Top Med Chem (2019) 32: 3–26
DOI: 10.1007/7355_2018_45

Published online: 9 June 2019

NS5A as a Target for HCV Drug Discovery

Donald R. O'Boyle II and Min Gao

Contents

Abstract Discovery and development of HCV inhibitors is one of the most successful stories in the history of antiviral research. After more than 30 years of effort by academic and pharmaceutical researchers, HCV infection is a curable disease. In fact, HCV is the first chronic infectious disease to be cured with combinations of direct antiviral agents. Among these antiviral agents, NS5A inhibitors are the most potent. The unprecedented low pM potency, pan-genotype coverage, and well-tolerated clinical profile have made NS5A inhibitors an essential component of all interferon-/ribavirin-free regimens in currently approved HCV therapies. Since NS5A has no known enzymatic activity and is not a traditional antiviral target, this review focuses on the challenges and concerns that arose during the discovery of this class of inhibitor, the mode of action/inhibition, and the value of NS5A inhibitors in the treatment of HCV infection.

Keywords Combination therapy, Daclatasvir, Genotype coverage, NS5A inhibitors, Resistance, Synergistic effect

D. R. O'Boyle II
Bristol-Myers Squibb Company, Pennington, NJ, USA
e-mail: Donald.OBoyle@bms.com

M. Gao (✉)
Arbutus BioPharma, Warminster, PA, USA
e-mail: mgao@arbutusbio.com

1 General Properties of NS5A

The nonstructural protein 5A (NS5A) is a phosphoprotein required for HCV RNA replication and virion assembly in vitro and in vivo [1, 2]. NS5A from genotype 1 (GT1) is comprised of approximately 447 amino acids (aa) that can be divided into several distinct domains (Fig. 1). The first 33 residues of NS5A are highly conserved in all genotypes and form an amphipathic alpha helix [3] that is essential to modulate the association between NS5A and the endoplasmic reticulum membrane (ER) for recruitment to lipid droplets [4, 5]. The remaining NS5A monomer consists of three structural domains (I, II, and III) separated from each other by two inter-domain regions called low-complexity sequences (LCS) [6]. NS5A has the ability to bind RNA, preferentially the polypyrimidine tracks of 3′ untranslated regions (UTR) [7]. Although each domain is able to bind independently to 3′-UTR [6], the distinct genetically defined functions of these domains suggest that differential binding to other targets may enable NS5A to play diverse roles at different stages of the HCV life cycle (RNA replication and virion assembly).

Domain I (aa 34-213) is the most conserved region among the different HCV genotypes, is essential for replication, and has been crystalized as a dimer in different conformations (Fig. 2) [8–10]. The dimer is oriented to form a groove between two monomers. The groove has been suggested to be an RNA-binding site [8]; however, NS5A dimerization appears to occur via direct contacts between NS5A monomers and not via RNA [5]. Mutation analysis showed that the first LCS (Fig. 1) is important for NS5A dimerization [5]. The various dimeric forms of NS5A were found using different expression/purification and crystallization conditions (Fig. 2a–c); however, the differing NS5A crystals indicate the protein monomers can interact in multiple ways to form dimeric complexes (Fig. 2a–d). Since NS5A protein performs multiple functions in vivo, different NS5A functions may require different conformations to accommodate each role: protein-protein interactions (dimer formation, host protein interactions), protein-membrane interactions (with NS5A amphipathic helix), protein-nuclei acid interactions (RNA binding), and regulatory posttranslational modifications (serine phosphorylation) [5, 11, 12]. Additional NS5A conformational changes may occur prior to the release of the

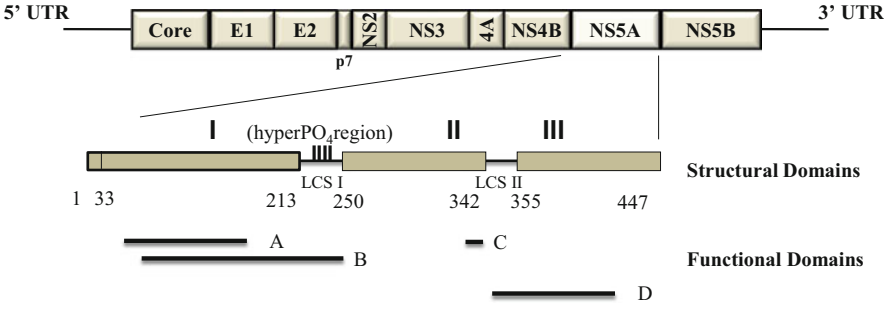

Fig. 1 Schematic drawing of HCV and NS5A. Structural and functional domains of NS5A

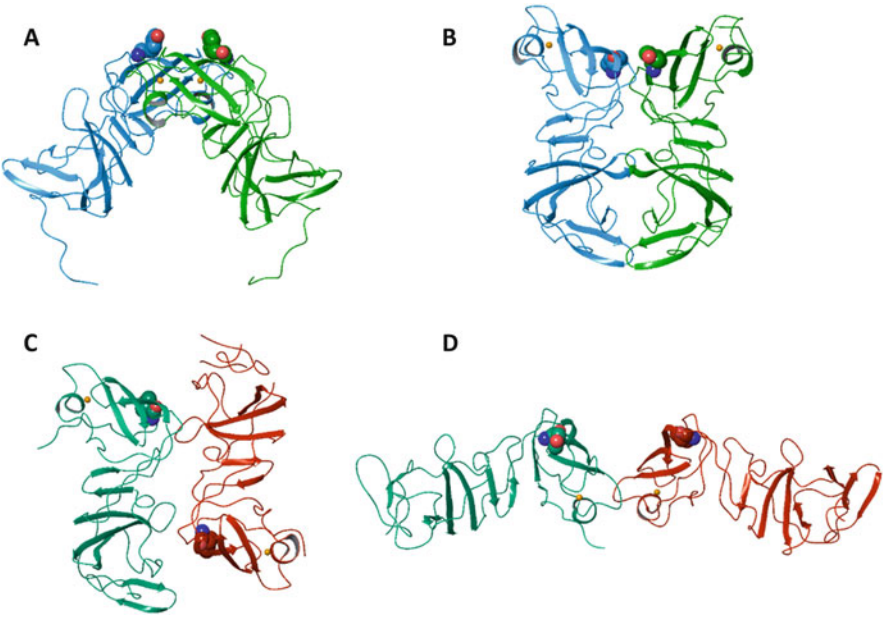

Fig. 2 The crystal structures for GT1b and 1a NS5A domain 1 dimers are displayed in ribbon representation. Tyrosine 93, a major resistant mutation, and Zinc (orange) are displayed as spheres. The GT1b monomers are blue and green, while the GT1a monomers are teal and red. (**a**) The first GT1b dimer structure [8] forms a potential RNA-binding pocket. (**b**) The 1b monomers in the second dimer [9] structure are in parallel to form an extensive interface. The first genotype GT1a NS5A domain 1 structure [10] contains two dimers. (**c**) The A and B monomers share the same interface as the dimer shown in (**b**); however, the monomers are antiparallel. (**d**) Monomers C and D form an extended N-terminal, head-to-head dimer

NS5A protein from the HCV poly-protein during replication complex formation. These conformational changes in NS5A are likely to be essential and represent multiple drug discovery targets that can be blocked via inhibitor binding.

Domain I also coordinates a single zinc (Zn++) atom per protein molecule. The coordination of the Zn++ by four NS5A cysteine residues (Cys^{39}, Cys^{57}, Cys^{59}, and Cys^{80}) [7, 8] suggests this is a structurally important metal ion required for NS5A folding and stability. Genetic data demonstrated that Zn++ binding is essential for multiple NS5A functions [7]. The coordination of Zn++ may also be important for the formation of higher-order structures such as oligomers/polymers. Domains II (aa 250–342) and III (aa 355–447) are less conserved among HCV genotypes than domain I [13] and natively unfolded [14, 15]. Domain II interacts with cyclophilin A (CypA), a cellular protein that stimulates RNA binding and is required for HCV replication [16]. This is consistent with the observation that CypA inhibitors such as cyclosporine (CsA) inhibit HCV replication [17]. Domain III is not required for HCV RNA replication but is essential for virion assembly [18, 19].

In addition to these structural domains, four functional domains (A, B, C, and D) of NS5A were mapped genetically using in vitro intragenic complementation experiments [20]. Domains A, B, and C have distinct roles in HCV RNA replication, while domain D is associated with virion assembly [18, 19].

NS5A has two phosphorylated forms, p56 and p58, that differ in electrophoretic mobility on SDS-PAGE. Basal phosphorylation of NS5A (p56) by host cellular protein kinases occurs at the center and near the C terminus (LCS II and domain III), whereas hyperphosphorylation of NS5A (p58) occurs in LCS I within a stretch of serine residues [21, 22] (Fig. 1). Ross-Thriepland and Harris [23] recently reviewed the cellular kinases involved in NS5A phosphorylation, the phosphorylated residues, and the functions of phosphorylation. The hyperphosphorylation of NS5A has a negative impact on HCV replicon replication in cell culture systems [21].

