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Discovery of the Pan-genotypic Hepatitis C Virus NS3/4A Protease Inhibitor Voxilaprevir (GS-9857): A Component of Vosevi ®

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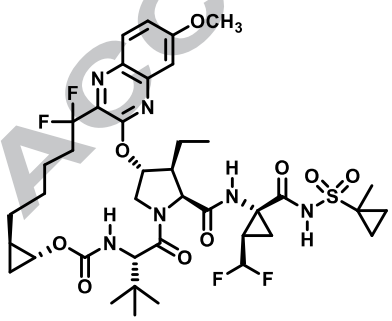
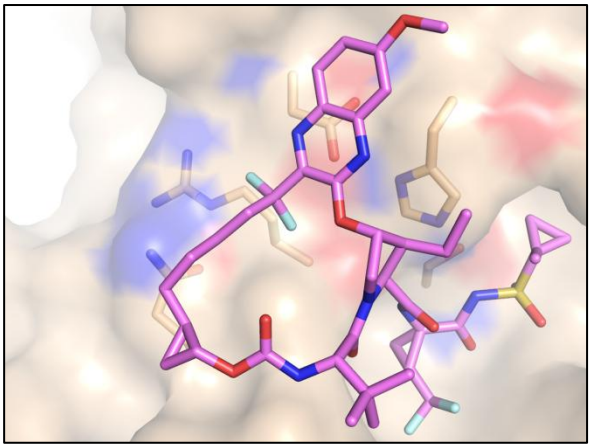
Keywords: Vosevi®, voxilaprevir, VOX, GS-9857, HCV, Hepatitis C virus, protein adducts

Abstract: Treatment of Hepatitis C virus (HCV) infection has been historically challenging due the high viral genetic complexity wherein there are eight distinct genotypes and at least 86 viral subtypes. While HCV NS3/4A protease inhibitors are an established treatment option for genotype 1 infection, limited coverage of genotypes 2 and/or 3 combined with serum alanine transaminase (ALT) elevations for some compounds has limited the broad utility of this therapeutic class. Our discovery efforts were focused on identifying an NS3/4A protease inhibitor with pan-genotypic antiviral activity, improved coverage of resistance associated substitutions, and a decreased risk of hepatotoxicity. Towards this goal, distinct interactions with the conserved catalytic triad of the NS3/4A protease were identified that improved genotype 3 antiviral activity. We further discovered that protein adduct formation strongly correlated with clinical ALT elevation for this therapeutic class. Improving metabolic stability and decreasing protein adduct formation through structural modifications ultimately resulted in voxilaprevir. Voxilaprevir, in combination with sofosbuvir and velpatasvir, has demonstrated pan-genotypic antiviral clinical activity. Furthermore hepatotoxicity was not observed in Phase 3 clinical trials with voxilaprevir, consistent with our design strategy. Vosevi ® (sofosbuvir, velpatasvir, and voxilaprevir) is now an approved pan-genotypic treatment option for the most difficult-to-cure individuals who have previously failed direct acting antiviral therapy.

Graphical Abstract:



Voxilaprevir
HCV GT1–6a EC_{50} = 1.5–6.6 nM
Human hepatocyte Pred CL = <0.07 L/h/kg
Protein adducts = <1 pmol equivalent/mg protein



Hepatitis C virus (HCV) infection remains a global health concern with an estimated 71 million people chronically infected worldwide[1]. Untreated, chronic HCV infection can result in cirrhosis, hepatocellular carcinoma and liver failure, contributing to approximately 400,000 annual deaths worldwide[2]. Thus, curative HCV treatment options can have a profound impact on the health of those infected. Despite this need, treatment of HCV infection has been particularly challenging due to the high viral genetic diversity wherein there are eight distinct genotypes (GTs) and at least 86 viral subtypes[3]. Consistent with this challenge, the initial wave of direct acting antiviral (DAA) agents were frequently most potent against HCV GT 1 while having compromised activity against GT 2 and/or 3.

