CHAPTER TWENTY-TWO

HCV Inhibition Mediated Through the Nonstructural Protein 5A (NS5A) Replication Complex

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1. INTRODUCTION

Hepatitis C virus (HCV) affects more than 170 million individuals worldwide. Despite increased monitoring of blood supply and prevention efforts, up to 4.7 million new infections take place each year.[1] While about 20% of all HCV infections clear spontaneously with time, the remaining ones become chronic[2] and potentially lead to steatosis, cirrhosis, and hepatocellular carcinoma.[3]

A combination of an injectable peginterferon and oral ribavirin has been the standard of care for the past decade. This 48-week, often grueling treatment, can be associated with very unpleasant side effects such as flu-like symptoms and fatigue, which, in turn, often leads to treatment discontinuation. The treatment options for HCV have expanded with the recent approval of HCV protease inhibitors (PI), boceprevir and telaprevir. New PI-inclusive treatment regimens have resulted in increased rates of sustained viral response (SVR) for both treatment naïve and experienced patients[4] and

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brought an all-oral HCV therapy closer to realization. To this end, other HCV targets[5] as well as small molecule inhibitors targeting host factors utilized by the virus for replication[6] have been pursued. Particularly noteworthy is the recently discovered class of NS5A inhibitors, due to their novel mechanism of action, high potency, and the promising clinical progression of several compounds.[7,8] Small-molecule NS5A drug discovery has been reviewed.[9,10] This chapter provides a brief historical perspective and a further update of recent activities in the field.

2. FIRST-GENERATION NS5A INHIBITORS

Initial inhibitors that targeted NS5A were peptides derived from an amphiphilic α-helical sequence in NS5A-termed amphiphilic α-helix (AH), thought to be responsible for NS5A binding to the membrane.[11] First-generation small molecule NS5A inhibitors included 4-aminoquinazolines **1**[12,13] from Arrow Therapeutics. Arrow and Astra-Zeneca subsequently advanced AZD-2836 and AZD-7295 to the clinic. Other early NS5A molecules included acetylene derivatives **2** from Presidio and XTL biopharmaceuticals[14] and piperazine-based inhibitors from Merck[15] (Fig. 22.1). Compound **3** in Merck series had replicon $EC_{50} = 160$ nM and caused mutations in NS5A, implicating it as a potential macromolecular target. Early compounds were generally not reported to be potent in the gt1a replicon assay.

3. CURRENT-GENERATION NS5A INHIBITORS

BMS scientists disclosed iminothiazolidinone HCV inhibitors,[16] which were susceptible to NS5A mutations. Optimization of the screening hit *BMS-858* led to *BMS-824* which showed a ~100-fold potency increase. *BMS-824* was found to undergo a spontaneous dimerization to potent compound **4**.[17] Subsequent structural explorations designed to define the minimum pharmacophore in **4** resulted in a picomolar inhibitor

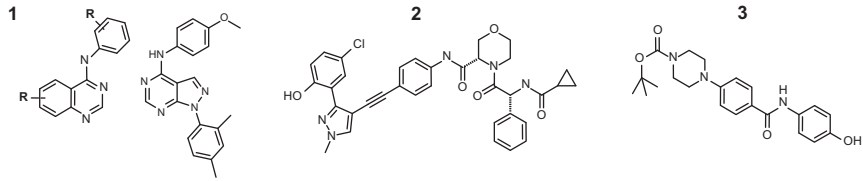

Figure 22.1 Structures of early NS5A small molecule inhibitors.

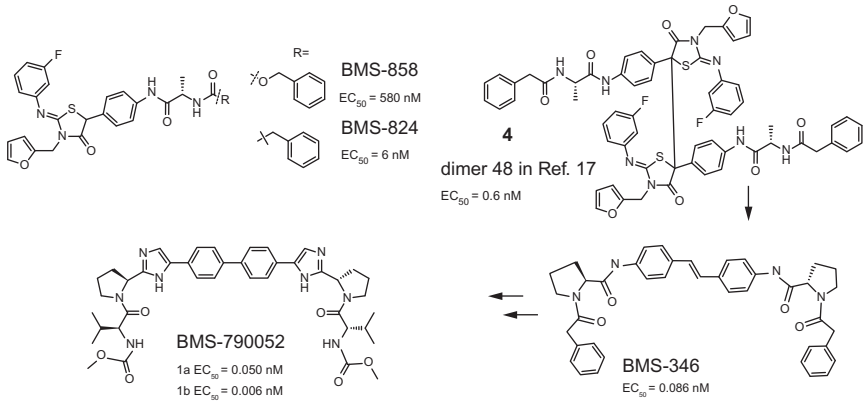

Figure 22.2 BMS progression from screening hit BMS-858 to clinical compound BMS-790052.