Adaptive mutations that greatly enhance RNA replication of GT1 HCV replicons [24] in vitro were identified by selection. Many of the mutations impact NS5A hyperphosphorylation: inhibition of p58 formation is associated with an increase in HCV RNA replication [11, 25–27]. The most effective adaptive mutation is S2204I, which impairs NS5A hyperphosphorylation. On the other hand, GT2a JFH-1 replicons replicate very well in cell culture without adaptive mutations [24]. To determine why adaptive mutations are needed for efficient replicon replication of genotypes other than GT2aJFH-1, a pooled lentivirus-based human cDNA library was screened. A single cellular protein, SEC14L2, was identified [25]. SEC14L2 promotes HCV infection by enhancing vitamin E-mediated protection against lipid peroxidation. It supports HCV replicon and infectious virus replication in cell culture without adaptive mutations. Observations that hyperphosphorylation is dependent on the presence of other nonstructural HCV proteins, such as NS4A [26, 28] and NS2 generated by NS2-3 auto-cleavage [29], or polyprotein consisting of N3-NS5A with active NS3 protease activity [30, 31] strongly suggest that a conformational change in NS5A may affect its hyperphosphorylation.

As a nonstructural protein without known enzymatic activity, NS5A relies on interactions with other viral and cellular proteins to exert multiple functions. These interactions do not occur simultaneously and must be temporally regulated to exert different essential roles during different stages of the HCV life cycle.

2 NS5A as a Target for HCV Drug Discovery

Based on clinical experience with HIV therapy, a combination regimen was predicted to be the most effective strategy for effective HCV treatments. Early on, it was noted that NS5A inhibitors possess several characteristics that make them attractive candidates as a component for combination therapy: (1) exceptional potency which drives a rapid initial viral RNA decline, (2) broad or pan-genotype coverage, and (3) mechanistically unique class with no cross resistance with other direct antiviral agents (DAA).

Traditional targets for antivirals are enzyme-based viral proteins, such as polymerase, protease, integrase, etc. In fact, more than half of the approved antiviral drugs are active site inhibitors represented by nucleos(t)ide analogs. Discovery of all new drugs requires the development of numerous in vitro assays (including binding, enzymatic, and cell-based) as well as co-crystallization with inhibitors and enzymes to insure the rational design of targeted inhibitors. Also, the data derived from in vitro assays are used routinely to predict the antiviral effects of inhibitors in the clinic. Since NS5A is not an enzyme target, development of assays enabling drug discovery was a challenge. The interactions of NS5A with many viral and cellular proteins could amplify the toxic effects as well as the antiviral effects. In addition, the host-cell environment affects the anti-HCV properties of NS5A inhibitors. Since the NS5A protein is a nontraditional antiviral target, sections that follow discuss the challenges and concerns that arose during the discovery of the first NS5A inhibitor daclatasvir (DCV, BMS-790052), the mode of action/inhibition, and the value of NS5A inhibitors in the treatment of HCV infection.

3 Discovery Challenges

The first NS5A inhibitor, BMS-858, was identified through an HCV replicon-based high-throughput screen of over 1 million compounds [32–34]. The path from BMS-858 to the discovery and development of DCV as a clinical candidate was littered with puzzles and questions. The astonishing in vitro potency of NS5A inhibitors was a puzzle that demanded focus on its mode of action (MOA). During the early stages of drug discovery, it was necessary to determine if the target of NS5A inhibitors was a cellular kinase or NS5A-kinase complex. Inhibition of p58 was associated with the activity of NS5A inhibitors (Fig. 3, left panel with compound BMS-529 [11, 34, 35]). The phenotype appeared to link with MOA since resistant NS5A lost sensitivity to p58 inhibition [34]. However, a similar phenotype was observed for NS3/NS4A protease inhibitors: the inhibition of NS5A p58 was lost with an NS3-resistant variant in the presence of a NS3 protease inhibitor [34]. These observations suggested that the inhibition of p58 is due to the NS3 protease inhibitor binding to its NS3 protease target and causing a conformational change of the kinase substrate, NS5A. Resistant variants selected with certain human kinase inhibitors also mapped to NS5A [36]. The caveat with these results is that under the selective pressure from a kinase inhibitor, it is easier to select resistance from a viral protein (the kinase substrate NS5A) than a cellular kinase itself. DCV-like molecules did not inhibit the activity of multiple kinases in vitro (Gao M, unpublished data).

The most convincing evidence that the target of DCV-like molecules is NS5A and not cellular kinase(s) was derived from the results of two experiments: (1) DCV inhibits p58 production of NS5A and replication of a JFH-1 replicon and virus without adaptive mutations [37] and (2) inhibition of p58 and HCV replicon activity can be separated (Fig. 3, right panel) [38]. Compound BMS-158 inhibited the HCV GT1b replicon with a median effective concentration (EC_{50}) of 0.5 nM; the resistant

	EC_{50} (nM)	
	WT	Resist (Y93H)
BMS-529	0.04	0.4
BMS-158	0.5	280

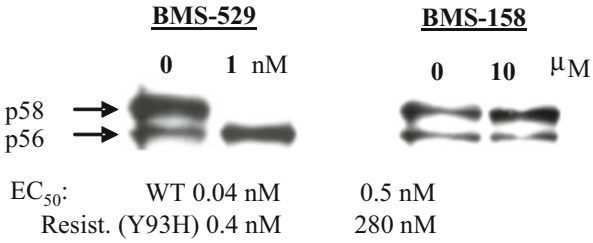

Fig. 3 Inhibition of HCV replicon replication and p58 production can be separated. EC_{50} values of compounds BMS-529 and BMS-158 were determined in HCV WT and Y93H replicons. Both compounds inhibited replicon through the same mechanism as shown by Y93H resistance. Western immunoblotting: GT1b plasmid was expressed in a vaccinia virus transient expression system treated with either DMSO (no compounds) or BMS-529 or BMS-158. Cell lysates were separated by SDS-PAGE, and NS5A proteins were identified by using an anti-NS5A antibody

variant NS5A Y93H is inhibited at EC_{50} of 280 nM. However, when NS5A without adaptive mutations was expressed from a vaccinia virus expression system compound, BMS-158 did not inhibit p58 (Fig. 3, right panel), in contrast to compound BMS-529 (left panel). This series of experiments convinced us that DCV did not inhibit kinase activity and enhanced our confidence that the target of DCV-like molecules is NS5A.

Drug discovery efforts also focused on whether inhibitors bind directly to NS5A. A biotinylated DCV-like molecule inhibited wild-type HCV replicon (EC_{50} of 33 nM) but was inactive toward the variant Y93H replicon ($EC_{50} > 10$ μM), whereas its diastereomer, used as a control, was inactive toward WT and Y93H ($EC_{50} > 10$ μM). NS5A was pulled down efficiently with the active inhibitor but not by the inactive diastereomer, suggesting selective binding to NS5A [35]. A different group reported a similar result [39]. Since the biotinylated DCV-like molecule binds WT NS5A and resistant NS5A with similar affinity, the correlation between specific inhibitor binding and antiviral activity was not firmly established using this approach. However, direct binding of NS5A inhibitors DCV and AZD7295 to bacterially expressed domain I with a Kd in the nM range has been reported [40]. Decreased binding affinity of these inhibitors to resistant variants L31V and Y93H confirmed specific binding and established a correlation between specific binding and anti-HCV effects. Interestingly, binding of these inhibitors does not affect NS5A dimerization, while RNA binding to NS5A inhibits inhibitor binding, suggesting that DCV-like molecules favor a dimeric structure of NS5A that does not

bind RNA [40]. Direct binding of ledipasvir (LDV) to a full-length NS5A containing a C-terminal His-tag produced from a baculovirus system was also reported [41]. Specificity was validated by (1) diminished binding of the resistant mutant NS5A (Y93H) to LDV and (2) competition of LDV binding to NS5A by DCV. Interestingly, LDV binding to NS5A was competed by DCV but not by the biotinylated compound BMS-671 [41], suggesting the binding mode of a monomer-like compound may be different from a dimer. These experiments establish the direct and specific binding of NS5A inhibitors to NS5A protein.

Phylogenetic analysis of nucleotide sequences identified at least six major genotypes [1 through 6] and many subtypes of HCV [42]. The highest priority for early HCV drug discovery was the development of an inhibitor with GT1a and 1b coverage, but the identification of an NS5A inhibitor with broad genotype coverage was the goal. The identification of NS5A inhibitors with broad genotype coverage required the development of many new research tools. When the first NS5A inhibitor was discovered, a GT1b replicon was the only genotype available. All the early structure-activity relationships (SAR) were established with the GT1b replicon [34, 43]. Although the major resistance residues of NS5A selected in GT1b replicon (L31 and Y93) are conserved in GT1a, none of the NS5A inhibitors discovered early (BMS-858, BMS-824, and BMS-346) were potent inhibitors of the GT1a replicon [34, 44]. The binding mode (dimeric vs. monomeric) and cap "structures" of the inhibitors were investigated to improve GT1a inhibition; however, the conservation of key resistance residues (L31 and Y93) provided the foundation for the design of potent inhibitors with broad or pan-genotype inhibition. Daclatasvir (DCV, BMS-790052) [35], a compound that preserves the symmetry present in BMS-346, inhibits most genotypes, and the second generation of NS5A inhibitors (velpatasvir (VEL), pibrentasvir (PIB), and elbasvir (ELB)) has significantly improved inhibition profiles for genotypes and resistance variants (Fig. 4 and Table 1).