Initially we advanced multiple compounds with potent GT1 antiviral activity into the clinic including inhibitors of the HCV NS3/4A protease (vedroprevir[4] and GS-9256[5]), NS5B polymerase (radalbuvir[6] and tegobuvir[7]) and NS5A protein (ledipasvir[8]). To address the therapeutic need to treat patients regardless of genotype, discovering compounds with potent pan-genotypic antiviral activity became a priority. Toward this goal, we initiated a comprehensive NS5A inhibitor program that resulted in the discovery of velpatasvir, which has potent picomolar activity against all gentoypes tested [9-10]. Upon combination of velpatasvir with sofosbuvir into the oral once-daily single tablet regimen (STR) Epclusa®, high sustained virological response (SVR) rates across all genotypes have been observed[4, 5]. Despite the generally high SVR rates obtained for the multiple HCV treatment options that are now approved, there are patients that fail these regimens with viral resistance associated substitutions (RASs, also referred to as mutants or variants) that are less susceptible to the individual DAA agents[11]. Thus, identifying next generation DAA compounds with antiviral activity against these RASs, while also having potent pan-genotypic activity, was considered essential to help treat this emerging high unmet medical need population. Voxilaprevir, the discovery of which described herein, is a pan-genotypic HCV NS3/4A protease inhibitor (PI) with improved antiviral activity against common RASs. Voxilaprevir has recently been approved as a component of the pan-genotypic STR Vosevi® (sofosbuvir, velpatasvir and voxilaprevir). Vosevi® provides a pan-genotypic treatment option for the most difficult-to-cure patients who have failed prior DAA therapies[12-13]. Additionally, for some DAA-naïve patients, Vosevi® provides an option of shortened treatment duration (8 weeks)[13].

NS3/4A PIs are a longstanding, important therapeutic class in the treatment of HCV infection. Reversible covalent α-ketoamide containing NS3/4A PIs, telaprevir and boceprevir, were the first DAAs approved for the treatment of HCV GT1 infection in combination with PEG and RBV. Second generation NS3/4A PIs containing terminal carboxylic acid or acyl sulfonamide groups, along with large aromatic hydrophobic groups extending from the central P2 amino acid, soon followed with improved GT1 cellular potency (**Figure 1**). However, reduced relative enzymatic and cellular potency against genotype 2 and/or 3[14-15] has limited their use in these genotypes. Serum alanine transaminase (ALT) elevations have also been identified as a potential hepatotoxicity signal with some NS3/4A PIs[16], contributing to clinical discontinuations (IDX-320)[17], clinical dose reductions (paritaprevir[18], asunaprevir[19], and grazoprevir[20]), and/or transaminase elevation warnings in the prescribing label (paritaprevir[21], asunaprevir[22], and grazoprevir[23]). Our internal discovery efforts focused on identifying a potent pan-genotypic HCV NS3/4A PI with improved coverage of RASs and a decreased risk of hepatotoxicity.