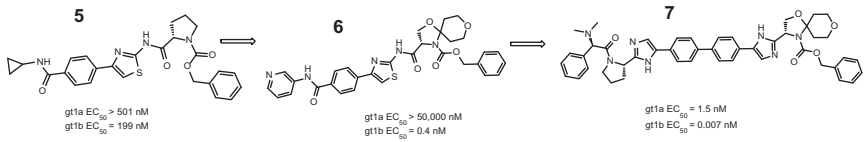

Figure 22.3 Genelabs and GSK discovery of spirocyclic NS5A inhibitor series.

BMS-346,[17,18] leading to BMS-790052. BMS-790052 was potent in both gt1b and gt1a replicon assays and was thus advanced to the clinic.[19] The structure of BMS-790052 (Fig. 22.2), anti-HCV properties, and single-dose monotherapy data in HCV-infected patients were described in 2010.[7]

Genelabs and GSK focused on optimizing novel thiazoles **5**.[20] Replacement of proline by the spiro tetrahydropyran-oxazolidine motif in **6** resulted in potency improvement in gt1b replicon.[21] Further extension of this motif to a pseudosymmetric biphenyl series (exemplified by compound **7**) improved both gt1b and, in particular, gt1a potencies[22–25] (Fig. 22.3).

4. NS5A STRUCTURAL BIOLOGY AND CURRENT INHIBITOR DESIGN

HCV NS5A is one of the six nonstructural viral proteins that coordinate intracellular viral replication. NS5A is very unique, having no known host or viral homolog, other than the NS5A homolog of the closely related GB virus B. It is reported to have multiple functions in the HCV life cycle and interact with a variety of viral and host proteins.[10] Recent studies

indicate that NS5A recruits lipid kinase PI4Ka to support the integrity of the membranous replication complex through increased local concentration of PI(4)P.[26,27] Though NS5A is required for viral RNA replication and is essential to virion production, much remains unknown about how it functions mechanistically at the molecular level.

NS5A contains three distinct domains, separated by relatively disordered segments. The C-terminal domain III regulates viral assembly and fitness[28] through a conserved cluster of Ser/Thr residues via phosphorylation,[29] while domain II contains elements that antagonize innate immune defenses[30] and is a site of cyclophilin inhibitors.[31] The N-terminal domain I contains two features: a conserved structural Zn-finger (ZnF) motif that is essential for viral RNA replication[32] and the AH terminus that is implicated in membrane association.

Two X-ray crystallographic structures of the ZnF unit (residues 33–245) have been reported.[33,34] While both structures suggest NS5A dimerization, the relative orientation of the ZnF units differs significantly. This discrepancy has been interpreted as multiple "active" NS5A orientations associated with different protein–protein and protein–RNA interaction roles.[34] Recent trends in NS5A inhibitor design utilize a dimeric pharmacophore, consistent with the premise that a dimeric form of NS5A is inhibited. NS5A inhibitors feature a linearly conjugated bis-aryl imidazole core, symmetrically terminated by peptidic caps, as found in BMS-790052. A variety of spiro and fused-ring variants of proline are also exemplified, as well as alcohol and ether derivatives of valine.

NS5A inhibitor-induced mutations arise predominantly at residue positions 28, 30, 31, and 93 in domain I, color-coded blue in Fig. 22.4. In HCV1b, Y93 is located at edge of the ZnF dimer interface, flanked by residues L28, R30, and L31, located in a flexible loop that connects the AH and ZnF units of domain 1. Modeling[10] suggests that this locus of residues functions as a "hinge" that regulates the orientation and large-amplitude motion of the AH relative to the ZnF unit. The O—O distance between Y93 residues in the monomer units is 19 Å, which serves as an estimate of the width of the dimer interface. This distance is roughly the length of dimeric NS5A inhibitor cores, defined by the intramolecular distance between the imidazole–proline junctions. Assuming resistant mutations are due to direct ligand contacts, symmetry implies dimeric inhibitors interact with NS5A by straddling the ZnF dimer interface, positioning the peptidic caps in proximity to either NS5A loop where mutational resistance is prevalent, modeled in Fig. 22.4. As independent fragments, the caps