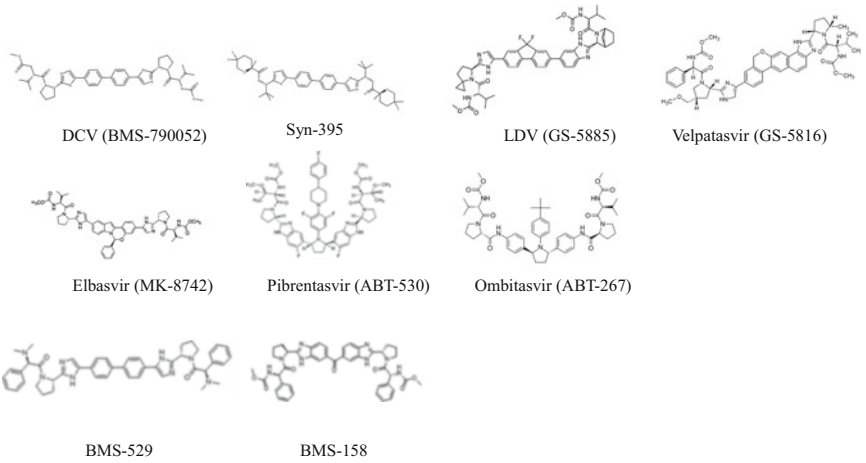

Fig. 4 Structures of different NS5A inhibitors used in this chapter

Table 1 In vitro replicon potency, genotype coverage, and resistance profiles of selected NS5A inhibitors[a]

Replicons (EC_{50}, nM)[a]	DCV (BMS-790052) [35, 45, 46]	LDV (GS-5885) [47, 48]	VEL (GS-5816) [49, 50]	EBR (MK-8742) [51, 52]	PIB (ABT-530) [53]	OMB(ABT-267) [54]
GT1a WT	0.05, *0.006*	0.031, *0.051*	0.031, *0.014*	0.004, *0.007*	0.002, *0.0007*	0.014, *0.003*
M28T	*4.1*	*1.8*	*0.11*	*0.11*	*0.0015*	*24.5*
M28V	*0.007*	*0.08*		*0.009*	*0.002*	*0.16*
Q30E	*150*	*44.1*	*0.25*		*0.002*	
Q30H	*8.7*	*5.3*	*0.032*	0.03	*0.0007*	*0.008*
Q30K	*146*		*0.15*			
Q30R	*7.3*	*12.4*	*0.031*	0.5	*0.001*	*2.2*
L31M	*2.1*	*14.6*	*0.22*	*0.07*	*0.0008*	*0.005*
L31V	*20*		*0.95*	0.5	*0.001*	
H58D	*3*	*32.7*	*0.10*	*0.04*	*0.0008*	*0.67*
Y93C	*11.1*	*48.7*	*0.052*	0.2	*0.001*	*4.6*
Y93H	*32*	*86.4*	*8.5*	2.4	*0.005*	*113*
Y93N	*282*	*>500*	*38.6*	*6.6*	*0.005*	*182*
Q30H-Y93H	553		*39.7*			
Q30R-Y93H	>1,000		*2.8*		*0.19*	
GT1b WT	0.009, *0.003*	0.004	0.015, *0.016*	0.003	0.004, *0.002*	0.005, *0.0008*
L31F	*0.013*			0.05		*0.008*
L31V	*0.061*		*0.037*	0.01		*0.007*
Y93H	*0.049*	*5.3*	*0.057*	0.05	0.001	0.06
L31F-Y93H	*14.9*					*8.1*
L31V-Y93H	*21.7*				0.002	*9.7*
GT2a (JFH)	0.071, *0.049*	21	*0.009*	0.003	0.005, 0.*001*	0.0008
GT2 (L31M)	6.9–13	*249*	*0.008–0.017*	3	*0.002*	0.012, *0.001*
T24A					*0.001*	*0.05*
F28S	>2,000				*0.001*	

GT3a	0.15, *0.25*	*168*	*0.004*	0.14	0.002, *0.0007*	0.019, *0.004*
M28T					*0.001*	*2.9*
A30K	*15.4*		*0.20*	7.0		
L31F	*80*			20		
Y93H	*688*		*2.9*	68	*0.002*	*30*
GT4a	0.012	0.39	*0.009*	0.003	0.002, *0.0008*	0.002, *0.0004*
L28V					*0.0009*	*0.008*
GT5a	0.033, 0.005	*0.15*	*0.059*		0.001, *0.0009*	0.003, *0.0009*
L28I	6.9				*0.001*	*0.072*
L31V	2.2				*0.0008*	*0.22*
L31F					*0.002*	*0.26*
GT6a	0.054, 0.05	1.1	0.007		0.003, *0.001*	0.37, *0.082*
L31M	43.9					
L31V					*0.001*	*0.56*

[a]EC_{50} values in italic were derived from transient replication assays

Cytotoxicity, an indicator of off-target activity, is generally easy to monitor and well separated from antiviral activity for enzyme targets such as polymerase and protease. For NS5A inhibitors, many different in vitro cell-based counterscreens (including a panel of DNA and RNA viruses and multiple cell lines derived from a variety of origins) as well as in vivo preclinical animal toxicity studies were used to evaluate off-target activity [34, 35]. The unprecedented potency and specificity of NS5A inhibitors yielded remarkable in vitro therapeutic indices (CC_{50}/EC_{50}) and in vivo safety margins. Indeed, clinical data have shown that NS5A inhibitors are well tolerated with a favorable adverse effect profile and low potential for drug-drug interactions [55, 56].

4 Anti-HCV Effects In Vitro and In Vivo

All NS5A inhibitors have been identified and evaluated with in vitro replicon or infectious virus assays. This class of inhibitors has produced the most potent antiviral agents reported to date, with EC_{50} values in the low pM range for all HCV genotypes (Table 1). DCV, the first NS5A inhibitor to enter the clinic, has broad genotype coverage with low pM potency in in vitro replicons, except for the GT2a variant containing the NS5A substitution L31M (EC_{50} 6.9–13 nM, Table 1). Analysis of baseline sequences of GT2a NS5A from >400 clinical specimens indicated that the most prevalent NS5A polymorphism associated with resistance is L31M (88%) [57]. Therefore, GT2a NS5A L31M can be considered "wild type (WT)" for NS5A inhibitor drug discovery. Indeed, the second-generation NS5A inhibitors, represented by VEL, PIB, and OMB, have true pan-genotype coverage with $EC_{50} < 0.5$ nM against all genotypes including GT2a expressing the NS5A L31M variant (Table 1). In addition to potency, the kinetics of antiviral suppression by NS5A inhibitors was found to vary based on the genotype- or strain-specific stability or half-life of the functional HCV replicase complex [58, 59]. This also modulates the effectiveness of NS5A inhibitors.

The in vitro replicon potency of NS5A inhibitors appears to correlate well with the initial HCV decline observed in infected patients treated with NS5A inhibitors. From a virology point of view, the antiviral effect of a specific inhibitor is determined mainly by two factors: intrinsic potency and resistance barrier. Because of the exceptional potency of NS5A inhibitors, patients generally experienced an initial sharp HCV RNA decline, indicative of the inhibition of wild-type virus. For example, the EC_{50} values of DCV for GT1a and GT1b replicons are 0.050 nM and 0.004 nM, respectively. The difference observed in GT1a and GT1b replicon potency was mirrored in a 14-day multiple ascending dose study, where the mean maximal decline in viral load was 3.6 $\log_{10}$ for GT1a-infected patients and 4.5 $\log_{10}$ for GT1b-infected patients who received 100 mg DCV once a day (QD) (Table 2). Rapid and sharp declines in HCV at early treatment time points in patients receiving NS5A inhibitors is another characteristic of this class of inhibitors. A 2 $\log_{10}$ viral RNA decline was reached 4 h after the first 60 mg dose of DCV [45]. This marked

Table 2 HCV RNA decline observed in monotherapies with selected NS5A inhibitors

Inhibitor	Dose	# of patients	Duration (days)	Max. viral decline				
				GT1a	GT1b	GT2	GT3	GT4
DCV (BMS-790052) [45]				Mean (log_{10})				
	60 mg QD	4 for GT1a	14	3.8				
	100 mg, QD	3/1 for GT1a/1b	14	3.6	4.5			
	30 mg, BID	2/2 for GT1a/1b	14	2.6	5.7			
LDV (GS-5885) [60]				Median (log_{10})				
	10 mg, QD	10/10 for GT1a/1b	3	3.2	3.3			
	90 mg, QD	10 for GT1a	3	3.1				
VEL (GS-5816) [61]								
	50 mg, QD	8/4 for GT1a/3	3	3.6			2.6	
	100 mg QD	8 for GT1a	3	3.6				
	150 mg, QD	7/8/8/6/2 for GT1a/1b/2/3/4	3	4.0	4.0	4.4	3.3	3.5
EBR (MK-8742) [62]				Mean (log_{10})				
	50 mg QD	5/5/5 for GT1a/1b/3	5	4.2	5.1		3.1	
	100 mg, QD	5 for GT3	5				3.4	
FIB (ABT-530) [63]				Mean (log_{10})				
	40 mg, QD	8 for GT1	3	4.1				
	120 mg QD	8 for GT1	3	4.5				
OMB (ABT-267) [64]				Mean (log_{10})				
	5 mg, QD	4 for GT1	3	2.9				
	50 mg, QD	4 for GT1	3	2.8				