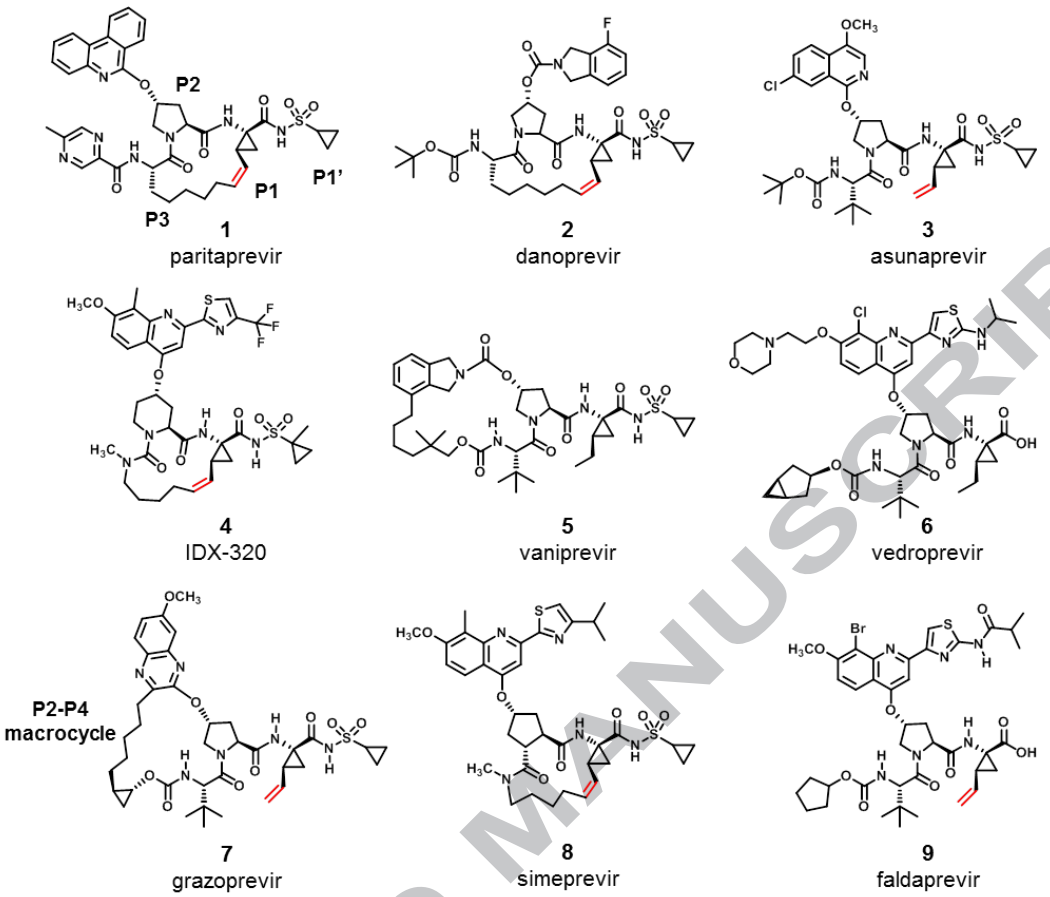


Figure 1. Representative HCV NS3/4A PIs

The HCV NS3/4A protein consists of an N-terminal serine protease domain and a C-terminal helicase domain. The NS3/4A protease cleaves the viral polyprotein at four distinct sites utilizing a classic catalytic triad (S139, H57 and D81) to generate the viral machinery required for viral replication. While the catalytic triad is conserved in all reported NS3/4A sequences, RASs elsewhere in the NS3/4A protease domain have rapidly emerged (including amino acid positions 155 and 168)[24]. In GT1, an electrostatic interaction between R155 and D168 is formed that creates an important surface for interaction with aromatic hydrophobic P2 groups present on many NS3/4A PIs. Amino acid substitutions at either position therefore have a significant impact on NS3/4A PI binding affinity. Amino acid 168 also plays an important role in GT3 wherein the wild-type Q168 contributes to a significant loss in GT3 potency for most NS3/4A PIs[15]. Removing steric bulk on the P2 region proximal to amino acid positions 155 and 168, along with modifications to the macrocycle, were strategies utilized in the discovery of grazoprevir[25]. Even with this design, grazoprevir loses 2-3 orders of magnitude in potency against GT3 compared to GT1 in both biochemical and cellular assays[15, 26]. To improve both GT3 potency and activity against common GT1 RASs, our internal discovery efforts were directed at modifications to the central pyrrolidine core with the goal of minimizing the dependence on interactions with the D168 and R155 residues and enhancing interactions with conserved catalytic triad region of the HCV NS3/4A protease.

We targeted direct interactions with the catalytic triad as it is conserved across genotypes and, due to its essential role in enzymatic activity, the catalytic triad is not a site of viral polymorphism or resistance. Realizing that the conserved H57 of the catalytic triad is positioned adjacent to the 3-position of the central pyrrolidine we investigated the potential of introducing alkyl, substituted alkyl and cycloalkyl

groups to establish a hydrophobic interaction with H57 (**Figure 2**). Consistent with this design principle, methyl substituted pyrrolidine **11 (Table 1)** improved cellular potency nearly 4-fold vs the unsubstituted comparator **10** (20 nM vs 74 nM, respectively). Ethyl substituted compound **12** possessed similarly improved cellular potency (EC_{50} = 23 nM), while also improving biochemical potency 2.5 fold vs compound **10** (67 pM vs 165 pM, respectively). A crystal structure of inhibitor **12** bound to the NS3/4A protease highlights that the ethyl substituent is positioned in van der Waals contact with the catalytic H57 residue as we intended (**Figure 2**). Extending beyond ethyl to n-propyl (**13)** was also beneficial compared to the unsubstituted pyrrolidine **10** (2-fold improvement in cellular potency), but was inferior in potency to the methyl or ethyl substituted pyrrolidine containing compounds **11** and **12**. Consistent with space constraints observed in the crystal structure, cyclopropyl (**14**), iso-butyl (**15**) or benzyl (**16**) substitution led to significantly compromised GT3 potency (all >1000 nM in GT3 EC_{50} assay). Since both the 3-methyl and 3-ethyl pyrrolidine substitutions contributed to significantly improved GT3 potency, these series were further pursued in parallel.

