

# The Discovery and Development of Daclatasvir: An Inhibitor of the Hepatitis C Virus NS5A Replication Complex



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**Abstract** The discovery of the hepatitis C virus (HCV) NS5A replication inhibitor daclatasvir (**1**) had its origins in a phenotypic screening lead. However, during the optimization campaign, it was observed that some members of the chemotype underwent a radical dimerization under the assay conditions. This redirected the effort to focus on palindromic molecules, a species subsequently shown to complement the dimeric nature of the NS5A protein. The most challenging aspect of the discovery program was extending antiviral activity to encompass GT-1a virus which was accomplished only after the development of extensive structure-activity relationships. In clinical trials, oral administration of daclatasvir (**1**) produced a profound effect on viral load with onset that was more rapid than had been seen previously with either NS3 protease or NS5B polymerase inhibitors. A groundbreaking clinical trial that combined daclatasvir (**1**) with the protease inhibitor asunaprevir (**52**) established that a chronic HCV infection could be cured with small molecule therapy in the absence of immune stimulation, setting the stage for approval of this regimen for the treatment of GT-1b-infected subjects by the Japanese health authorities on July 4, 2014.

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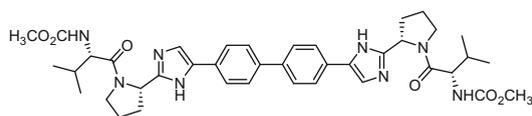
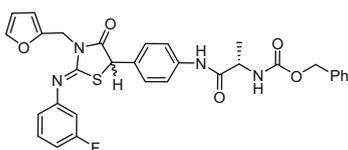
## 1 Introduction

The discovery of the hepatitis C virus (HCV) nonstructural 5A (NS5A) replication complex inhibitor daclatasvir (**1**) began with the development of a genotype 1b (GT-1b) replicon that was implemented as a phenotypic screen using a design that conferred a stringent triaging of hit molecules [1–11]. This screening campaign was part of a broader strategy that pursued the identification of mechanistically orthogonal inhibitors of HCV that could be used in combination therapy, an approach that anticipated clinical use of drug cocktails to minimize the selection of resistant viruses. More specifically, the replicon screen comprised of simultaneously assessing the antiviral activity of test molecules toward a sub-genomic HCV GT-1b construct and a bovine viral diarrhea virus (BVDV) replicon, both replicating in the same human liver Huh-7 cell line background and seeded in the same well of a 96-well plate [6]. HCV inhibition was determined indirectly using a fluorescence resonance energy transfer (FRET)-based assay that assessed NS3 protease activity toward a synthetic substrate incorporating both a fluorescence donor [5-((2-aminoethyl)amino)naphthalene-1-sulfonic acid (EDANS)] in the amino terminus and a fluorescence acceptor [4-((4-(dimethylamino)phenyl)azo)benzoic acid, succinimidyl ester (DABCYL)] at the carboxyl terminus. The BVDV replicon incorporated a firefly luciferase reporter gene that provided an orthogonal readout of replication activity based on the emission of light, reflecting the amount of enzyme present after adding a substrate. The third piece of information harvested from the assay, which was also the first experimental data obtained, was AlamarBlue cell viability staining which assessed mitochondrial function, providing an indication of the cytotoxicity of test compounds. This assay configuration was used to interrogate a representative selection of the Bristol-Myers Squibb proprietary compound collection and identified the iminothiazolidinone **2** as hit that met the criteria of exhibiting activity toward the HCV replicon at concentrations at least tenfold lower than either inhibitory activity toward the BVDV replicon or cytotoxicity [12]. The specific details of the antiviral profile of **2** in the screening assays and toward a panel of viruses are summarized in Table 1. Compound **2** had an interesting background since it had its origin in a prospective library that had been prepared as part of a campaign to embellish the Bristol-Myers Squibb proprietary compound collection. A notable structural feature of **2** is that it had been designed to include a phenyl substituent at C-5, unusual since C-5 benzylidene derivatives are far more prevalent in the literature, a function of convenient access by way of a Knoevenagel condensation reaction between a C-5 unsubstituted iminothiazolidinone and an aldehyde [13, 14].

**Table 1** Antiviral activity of **2** in the GT-1b HCV replicon and toward a panel of viruses

Assay	EC <sub>50</sub> (μM)	CC <sub>50</sub> (μM)
GT-1b HCV replicon	0.57	>50
BVDV replicon	24	>100
BVDV virus	17	>150
HIV-1	41	41
HRV	>100	>100
RSV	23	23

*HIV-1* human immunodeficiency virus-1, *HRV* human rhinovirus, *RSV* respiratory syncytial virus

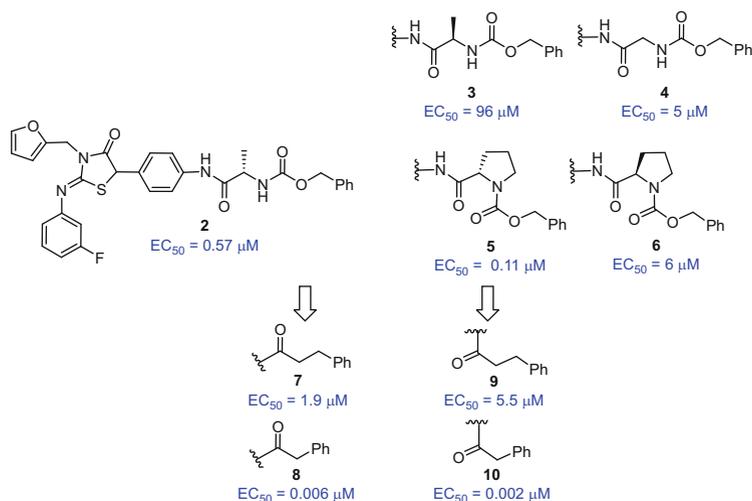
**1** (BMS-790052, daclatasvir)**2** (BMS-858)

The data accumulated for **2** that are compiled in Table 1 revealed a profile of significant and selective inhibitory potency toward the GT-1b HCV replicon with an EC<sub>50</sub> value of 570 nM, a finding that encouraged further study of the chemotype [15]. Adding to the intrigue with **2** as a lead inhibitor was the generation and validation of resistant mutants arising in the replicon in response to selective pressure that mapped to the amino terminus of the NS5A protein, specifically a Tyr98His and a Leu31Val/Gln54Leu combination [12]. At the time of this discovery, little was known about the specific functions of the NS5A protein in viral replication although, perhaps not surprisingly for such a small virus, it was known to be an essential protein [16–20].