Table 3 SVR12 for FDA approved combination therapies containing selected second-generation NS5A inhibitors

Combination	Non-NS5A inhibitor in the combination	Usage	Genotypes	Duration (weeks)
Harvoni (LDV/SOF) [65, 66]	SOF: nucleotide analog	90/400 mg, QD	1, 4–6	8–24
Epclusa (VEL/SOF) [67]		100/400 mg	1–6	12
Vosevi (VEL/SOF/VOX) [68]	VOX: NS3/4A protease inhibitor	100/400/ 100 mg	1–6	12
Zepatier (EIB/GRA) [69]	GRA: NS3/4A protease inhibitor	50/100 mg	1, 4	12–16
Mavyret (PIB/GLE) [70]	GLE: NS3/4A protease inhibitor	40/100 mg	1–6	12

and robust antiviral effect suggested the use of NS5A inhibitors could shorten treatment duration significantly, making therapy more tolerable. The first- and second-generation NS5A inhibitors (Table 2) have strong antiviral effects in monotherapy trials in different genotypes tested, confirming that the exceptional in vitro potency of NS5A inhibitors translated to in vivo efficacy.

NS5A inhibitors are the most potent antiviral agents developed to date and are components of all interferon/ribavirin-free regimens in currently approved HCV therapy (Table 3). FDA approved Harvoni (SOF-nucleotide/LDV-NS5A) as a once-daily single-tablet regimen to treat HCV in adults in 2014 and in children in 2017 [65, 66]. Subsequently, NS5A inhibitors were combined successfully with other DAAs, such as NS3 protease inhibitors. The treatments offer excellent efficacy and safety profiles, especially treatments containing the second generation of NS5A inhibitors (Table 3). The FDA approved Epclusa in 2016 and Vosevi and Mavyret in 2017 as fixed-dose combinations for treatment of HCV GT1–6 [67, 68, 70]. The "one-pill for all" regimen greatly simplified therapy by precluding the need to screen genotypes prior to treatment. The overall cure rates have reached $\geq$92% with 8–16 weeks of treatment for all genotypes (Table 3). With certain patient populations, the cure rate is almost 100% [71, 72].

5 Resistance

Infection with HCV results in a highly heterogeneous virus population, a consequence of its rapid replication turnover rate (~10^{12} virions/day) and the lack of a proofreading function in the NS5B polymerase. Therefore, mutations at every position of the HCV genome are possible, and variants resistant to individual DAAs are predicted to preexist at baseline (BL) in infected subjects.

In addition to intrinsic potency, the resistance barrier of an inhibitor determines its antiviral effect. A slow second phase of viral decline or a slight viral rebound was observed at later time points during the 14-day monotherapy study of DCV [45, 46]. This observation was consistent with an accumulation of resistant variants and suggested that the adaptation or selection of resistant variants enhanced their fitness. The emergence of resistance suggests that DCV, like NS3 protease inhibitors and NS5B polymerase allosteric inhibitors, may have a low genetic barrier to resistance. A single-nucleotide change (UAU or UAC to AAU or AAC) at residue 93 (Tyr to Asn) of GT1a NS5A is sufficient for HCV to acquire clinical resistance to DCV. Furthermore, the accumulation or acquisition of additional mutations generates linked substitutions such as Q30D/H/L/R-Y93C/H/N that confer higher levels of resistance [46, 47, 49, 52, 54].

All amino acid (aa) substitutions associated with resistance to this class of inhibitors have been mapped to the N-terminal 100 residues for all genotypes (Table 1). The most prevalent resistant substitutions for GT1–6 are shown in Fig. 5 [45–54, 60–64, 73–76]. Observation of the same substitutions in vitro and in vivo confirms the utility of the replicon system for assessing resistance in response to treatment with NS5A inhibitors. In general, GT1a variants conferred higher levels of resistance than GT1b variants, possibly explaining why viral breakthrough was more common among patients with GT1a. Some single amino acid substitutions confer low-to-moderate levels of resistance (Q30H: 1,450- and

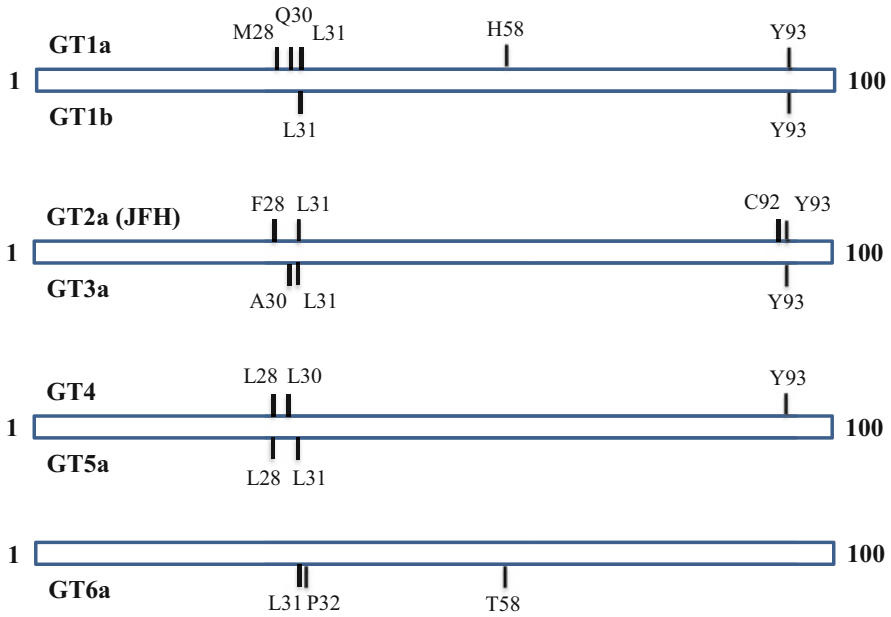

Fig. 5 Major NS5A resistance-associated substitutions observed in GT1-6. Most, if not all, substitutions are mapped to the first 100 aa of NS5A

2.3-fold; Y93H 5,333- and 607-fold resistance to DCV and VEL, respectively), but linked substitutions such as Q30H-Y93H confer higher levels of resistance (92,167- and 2,836-fold resistance to DCV and VEL, respectively; Table 1). However, the second-generation NS5A inhibitor, pibrentasvir (PIB, ABT-530), has not only a pan-genotype coverage profile but also a high barrier to resistance in vitro (Table 1). For example, the GT1a Y93N variant has high levels of resistance to DCV, LDV, OMB, and VEL (EC_{50} values of 282, >500, 182, and 38.6 nM, respectively) but is still very sensitive to PIB (EC_{50} of 0.005 nM). In fact, EC_{50} values of PIB for all tested variants from GT1–GT6 are less than 1 nM, including GT1a Q30R-Y93H variant with linked substitutions (Table 1).

Resistance of variants to different NS5A inhibitors varies significantly, mainly determined by inhibitory pressure, fitness of the variants, and genetic background of HCV before treatment. The inhibitory pressure of inhibitors and the fitness of variants are relatively easy to measure, while monitoring the genetic background of HCV replicons and patient specimens before treatment, especially minor variants, can be a challenge. A correlation between the influence of naturally occurring polymorphisms on DCV activity in vitro and in vivo has been observed [77]. A Q30R variant with a low level of in vitro resistance to DCV (EC_{50} ~7 nM) was observed at viral breakthrough in a GT1a-infected patient. Because the level of DCV observed in the plasma of the patient was high (Ctrough $\geq$117 nM), a rigorous investigation was initiated to determine the basis for resistance. A baseline polymorphism (E62D) found in this patient did not show resistance to DCV when it was introduced into a GT1a replicon; but the linked variant, Q30R-E62D, conferred high-level resistance in vitro (EC_{50} = 153 nM) and is likely to be responsible for viral breakthrough in vivo. These data showed that a BL polymorphism with minimal impact on the anti-HCV effect of DCV could enhance the emergence of resistance and significantly affect clinical outcome. Further support was obtained by evaluating hybrid replicons in which the entire NS5A coding region of GTla was replaced with the corresponding region of specimens collected from the infected patient. This work established a clear, systematic approach to monitor resistance to NS5A inhibitors in the clinic.