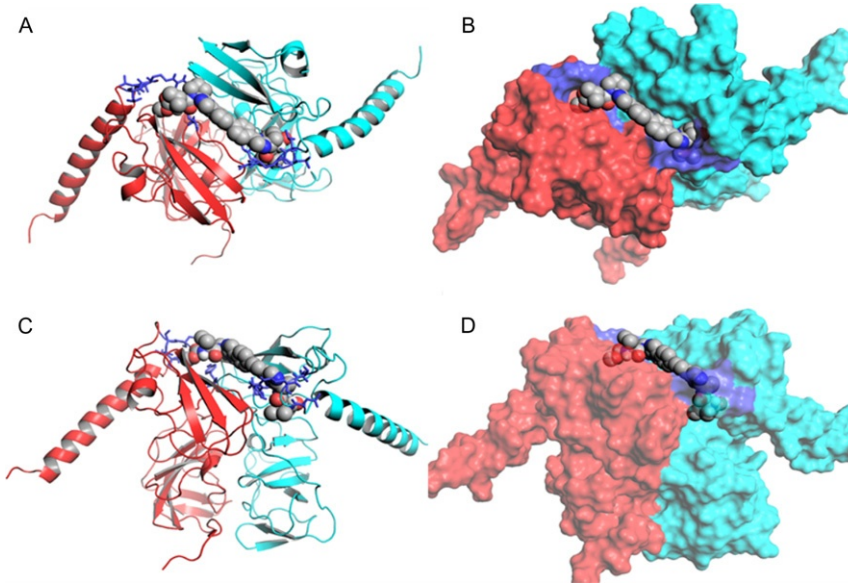

Figure 22.4 Putative binding mode of BMS-790052 with NS5A gt1b domain I, based on the crystal structure of the ZnF domain[34] and homology modeling of the AH and ZnF-AH loop units (residues 1–36).[10] Monomer units are color-coded red and cyan. BMS-790052-resistant mutant sites (gt1a: Met28, Gln30, Leu31, Tyr93) are color-coded blue. (A) Dimeric interaction mode of BMS-790052 (CPK spheres) looking down the C2-symmetry axis of the NS5A homodimer. The bis-phenyl imidazole core of BMS-790052 spans the NS5A dimer interface, positioning the Pro-Val-carbamate caps at the hinge loops, between the ZnF and AH units. (B) Transparent surface rendering of (A). (C) View of the dimer interface, perpendicular to the C2 axis. (D) Transparent surface rendering of (C).

are assumed to interact only weakly with the loop region ($K_{\mathrm{d,mono}} \sim 1\ \mu\mathrm{M}$), but by linking the caps to bind cooperatively, inhibition grows exponentially ($K_{\mathrm{d,dimer}} \sim K_{\mathrm{d,mono}} \cdot K_{\mathrm{d,mono}} \sim 1\ \mathrm{pM} = \exp[-\Delta G_{\mathrm{dimer}}/\mathrm{RT}] \sim \exp[-2\Delta G_{\mathrm{mono}}/\mathrm{RT}]$). This may explain the picomolar potency of dimeric NS5A inhibitors and the abrupt loss in potency that can be observed as dimeric NS5A inhibitors are truncated into monomeric analogues.

Notably, neither NS5A inhibitor cocrystal structures nor labeling studies have been published to date. Thus, the direct binding modes of NS5A inhibitors, dimeric or monomeric, remain elusive. Conceivably, the absence of the connecting loop between helix and ZnF domains, where mutations arise, could preclude the formation of a cocrystal complex with truncated protein constructs used thus far in structural biology. Given the variety of NS5A functions reported in the literature, multiple inhibitory mechanisms

of action could also complicate the interpretation of SAR. Future experimental structure-based studies, as well as NS5A functional studies, will hopefully provide clearer insight into the SAR and functions of NS5A.