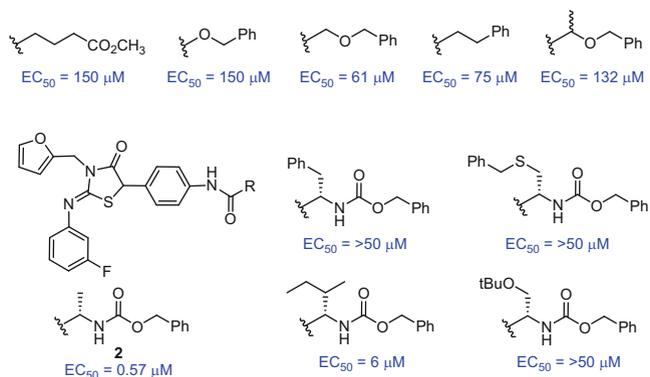
The Z-configuration of the 2-arylimino moiety of **2** was assigned based on prior studies which indicated that this topology minimized steric effects. However, attempts to separate the individual stereoisomers at C-5 were thwarted by facile racemization of the benzylic center after chiral chromatographic separation [15, 21]. Seminal insights into structure-activity relationships (SARs) were gleaned from a survey that explored variation of the three major peripheral elements of the molecule – the furanylmethyl substituent, the arylimino moiety, and the amino acid amide. Changes to both the furanylmethyl and arylimino moieties in the context of the Cbz-alanine amide were found to influence potency, with compounds incorporating polar heterocycles being the more potent, while more lipophilic substituents

generally exhibited poorer replicon inhibitory activity. Overall, a good dynamic range of potency was observed with the structural variations sampled, with  $EC_{50}$  values ranging from 10 to 2.5  $\mu\text{M}$ . However, more profound and precise effects on HCV GT-1b inhibitory potency were associated with changes to the Cbz-alanine amide element, with the key findings summarized in Fig. 1. The unnatural alanine analogue **3** was 170-fold less potent, while the glycine homologue **4** was eightfold weaker than **2**. The strong dependence of antiviral activity on the absolute configuration of the amino acid element was reproduced in the proline derivatives **5** and **6**, both of which were fivefold more potent than their alanine congeners [15]. Replacing the Cbz element with a dihydrocinnamate moiety resulted in an erosion of potency in both series, as exemplified by **7** and **9**. However, a significant increase in potency was observed with the phenylacetamide moiety found in **8** and **10**, with both compounds expressing single digit nanomolar  $EC_{50}$  values in the replicon and confirming the inherent advantage offered by proline. Only weak antiviral effects were observed with the many other amino acids and additional structural variations that were explored at this site of the pharmacophore, with some representative examples of those sampled compiled in Fig. 2 [15].

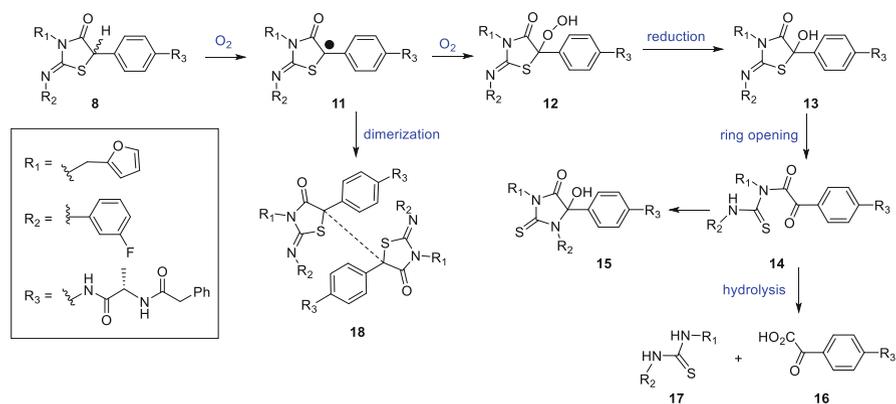
While these collective SARs appeared to be coherent and were readily interpretable, as highlighted by the sensitivity of potency to changes to the amino acid element, as exploration of **2** and its analogues continued, it became apparent that the iminothiazolidinone chemotype was unstable under some conditions [15, 22]. The first indication of a problem was the observation that a sample of **2** degraded upon standing as a solution in DMSO for several days, with the thiohydantoin **15** ( $R_3 = \text{NHCO}_2\text{CH}_2\text{Ph}$ ) and thiourea **17** ( $R_3 = \text{NHCO}_2\text{CH}_2\text{Ph}$ ) determined to be two of the degradation products after a preparative experiment (Scheme 1). In the replicon, **15** exhibited a modest inhibitory activity,  $EC_{50} = 13 \mu\text{M}$ ,



**Fig. 1** Structure-activity relationships associated with variation of the amino acid moiety of **2**



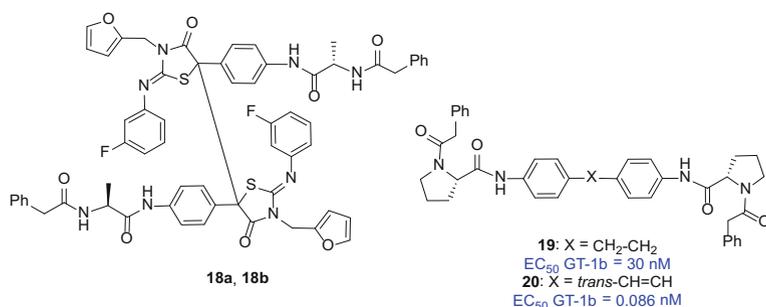
**Fig. 2** Structure-activity relationships associated with the amino acid element of **2**



**Scheme 1** Chemical degradation path elucidated for **2**

while **17** was essentially inactive. Compounding the intrigue, incubating the more potent **8** in replicon media until degradation was complete and then assessing the antiviral activity of the preparation in the GT-1b replicon demonstrated that the HCV inhibitory activity and potency were fully preserved [15, 22]. A more detailed analysis of the degradation products from **8** guided by high-performance liquid chromatography (HPLC) biogram analysis, in which fractions taken from the chromatography column are concentrated and assessed for replicon inhibition, led to the identification of two potent constituents present in just trace amounts in the cell media [22, 23]. A preparative experiment allowed isolation of a sufficient quantity of each component to allow for a more detailed characterization.  $^1\text{H-NMR}$  and mass spectrum analyses indicated that the two compounds were dimers with an isomeric relationship and assigned as **18** based on the absence of the C-5 hydrogen atom of the iminothiazolidinone ring in the  $^1\text{H-NMR}$  spectrum and connectivity that was

confirmed after the analysis of the  $^{13}\text{C}$ -NMR spectrum. Although neither the exact stereo-composition of the benzylic centers of **18** nor the precise relationship between these two compounds was determined, it is of note that the isomer that was less mobile on a reversed phase column converted to the more mobile isomer upon heating in  $\text{CD}_3\text{CN}$  at  $50^\circ\text{C}$ , an observation made while conducting an NMR experiment as part of the structure determination process [22]. With the acquisition of these data, the mechanistically holistic picture of the degradation process that is presented in Scheme 1 was proposed. Abstraction of the C-5 hydrogen atom of **8** by oxygen, which is a diradical in the ground state, would lead to the C-5 radical **11** that is stabilized in a classic captodative fashion by the adjacent  $\text{C}=\text{O}$  moiety, the sulfur atom, and the phenyl substituent [24, 25]. Combination of **11** with  $\text{O}_2$  and hydrogen atom acquisition would lead to the hydroperoxide species **12** which in DMSO would be reduced to alcohol **13**, an unstable ring system that would be expected to undergo ring opening to give **14**. Reclosure of **14** would then afford the thiohydantoin **15**, which had been isolated in the original DMSO degradation experiment, while hydrolytic decomposition of **14** would afford acid **16** and the thiourea **17**, the latter of which had also been isolated. However, in cell culture media, the stability of radical **11** is presumably such that dimerization can occur, a process that may well be facilitated by aggregative association of **8** and/or **11** in the aqueous medium.