Although NS5A inhibitors have a relatively low resistance barrier compared to *sofosbuvir*, the resistance barrier becomes less important with combination treatment/therapy. Effective control of HIV infection/resistance using combination therapies provided a clear path for the development of HCV inhibitors. From the beginning of HCV drug discovery, development of combination therapies was the goal for an HCV cure. To be an effective combination therapy, individual inhibitors should (1) target different viral proteins or different stages of the viral life cycle, (2) have no detectable overlapping toxicity in preclinical animal studies, and (3) have minimal or no drug-drug interactions. To identify effective combination treatments that included DCV, in vitro combination studies were performed. As shown in Fig. 6, numerous resistant colonies were observed (Fig. 6a, b) when HCV replicon cultures were treated with a single agent, DCV, ASV (asunaprevir, NS3 protease inhibitor), or BCV (beclabuvir, NS5B non-nucleoside polymerase inhibitor), at a concentration 30-fold above the inhibitor EC_{50} [78]. Dual

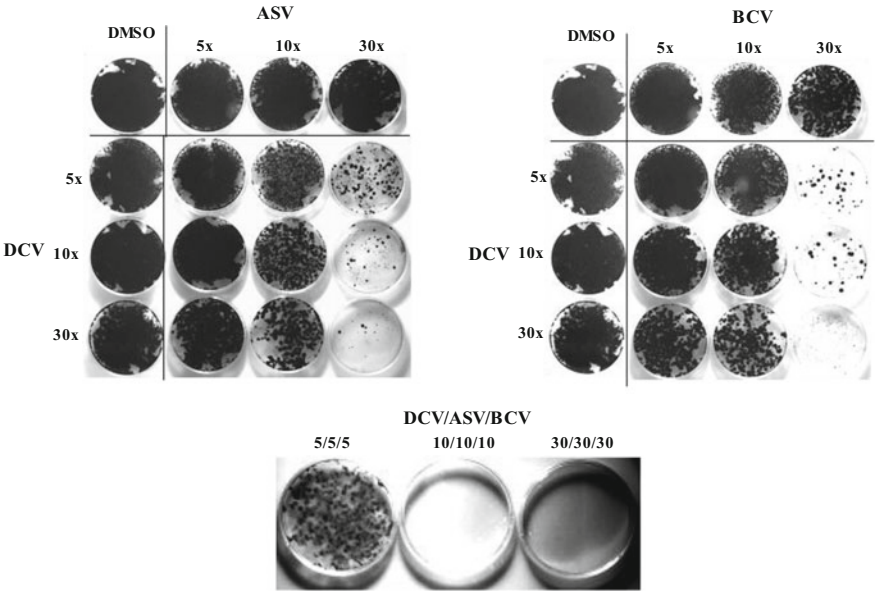

Fig. 6 Combination treatment reduces the emergence of resistant colonies. GT1b HCV replicon cells were incubated for 4 weeks with BMS-790052 (DCV), BMS-791325 (BCV), or BMS-650032 (ASV) as monotherapy and dual therapy (top left and right) and triple therapy (bottom) at 5×, 10×, and 30× EC_{50}. Colonies were visualized by crystal violet staining. Data shown are representative of the results of three independent experiments

combinations, DCV + ASV or DCV + BCV (concentrations 15-fold above each inhibitor EC_{50}), reduced the number of resistant colonies compared to the single agents. A triple combination of DCV + ASV + BCV at concentrations tenfold above each inhibitor EC_{50} eliminated HCV replicon (Fig. 6c). The power of combination therapy for curing HCV has been confirmed in clinical studies (Table 3).

6 Mode of Action

The mode of action (MOA) of NS5A inhibitors is not fully understood; however, several models have been proposed based on experimental results and mathematical modeling. The models provide insights into how these inhibitors affect the biologic functions of NS5A and HCV.

The pM potency of NS5A inhibitors in vitro translated to remarkable initial viral decline in vivo, suggesting the MOA of NS5A inhibitors could be related to the roles of NS5A during the HCV life cycle. Clinical data indicated that the initial viral decline observed with NS5A inhibitor treatment was faster than any other antiviral agents reported. This led to mathematical modeling that predicted DCV efficiently

blocks two distinct stages of the viral life cycle, RNA synthesis and virion assembly/secretion, with mean effectiveness of 99% and 98%, respectively. The model also yielded a more precise estimate of 45 min for the HCV serum half-life [79–81]. Experiments done in vitro with infected cells corroborated a prediction based on the model: intracellular HCV RNA had a similar pattern of decline when cells were treated with either DCV or an HCV polymerase inhibitor (NM107), but only DCV treatment yielded a rapid initial decline of extracellular viral titers compared to a delayed and slow decline with NM107 [80]. A second in vitro study confirmed these results [80]. In this study, the kinetics of antiviral suppression by NS5A inhibitors were compared to protease inhibitors and NS5B polymerase inhibitors. Despite their potency, NS5A inhibitors were slow to inhibit HCV RNA synthesis, compared to the protease and polymerase inhibitors. However, NS5A inhibitors rapidly depleted intracellular infectious virus and RNA-containing HCV particles, indicating the inhibition of intracellular virion assembly. Inhibition of virion assembly has not been confirmed by resistance analysis; no NS5A-resistant variants associated with virion assembly have been reported. In addition, the inhibition of different stages of the HCV life cycle (RNA replication and virion assembly) does not explain the potency of NS5A inhibitors since HCV virion assembly is not present in the replicon system in cell culture.

HCV replication in replicon cells is inhibited by a ratio of DCV to NS5A estimated to be approximately 1 to 47,000. This ratio suggests that a small number of inhibitor molecules impact the function of a large number of NS5A protein molecules, and it may be related to the potency of NS5A inhibitors [82–85]. Based on the crystal structures of NS5A protein dimers and the structural analysis of NS5A inhibitor and NS5A protein co-crystals, it has been proposed that NS5A forms polymers and/or oligomers requiring only small amounts of NS5A inhibitors to affect the higher-order forms [8, 9, 41].

Biophysical methods were used to observe the intrinsic self-association of NS5A domain 1 (GT1a and GT1b) which existed as a heterogeneous mixture in solution and exhibited dynamic equilibria between monomers and higher-order structures [85]. The formation of large and irreversible protein aggregates was induced by DCV [85]. NS5A inhibitor binding to a variety of NS5A species inside cells (monomers, dimers, and multiples of NS5A dimers) was observed using NS5A compounds containing cross-linking functionalities (azide, bis-azide) [86]. NS5A inhibitor binding to HCV processing intermediates was observed using elution studies performed with the biotin affinity compound BMS-671 [86]. Release of NS5A proteins from BMS-671 required detergent with heating. The harsh elution conditions suggest BMS-671 is "wrapped up" by the NS5A proteins during the folding process [10, 86]. Lending support to this hypothesis are (1) the targeted disruption of only new HCV membranous-web replication centers by NS5A inhibitors, with little to no effect on existing replication centers, and (2) the detection of NS5A inhibitors associated with monomeric NS5A during SDS-PAGE [39, 86].

A series of experiments exploring the possibility that a single inhibitor may inhibit the function(s) of NS5A oligomers revealed a novel synergy mechanism between specific pairs of NS5A compounds of similar structure (Fig. 7) [84, 87].

