included replacement of the two "A" phenyl rings with shorter, nonaromatic linkers, but these substitutions resulted in >1,000-fold drop in potency. However, the straight aromatic linkers could be extended by up to five rings and still retain picomolar potency. The addition of specific halogen substitution at ring "B" was able to rescue the potency of an inactive parent compound with bent linkers, which suggests a key role for conformational.
stability related to potency. For series 2, symmetric halogen substitutions (Cl, Br, I, or F) at the x and x’ positions produced compounds with similar EC_{50} to the parent compound. Surprisingly, an aryl substitution at the imidazole region B maintained activity at 200 pM, and the oxaryl group improved potency to 26 pM, suggesting an open binding pocket in that region. In addition, an azido group substitution of the proline-like ring “C” at position y or z did not alter the activity (~20 pM), while larger 1,4 triazoles were only tolerated at position z, suggesting stereochemical restraints of the binding pocket. Series 3 explored class 2 monomer activity. While the nonsubstituted scaffold was found to be completely inactive, a dual substitution with an azido group at position w and a bromo group at position x produced a highly active monomer, compound 6, with picomolar inhibition of genotype 1b replicons (Figure 3). Resistance selection with both class 1 and 2 compounds in this series identified the emergence of NS5A mutations at positions 93 and 31, and modeling has also supported the potential of shared binding site(s) for highly active members of both classes (Schinazi, unpublished data, 2013).

Researchers at Pharmasset Inc (now Gilead Sciences, Foster City, CA, USA) used computational modeling to investigate the key pharmacophore responsible for the high activity of the class 1 dimers and theorized the importance of an intramolecular hydrogen bond between regions B and C to stabilize a conformation resembling a peptide “gamma-turn.”\textsuperscript{94} Their development of a low picomolar active fluoroolefin-based mimetic supports that hypothesis and may provide a structural clue about the biological site of action. Although daclatasvir is both symmetric and dimeric by design, newer asymmetric and monomeric analogs have also been found to have high activity, suggesting that symmetry and high molecular weight dimers may not be a critical requirement for productive interactions with NS5A, in the next-generation compounds.

**Resistance and biological space of NS5A-directed inhibitors**

Overcoming acquired resistance is a major challenge for all antiviral targets, but for NS5A, acquired resistance defines the target. Daclatasvir, for example, was discovered using HCV replicon systems, which identified the rapid emergence of key resistance-bearing mutations at residues 31 and 93 in the NS5A coding region for genotypes 1a and 1b. Site-directed mutagenesis at those positions resulted in significant loss of drug activity and correlated with selection of the same mutations in clinical trials.\textsuperscript{86,95,96} The genotype 1b mutations Y93H and L31V are striking examples of aa changes that individually increase resistance 24- and 28-fold, respectively, but which increase resistance 14,789-fold when combined\textsuperscript{97} (Table 2), suggesting a specific biological site for drug binding. The locations of these drug-resistant mutations within the AH-D1a linker and near the D1a/D1b interface are highlighted for genotype 1b in Figure 1B.

The structure of aa 1–31 at the N-terminal of NS5A was elegantly solved by NMR in lipid bilayer models. It was found to form a flexible amphipathic α-helix between residues 5–25 that inserted along the membrane surface with hydrophobic residues buried, polar residues exposed, and amphiphilic tryptophan residues aligned in plane (PDB ID 1R7G).\textsuperscript{32} The two 3D X-ray crystal structure solutions for subdomains D1a and D1b reveal very different dimer alignments (Figure 1).\textsuperscript{33,34} Because the deletion of AH was required for crystallization in each case, computational modeling has been employed to visualize the potential drug binding region at the AH–D1a
Table 2 Resistance profile and fitness of daclatasvir, in genotype 1a and 1b replicon systems