The replicon inhibitory potency of the dimers **18a** and **18b** was striking, with the less mobile isomer eluting from the reversed phase chromatography column exhibiting an  $\text{EC}_{50}$  value of 600 pM in the GT-1b replicon, while the thermodynamically more stable and chromatographically more mobile isomer was 70-fold weaker,  $\text{EC}_{50} = 43$  nM [15, 22]. With the elucidation of the structures of **18a** and **18b**, consideration was given to the concept that the NS5A-inhibiting pharmacophore represented by these dimers may be the embedded symmetrical bibenzyl element. This notion was based on an appreciation of the precise SARs surrounding the amino acid moiety in contrast to the relatively more nebulous effects associated with structural variation to the iminothiazolidinone substituents. Under this concept, the iminothiazolidinone ring system of **2** was suggested to act as a scaffolding element by which a bivalent NS5A inhibitor pharmacophore was convened through a radical-mediated dimerization process [15, 22]. This hypothesis was readily tested in the context of the proline-based chemotype, with **19** demonstrating an  $\text{EC}_{50}$  value of 30 nM and confirming the pharmacophore proposal. More interestingly, the unsaturated synthetic precursor **20**

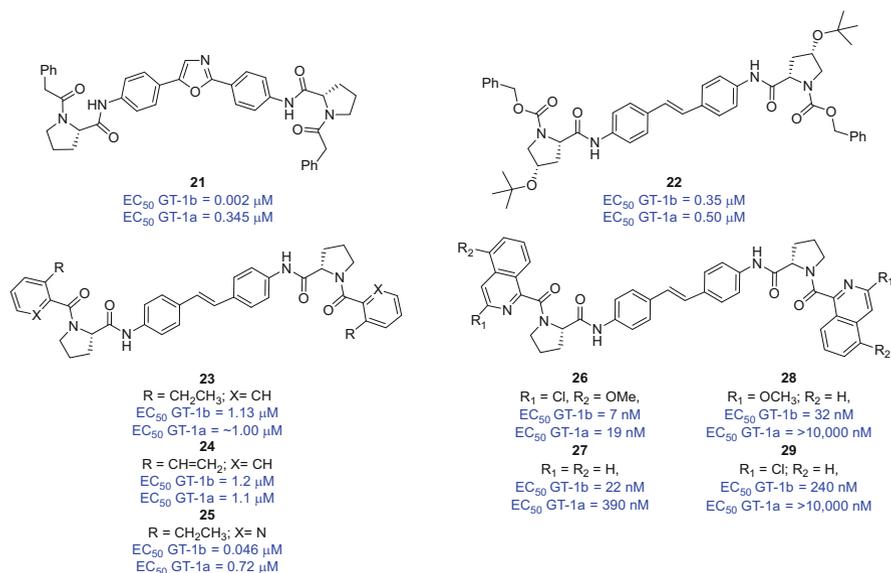
was almost 350-fold more potent than **19**,  $EC_{50} = 0.086$  nM, providing further insight into the topography of the NS5A-inhibiting pharmacophore.

## 2 Optimization of Dimeric NS5A Replication Complex Inhibitors to Daclatasvir

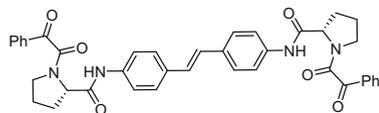
The discovery of the structurally simpler symmetrical pharmacophore represented by **19** and **20** turned out to be prescient since X-ray crystallographic structure data for NS5A constructs that were published several years after this discovery revealed a dimeric, C<sub>2</sub>-symmetric protein complex [26–28]. Of note, this structure was determined using a protein construct lacking the membrane-associating amino terminus but, nevertheless, containing some of the key elements of the anticipated binding site for NS5A inhibitors based on the location of resistance mutations. The availability of a structure of the amino terminus of NS5A, acquired by NMR methodology, facilitated the construction of models of NS5A that, when combined with the resistance mutation data, suggested that these compounds bound across the NS5A dimer interface at a site residing between the membrane and the core of the protein. Dimerization of the NS5A protein in cells was subsequently confirmed as was association of NS5A with RNA, a prediction from the X-ray data based on the preponderance of basic amino acids lining the inner surface of the U-shaped dimeric form of NS5A in the solid state [29–32].

While the antiviral activity of **20** was attractive, there were concerns around elements embedded within the structure, with the olefin observed to be configurationally unstable in some analogues, while the potential for release of one or both aniline moieties after the action of an amidase or protease in vivo raised the specter of toxicity. However, a considerably more challenging problem arose when **20** was evaluated in a newly developed GT-1a replicon where the compound was found to be poorly active, with an  $EC_{50}$  value that was in excess of 10  $\mu$ M. The GT-1a strain of HCV is clinically relevant, with prevalence that varies across the globe; consequently, securing activity toward this genotype was considered to be a critical objective. Enhancing GT-1a inhibitory activity became the immediate focus of further study, and the approach adopted, while primarily directed toward the peripheral elements, was broad-based in prosecution. Modifications to all facets of the molecule were explored, some of which were also directed toward simultaneously addressing the structural liabilities highlighted above. However, as described below, introducing and retaining GT-1a coverage while optimizing ADME properties would end up posing a considerable challenge. While deeper insight into SARs for GT-1b inhibition emerged from the initial phases of this effort, only a small number of compounds were identified that exhibited modest but reproducible GT-1a inhibition. Among these were the oxazole **21** and the substituted proline derivative **22**, both of which were inhibitory in the GT-1a replicon with  $EC_{50}$  values of less than 1  $\mu$ M [33]. However, attempts to optimize these molecules were unproductive, in each case leading to SAR cul-de-sacs. As the studies progressed, the 2-ethyl-substituted benzamide derivative **23** and its unsaturated homologue **24** were discovered to

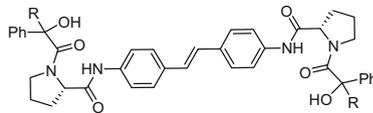
exhibit weak inhibition of GT-1a and GT-1b replicons, with modeling studies suggesting that the effect of substitution was not a function of modulating the conformation between the phenyl ring and proline carbonyl [34]. The pyridine derivative **25** further advanced the SAR associated with this cap element but, more importantly, formed the basis of the discovery of the isoquinoline derivative **26**, in which the ethyl substituent was incorporated into a fused ring, as the first compound to exhibit potent and balanced antiviral activity toward GT-1a and GT-1b replicons [34]. The promise of this compound was further underscored when it was screened in replicons or hybrid replicons representing genotypes 2a, 3a, and 5a where the EC<sub>50</sub> values ranged from 2.2 to 14 nM.



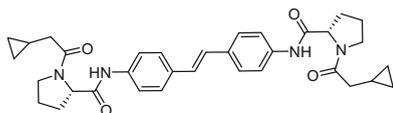
Further examination of the SARs revealed that GT-1a inhibitory activity was much more sensitive to the nature and the substitution pattern of the isoquinoline ring than GT-1b [34]. For example, **27**, the parental analogue of **26**, exhibited GT-1a and GT-1b EC<sub>50</sub> values that were 20- and 3-fold weaker than that of **26**, respectively. In addition, the methoxy-substituted derivative **28** and its chloro-substituted analogue **29** retained potent GT-1b inhibition, but their GT-1a EC<sub>50</sub> values were >10 μM. However, a more fruitful avenue of investigation was found when the effects of deannulating the isoquinoline ring were probed [35]. The α-keto amide **30** preserved the GT-1a inhibition exhibited by **26**, while the derived secondary alcohols **31** and **32** demonstrated that planarity at this site was not a specific requirement. The tertiary alcohol homologues **33** and **34** added further to the SARs, with the (*S,S*)-analogue **34** the more potent isomer, particularly toward the GT-1b replicon where the EC<sub>50</sub> value was 6 pM. Another encouraging observation was made with **35** which, although poorly active in the GT-1a replicon, demonstrated 64% bioavailability following oral dosing to rats, indicating that securing systemic exposure after oral delivery of these symmetrical stilbene derivatives was feasible.