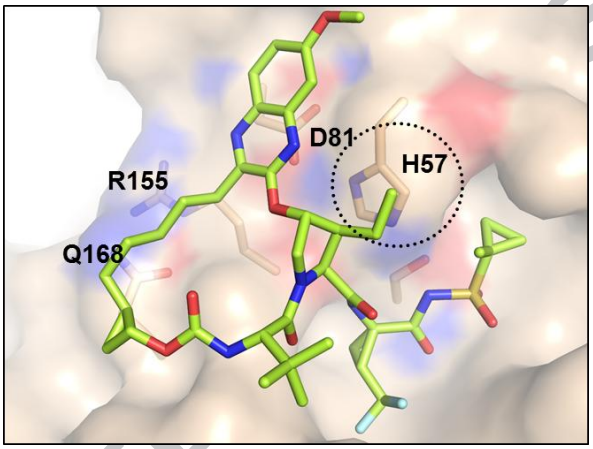
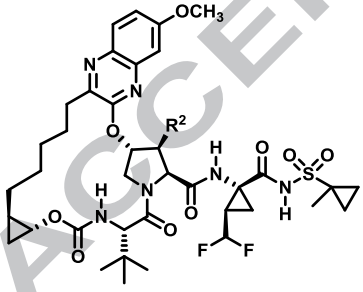
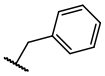


Figure 2. Crystal structure of 3-ethyl substituted pyrrolidine compound **12** bound to GT3 surrogate (GT1 D168Q) NS3/4A protease (PDB code: 6NZV)

Table 1
Genotype 3 biochemical and cellular potency of 3-substituted pyrrolidine core containing macrocycles