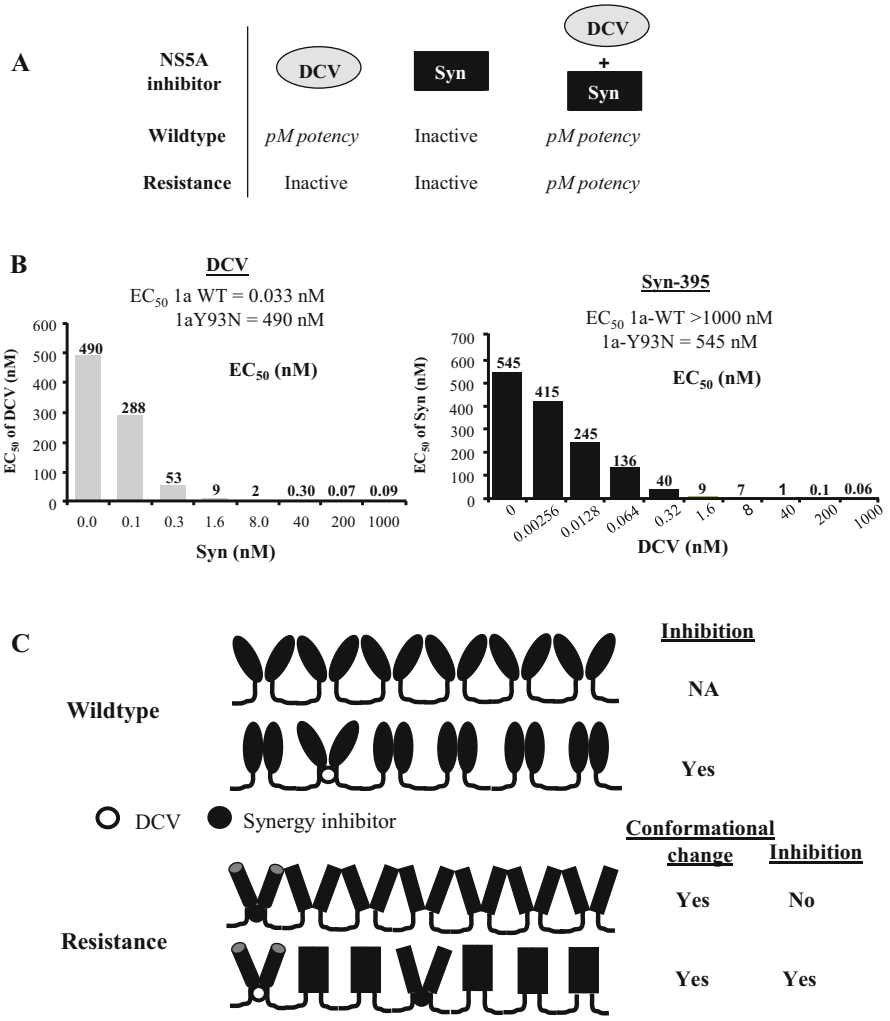

Fig. 7 Synergistic anti-HCV effect of NS5A compounds. (**a**) Cartoon representation of the synergistic effect of a pair of NS5A compounds, DCV and Syn-395. (**b**) Synergistic effect in a GT1a Y93N replicon. Left panel, EC_{50} values of DCV were determined in the absence or presence of different concentrations of Syn-395; right panel, EC_{50} values of Syn-395 were determined in the absence or presence of different concentrations of DCV. (**c**) Working model of the synergistic effect of a pair of NS5A compounds. NS5A molecules form oligomers in the replication complex and communicate with each other. For WT NS5A, DCV binds to NS5A, affecting multiple NS5As and disrupting the function(s) of the entire oligomer. Although DCV can bind resistant NS5A, it does not disrupt NS5A function(s); however, DCV binding causes a conformational change that accommodates the binding of the second inhibitor on adjacent NS5A molecules and disrupts function(s) of the oligomer

DCV exhibits an EC50 of 0.033 nM against a WT GT1a replicon, but it has no apparent activity against some NS5A-resistant variants such as Y93N ($EC_{50} > 100$ nM, Fig. 7b). A structurally related compound, referred to as a synergist (Syn-395, Fig. 7a), is inactive against both WT and resistant variants ($EC_{50} > 100$ nM). However, the combination of DCV and Syn-395 has remarkably potent inhibitory activity, with EC_{50} values in the pM range against DCV-resistant variants as well as the WT replicon (Fig. 7a). Specifically, the presence of Syn-395 enhances the potency of DCV approximately 2,600-fold against the Y93N variant, from 339 nM in the absence of Syn-395 to 0.13 nM in the presence of 40 nM Syn-395 (Fig. 7b, left panel). A similar synergistic effect was observed in a reciprocal experiment: Syn-395 is inactive against both WT and resistant variants ($EC_{50} > 500$ nM). The presence of DCV enhances the potency of Syn-395 against the Y93N variant from 545 nM in the absence of DCV to 1 nM in the presence of 40 nM DCV (Fig. 7b, right panel). This result illustrates a cooperative interaction between DCV and Syn-395 inhibiting HCV replication in the presence NS5A protein carrying a resistance substitution. This synergistic effect of a pair of NS5A inhibitors observed in the GT1a replicon is conserved among different HCV genotypes [81, 87]. The synergistic inhibitory effect of specific pairs of NS5A compounds, such as DCV and Syn-395, suggests that the conformational changes in NS5A protein induced by DCV only accommodate the binding of synergists of specific structure. The results indicate that Syn-395 binds to both WT- and DCV-resistant NS5A, but the binding of Syn-395 is not able to inhibit virus replication. However, Syn-395 binding, either adjacent to or a few NS5A dimers to either side of DCV, potentiates the effect of DCV by introducing a conformational change that resensitizes the resistant NS5A toward DCV inhibition (Fig. 7c). These experiments clearly demonstrated the functional communication between NS5A molecules during HCV replication.

7 Conclusion

A new class of anti-HCV inhibitor targeting the NS5A protein has become an essential component of combination therapies for eradicating this chronic viral disease. Lessons learned during the discovery of the class illustrate the general challenges of developing drugs for novel targets. Understanding the mode of inhibition of HCV NS5A inhibitors may illuminate specific opportunities to discover drugs with a similar mechanism for diseases other than HCV.

Acknowledgments We thank Drs David Langley for providing Fig. 1 and Susan Roberts for critical review and editing the manuscript.

Compliance with Ethical Standards

Conflict of Interest: Dr. Gao worked for Bristol Myers Squibb.

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

References

1. Macdonald A, Harris M (2004) Hepatitis C virus NS5A: tales of a promiscuous protein. J Gen Virol 85:2485–2502
2. He Y, Staschke KA, Tan SL (2006) HCV NS5A: a multifunctional regulator of cellular pathways and virus replication. In: Tan SL (ed) Hepatitis C virus genome and molecular biology. Horizon Bioscience, Norfolk, pp 267–292 Chapter 9
3. Brass V, Bieck E, Montserret R, Wolk B, Hellings JA, Blum HE, Penin F, Moradpour D (2002) An amino-terminal amphipathic a-helix mediates membrane association of the hepatitis C virus nonstructural protein 5A. J Biol Chem 277:8130–8139
4. Penin F, Brass V, Appel N et al (2004) Structure and function of the membrane anchor domain of hepatitis C virus nonstructural protein 5A. J Biol Chem 279(39):40835–40843
5. Lim PJ, Chatterji U, Cordek D et al (2012) Correlation between NS5A dimerization and hepatitis C virus replication. J Biol Chem 287:30861–30873
6. Foster TL, Belyaeva T, Stonehouse NJ, Pearson AR, Harris M (2010) All three domains of the hepatitis C virus nonstructural NS5A protein contribute to RNA binding. J Virol 84:9267–9277
7. Tellinghuisen TL, Marcotrigiano J, Gorbalenya AE, Rice CM (2004) The NS5A protein of hepatitis C virus is a zinc metalloprotein. J Biol Chem 279:48576–48587
8. Tellinghuisen TL, Marcotriqiano J, Rice CM (2005) Structure of the zinc-binding domain of an essential component of the hepatitis C virus replicase. Nature 435:374–379
9. Love RA, Brodsky O, Hickey MJ, Wells PA, Cronin CN (2009) Crystal structure of a novel Dimeric form of NS5A domain I protein from hepatitis C virus. J Virol 83:4395–4403
10. Lambert SM, Langley DR, Garnett JA, Angell R, Hedgethorne K, Meanwell NA, Matthews SJ (2014) The crystal structure of NS5A domain 1 from genotype 1a reveals new clues to the mechanism of action for dimeric HCV inhibitors. Protein Sci 23:723–734
11. Blight KJ, Kolykhalov AA, Rice CM (2000) Efficient initiation of HCV RNA replication in cell culture. Science 290:1972–1974
12. Lohmann V, Korner F, Dobierzewska A, Bartenschlager R (2001) Mutations in hepatitis C virus RNAs conferring cell culture adaptation. J Virol 75:1437–1449
13. Tellinghuisen TL, Foss KL, Treadaway JC, Rice CM (2008) Identification of residues required for RNA replication in domains II and III of the hepatitis C virus NS5A protein. J Virol 82:1073–1083
14. Liang Y, Ye H, Kang CB, Yoon HS (2007) Domain 2 of nonstructural protein 5A (NS5A) of hepatitis C virus is natively unfolded. Biochemistry 46:11550–11558
15. Hanoulle X, Verdeqem D, Badillo A, Wieruszeski JM, Penin F, Lippens G (2009) Domain 3 of non-structural protein 5A from hepatitis C virus is natively unfolded. Biochem Biophys Res Commun 381:634–638
16. Foster TL, Gallay P, Stonehouse NJ, Harris M (2011) Cyclophilin A interacts with domain II of hepatitis C virus NS5A and stimulates RNA binding in an isomerase-dependent manner. J Virol 85:7460–7464
17. Fischer G, Gallay P, Hopkins S (2010) Cyclophilin inhibitors for the treatment of HCV infection. Curr Opin Investig Drugs 11:911–918
18. Tellinghuisen TL, Foss KL, Treadaway JC (2008) Regulation of hepatitis C virion production via phosphorylation of the NS5A protein. PLoS Pathog 4:e1000032