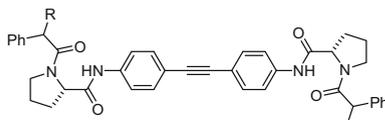
**30**  
 $EC_{50}$  GT-1b = 8 nM  
 $EC_{50}$  GT-1a = 447 nM



**31:** R = H, (R,R)  
 $EC_{50}$  GT-1b = 0.022 nM  
 $EC_{50}$  GT-1a = 640 nM  
**32:** R = H, (S,S)  
 $EC_{50}$  GT-1b = ~4.2 nM  
 $EC_{50}$  GT-1a = 1020 nM  
**33:** R = CH<sub>3</sub>, (R,R)  
 $EC_{50}$  GT-1b = <4.6 nM  
 $EC_{50}$  GT-1a = 6850 nM  
**34:** R = CH<sub>3</sub>, (S,S)  
 $EC_{50}$  GT-1b = 0.006 nM  
 $EC_{50}$  GT-1a = 84 nM



**35**  
 $EC_{50}$  GT-1b = 62 nM  
 F in rats = 64%



**36:** R = (R,R)-N(CH<sub>3</sub>)<sub>2</sub>  
 $EC_{50}$  GT-1b = <4.6 nM  
 $EC_{50}$  GT-1a = 8.8 nM  
**37:** R = (S,S)-N(CH<sub>3</sub>)<sub>2</sub>  
 $EC_{50}$  GT-1b = <4.6 nM  
 $EC_{50}$  GT-1a = 628 nM  
**38:** R = (R,R)-NHCO<sub>2</sub>CH<sub>2</sub>  
 $EC_{50}$  GT-1b = 0.013 nM  
 $EC_{50}$  GT-1a = 26 nM

Replacing the stilbene element with an alkyne, which resolved a *cis-trans* isomerization issue observed with some analogues, added further to the understanding of the topography of the pharmacophore. Additional probing of the amino acid terminal region using this alkyne-based scaffold identified potent arylglycine-based analogues for which the GT-1a  $EC_{50}$  values for some compounds, including **36–38**, were single digit nM [35]. Notably, the change in stereochemical preference in evolving the chemotype from the mandelamide analogues **33** and **34** to the phenylglycine analogues **36** and **37** further highlighted the relatively intricacy and sensitivity of the GT-1a inhibition SARs that were being uncovered during this phase of the project. Equally intriguing was the accumulating evidence indicating that the GT-1b virus was highly tolerant of many structural changes, an observation that could not readily be explained based on the differences in amino acid composition at the putative binding site of the compounds.

A concurrent effort examined scaffold modifications directed toward the identification of a less problematic replacement for the anilide moiety that would decrease or eliminate the potential for aniline release *in vivo* which led to the emergence of two noteworthy SAR findings. Firstly, the replacement of the anilide moieties of **36** and **38** with a benzimidazole, a design intended to preserve both the H-bond donor and acceptor properties of the anilide, resulted in a 4- to 30-fold reduction in potency toward the GT-1a replicon, as exemplified by **39** and **40** [36]. Secondly, a mix and match SAR exercise accentuated the sensitivity of GT-1a inhibitory potency to topological parameters, exemplified by the 70-fold difference in GT-1a inhibitory potency between regioisomers **41** and **42**.

These SAR findings were attributed to the altered topology of the peripheral pharmacophoric elements with respect to the core, a shortcoming that was ultimately addressed by the biphenyl derivatives **43** and **44** which recapitulate the linearity

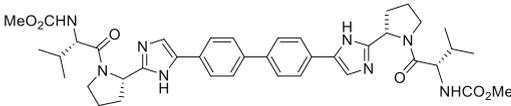
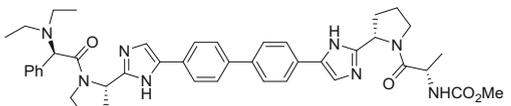
associated with the core alkynes in **36–40**. In **43** and **44**, a motif that was arrived at only after considerable experimentation, deannulation of the benzimidazole ring provided a structural arrangement that compensated for the reduced length of the core of the pharmacophore relative to **39** and **40**. The success of this design strategy is readily apparent since both **43** and **44** are exquisitely potent HCV antiviral agents with balanced GT-1a and GT-1b inhibition, with EC<sub>50</sub> values ranging from 7 to 42 pM [4]. However, the oral bioavailability and systemic exposure of both **43** and **44** in the rat were poor, a result attributed, in part, to the high molecular weight (747 and 807 Da, respectively) and structural composition. This notion was reinforced by PK studies with the smaller (MW = 713) and unsymmetrical tetrahydrofuran **45** which divests of a H-bond donor. Although the oral bioavailability of **45** was low in rodents (F in mouse = 17%, F in rat = 6.8%), its exposure in the dog was much improved, with bioavailability measured at 45%. In an effort to reduce the molecular weight of the carbamate **44**, the two aromatic rings of the phenyl glycine moiety were curtailed to isopropyl substituents affording the D-valine derivative **46**. However, this structural modification resulted in a significant reduction in potency toward both HCV genotypes, with the GT-1a inhibition particularly sensitive, eroding by 44,000-fold. This SAR observation took some time to understand and was resolved only after further study of the chemotype, which revealed that the preferred absolute configuration of the alkyl-glycine caps was the opposite of that of the aryl-glycine caps. The initial observation in this direction was made when the tetrahydrofuran ring of **45** was replaced with L-alanine to provide **47**, which restored potency to sub-nanomolar levels. In an observation that proved to be pivotal, the symmetrical alanine derivative **48** performed similarly, and further optimization of the amino acid appendage led to the discovery of the *bis*-L-valine derivative **1**, an exercise that also included assessing the potential of unsymmetrical derivatives. The decision to advance **1** into IND-enabling toxicology evaluation was taken after a careful comparison with **49**, an analogue with two changes to the periphery that demonstrated similar antiviral properties to **1** (Table 2) but a different PK profile (Table 3). The decision to select **1** for development rather **49** was based on the observation of a twofold accumulation of the latter compound in the plasma, livers, and hearts of mice after 4 days of daily drug administration which occurred at all of the dose levels (15, 50, and 100 mg/kg) examined.

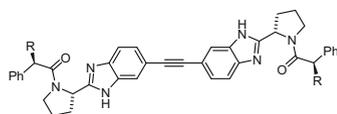
**Table 2** Inhibition of replicons and hybrid replicons by **1** and **49**

Replicon genotype <sup>a</sup>	EC <sub>50</sub> value for <b>1</b> (nM)	EC <sub>50</sub> value for <b>49</b> (nM)
GT-1a H77	0.050	0.036
GT-1b Con1	0.009	0.012
GT-2a JFH-1	0.071	0.020
GT-3a	0.146	0.008
GT-4a	0.012	0.014
GT-5a	0.033	0.021
GT-6	0.054	ND

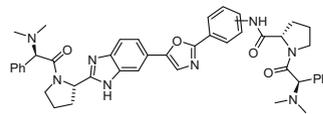
<sup>a</sup>With the exception of GT-2a JFH-1, all data are from hybrid replicons in either a GT-2a JFH-1 or GT-1b Con1 backbone: GT-3a (1–100 NS5A amino acid in Con1); GT-4a (full-length NS5A in Con1); GT-5a (1–110 NS5A amino acid in JFH-1); GT-6a (full-length NS5A in JFH-1)

**Table 3** Pharmacokinetic profile of **1** and **49** in preclinical species

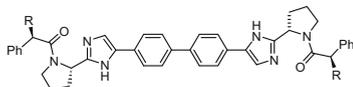
Species	Dose (mg/kg)	Plasma AUC ( $\mu\text{M h}$ )	Plasma concentration at 24 h (nM)	Oral bioavailability
<b>1</b>				
				