Since recent NS5A inhibitors are dimeric, their physical properties can exceed optimal lead-like metrics for small molecules by roughly a factor of two. The median MW, *dogP*, and *dogD* are approximately 787, 4.9, and 4.6, respectively, which can present a challenge for achieving *in vivo* oral bioavailability. Nonetheless, encouraging clinical results for BMS-790052 have demonstrated the viability of this dimeric NS5A inhibitor for treating HCV, as well as the caveats of strictly imposing widely accepted, but crude, cutoffs that can subvert discovery and development of promising therapeutics.

Figure 22.5 captures the distribution of patents exemplifying dimeric NS5A inhibitors. Following BMS-790052, a number of companies are in the clinic with reported NS5A inhibitors. While the chemical structures of most NS5A clinical candidates have not been disclosed, Fig. 22.6 illustrates the diversity of several inhibitors reported in the NS5A patent space

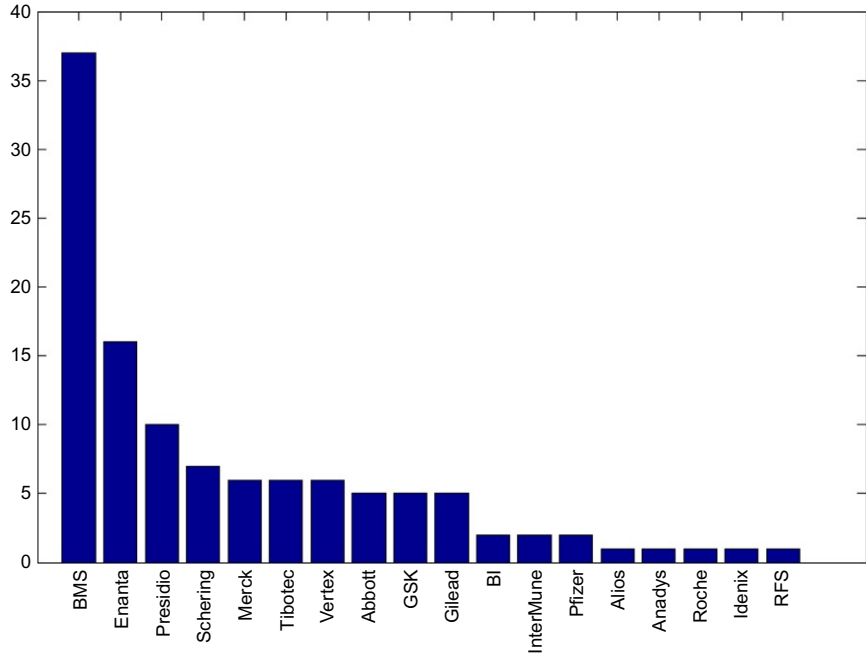

Figure 22.5 The distribution of patent applications associated with dimeric HCV NS5A inhibitors.

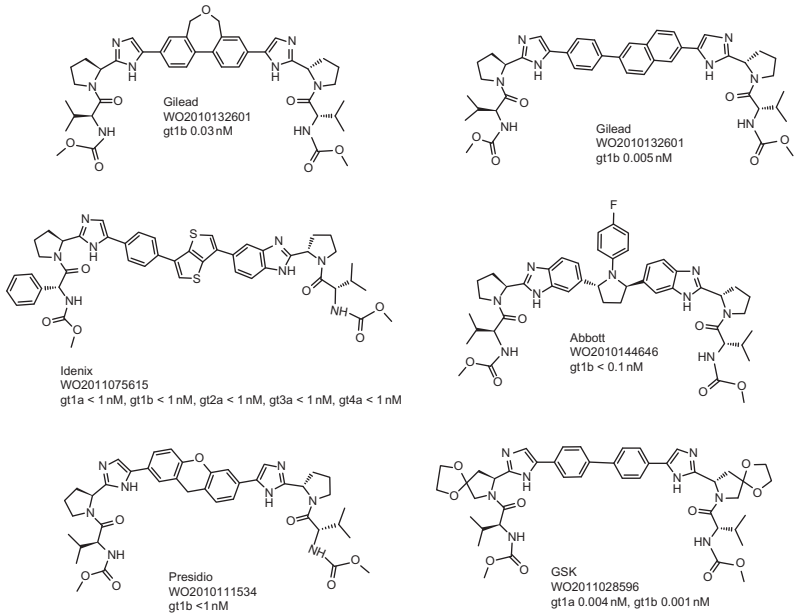

Figure 22.6 Selected diversity of NS5A inhibitors from the recent patent literature.

from companies currently pursing HCV clinical trials. The Abbott core motif is notable in that it suggests an orthogonal dimension for scaffold modification, along the C_2 axis of the core.