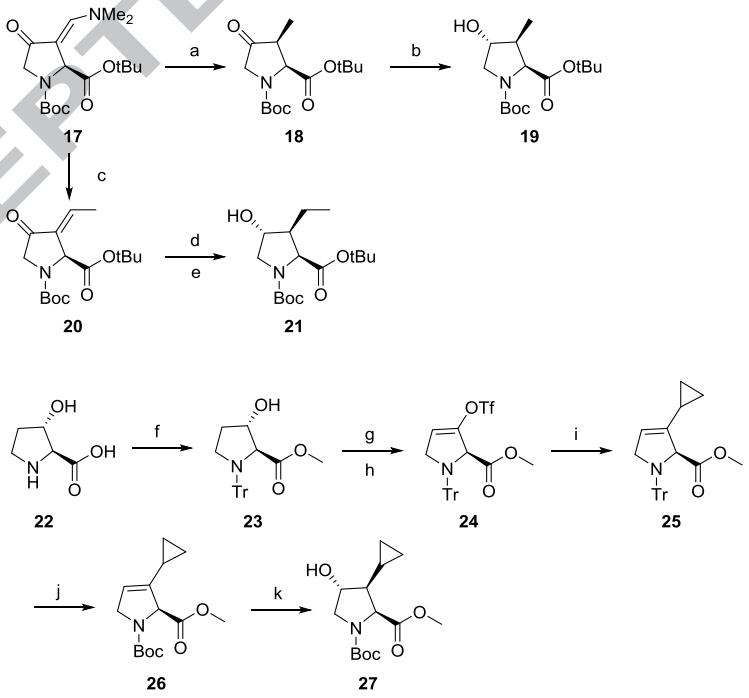


Cpd	R^2	GT3 Ki[a] (nM)	GT3a EC_{50}[b] (nM)
10	H	0.165	74
11	Me	0.100	20

12	Et	0.067	23
13	n-Pr	0.120	36
14		4.9	1164
15		0.630	1125
16		0.870	1107

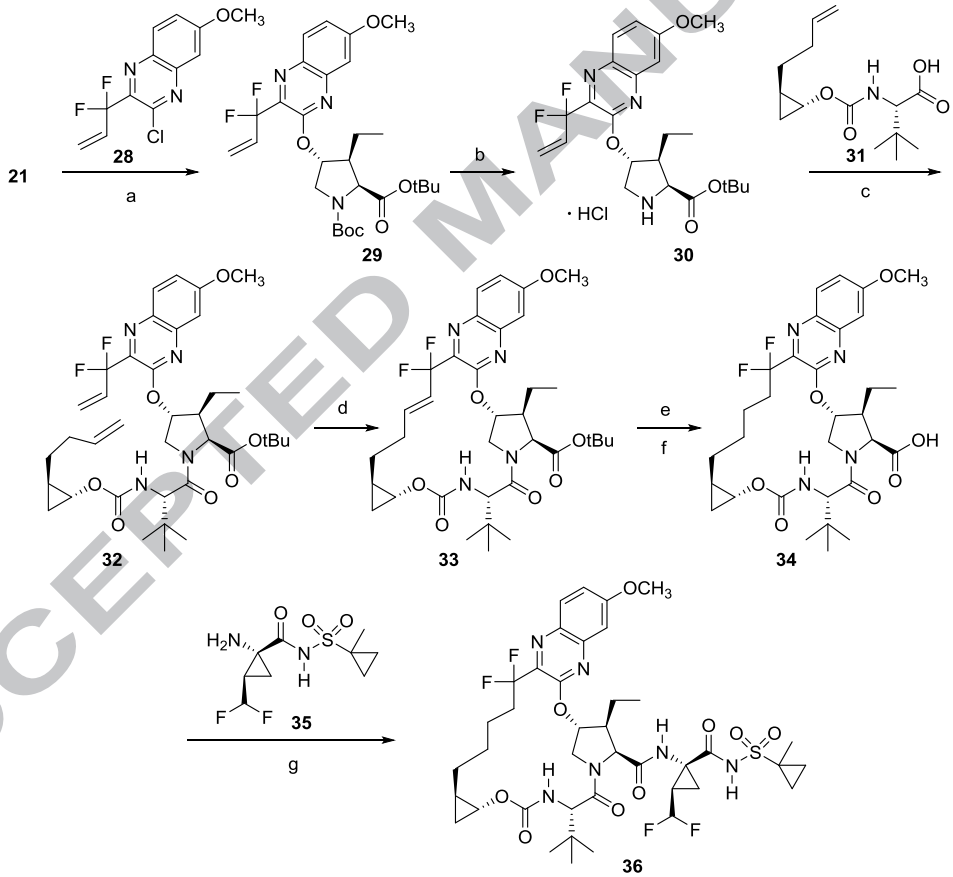
[a]Ki determined by enzymatic assay using an HCV genotype 3a NS3/4A protein
[b]EC_{50} determined by cell based assay using RLuc cells harboring subgenomic genotype 3a replicon

Three synthetic routes were employed to prepare pyrrolidine cores derivatized at the 3-position with alkyls, substituted alkyls, and cycloalkyls (**Scheme 1**). Enaminone **17**[27] served as a common precursor utilized for the synthesis of both alkyl and substituted alkyl derivatives, with the synthesis of both 3-methyl and 3-ethyl substituted pyrrolidines highlighted in **Scheme 1**. Hydrogenation of enaminone **17** with Pd/C yielded the desired 3-methyl pyrrolidine **18** along with the undesired diastereomer in a ratio of 2:1, which was enriched to 93:7 following recrystallization. Ketone **18** was then stereoselectively reduced with the CBS catalyst, affording 4-hydroxy-3-methyl pyrrolidine **19** as a single diastereomer. Synthesis of 3-ethyl-4-hydroxy-pyrrolidine **21** proceeded through initial treatment of enaminone **17** with MeMgBr to generate enone **20**. Stereoselective CBS reduction of the ketone followed by hydrogenation of the olefin generated 3-ethyl-4-hydroxy-pyrrolidine **21**. Synthesis of 3-cyclopropyl-4-hydroxy-pyrrolidine **27** initiated from 3-hydroxy-pyrrolidine **22**, which was first esterified and protected with a trityl group to generate **23**. Oxidation of **23** with NMO and TPAP, followed by treatment with LiHMDS and Comins' reagent, selectively generated enol triflate **24**. Negishi coupling of **24** using cyclopropyl zinc bromide furnished intermediate **25**. Amine protecting group exchange yielded **26**, which underwent stereoselective hydroboration-oxidation to provide 3-cyclopropyl-4-hydroxy-pyrrolidine **27**.