Rat	5.0	4.8	18	50%
Dog	2.3	11	26	108%
Cynomolgus monkey	2.8	1.93	6.5	38%
<b>49</b>				
				
Rat	5.0	0.17	Below detection	3.6%
Dog	3.5	1.2	9.0	66%
Cynomolgus monkey	3.0	0.5	4.2	21%



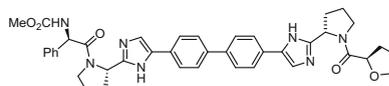
**39:** R = N(CH<sub>3</sub>)<sub>2</sub>  
 EC<sub>50</sub> GT-1b = 0.09 nM  
 EC<sub>50</sub> GT-1a = 260 nM  
**40:** R = NHCO<sub>2</sub>CH<sub>3</sub>  
 EC<sub>50</sub> GT-1b = 0.055 nM  
 EC<sub>50</sub> GT-1a = 108 nM



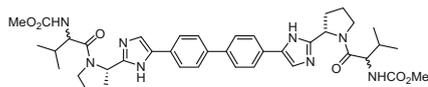
**41** (para)  
 EC<sub>50</sub> GT-1b = 0.077 nM  
 EC<sub>50</sub> GT-1a = 251 nM  
**42** (meta)  
 EC<sub>50</sub> GT-1b = 0.14 nM  
 EC<sub>50</sub> GT-1a = 3.5 nM



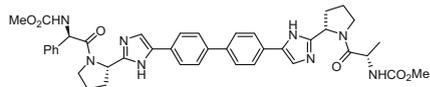
**43:** R = N(CH<sub>3</sub>)<sub>2</sub>  
 EC<sub>50</sub> GT-1b = 0.042 nM  
 EC<sub>50</sub> GT-1a = 0.038 nM  
**44:** R = NHCO<sub>2</sub>CH<sub>3</sub>  
 EC<sub>50</sub> GT-1b = 0.007 nM  
 EC<sub>50</sub> GT-1a = 0.028 nM



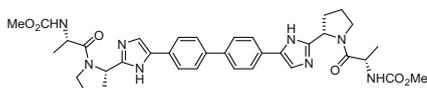
**45**  
 EC<sub>50</sub> GT-1b = 0.015 nM  
 EC<sub>50</sub> GT-1a = 138/ nM  
 F: rat (6.8%), mouse (17%), dog (45%)



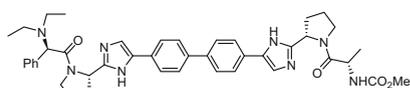
**46** (R,R)  
 EC<sub>50</sub> GT-1b = 1.22 nM  
 EC<sub>50</sub> GT-1a = 1240 nM  
**1** (S,S)  
 EC<sub>50</sub> GT-1b = 0.009 nM  
 EC<sub>50</sub> GT-1a = 0.050 nM



**47**  
 EC<sub>50</sub> GT-1b = 0.013 nM  
 EC<sub>50</sub> GT-1a = 0.112 nM



**48**  
 EC<sub>50</sub> GT-1b = 0.090 nM  
 EC<sub>50</sub> GT-1a = 0.282 nM



**49**  
 EC<sub>50</sub> GT-1b = 0.012 nM  
 EC<sub>50</sub> GT-1a = 0.036 nM

The antiviral profiles of **1** and **49** toward wild-type and hybrid replicons representing all of the genotypes and subtypes that were available at the time are summarized in Table 2 [1–4, 37–40]. Adding to confidence in the potential of **1** as it negotiated the development path toward clinical evaluation was the potent inhibition observed in a newly established GT-2a JFH-replicating virus assay. The EC<sub>50</sub> value of 28 pM in this assay exhibited a good correlation with the inhibitory potency toward the GT-2a JFH replicon [1–4].

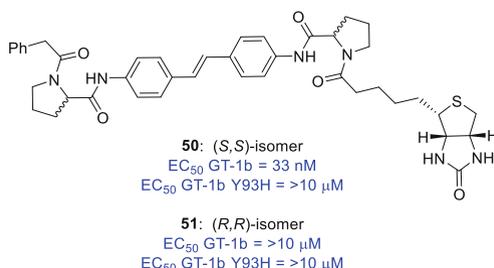
The pharmacokinetic parameters of **1** and **49** in rat, dog, and cynomolgus monkey are compiled in Table 3 and demonstrate good systemic exposure following oral administration, with the drug concentration measured at 24 h post-dose well in excess of the EC<sub>50</sub> values for the GT-1a and GT-1b replicons and the protein-binding-adjusted EC<sub>90</sub> value of 383 pM determined for the GT-1a replicon. More importantly, target organ exposure was also demonstrated, with liver levels of 103 nM measured in the rat 24 h following a 5 mg/kg dose of **1**, a concentration that was fivefold higher than that in plasma. The favorable absorption properties of **1** have been attributed, in part, to the formation of an intramolecular H-bond between the carbamate C=O and imidazole N-H moieties that enhances lipophilicity and reduces the apparent PSA of the molecule based on a chromatographic analysis and which is supported by modeling studies [41].

### 3 Mode of Action Studies with Daclatasvir

Despite its high potency and broad genotype inhibitory activity, the precise mode of inhibition of HCV replication by **1** remains as enigmatic as does the biochemical function of the NS5A protein [16, 18, 42–48]. HCV NS5A has no known enzymatic activity but is a critical element in the assembly and function of the replication complex on intracellular membranes and also in virion assembly [16, 42–48]. HCV NS5A is a 447-residue phosphoprotein that is comprised of three functional domains and an amphipathic helix at the amino terminus that associates with but does not traverse to biological membranes. Domain 1 contains a Zn<sup>2+</sup> binding motif and several serine residues that are sites of basal phosphorylation and hyperphosphorylation. The phosphorylation state of NS5A may modulate its function in virus replication and assembly with the hyperphosphorylated form, which can be produced by the action of the host cell lipid kinase, phosphatidylinositol 4-kinase, involved in the assembly of virions. Domain 2 has been shown to bind to the NS5B RNA-dependent RNA polymerase and has been associated with the sensitivity of the virus to interferon therapy although that function is controversial. While domain 3 appears to play a role in virus replication, it has most prominently been associated with virion assembly. The mapping of resistance mutations arising in response to selective pressure exerted by **1** and related analogues to domain 1 of NS5A is consistent with the effect on virus replication, but studies with infectious virus have demonstrated that **1** also interferes with the assembly of virions [42–50]. The

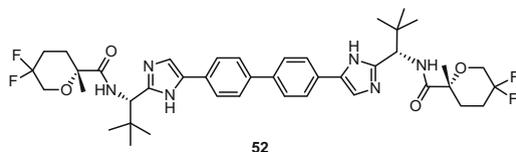
latter effect has been postulated to explain the rapid decline in viremia observed in HCV-infected patients administered clinically effective doses of **1** (vide infra) [3, 51]. In addition to associating with all of the viral nonstructural proteins, HCV NS5A has also been shown to bind to an extensive repertoire of host cell proteins that exceeds 130 entities [52–57]. As a consequence, the NS5A protein is typically viewed as a master regulator of virus replication and virion production, orchestrating both viral proteins and the host cell environment to ensure the successful production and release of progeny virus [42–48].