5. CLINICAL PROGRESS OF NS5A INHIBITORS

NS5A inhibitors have been evaluated in single and multiple dose studies in healthy volunteers to assess pharmacokinetics and safety. Evaluation in HCV-infected subjects has been done in monotherapy studies using single dose or multiple daily doses or in combination with other regimens in longer-term studies. Endpoints that are commonly used in HCV clinical trials are rapid virological response (RVR, undetectable HCV RNA after 4 weeks), extended RVR (eRVR, undetectable HCV RNA at weeks 4 and 12), and SVR (undetectable HCV RNA 12 weeks after treatment cessation, SVR12, or 24 weeks after treatment cessation, SVR24). SVR24 is considered a virological cure for HCV although regulatory agencies are now accepting SVR12 as an endpoint.

Bristol–Myers Squibb's Daclatasvir (BMS-790052) is the most advanced NS5A inhibitor in the clinic. It was first disclosed at the 59th annual meeting

of the American Association for the Study of Liver Diseases and generated substantial interest in this class of inhibitors due to the very rapid and profound viral load reductions resulting from administration of single dose to HCV-infected patients.[7] A 14-day monotherapy trial has confirmed the potency of daclatasvir with a maximum mean log viral load reduction of 2.8 logs achieved with a 1-mg dose administered once a day.[35] However, a low genetic barrier to resistance was displayed by daclatasvir as viral breakthroughs occurred by day 7 at doses as high as 100 mg QD necessitating its combination with an inhibitor with a different mechanism of action. BMS has initiated multiple clinical trials with daclatasvir in combination with other regimens, and the early results are encouraging although these studies are small and have not reached their final endpoints.

In a Phase 2a study,[36] 48 treatment naïve genotype 1 HCV-infected patients were randomized to receive placebo, 3, 10, or 60 mg daclatasvir for 48 weeks in combination with pegylated interferon α2a and ribavirin (PR). In the 3-mg group, 5/12 patients had an RVR and SVR12. In the 10-mg group, 11/12 patients had an RVR and SVR12. In the 60-mg group, 10/12 patients had an RVR and SVR12. These responses were much better than the PR plus placebo group who had RVR in 1/12 patients and SVR12 in 3/12 patients. There were 7, 3, 2, and 9 virologic failures in the 3, 10, 60, and placebo groups, respectively, which highlights the ability of robust combination regimens to reduce virologic failures.

In a study in treatment naïve genotype 1-infected Japanese patients,[37] daclatasvir at 10 or 60 mg QD was given in combination with pegylated interferon α2a and ribavirin for 12 weeks. Patients who had a protocol-defined response (PDR) of HCV RNA < LOQ at week 4 and undetectable at week 12 had an additional 12 weeks of triple therapy, while those who did not achieve PDR had an additional 36 weeks of triple therapy. 7/9 patients in the 10-mg group achieved PDR as did 8/8 patients in the 60-mg group. All patients who achieved PDR and who received 24 weeks of total treatment had an SVR24. The combination of daclatasvir and PR was also tested in patients who had previously undergone treatment with PR but were partial or null responders. 8/9 patients in the 10-mg group achieved PDR and four of these achieved SVR24. In the 60-mg group, 7/9 patients achieved PDR and 6 of these had SVR24. SVR24 rates on the patients who did not achieve PDR and went on to a longer course of treatment are not yet available.

In a similar study using pegylated interferon α2b and ribavirin in combination with 10 or 60 mg daclatasvir,[38] 7/9 treatment naïve patients in the 10-mg group and 10/10 in the 60-mg group achieved PDR and received

the shorter treatment course. Of these, six patients in the 10-mg group and nine in the 60-mg group had an SVR24. This study also looked at prior PR nonresponders. The efficacy of daclatasvir in combination with pegylated interferon α2b and ribavirin was not as substantial in this difficult to treat group. Only 5/9 patients in the 10-mg group achieved PDR and only 2 of these had SVR24. In the 60-mg group, 3/9 patients achieved PDR and 2 of these had SVR24. For the overall nonresponder population, SVR24 was achieved in 22% of the 10-mg group and 33% of the 60-mg group.[39]