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Amino acid substitutions</th>
<th>Replication fitness (%) average ± SD</th>
<th>Potency EC_{50} (ng/mL), average ± SD</th>
<th>FR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype 1a</td>
<td>WT</td>
<td>100</td>
<td>0.0044±0.0028</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>M28A</td>
<td>27±8</td>
<td>20.2±10.3</td>
<td>4,591</td>
</tr>
<tr>
<td></td>
<td>M28T</td>
<td>14±3</td>
<td>3.0±0.3</td>
<td>662</td>
</tr>
<tr>
<td></td>
<td>M28V</td>
<td>16±11</td>
<td>0.0055±0.0019</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>Q30E</td>
<td>130±56</td>
<td>110.9±66.0</td>
<td>25,205</td>
</tr>
<tr>
<td></td>
<td>Q30H</td>
<td>75±31</td>
<td>6.5±1.4</td>
<td>1,477</td>
</tr>
<tr>
<td></td>
<td>Q30R</td>
<td>41±16</td>
<td>5.4±1.8</td>
<td>1,227</td>
</tr>
<tr>
<td></td>
<td>Q30K</td>
<td>19±9</td>
<td>108±52</td>
<td>24,545</td>
</tr>
<tr>
<td></td>
<td>L31M</td>
<td>55±15</td>
<td>1.3±0.5</td>
<td>341</td>
</tr>
<tr>
<td></td>
<td>L31V</td>
<td>117±29</td>
<td>14.9±4.4</td>
<td>3,386</td>
</tr>
<tr>
<td></td>
<td>H58D</td>
<td>92±9</td>
<td>2.2±0.3</td>
<td>500</td>
</tr>
<tr>
<td></td>
<td>H58P</td>
<td>266±261</td>
<td>0.0003±0.0006</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>Y93C</td>
<td>11±7</td>
<td>8.2±3.0</td>
<td>1,864</td>
</tr>
<tr>
<td></td>
<td>Y93H</td>
<td>18±11</td>
<td>23.9±7.0</td>
<td>5,432</td>
</tr>
<tr>
<td></td>
<td>Y93N</td>
<td>13±8</td>
<td>208.9±47.9</td>
<td>47,477</td>
</tr>
<tr>
<td></td>
<td>M28V + Q30R</td>
<td>147±55</td>
<td>1.4±0.13</td>
<td>350</td>
</tr>
<tr>
<td></td>
<td>Q30H + Y93H</td>
<td>20±6</td>
<td>409.8±153.6</td>
<td>93,136</td>
</tr>
<tr>
<td></td>
<td>Q30R + H58D</td>
<td>60±12</td>
<td>1.86±0.46</td>
<td>424,318</td>
</tr>
<tr>
<td>Genotype 1b</td>
<td>WT</td>
<td>100</td>
<td>0.0019±0.0007</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>L31M</td>
<td>99±23</td>
<td>0.0062±0.0014</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>L31V</td>
<td>158±54</td>
<td>0.053±0.015</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>Q54H</td>
<td>83±18</td>
<td>0.002±0.0003</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Q54N</td>
<td>83±29</td>
<td>0.002±0.0006</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Y93H</td>
<td>27±16</td>
<td>0.046±0.018</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>L31M + Y93H</td>
<td>70±68</td>
<td>13.5±12.2</td>
<td>7,105</td>
</tr>
<tr>
<td></td>
<td>L31V + Y93H</td>
<td>50±38</td>
<td>28.1±24.7</td>
<td>14,789</td>
</tr>
<tr>
<td></td>
<td>Q54H + Y93H</td>
<td>22±7</td>
<td>0.018±0.005</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>L31V + Q54H + Y93H</td>
<td>189±25</td>
<td>36.1±7.7</td>
<td>19,000</td>
</tr>
</tbody>
</table>


Abbreviations: EC_{50}, 50% effective concentration; FR, fold resistance; SD, standard deviation.

interface. Cordek et al reported the use of a full-length NS5A AH-D1 homology model (combining the α-helix from NMR with the dimer from PDB ID 3FQQ) to dock both class 1 and class 2 NS5A inhibitor compounds developed at GlaxoSmithKline, PLC (Brentford, UK).21 While residues 28, 30, and 31 were described to be in close proximity to Tyr 93 at the dimer interface of the model, the direct binding mode was reported as “elusive.”22 More recently, a group from Bristol-Myers Squibb (New York, NY, USA) has proposed a symmetric binding mode at the dimer interface of their PDB ID 1ZH1-based structure; however, it does not easily explain the high activity of some class 2 compounds.23 A new structural review has also reported the potential for connecting AH from NMR to either of the X-ray crystal structures, but with ambiguity of the drug-binding detail.24 Our group has studied both dimer forms as platforms for building full-length NS5A AH–D-I homology models and tested multiple possible AH–D1a linker conformations related to drug binding. We found the PDB ID 1ZH1 dimer template to provide better AH alignments, based on chemical genetics-derived distance constraints (J Nettles, unpublished data, 2013). Our analysis for docking NS5A inhibitors to both genotype 1a and 1b models predicts the asymmetric drug binding of one cap between the pair of Tyr 93 residues at the N-terminal AH/dimer/membrane interface, with a secondary cap interaction between 93 and 31 of the two monomers (Figure 5A). This binding mode can accommodate both class 1 and class 2 binding (J Nettles, unpublished data, 2013). These results further suggest a potential for conformational changes in the AH linker region that allows higher affinity asymmetric binding of the class 1 dimers to a membrane morphology associated with LDs (Figure 5B). The “open” 93/31 site allows the second cap to penetrate closer to the D1a core packing near residue 30, consistent with the resistance data of Table 2 and the LD sequestering mechanism.
which in humans, has demonstrated a rapid and robust HCV in-class NS5A inhibitor of the HCV replication complex, Daclatasvir/BMS-790052.