An association of NS5A inhibitors with the viral protein was originally demonstrated by studies with the biotin-labeled derivative **50** which is an effective inhibitor of GT-1b replication [3]. The antiviral activity of **50** is highly sensitive to the absolute configuration of the proline moiety since the (*R,R*)-isomer **51** is inactive, while inhibition is substantially reduced by the Tyr93His resistance mutation that arises in response to virus passaging in the presence of **1**. This SAR profile is strictly analogous to that established for the stilbene chemotype, and **50** was thus viewed as a useful tool molecule with which to probe drug-target binding interactions. In an initial experiment, GT-1b replicon lysate was incubated with **50**, and the mixture passed over streptavidin immobilized on beads; however, this experimental protocol failed to pull down any viral protein. In contrast, incubating replicon cells with **50** for 18 h before lysing and passing the lysate over streptavidin beads identified only NS5A as a binding partner, while a control experiment with inactive diastereomer **50** failed to isolate any viral proteins. These results indicate that **50** binds to HCV NS5A and that binding is dependent on the absolute configuration of the proline element, an observation concordant with the SARs developed in the stilbene-based series.



While the experiments conducted with **50** indicate that the binding of inhibitors to the NS5A is choreographically somewhat complex, the binding of inhibitors of NS5A to both domain 1 and the full-length viral protein was subsequently demonstrated in a series of independent biochemical experiments [58, 59]. These studies have suggested that the binding of inhibitors to NS5A interferes with the association of viral RNA with the protein, with the binding of compounds competed out by other NS5A inhibitors and demonstrating diminished affinity for the Tyr93His mutant protein [58, 59]. However, profiling of inhibitors in cell-based assays has indicated that disruption of RNA binding to NS5A does not appear to occur and that the introduction of key resistant mutations leads to only a modest reduction in the

binding of inhibitors [60, 61]. Studies with **50** in resistant GT-1b replicons indicated that while the Tyr93His-resistant mutation reduced inhibitory potency by 220-fold, an estimate of the amount of NS5A protein pulled down by the chemical probe, as determined by an analysis of Western blots, suggested similar levels of protein-drug association for the resistant and wild-type strains [60, 61]. Adding further to the complexity of the biochemistry was the observation that in a protein pull-down experiment, an attempt to outcompete the biotinylated tool compound **50** with a non-biotinylated analogue in a GT-1b Tyr93His-containing mutant ( $EC_{50} = 290$  nM for stated analogue vs  $>7$   $\mu$ M for **50**) not only failed but, at low concentration, appeared to have incrementally enhanced the amount of NS5A pulled down. From these data, it was inferred that the development of resistance to **1** does not lead to exclusion of binding to the NS5A protein, as is often observed with other classes of antiviral agents. Rather, these observations suggested a scenario in which HCV NS5A develops resistance by accommodating rather than expelling inhibitors, with the mutations presumably allowing restoration of protein function in the presence of the bound inhibitor. Consistent with this suggestion, several of the resistant mutations incorporate smaller or more flexible amino acid side chains, exemplified by Tyr93His, Tyr93Cys, Leu31Met, and Leu31Val, which may restore conformational flexibility compromised by the binding of inhibitors. These observations stimulated an experiment designed to evaluate the effect of combining **1** with structurally related compounds on the function of HCV NS5A incorporating resistance mutations. Two possible outcomes were contemplated: in the first scenario, a molecule would simply compete with bound **1** and the observed effect would be one of silence. However, the alternative scenario speculated on the potential of a second molecule to act in conjunction with **1** to restore inhibition by binding to an adjacent site on the NS5A molecule. A screen of compounds selected from the library of HCV NS5A inhibitors assessed in the presence of **1** using the Tyr93Asn GT-1a-resistant replicon, followed by SAR optimization, identified Syn-395 (**52**) as a molecule capable of restoring the sensitivity of resistant virus to the inhibitory effects of **1**. For example, in a typical experiment, **1** exhibited  $EC_{50}$  values of 33  $\mu$ M and 339 nM toward the wild type and Tyr93Asn mutant replicons, respectively, whereas **52** was poorly active in both replicons, with  $EC_{50}$  values of 214 and 215 nM, respectively. However, the  $EC_{50}$  of **1** toward the Tyr93Asn mutant replicon improved to 0.13 nM when titrated in the presence of a suboptimal concentration (40 nM) of **52**. This result represented a 2,600-fold increase in the sensitivity of the Tyr93Asn replicon to **1** in the presence of **52**. The synergistic relationship between **1** and **52** was confirmed in a reciprocal experiment where **52** was titrated in the presence of suboptimal amount of **1** affording a similar outcome [60, 61].



These observations, taken together with the absence of structural data that holistically captures the HCV NS5A drug-binding sites in the context of the membrane-

bound replication complex, confer considerable complexity on the nature of drug-target interactions, the mode of inhibition, and the function of the NS5A protein. This has presented a significant challenge to developing a more coherent and detailed understanding of the mechanism of action of HCV NS5A inhibitors and the modeling of drug-target interactions of these potent antiviral agents and the allosteric modulators [60–67]. While the bivalent nature of NS5A inhibitors, including the allosteric modulators represented by **52**, complements the dimeric form of the protein observed in solid-state structures of elements of domain I, the binding interfaces between the proteins vary [26–28, 60, 61]. One interpretation of this observation anticipates an oligomeric form of HCV NS5A in cells that can bind to the viral RNA and protect it from chemical and enzymatic degradation while providing a platform for RNA presentation to the polymerase and translocation to the developing virion [68–70]. However, the biochemical pharmacological effects of NS5A inhibitors are multifaceted and complex and include altering the subcellular distribution of NS5A, modulating the phosphorylation state of the protein, interfering with the formation of the membranous factories where virus replication occurs, and blocking the transfer of the viral genome to assembly proteins, leading to a clustering of HCV proteins at endoplasmic reticulum membranes [71–76].

## 4 Clinical Trials with Daclatasvir

The phase I clinical trial with **1** comprised of a randomized, double-blind, placebo-controlled, single ascending dose study involving administration of 1, 10, 25, 50, 100, and 200 mg of the drug to normal healthy volunteers (NHVs) [3]. A dose-proportional increase in plasma exposure was observed over the dosing range, and the concentration of **1** in plasma 24 h after dosing exceeded the protein-binding-adjusted EC<sub>90</sub> values of 49 pM (0.04 ng/mL) and 383 pM (0.28 ng/mL) recorded for the GT-1b and GT-1a replicons, respectively [3]. Compound **1** was quickly absorbed and exposure extended beyond 24 h, with plasma drug concentration maintained above the less sensitive GT-1a protein-binding-adjusted EC<sub>90</sub> value of 383 pM at 72 h for all but the 1 mg dose, predicting the potential for once-daily dosing [51]. The absolute oral bioavailability of **1** in humans is 67%, and the compound is ~99% bound to plasma proteins [77–80]. In this trial, **1** was safe and well tolerated at all of the administered doses with no clinically significant adverse effects observed, a profile that set the stage for a proof-of-concept study in HCV GT-1-infected subjects. Doses of 1, 10, and 100 mg were administered in a randomized, double-blind, placebo-controlled, single ascending dose format similar to that used for the NHV study, and plasma HCV RNA levels were monitored until 172 h post-dose. The results of this study are compiled in Table 4 with mean plasma HCV RNA declining by 1.8 log<sub>10</sub> IU/mL 24 h following the 1 mg dose, while the 10 and 100 mg doses provided increased efficacy, with viral load declines of 3.2 and 3.3 log<sub>10</sub> IU/mL, respectively, measured at 24 h. The mean maximal viral load reduction in the 100 mg dose cohort was 3.6 log<sub>10</sub> IU/mL with HCV RNA measured at 35 IU/mL in one of the GT-1b-infected subjects, while plasma RNA in another was below the