The SVR24 rates for the treatment naïve patients who had PDR for these two studies (30/32 patients) were similar to the SVR24 rate of 89% for treatment naïve patients who received telaprevir in combination with PR for 12 weeks and, using response guided therapy (undetectable HCV RNA at weeks 4 and 12), an additional 12 weeks PR.[40]

Daclatasvir is currently in a phase 2b study in combination with pegylated interferon α2a and ribavirin in prior PR nonresponders. Interim week 12 data was presented at the 2012 International Liver Congress.[41] In the 20-mg daclatasvir group, 18% of null responders and 26% of partial responders met the primary endpoint of eRVR. In the 60-mg daclatasvir group, 20% of null responders and 36% of partial responders had eRVR. Although the SVR rates are not yet available, these eRVR rates are not encouraging for the use of NS5A inhibitors as an add-on to retreatment with PR of prior nonresponders.

Daclatasvir has also been investigated in combination with other direct acting antivirals (DAAs) in interferon α free regimens. Daclatasvir has been combined with GS-7977, a nucleotide NS5B inhibitor, in treatment naïve patients infected with HCV genotype 1, 2, or 3. In genotype 1a/1b patients, 100% of patients receiving 60 mg daclatasivr and 400 mg GS-7977 for 24 weeks had SVR4. An SVR4 rate of 100% was also achieved in genotype 2 or 3 infected patients. When ribavirin was added on to the combination of daclatasvir and GS-7977, an SVR4 of 100% was obtained in the genotype 1/1b patients and an SVR4 of 86% in genotype 2/3 patients.[42]

An exploratory study in genotype 1 HCV-infected patients who were prior PR nonresponders dosed 11 patients with 60 mg daclatasvir once daily and 600 mg NS3 PI asunaprevir twice daily for 24 weeks. Another group of 10 patients received daclatasvir and asunaprevir in combination with PR for 24 weeks.[43] Four of 11 patients in the dual therapy group achieved the primary endpoint of SVR12, while 10/10 patients in the quadruple therapy group had SVR12. This was the first indication that an interferon free, all DAA regimen can effect a cure. The primary reason for treatment failure

in the all DAA regimen was viral breakthrough which occurred in six patients who were infected with genotype 1a HCV. The two genotype 1b-infected patients in this group both had SVR12 consistent with the higher genetic barrier to resistance of NS5A inhibitors against genotype 1b HCV. A recently published study has confirmed the impressive efficacy that can be achieved against genotype 1b.[44] Ten genotype 1b HCV-infected patients who were prior PR nonresponders were treated with 60 mg daclatasvir once daily and initially 600 mg asunaprevir twice daily for 24 weeks. The dose of asunaprevir was reduced to 200 mg twice daily during the study after hepatic enzyme elevations occurred in an asunaprevir plus PR clinical trial. 9/10 patients completed 24 weeks of treatment and all 9 had SVR12 and SVR24. Additional null responder patients were added to this study resulting in an SVR24 for 19 of 21 patients.[45] This is an unprecedented response in the difficult to treat nonresponder population which has a 41% SVR rate when treated with the approved PI telaprevir in combination with PR.[46]

Gilead Sciences' GS-5885 is an NS5A inhibitor that showed clinical proof of concept in a 3-day monotherapy trial in genotype 1 patients.[47] HCV-infected subjects dosed with 1, 3, 10, 30, or 90 mg had median maximal reductions >3 logs in dose groups ≥ 3 mg. The dosing period was too short to detect viral breakthroughs during treatment, but genotypic and phenotypic analysis detected the presence by population sequencing of variants resistant to GS-5885 in all patients dosed ≥ 3 mg at day 4 or 14. Interestingly, three patients whose maximal viral load reductions were <1.6 logs had viral variants at baseline with resistance to NS5A inhibitors (Q30E and L31M and Y93C in genotype 1a). GS-5885 was well tolerated in this study and has progressed to Phase 2 studies. GS-5885 has also been investigated in an interferon free regimen in treatment naïve genotype 1-infected patients. Patients in Arm 1 received 30 mg QD GS-5885, 200 mg QD GS-9451, an NS3 PI, 30 mg BID GS-9190, an NS5B nonnucleoside inhibitor, and ribavirin for 24 weeks. In Arm 2, the dose of GS-5885 was increased to 90 mg QD and patients were randomized after 12 weeks to stop treatment or continue treatment through week 24. Not all patients have completed the study, but interim results presented at the 2012 International Liver Congress are promising.[48] In Arm 1, 80% of the patients who completed the study had an SVR12. In Arm 2, 81% of the patients who received 12 weeks of treatment had SVR12 and 100% of the patients who received 24 weeks of treatment had SVR12. These numbers could change as there are still a number of patients in follow-up, but the prospects for replacing interferon with an all-oral regimen are encouraging based on these early results.