19. Appel N, Zayas M, Miller S, Krijnse-Locker J, Schaller T, Friebe P, Kallis S, Engel U, Bartenschlager R (2008) Essential role of domain III of nonstructural protein 5A for hepatitis C virus infectious particle assembly. PLoS Pathog 4:e1000035
20. Fridell RA, Valera L, Qiu D, Kirk MJ, Wang C, Gao M (2013) Intragenic complementation of hepatitis C virus NS5A replication-defective alleles. J Virol 87:2320–2329
21. Evans MJ, Rice CM, Goff SP (2004) Phosphorylation of hepatitis C virus nonstructural protein 5A modulates its protein interactions and viral RNA replication. Proc Natl Acad Sci U S A 101:13038–13043
22. Neddermann P, Quintavalle M, Di Pietro C, Clementi A, Cerretani M, Altamura S et al (2004) Reduction of hepatitis C virus NS5A hyperphosphorylation by selective inhibition of cellular kinases activates viral RNA replication in cell culture. J Virol 78:13306–13314
23. Ross-Thriepland D, Harris M (2015) Hepatitis C virus NS5A: enigmatic but still promiscuous 10 years on. J Gen Virol 96:727–738
24. Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, Murthy K, Habermann A, Kräusslich HG, Mizokami M, Bartenschlager R, Liang TJ (2005) Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med 11:791–796
25. Saeed M, Andreo U, Chung HY, Espiritu C, Branch AD, Silva JM, Rice CM (2015) SEC14L2 enables pan-genotype HCV replication in cell culture. Nature 524:471–475
26. Kaneko T, Tanji Y, Satoh S, Hijikata M, Asabe S, Kumura K, Shimotohno K (1994) Production of two phosphoproteins from the NS5A region of the hepatitis C viral genome. Biochem Biophys Res Commun 205:320–326
27. Appel N, Pietschmann T, Bartenschlager R (2005) Mutational analysis of hepatitis C virus nonstructural protein 5A: potential role of differential phosphorylation in RNA replication and identification of a genetically flexible domain. J Virol 79:3187–3194
28. Asabe SI, Tanji Y, Satoh S, Kaneko T, Kimura K, Shimotohno K (1997) The N-terminal region of hepatitis C virus-encoded NS5A is important for NS4A-dependent phosphorylation. J Virol 71:790–796
29. Liu QY, Bhat RA, Prince AM, Zhang P (1999) The hepatitis C virus NS2 protein generated by NS2-3 autocleavage is required for NS5A phosphorylation. Biochem Biophys Res Commun 254:572–577
30. Neddermann P, Clementi A, De Francesco R (1999) Hyperphosphorylation of the hepatitis C virus NS5A protein requires an active NS3 protease, NS4A, NS4B, and NS5A encoded on the same polyprotein. J Virol 73:9984–9991
31. Koch JO, Bartenschlager R (1999) Modulation of hepatitis C virus NS5A hyperphosphorylation by nonstructural proteins NS3, NS4A, and NS4B. J Virol 73:7138–7146
32. Lohmann V, Korner F, Koch J, Herian U, Theilmann L, Bartenschlager R (1999) Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 285:110–113
33. O'Boyle 2nd DR, Nower PT, Lemm JA, Valera L, Sun JH, Rigat K, Colonno R, Gao M (2005) Development of a cell-based high-throughput specificity screen using a hepatitis C virus-bovine viral diarrhea virus dual replicon assay. Antimicrob Agents Chemother 49:1346–1353
34. Lemm JA, O'Boyle II DR, Liu M, Nower PT, Colonno R, Deshpande MS, Snyder LB, Martin SW, St Laurent DR, Serrano-Wu MH et al (2010) Identification of hepatitis C virus NS5A inhibitors. J Virol 84:482–491
35. Gao M, Nettles RE, Belema M, Snyder LB, Nguyen VN, Fridell RA, Serrano-Wu MH, Langley DR, Sun JH, O'Boyle II DR et al (2010) Chemical genetics strategy identifies an HCV NS5A inhibitor with a potent clinical effect. Nature 465:96–100
36. Quintavalle M, Sambucini S, Di Pietro C, De Francesco R, Neddermann P (2006) The alpha isoform of protein kinase CKI is responsible for hepatitis C virus NS5A hyperphosphorylation. J Virol 80:11305–11312
37. Fridell RA, Qiu D, Valera L, Wang C, Rose RE, Gao M (2011) Distinct functions of NS5A in hepatitis C virus RNA replication uncovered by studies with the NS5A inhibitor BMS-790052. J Virol 85:7312–7320

38. Gao M, Fridell R, O'Boyle II D, Qiu D, Sun JH, Lemm J, Nower P, Valera L, Voss S, Liu M, Belema M, Nguyen V, Romine J, Martin S, Serrano-Wu M, St. Laurent D, Snyder L, Colonno R, Hamann L, Meanwell N (2008) HCV NS5A inhibitors: from screen lead to clinic. In: 15th international symposium on HCV and related virus, San Antonio
39. Berger C, Romero-Brey I, Radujkovic D, Terreux R, Zayas M, Paul D, Harak C, Hoppe S, Gao M, Penin F, Lohmann V, Bartenschlager R (2014) Daclatasvir-like inhibitors of NS5A block early biogenesis of HCV-induced membranous replication factories, independent of RNA replication. Gastroenterology 147:1094–1105
40. Ascher DB, Wielens J, Nero TL, Doughty L, Morton CJ, Parker MW (2014) Potent hepatitis C inhibitors bind directly to NS5A and reduce its affinity for RNA. Sci Rep 4:4765
41. Kwon HJ, Xing W, Chan K, Niedziela-Majka A, Brendza KM, Kirschberg T, Kato D, Link JO, Cheng G, Liu X, Sakowicz R (2015) Direct binding of ledipasvir to HCV NS5A: mechanism of resistance to an HCV antiviral agent. PLoS One 10:e0122844. https://doi.org/10.1371/journal.pone.0122844
42. Zein NN (2000) Clinical significance of hepatitis C virus genotypes. Clin Microbiol Rev 13:223–235
43. Conte I, Giuliano C, Ercolani C, Narjes F, Koch U, Rowley M, Altamura S, De Francesco R, Neddermann P, Migliaccio G, Stansfield I (2009) Synthesis and SAR of piperazinyl-*N*-phenylbenzamides as inhibitors of hepatitis C virus RNA replication in cell culture. Bioorg Med Chem Lett 19:1779–1783
44. Lemm JA, Leet JE, O'Boyle II D, Romine L, JL X, Huang SX, Alberts J, Sun JH, Nower PT, Martin SW, Serrano-Wu MH, Meanwell NA, Snyder LB, Gao M (2011) Discovery of potent NS5A inhibitors with dimeric structures. Antimicrob Agents Chemother 55:3795–3802
45. Nettles RE, Min Gao M, Bifano M, Chung E, Persson A, Marbury TC, Goldwater R, DeMicco MP, Rodriguez-Torres M, Vutikullird A et al (2011) Multiple ascending dose study to evaluate BMS-790052, a novel NS5A inhibitor, in patients infected with hepatitis C virus genotype 1. Hepatology 54:1956–1966
46. Fridell RA, Wang C, Sun JH, O'Boyle II DR, Nower P, Valera L, Qiu D, Roberts S, Huang X, Kienzle B et al (2011) Genotypic and phenotypic analysis of variants resistant to HCV NS5A replication complex inhibitor BMS-790052: in vitro and in vivo correlations. Hepatology 54:1924–1935
47. Wang KA, Worth A, Martin R, Svarovskaia E, Brainard DM, Lawitz E, Miller MD, Mo H (2013) Characterization of hepatitis C virus resistance from a multiple-dose clinical trial of the novel NS5A inhibitor GS-5885. Antimicrob Agents Chemother 57:6333–6340
48. Cheng G, Tian Y, Doehle B, Peng B, Corsa A, Lee Y-J, Gong R, Yu M, Han B, Xu S et al (2016) In vitro antiviral activity and resistance profile characterization of the HCV NS5A inhibitor ledipasvir. Antimicrob Agents Chemother 60:1847–1853
49. Lawitz EJ, Dvory-Sobol H, Doehle B, Worth AS, McNally J, Brainard DM, Link JO, Miller M, Mo H (2016) Clinical resistance to velpatasvir (GS-5816), a novel pan-genotypic inhibitor of the hepatitis C virus NS5A protein. Antimicrob Agents Chemother 60:5368–5378
50. Chen G, Yu M, Peng B, Lee YJ, Trejo-Martin A, Gong R, Bush C, Worth A, Nash M, Chan K, Yang H, Beran R, Tian Y, Perry J, Taylor J, Yang C, Paulson M, Delaney W, Link LO (2013) GS-5816, a second generation HCV NS5A inhibitor with potent antiviral activity, broad genotypic coverage and a high resistance barrier. J Hepatol 58:S484
51. Coburn CR, Meinke PT, Chang W, Fandozzi CM, Graham DJ, Hu B, Huang Q, Kargman S, Kozlowski J, Liu R, McCauley JA, Nomeir AA, Soll RM, Vacca JP, Wang D, Wu H, Zhong B, Olsen DB, Ludmerer SW (2013) Discovery of MK-8742: an HCV NS5A inhibitor with broad genotype activity. ChemMedChem 8:1930–1940
52. Liu R, Curry S, McMonagle P, Yeh WW, Ludmerer SW, Jumes PA, Marshall WL, Kong S, Ingravallo P, Black S et al (2015) Susceptibilities of GT 1a, 1b, and 3 HCV variants to the NS5A inhibitor Elbasvir. Antimicrob Agents Chemother 59:6922–6929
53. Ng TI, Krishnan P, Pilot-Matias T, Kati W, Schnell G, Beyer J, Reisch T, Lu L, Dekhtyar T, Irvin M, Tripathi R, Maring C, Randolph JT, Wagner R, Collins C (2017) In vitro antiviral activity and resistance profile of the next-generation hepatitis C virus NS5A inhibitor Pibrentavir. Antimicrob Agents Chemother 61(5):e02558–e02516