**Table 4** Dose, HCV genotype distribution, and plasma HCV RNA levels following administration of **1** to HCV-infected subjects

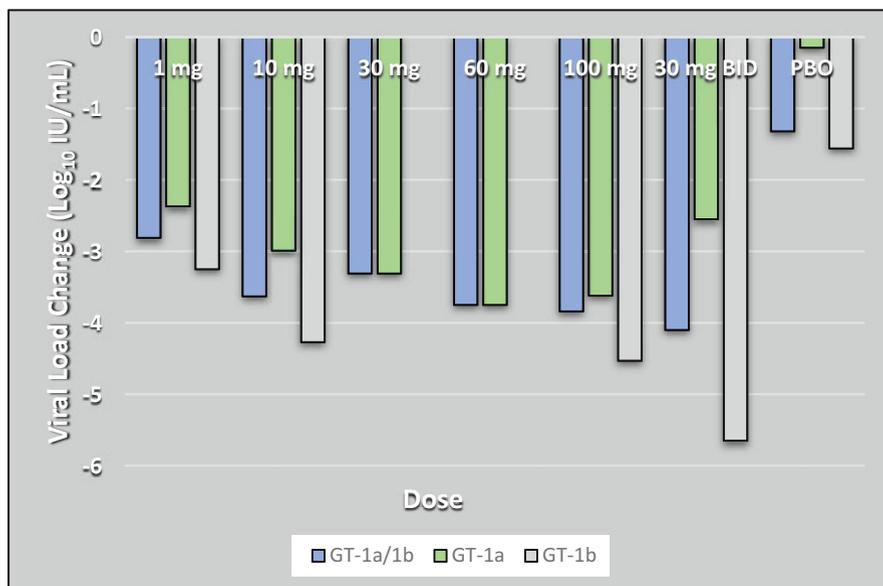
Dose of <b>1</b>	1 mg	10 mg	100 mg
GT-1a/1b	6/0	3/2	2/3 <sup>a</sup>
Mean viral load reduction at 24 h (range)	1.8 log <sub>10</sub> (0.2–3.0 log <sub>10</sub> ) IU/mL	3.2 log <sub>10</sub> (2.9–4.0 log <sub>10</sub> ) IU/mL	3.3 log <sub>10</sub> (2.7–3.6 log <sub>10</sub> ) IU/mL
Mean maximal reduction in viral load			3.6 log <sub>10</sub> (3.0–4.1 log <sub>10</sub> ) IU/mL

<sup>a</sup>One subject withdrew 8 h after dosing of **1**

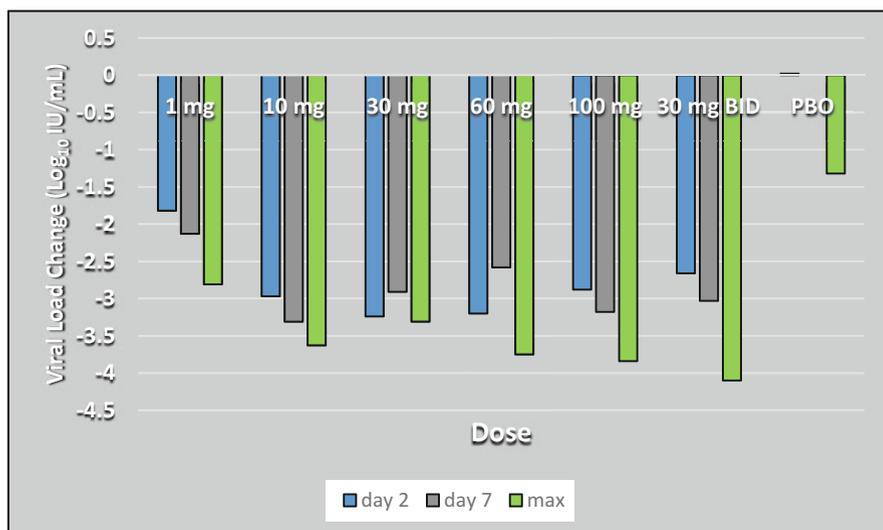
lower limit of quantification (25 IU/mL) at 144 h post-dose. The decline in plasma viral RNA concentration following administration of the 10 and 100 mg doses of **1** was both rapid and profound in nature, with a mean reduction of 1.95 log<sub>10</sub> IU/mL measured at 6 h post-dose for nine of the patients [49, 51]. The steep decline in viral load was subsequently explained after the development of a multiscale model of viral kinetics that took into account the effects of **1** on both viral replication and virus assembly and secretion, with the latter being the source of an immediate effect on virion production. The mean effectiveness of **1** on virus RNA production and virion assembly/secretion was estimated to be 99 and 99.8%, respectively, and yielded an estimate of plasma HCV half-life of 45 min, significantly shorter than the 2.7 h half-life estimated from an analysis of viral kinetics during treatment with older, interferon-based therapies [49].

The profile of **1** was further explored in a double-blind, placebo-controlled multiple ascending dose study in which the drug was administered for 14 consecutive days to GT-1-infected subjects at doses of 1, 10, 30, 60, and 100 mg once daily and 30 mg twice daily [51]. Each panel comprised of five patients randomized such that four received drug and one was administered a placebo control, with drug PK parameters determined on days 1 and 14. Median peak plasma concentrations of **1** occurred 1–2 h after dosing, and the PK profile was supportive of once-daily dosing with a mean terminal half-life of 12–15 h and steady state achieved after 3–4 days of drug administration. The mean maximal reduction in HCV RNA levels in plasma are compiled in Fig. 3 with 30 and 60 mg QD cohorts comprised solely of GT-1a-infected subjects. In the other dosing cohorts, those infected with GT-1b virus exhibited a greater response compared to those infected with GT-1a virus. However, most patients experienced viral rebound on or before day 7 of therapy with viral RNA levels below the mean maximal decline except in the 30 mg BID cohort (Fig. 4). Rebound was typically more rapid in the GT-1a-infected subjects which can be explained by a lower genetic barrier to resistance in this subtype [81–83]. In HCV GT-1a, only a single base pair change in the viral RNA is typically required to code for an alternative amino acid, while GT-1b frequently requires two base pair changes for coherent coding [81–83]. Population sequencing indicated the appearance of mutations at Met28, Gln30, Leu31, and Tyr93 all of which had been identified as resistance mutation hotspots in response to selective pressure by **1** in replicon studies in vitro [84, 85].