GlaxoSmithKline is developing an NS5A inhibitor with a preclinical profile that is more potent on genotype 1b Y93N and Y93H mutants than the BMS compound daclatasvir.[49] In a Phase 1 study, GSK2336805 showed good PK in single and multiple ascending dose studies and was well tolerated up to 60 mg as a single dose and 75 mg for 14 days in multiple doses. Genotype 1 HCV-infected patients were dosed with 1, 10, 30, 60, and 120 mg of GSK2336805 and HCV viral load was monitored. 2.0–3.9 log reductions were achieved after single dose of $\geq$10 mg of GSK2336805.[50] Genotypic analysis of posttreatment samples detected the emergence of variants associated with resistance to GSK2336805 in 12 of 14 subjects dosed with $\geq$10 mg GSK2336805.

ABT-267 is Abbott Laboratories' NS5A inhibitor in clinical development for HCV. Single and multiple ascending dose studies in healthy volunteers have shown good PK and tolerability.[51] In a 3-day monotherapy study conducted in HCV genotype 1-infected patients, doses of 5, 50, and 200 mg QD of ABT-267 resulted in mean maximal log reductions in viral loads of 2.89, 2.77, and 3.1, respectively.[52] These same doses were given for 12 weeks in combination with PR followed by PR alone for an additional 36 weeks. One of the primary endpoints was RVR which was achieved in 33% of the 5-mg group, 56% of the 50-mg group, and 70% of the 200-mg group. SVR24 is not yet available from this ongoing study.[53]

Presidio Pharmaceuticals, Inc. is developing two NS5A inhibitors. PPI-461 is their lead compound which has shown proof of concept in the clinic, and PPI-668 is their back-up compound currently in a Phase 1b study. PPI-461, like many of the NS5A inhibitors in development, is an extremely potent inhibitor of genotype 1b HCV with slightly lower activity against genotype 1a. PPI-668 is more active against replicons containing the NS5A gene from genotypes 3a and 6a than PPI-461. PPI-461 was dosed at 50, 100, and 200 mg daily for 3 days and achieved mean log viral load reductions of 2.65, 3.65, and 3.62 logs, respectively.[54] One patient in the 50-mg cohort only had a 0.4-log reduction, and genotypic analysis showed the presence at baseline of HCV with four linked NS5A-resistance mutations. Resistance substitutions in NS5A were detected in 17/18 subjects after 3 days of monotherapy.[55]

Achillion Pharmaceuticals, Inc. has ACH-2928 in clinical development. ACH-2928 was dosed at 10 and 60 mg QD for 3 days in a monotherapy study in HCV genotype 1-infected patients. The 10-mg group had a mean maximal log viral load reduction of 2.79 logs, and the 60-mg group had a mean maximal log viral load reduction of 3.68 logs.[56] Achillion also has

another NS5A inhibitor in development. ACH-3102 has improved potency against HCV replicons containing mutations associated with resistance to other NS5A inhibitors. Unlike ACH-2928 and daclatasvir, ACH-3102 also retains potency against chimeric replicons containing NS5A from genotype 2a and 2b patient isolates.[57]

6. FUTURE PROSPECTS

The chemical exploration of small molecules targeting NS5A has increased in the past 2–3 years. The clinical progression of NS5A inhibitors has also increased in terms of the number of candidates in the clinic and in the number of novel regimens that are being investigated with them. The addition of NS5A inhibitors to the clinician's armamentarium will improve SVR rates in difficult to treat populations such as interferon nonresponders. More importantly, the success seen in combinations of these very potent inhibitors with other DAAs shows the potential to completely replace interferon containing therapies with all oral drugs. The current NS5A inhibitors in clinical development are very potent inhibitors of genotype 1 HCV but are less potent against some of the other genotypes. Designing NS5A inhibitors with potent pan-genotype activity will allow all oral regimens to be extended to all HCV-infected patients regardless of genotype.

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