Drugs currently in Phase III clinical trials summarized in Table 3. Among them, three drugs are currently in Phase III clinical trials, including daclatasvir, ledipasvir, and ABT-267; seven drugs are in Phase II, and three drugs are in Phase I. To date, only three structures have been released and are planned to start shortly.

Figure 5 Theoretical models of daclatasvir (turquoise) binding to NS5A and potential AH mediated effect on membrane morphology. Notes: The models suggest the drug (turquoise) binds simultaneously to two asymmetric sites at the NS5A A/B dimer/membrane interface (blue/gold). A core site between Y93 of each monomer binds one cap of the drug, while the second cap fits between residues 93 and 31 of the aligned subunits. (A) The aromatic linker provides favorable interactions and positions the caps simultaneously within the two LA sites formed by AH aligned in the membrane plane. (B) Conformational change of AH exposes an HA “open” drug site between 93 and 31 of the different subunits. Binding to this state may lock NS5A into a conformation conducive to lipid droplet formation and release to cytosol, and is thought to impair assembly of other viral oligomers. (C) NS5A binding with RNA, NS5B, and other proteins induces replication complex formation in a membranous web and significantly lowers the affinity for drug binding. Reproduced with permission of James H Nix. Copyright © 2013.

Abbreviations: AH, amphipathic helix; HA, higher affinity; LA, lower affinity; Leu, leucine; NS5A, nonstructural protein 5A; RNA, ribonucleic acid; Tyr, tyrosine.

NS5A inhibitors in clinical trials

According to http://www.clinicaltrials.gov, at least 13 potent NS5A DAAs have been evaluated in clinical trials (see Table 3). Among them, three drugs are currently in Phase III clinical trials, including daclatasvir, ledipasvir, and ABT-267; seven drugs are in Phase II, and three drugs are in Phase I. To date, only three structures have been released and are summarized in Figure 3.

Drugs currently in Phase III clinical trials

Daclatasvir/BMS-790052

Daclatasvir/BMS-790052 (Bristol-Myers Squibb) is the first-in-class NS5A inhibitor of the HCV replication complex, which in humans, has demonstrated a rapid and robust HCV RNA decline (3.6 log10), with no signs of adverse effects.99 This compound blocks NS5A hyperphosphorylation and has the potential of shifting the subcellular localization of the viral protein.78,97 Daclatasvir has been shown to be highly effective against genotypes 1 and 2, and when in combination with IFN plus RBV, daclatasvir demonstrated a 100% SVR at 12 weeks posttreatment in persons infected with genotype 4. Impressively, in a Phase II study using IFN-free regimens with or without RBV, daclatasvir plus sofosbuvir (GS-7977) demonstrated a 100% SVR at 4 weeks (SVR4) and a 100% SVR12 in genotype 1–infected subjects who were either treatment naïve or who had failed prior treatment with telaprevir or boceprevir plus PEG-IFN/RBV, and 91% for genotypes 2– and 3–infected persons.100 Furthermore, when combined with NS3PI, this was the first compound to demonstrate the proof of concept that an IFN-free regimen could eradicate HCV.101 Based on the Phase II study results, which look promising, Phase III studies of the use of daclatasvir in combination with asunaprevir in genotype 1b treatment-naïve individuals are currently ongoing, and studies of the combination with asunaprevir and BMS-791325 are planned to start shortly.102