While the results of this trial further confirmed the potential of HCV NS5A as a therapeutic target, the rapid emergence of resistance to **1** anticipated that optimal

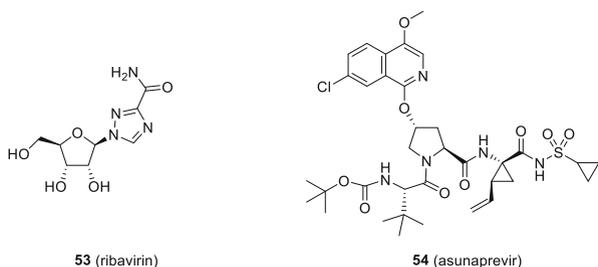


**Fig. 3** Mean maximum decrease in plasma viral RNA in HCV GT-1-infected subjects following dosing of **1** for 14 days



**Fig. 4** Mean viral load reductions on days 2 and 7 compared with the maximal viral RNA decline following dosing of **1**

clinical application would be as part of combination therapy [79, 81–91]. The combination of **1** as add-on therapy to the extant standard of care, pegylated IFN $\alpha$  and ribavirin (**53**), was explored clinically in patients infected with HCV genotypes 1–4 and a subset of patients who were co-infected with HIV-1. The results indicated that sustained virologic responses could be achieved with shorter 24-week durations of therapy with a burden of side effects comparable to that of pegylated IFN $\alpha$  and **53** [92–96]. However, it was the opportunity availed by the contemporaneous development of the HCV NS3 protease inhibitor asunaprevir (**54**) that allowed the pursuit of a parallel clinical program that had a significant impact on the course of the clinical development of HCV therapeutic agents [97–102]. In a small open-label clinical trial conducted in HCV GT-1-infected subjects who had no evidence of cirrhosis and who had previously failed to respond to peg-IFN/**53** therapy (referred to as null responders), a combination of **1** (60 mg QD PO) and **54** (600 mg BID PO) with peg-interferon  $\alpha$ 2a (180  $\mu$ g per week subcutaneously) and **53** (1,000 or 1,200 mg QD PO, depending on body weight) administered for 24 weeks was compared with a regimen comprised of only the two direct-acting antiviral agents (DAAs) [99, 102]. All of the ten patients receiving the quadruple drug regimen had undetectable levels of HCV RNA in plasma measured at 12 weeks following the last dose (SVR<sub>12</sub>), while nine also achieved SVR<sub>24</sub>. One patient in this group had detectable HCV RNA in plasma at week 24, but this was not quantifiable, and viral RNA was not detected in plasma 5 weeks later [99, 102]. Of the 11 patients receiving only the dual DAA combination, five had undetectable levels of HCV RNA in plasma at the end of therapy, and four maintained this status at weeks 4, 12, and 24 after the last drug dose. This cohort was comprised of nine subjects infected with GT-1a and two infected with GT-1b, with both GT-1b-infected patients achieving SVR<sub>24</sub>, while six patients infected with GT-1a virus experienced virological breakthrough while on therapy and the remaining patient infected with GT-1a virus relapsed after completing drug therapy. This study, which was conducted in a very challenging patient population, provided the first indication that a chronic HCV infection could be cured solely by treatment with DAAs in the absence of the immune stimulation provided by peg-interferon  $\alpha$ 2a and **53** [100].



The successful treatment of HCV GT-1b infections with **1** and **54** redirected the clinical development plan for this dual combination to Japan where GT-1b is the

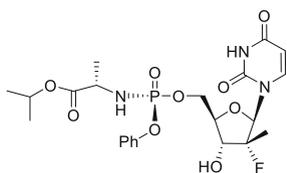
most prevalent, accounting for approximately 70% of the estimated two million infections at the time [103, 104]. The combination of **1** and **54** has been studied extensively in GT-1b-infected Japanese patients, leading to approval of the drug combination by the Japanese Pharmaceutical and Medical Devices Agency (PMDA) on July 4, 2014 [105–119]. The marketing authorization of **1** as Daklinza™ and **54** as Sunvepra® in Japan represented the first approval of a combination of DAAs for the treatment of HCV infection although the combination of sofosbuvir (**55**) and **53** had been approved by the FDA in December of 2013 [107]. The phase III Japanese clinical trials of **1** (60 mg QD) and **54** (100 mg BID) in GT-1b-infected subjects that subtended marketing approval evaluated 24 weeks of therapy and were associated with SVR<sub>12</sub> rates of 81% in non-responders and 87% in those intolerant of or ineligible for pegylated interferon therapy. In a multinational study conducted in a broad GT-1b patient population, the SVR<sub>12</sub> rates were 90% in those naïve to therapy and 82% in those intolerant of or ineligible for interferon-based therapies. The pre-existence of the Tyr93His polymorphism in the HCV NS5A gene was a predictor of lower clinical efficacy, with the SVR rates declining to 45 from 95% in those patients that harbored this mutation at baseline, which has a 15% prevalence in the Japanese patient population.

Broadening the utility of **1** and **54** to treat HCV GT-1a infections required the addition of a third agent, the allosteric RNA-dependent RNA polymerase inhibitor beclabuvir (**56**) which was developed as a fixed-dose combination comprising of 30 mg of **1**, 200 mg of **54**, and 75 mg of **56** administered as a BID regimen for 12 weeks [120–126]. In the UNITY 1 international study which was conducted in 415 non-cirrhotic patients with HCV GT-1 infection, 91% of the patients achieved SVR<sub>12</sub>. SVR<sub>12</sub> rates of 92% were observed in treatment-naïve patients and 89% in treatment-experienced patients, while virologic failure occurred in 8% of the patients. In the UNITY-2 phase III study conducted in the United States, Canada, France, and Australia in 202 GT-1-infected patients with compensated cirrhosis, the SVR<sub>12</sub> rates were 93% for patients in the treatment-naïve group and 87% for those in the treatment-experienced group. SVR<sub>12</sub> rates were improved to 98% for patients in the treatment-naïve group and 93% for those in the treatment-experienced group when **53** was included in the regimen. In a phase III trial (UNITY 3) conducted in 217 Japanese patients infected with GT-1 HCV, SVR<sub>12</sub> rates of ≥95% were achieved in both treatment-naïve ( $n = 152$ ) and interferon-experienced ( $n = 65$ ) cohorts after 12 weeks of therapy. The SVR<sub>12</sub> rates were similar across the patient subgroups evaluated that included patients with cirrhosis and those aged ≥65 years. These studies contributed to the approval of the fixed-dose combination of **1**, **54**, and **56** for marketing in Japan on December 20, 2016.

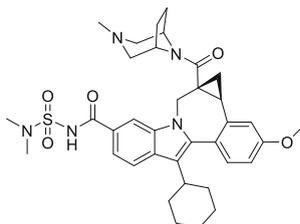
A number of clinical studies have demonstrated that co-dosing of **1** with the nucleoside-based NS5B RNA-dependent RNA polymerase inhibitor **55**, with and without **53**, achieves a high cure rate across HCV genotypes and patient population groups, including in those with comorbidities such as HIV-1 infection [127–129]. In a compassionate use program that reflected a real-world experience, including some subjects with advanced liver disease that would have been excluded from phase III studies, the combination of **1** and **55** (with and without **53**) demonstrated a high

efficacy [130]. In addition, long-term follow-up studies have demonstrated a 99% durability for the SVR<sub>12</sub> associated with various regimens that include **1** [131].

Daclatasvir (**1**) has been approved in more than 60 countries for use in combination with **54**, **56**, or other HCV inhibitors, including **55** [132]. A combination of all four of these agents has been evaluated in GT-1-infected patients as a drug intensification approach to shortening the duration of therapy to 4 or 6 weeks [133]. However, while the majority (96%) of patients experienced undetectable levels of HCV RNA at the end of therapy, relapse occurred in 77% of those treated for 4 weeks and 43% of those subject to 6 weeks of treatment with quadruple therapy [133].



**55** (sofosbuvir)



**56** (beclabuvir)

## 5 Conclusion

The NS5A replication complex inhibitor class of HCV inhibitor has become established as a critical component of all of approved pan-genotypic DAA combination therapies [132]. The discovery of **1**, the prototype NS5A inhibitor that is the founding member of the class, was identified only after considerable optimization of a lead discovered by phenotypic screening, a powerful approach to drug discovery that has proven to be well-suited as a means of identifying mechanistically novel antiviral agents [134–138].

### Compliance with Ethical Standards

**Conflict of Interest** The authors are employees of Bristol-Myers Squibb and own company stock.

**Ethical Approval** This article does not contain any studies with human participants or animals performed by any of the authors.

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