Scheme 1. (a) H_2, Pd/C, acetone, 62% following recrystallization at 93:7 d.r; (b) BH_3•Me_2S, (*R*)-Me-CBS, THF, -78 °C to rt, 68% as single diastereomer; (c) MeMgBr, MeTHF/toluene, -12 °C; (d) (*R*)-Me-CBS, BH_3-$PhNEt_2$, 80-90% over 2 steps; (e) H_2, Pd/C, Cs_2CO_3, IPAc, 80%; (f) i. $SOCl_2$, MeOH; ii. TrCl, Et_3N, DCM, 0 °C to rt, 72% over 2 steps; (g) NMO, TPAP, 4 Å MS, DCM, 81%; (h) N-(5-chloro-2-pyridyl)bis(trifluoromethanesulfonimide), LiHMDS, THF, -78 °C to rt, 63%; (i) cyclopropyl zinc bromide, $Pd(Ph_3)_4$, THF, 50 °C, 60%; (j) i. HCl, MeOH, DCM; ii. Boc_2O, DMAP, Et_3N, DCM, 68% over 2 steps; (k) BH_3•Me_2S, H_2O_2, NaOH, THF, H_2O, 45%.

The general route to prepare 3-substituted pyrrolidine core containing macrocyclic NS3/4A PIs is shown in **Scheme 2**. Base promoted S_nAr reaction between 3-ethyl-4-hydroxy-pyrrolidine **21** and quinoxaline **28** generated intermediate **29**, which was then subjected to acidic Boc deprotection to afford amine **30**. Amide bond formation with acid **31** employing standard peptide coupling conditions yielded **32**, which underwent ring closing cross-metathesis using Zhan 1b catalyst to generate macrocycle **33**. Hydrogenation of the resulting macrocyclic olefin over Rh/alumina followed by t-butyl ester deprotection afforded acid **34**. Coupling of the cyclopropyl sulfonamide **35** using HATU completed the synthesis of the 3-ethyl-pyrrolidine containing macrocyclic NS3/4A PI **36**.



Scheme 2. Reagents and conditions. (a) Cs_2CO_3, MeCN, 85 °C, 68%; (b) HCl, dioxane, rt, 75-100% (c) HATU, DIPEA, DMF, rt, 74%; (d) Zhan 1b catalyst, DCE, 80 °C, 86%; (e) Rh/Alumina, H_2, DCM, 86-88%; (f) TFA, DCM, 95–97%; (g) HATU, DIPEA, DMF, 70–87%.

After our initial success in improving GT3 activity, we turned our attention to addressing a toxicity concern that plagues some NS3/4A PIs. Antiviral therapy has been associated with ALT elevations, as a

potential biomarker for hepatotoxicity, contributing to label warnings and recommendations for ALT monitoring for a number of NS3/4A PIs. While drug-induced hepatotoxicity is always a concern, the concern is heightened when treating a liver disease. A number of factors have been associated with drug-induced liver injury (DILI) including reactive metabolite formation, mitochondrial toxicity, host immune-response pathways and inhibition of biliary transporters[28-32]. Consistent with reactive metabolites contributing to ALT elevation with NS3/4A PIs, co-administration of the cytochrome P450 3A (CYP3A) inhibitor ritonavir (to suppress reactive metabolite formation) with danoprevir (**2**) resulted in reduced clinical ALT elevations[33-34]. To further investigate the association of reactive metabolites with ALT elevation across multiple NS3/4A PIs, we synthesized and then evaluated radiolabeled (^{3}H or ^{14}C) paritaprevir, danoprevir, asunaprevir, IDX-320, vaniprevir, vedroprevir, grazoprevir, simeprevir and faldaprevir in protein adduct assays and correlated these results with reports of clinical ALT elevations. Overall, protein adduct levels had a strong correlation with clinical ALT elevations (**Table 3**). All compounds generating >50 pmol equivalent/mg protein adducts experienced clinical ALT elevations, while four of the five compounds generating <50 pmol equivalent/mg protein adducts did not show significant ALT elevations. These studies are consistent with the formation of reactive metabolites as an important correlator with clinical ALT elevations and potential hepatotoxicity. We were additionally concerned the reactive metabolites generated in an HCV infected patient's liver might be an additional driver of idiosyncratic drug reactions based on the "danger hypothesis" (that cell stress is a factor in idiosynchratic drug reactions).[36] Therefore, minimizing protein adduct formation became an essential element in our optimization efforts.