Ledipasvir/GS-5885

Ledipasvir/GS-5885 (Gilead) demonstrated a high potency for HCV genotypes 1a (34 pM), 1b (4 pM), 4a (110 pM), and 6a (110 pM) but lower activity against genotypes 2a (21 nM) and 3a (41 nM), with an excellent overall selectivity index (>800,000 in replicon cells).103 A Phase I clinical study104 of two doses showed a 2.3 and 3.3 log10 HCV genotype 1 load reduction after 3 days of monotherapy (1 and 10 mg QD [daily], respectively) but with quick emergence of resistance (residues 30 and 31 in genotype 1a and residue 93 in genotype 1b). During a first Phase II trial (ELECTRON), the combination of ledipasvir, sofosbuvir, and RBV led to 100% SVR for genotype 1 after 12 weeks, in treatment-naïve individuals and prior nonresponders. Overall, the regimen was well tolerated: anemia (20%), depression (8%), and headache (4%).105 A Phase II trial (LONESTAR) involving the combination of ledipasvir (QD) and sofosbuvir (QD) without RBV, for 12 weeks led to SVR4 in 19/19 treatment-naïve and in 18/19 treatment-experienced genotype 1 subjects.106 In another study, the combination of ledipasvir (30 mg QD) with PEG-IFN (180 mg/week) plus RBV (1,000–1,200 µg/day) with or without GS-9451 (a NS3 PI, 200 mg QD) led to a high SVR4 in treatment-naïve and treatment-experienced subjects infected with HCV genotype 1. The same combination led also to
Table 3 NS5A agents in clinical development for chronic HCV infection

<table>
<thead>
<tr>
<th>Drug name (company)</th>
<th>Phase</th>
<th>In vitro activity E_{50} (pm)</th>
<th>Genotype specificity</th>
<th>Development of resistance</th>
<th>Drug combination Phase I, II, or III</th>
<th>Adverse-effect profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daclatasvir/BMS-790052 (Bristol-Myers Squibb)</td>
<td>III</td>
<td>9–50</td>
<td>1b &gt; 2a &gt; 1a</td>
<td>Low/intermediate</td>
<td>Dual or triple with DAAs SVR4/12/24 (91%–100%)</td>
<td>Headache, diarrhea, nausea</td>
</tr>
<tr>
<td>Ledipasvir/GS-5885 (Gilead)</td>
<td>III</td>
<td>4–2.1 × 10^4</td>
<td>1b &gt; 4a, 6a &gt; 2a, 3a</td>
<td>30 and 31 in GT1a and 93 in GT1b</td>
<td>+ Sofosbuvir (GS7977) ± RBV + PEG/RBV ± GS-6451 (NS3 PI)</td>
<td>Phase I: no significant adverse effects</td>
</tr>
<tr>
<td>ABT-267 (AbbVie)</td>
<td>III</td>
<td>5–14</td>
<td>1 &gt; 2–6</td>
<td>Low/intermediate</td>
<td>Dual or triple or quadruple with DAAs/±RBV, SVR8/12 (87%–98%)</td>
<td>Anemia, depression, headache</td>
</tr>
<tr>
<td>ACH-3102 (Achillion)</td>
<td>II</td>
<td>4–10</td>
<td>1–5</td>
<td>Low</td>
<td>Single dose: HCV RNA levels declined &gt;3.7 log_{10} in GT1a. Dual with RBV initiated</td>
<td>Headache, nausea, fatigue</td>
</tr>
<tr>
<td>IDX-719 (Idenix)</td>
<td>II</td>
<td>2–24</td>
<td>1–5</td>
<td>Y93H</td>
<td>3-day monotherapy: HCV levels declined &gt;3.7 log_{10} for GT1–4.</td>
<td>No significant adverse effects</td>
</tr>
<tr>
<td>MK-8742 (Merck)</td>
<td>II</td>
<td>1a/1b, 2a, 3a, and 4a: 3–20</td>
<td>1–4</td>
<td>Low</td>
<td>Phase I – unavailable</td>
<td>Unavailable</td>
</tr>
<tr>
<td>AZD-7295/A-831 (AstraZeneca/Arrow)</td>
<td>II</td>
<td>1b: 7 × 10^4</td>
<td>No detectable effect on 1a or 3</td>
<td>Unavailable</td>
<td>Unavailable</td>
<td>Unavailable</td>
</tr>
<tr>
<td>GS2336805 (GlaxoSmithKline)</td>
<td>II</td>
<td>1.8–44</td>
<td>4a, 5a &gt; 1b, 6ab &gt; 1a &gt; 2a, 3a, 6c–g, 6h–n</td>
<td>Unavailable</td>
<td>+ VX-135 ± RBV</td>
<td>Mild headache</td>
</tr>
<tr>
<td>PPI-668 (Presidio)</td>
<td>Ila</td>
<td>20–1.3 × 10^4</td>
<td>1–7</td>
<td>Unavailable</td>
<td>+ BI201335 (faldaprevir) and BI207127 ± RBV</td>
<td>None</td>
</tr>
<tr>
<td>GS-5816 (Gilead)</td>
<td>II</td>
<td>7–59</td>
<td>1–6</td>
<td>Unavailable</td>
<td>+ Sofosbuvir (GS-7977)</td>
<td>None</td>
</tr>
<tr>
<td>EDP-239 (Novartis)</td>
<td>I</td>
<td>4–34</td>
<td>1b &gt; 1a</td>
<td>Unavailable</td>
<td>Currently undergoing Phase I</td>
<td>Unavailable</td>
</tr>
<tr>
<td>ACH-2928 (Achillion)</td>
<td>I</td>
<td>2–46</td>
<td>1 &gt; 2–6</td>
<td>Intermediate</td>
<td>3-day monotherapy</td>
<td>Anemia, fatigue</td>
</tr>
<tr>
<td>PPI-461 (Presidio)</td>
<td>I</td>
<td>Unavailable</td>
<td>1–6</td>
<td>28, 30, 31, and 93</td>
<td>NA</td>
<td>None</td>
</tr>
</tbody>
</table>