54. Krishnan P, Beyer J, Mistry N, Koev G, Reisch T, DeGoey D, Kati W, Campbell A, Williams L, Xie W, Setze C, Molla A, Collins C, Pilot-Matias T (2015) In vitro and in vivo antiviral activity and resistance profile of ombitasvir, an inhibitor of hepatitis C virus NS5A. Antimicrob Agents Chemother 59:979–987
55. Pol S (2013) Daclatasvir, an effective inhibitor of the hepatitis C virus replication complex protein NS5A: review of virologic data, treatment rationale and clinic trials. Clin Invest 3:191–207
56. Pawlotsky JM (2013) NS5A inhibitors in the treatment of hepatitis C. J Hepatol 59:375–382
57. Zhou N, Han Z, Hartman-Neumann S, DeGray B, Ueland J, Vellucci V, Hernandez D, McPhee F (2016) Characterization of NS5A polymorphisms and their impact on response rates in patients with HCV genotype 2 treated with daclatasvir-based regimens. J Antimicrob Chemother 71:3495–3505
58. Benzine T, Brandt R, Lovell WC, Yamane D, Neddermann P, De Francesco R, Lemon SM, Perelson AS, Ke R, McGivern DR (2017) NS5A inhibitors unmask differences in functional replicase complex half-life between different hepatitis C virus strains. PLoS Pathog 13:e1006343. https://doi.org/10.1371/journal.ppat.1006343
59. Fridell RA, Qiu D, Wang C, Valera L, Gao M (2010) Resistance analysis of the HCV NS5A inhibitor, BMS-790052, in the in vitro replicon system. Antimicrob Agents Chemother 54:3641–3650
60. Lawitz EJ, Gruener D, Hill JM, Marbury T, Moorehead L, Mathias A, Cheng G, Link JO, Wong KA, Mo H, McHutchison JG, Brainard DM (2012) A phase 1, randomized, placebo-controlled, 3-day, dose-ranging study of GS-5885, an NS5A inhibitor, in patients with genotype 1 hepatitis C. J Hepatol 57:24–31
61. Lawitz E, Freilich B, Link J, German P, Mo H, Han L, Brainard DM, McNally J, Marbury T, Rodriguez-Torres M (2015) A phase 1, randomized, dose-ranging study of GS-5816, a once-daily NS5A inhibitor, in patients with genotype 1-4 hepatitis C virus. J Viral Hepat 22:1011–1019
62. Yeh WW, Lipardi C, Jumes P, De Lepeleire I, Caro L, Huang X, Mangin E, Nachbar RB, Gane E, Popa S, Ghicavii N, Wagner F, Butterton JR (2013) MK-8742, an HCV NS5A inhibitor with a broad Spectrum of HCV genotypic activity, demonstrates potent antiviral activity in Genotype-1 and -3 HCV-infected patients. In: 64th AASLD, Washington
63. Lawitz EJ, O'Riordan WD, Asatryan A, Freilich BL, Box TD, Overcash JS, Lovell S, Ng TI, Liu W et al (2015) Potent antiviral activity of ABT-493 and ABT-530 with 3-day monotherapy in patients with and without compensated cirrhosis with HCV GT1a infection. Antimicrobial. https://doi.org/10.1128/AAC.02524-15.AAC
64. Lawitz E, Marbury T, Campbell A, Dumas E, Kapoor M, Pilot-Matias T, Krishnan P, Setze C, Xie W, Podsadecki T et al (2012) Safety and antiviral activity of ABT-267, a novel NS5A inhibitor, during 3-day monotherapy: first study in HCV genotype-1 (GT1)-infected treatment-naïve patients. J Hepatol 56:S469–S470
65. FDA News Release: FDA approves Gilead's Harvoni® (Ledipasvir/Sofosbuvir) for the treatment of genotype 1 chronic hepatitis C (released by gild), 10 Oct 2014
66. FDA News Release: FDA approves two hepatitis C drugs for pediatric patients, 7 Apr 2017
67. FDA News Release: FDA approves Epclusa for treatment of chronic hepatitis C virus infection, 28 June 2016
68. FDA News Release: FDA approves Vosevi for hepatitis C, 18 July 2017
69. FDA News Release: FDA approves Zepatier for the treatment of chronic hepatitis C virus (HCV) genotypes 1 and 4 infections, 28 Jan 2016
70. FDA News Release: FDA approves Mavyret for hepatitis C, 3 Aug 2017
71. Feld JJ, Jacobson IM, Hézode C, ASTRAL-1 Investigators et al (2015) Sofosbuvir and velpatasvir for HCV genotype 1, 2, 4, 5, and 6 infection. N Engl J Med 373:2599–2607
72. Zeuzem S, Ghalib R, Reddy KR et al (2015) Grazoprevir-elbasvir combination therapy for treatment-naive cirrhotic and noncirrhotic patients with chronic hepatitis C virus genotype 1, 4, or 6 infection: a randomized trial. Ann Intern Med 163:1–13

73. McPhee F, Hernandez D, Zhou N (2016) Effect of minor populations of NS5A and NS5B resistance-associated variants on HCV genotype-3 response to daclatasvir plus sofosbuvir, with or without ribavirin. Antivir Ther 22:237–246
74. Zhou N, Hernandez D, Ueland J, Yang X, Yu F, Sims K, Yin PD, McPhee F (2016) NS5A sequence heterogeneity and mechanisms of Daclatasvir resistance in hepatitis C virus genotype 4 infection. J Infect Dis 213:206–215
75. Camus G, Han B,| Asselah T,| Hsieh D, Dvory-Sobol H, Lu J, Svarovskaia E, Martin R, Parhy B, Miller MD, Brainard DM, Kersey K, Abergel A Mo H. Resistance characterization of ledipasvir and velpatasvir in hepatitis C virus genotype 4. J Viral Hepat 2018;25:134-143
76. Wang C, Jia L, O'Boyle II DR, Sun JH, Rigat K, Valera L, Nower P, Huang X, Kienzle B, Roberts S, Gao M, Fridell RA (2014) Comparison of Daclatasvir resistance barriers on NS5A from hepatitis C virus genotypes 1 to 6: implications for cross-genotype activity. Antimicrob Agents Chemother 58:5155
77. Sun JH, O'Boyle II DR, Zhang DR, Wang Y, Nower C, Valera P, Roberts L, Nettles S, Fridell RA, Gao M (2012) Impact of a baseline polymorphism on the emergence of resistance to the hepatitis C virus nonstructural protein 5A replication complex inhibitor BMS-790052. Hepatology 55:1692–1699
78. Pelosi LA, Voss S, Liu M, Gao M, Lemm JA (2012) Effect on hepatitis C virus replication of combinations of direct-acting antivirals, including NS5A inhibitor Daclatasvir. Antimicrob Agents Chemother 56:5230
79. Guedj J, Dahari H, Uprichard SL, Perelson AS (2013) The rapid viral decline with the HCV NS5A inhibitor daclatasvir reveals a dual mode of action and leads to a new HCV half-life estimate. Exp Rev Gastroenterol Heoatol 7:397–399
80. Guedj J, Dahari H, Rong L, Sansone ND, Nettles RE, Cotler SJ, Layden TJ, Uprichard SL, Perelson AS (2013) Modeling shows that the NS5A inhibitor daclatasvir has two modes of action and yields a shorter estimate of the hepatitis C virus half-life. Proc Natl Acad Sci U S A 110:3991–3996
81. McGivern DR, Masaki T, Williford S, Ingravallo P, Feng Z, Lahser F, Asante-Appiah E, Neddermann P, De Francesco R, Howe AY, Lemon SM (2014) Kinetic analyses reveal potent and early blockade of hepatitis C virus assembly by NS5A inhibitors. Gastroenterology 147:453–462
82. Quinkert D, Bartenschlager R, Lohmann V (2005) Quantitative analysis of the hepatitis C virus replication complex. J Virol 79:13594–13605
83. Pietschmann T, Lohmann V, Rutter G, Kurpanek K, Bartenschlager R (2001) Characterization of cell lines carrying self-replicating hepatitis C virus RNAs. J Virol 75:1252–1264
84. Sun JH, O'Boyle II DR, Fridell RA, Langley DR, Wang C, Roberts SB, Nower P, Johnson BM, Moulin F, Nophsker MJ, Wang YK, Liu M, Rigat K, Tu Y, Hewawasam P, Kadow J, Meanwell NA, Cockett M, Lemm JA, Kramer M, Belema M, Gao M (2015) Resensitizing daclatasvir-resistant hepatitis C variants by allosteric modulation of NS5A. Nature 527(7577):245–248
85. Beldar S, Manimekalai MSS, Cho N-J, Baek K, Grüber G, Yoon HS (2018) Self-association and conformational variation of NS5A domain 1 of hepatitis C virus. J Gen Virol 99:194. https://doi.org/10.1099/jgv.0.001000
86. O'Boyle II DR, Sun JH, Nower PT, Lemm JA, Fridell RA, Wang C, Romine JL, Belema M, Nguyen V, St. Laurent DR, Serrano-Wu M, Snyder LB, Meanwell NA, Langley DR, Gao M (2013) Characterizations of HCV NS5A replication complex inhibitors. Virology 444:343–354
87. O'Boyle 2nd DR, Nower PT, Gao M, Fridell R, Wang C, Hewawasam P, Lopez O, Tu Y, Meanwell NA, Belema M, Roberts SB, Cockett M, Sun JH (2015) Synergistic activity of combined NS5A inhibitors. Antimicrob Agents Chemother 60(3):1573–1583