Notes: Bristol-Myers Squibb (New York, NY, USA); Gilead (Foster City, CA, USA); AbbVie (North Chicago, IL, USA); Achillion Pharmaceuticals (New Haven, CT, USA); Idenix Pharmaceuticals, Inc (Cambridge, MA, USA); Merck and Co, Inc (Whitehouse Station, NJ, USA); AstraZeneca (London, UK); Arrow Therapeutics Limited (London, UK); GlaxoSmithKline, PLC (Brentford, UK); Presidio Pharmaceuticals, Inc (San Francisco, CA, USA); Novartis Pharmaceuticals Corp (Basel, Switzerland).

Abbreviations: DAA, direct-acting antiviral agent; E_{50}, median effective concentration; GT, genotype(s); HCV, hepatitis C virus; NA, not available; NS5A, nonstructural protein 5A; PeG, peginterferon; PI, protease inhibitor; RBV, ribavirin; RNA, ribonucleic acid; SVR4/12/24, sustained virologic response at week 4, 12, and 24 posttreatment; SVR8/12, sustained virologic response at week 8 and 12 posttreatment.

��SVR12率是在治疗-naïve的IL28B CC患者中，这些患者与24周治疗与PEG-IFN plus RBV的结果一致。四药疗法（ledipasvir plus GS-9451 plus PEG-IFN plus RBV）的安全性和耐受性良好，但治疗因严重的中性粒细胞减少而终止。治疗-naïve患者在小样本量的研究中，治疗-naïve HCV genotype 1–infected subjects中\textsuperscript{106}。

**ABT-267**

A Phase III clinical trial involving the combination of three DAAs plus a protease booster and RBV is currently underway. In a Phase IIb clinical trial with a quintuple twice-a-day drug combination of ABT-267 (AbbVie, North Chicago, IL, USA) plus the PI ABT-450 plus ritonavir plus the nonnucleoside inhibitor ABT-333 plus RBV, a SVR12 of 98% and 93% was achieved in treatment-naïve persons and null responders infected with HCV genotype 1, respectively (Table 3).\textsuperscript{111}
**Drugs currently in Phase II clinical trials**

**ACH-3102**

ACH-3102 (Achillion Pharmaceuticals) is a potent second-generation NS5A inhibitor with a demonstrated high pharmacologic barrier to resistance and a low potential for the emergence of resistant variants in genotype 1 replicon systems. In a Phase Ia clinical trial, ACH-3102 demonstrated a rapid and robust HCV RNA decline (3.6–4.6 log₁₀ range) in genotype 1–infected subjects. A Phase II clinical trial of ACH-3102 with RBV has been initiated (Table 3).

**Samatasvir (IDX719)**

Samatasvir (Idenix Pharmaceuticals, Inc, Cambridge, MA, USA) has been shown to be highly potent against genotypes 1–4 in vitro; however, a low-resistance barrier to the Y93H variant has been demonstrated with this NS5A inhibitor. Nevertheless, a Phase II clinical trial is planned with samatasvir plus two other DAAs (TMC647055, a nonnucleoside polymerase inhibitor; and simeprevir, a PI) from Janssen (Janssen Pharmaceuticals, Inc, Titusville, NJ, USA).

**MK-8742**

MK-8742 (Merck) is a highly potent compound against genotype 1. It appears to function as a disruptor of the replication complex. In combination with MK-5172 (a PI), this candidate drug exhibited a high barrier to the development of escape mutants. Phase II clinical trials are underway.

**AZD-7295/A-831**

AZD-7295 (AstraZeneca, London, UK; Arrow Pharmaceutical, Inc) initially demonstrated promising potency against genotypes 1b and 1a replicons (EC₅₀ = 7 nM and 1.24 μM, respectively). In 2008, a Phase II trial of AZD7295 was initiated. The status of this compound remains unknown.

**GSK2336805**

GSK2336805 (AstraZeneca/Arrow) is a highly potent NS5A inhibitor displaying low EC₅₀ values (7–59 pM) against genotypes 1–6. GS-5816 has also shown a high-resistance barrier and retains potency against common NS5A polymorphisms and resistance mutations (EC₅₀ = 130 pM against the L31M mutation and 121 pM against the Y93C mutation). In addition, GS-5816 was shown to remain active against mutations from other classes of HCV inhibitors and to show additive to moderately synergistic activities when combined with other anti-HCV agents (including sofosbuvir).

**Drugs currently in Phase I clinical trials**

**ACH-2928**

ACH2928 (Achillion Pharmaceuticals, New Haven, CT, USA) is a highly potent NS5A inhibitor. In a 3-day, monotherapy Phase I clinical trial, this compound demonstrated an impressive and rapid reduction of HCV RNA levels (3.7 log₁₀) in persons infected with genotype 1.
is a potent NS5A-targeted molecule, which in vitro, has demonstrated picomolar activity against genotypes 1b (EC$_{50}$ = 7 pM) and 1a (EC$_{50}$ = 31 pM). The combination of EDP-239 with the cyclophilin inhibitor alisporivir resulted in a strong synergistic anti-HCV effect against genotypes 1–3.$^{27,122}$ Novartis has recently initiated Phase I clinical trials of EDP-239.

**PPI-461**

PPI-461 (Presidio Pharmaceuticals, Inc), Presidio’s first-generation NS5A inhibitor has shown potent in vitro activity for the HCV genotypes 1–6 and up to a 3.6 log$_{10}$ load reduction after 3 days of monotherapy (100 and 200 mg oral doses) in HCV genotype 1–infected subjects.$^{123}$ However, a rapid emergence of resistance was observed (at residues 28, 30, 31, and 93). Presidio’s second-generation NS5A inhibitor, PPI-668, also has pangenotypic activity (described above).

**Conclusion**

Despite the current gaps in our understanding of NS5A, this NS protein has been implicated as an important modulator of critical viral functions, including viral RNA binding and replication, virus assembly, and regulation of the antiviral IFN response. Together, these roles present a unique platform for NS5A inhibitors, to be combined with more traditional target-based drug discovery. NS5A-directed compounds are considered to be the most potent anti-HCV molecules ever discovered. However, their Achilles heel is that they select drug-resistant mutants fast and to be effective, must be used with other potent DAA drugs, to prevent clinical resistance. Structure-based models have demonstrated the potential for identifying target domains and spatial interactions that rationalize the selection of resistance mutations at specific positions within NS5A and potential binding partners (eg, residues 93 and 31). Continued SAR studies will further elucidate the mechanism(s) of action of these small molecules and facilitate the discovery of new drugs with higher barrier to resistance.

NS5A-targeted therapies demonstrate a remarkable capacity to inhibit HCV RNA replication, with minimal in vivo toxicity. At least two molecules (daclatasvir and ledipasvir) are being clinically evaluated in IFN-free combination therapies. NS5A inhibitors appear to be emerging as important components of HCV DAA regimens with dual functionality. In addition to having picomolar potency, they can synergize with other DAA, targeting different viral proteins, to prevent and suppress the emergence of resistant viruses. If NS5A inhibitors can exercise their remarkable potency to rapidly drive down virus replication within the protection offered by other DAAs, then resistance to any one DAA component may be suppressed by other components in the combination regimens. Thanks to these advances, the future is looking promising for the millions of HCV-infected individuals. An IFN-free, oral, once-daily, pangenotypic drug combination of a nucleoside analog, such as sofosbuvir, with a next-generation pangenotypic NS5A drug, such as GS-5816, may play a pivotal role and could potentially cure millions of chronically infected HCV individuals and result in global HCV eradication in the near future.

**Acknowledgments**

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**Disclosure**

The authors report no conflicts of interest in this work.

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