Table 3
Protein adduct formation and reported ALT elevation for representative HCV NS3/4A PIs.

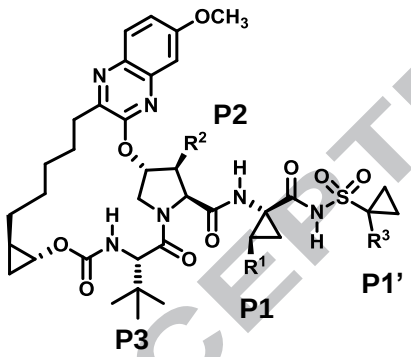
Cpd	Protein adducts (pmol equivalent/mg protein)	ALT elevation[35]
parataprevir (**1**)	295	Yes[18, 21, 36]
danoprevir (**2**)	202	Yes[33-34]
asunaprevir (**3**)	114	Yes[19, 22]
IDX-320 (**4**)	96	Yes[17]
vaniprevir (**5**)	29	No[37]
vedroprevir (**6**)	26	No[38-39]
grazoprevir (**7**)	24	Yes[20, 23]
simeprevir (**8**)	22	No[40]
faldaprevir (**9**)	15	No[41]

The cyclopropyl olefin P1 moiety is a common structural feature on HCV NS3/4A PIs (**Figure 1**), with all five HCV PIs that demonstrate clinical ALT elevation containing this olefin functional group either as a terminal vinyl or a macrocyclic olefin (olefin highlighted in red in **Figure 1**). Mechanistically, we hypothesized this cyclopropyl olefin could undergo metabolic activation to form a reactive epoxide that could then react with nucleophiles (e.g. proteins and glutathione), contributing to transaminase elevation[42]. To explore this mechanistic hypothesis, we synthesized the vinyl containing compound **37** in our methyl pyrrolidine series (**Table 4**) and then incubated this compound in human liver microsomes in the presence of glutathione followed by metabolite identification (Met ID). Two of the metabolites generated supported our mechanistic hypothesis; one was direct oxidation at P1 (consistent with an epoxide being formed from the olefin) and the second was olefin oxidation followed by epoxide opening with glutathione. Additional olefin P1 containing molecules were also evaluated in human microsomal Met ID assays with similar resultant metabolite profiles involving olefin activation (data not shown). Compound **37** was also evaluated in a human liver microsomal (HLM) metabolic stability assay to

determine the predicted clearance (Pred CL). Moderate metabolic instability was observed (Pred CL = 0.64 L/h/kg). The P1 vinyl was also synthesized in the ethyl pyrrolidine (**41**) series and similarly demonstrated poor metabolic stability (Pred CL = 0.84 L/h/kg). Of particular concern, compound **41** generated 117 pmol equivalent/mg protein adducts further highlighting the potential risk with this functional group. Thus, identifying a replacement for the P1 vinyl with acceptable potency and an improved drug metabolism and pharmacokinetic profile became our next challenge.

The natural peptide substrates of the HCV NS3/4A protease contain a cysteine or threonine at P1, highlighting the small nature of the S1 pocket[43]. While replacing the P1 vinyl (**37**) with an ethyl (**38**) on the 3-methyl pyrrolidine series improved the predicted clearance from 0.64 to 0.40 L/h/kg, the larger ethyl group resulted in an approximate 5-fold loss in both biochemical and cellular GT3 potency. We considered that improved oxidative metabolic stability with replacement of the vinyl group at P1 was an initial indicator of a positive direction for removing reactive metabolites. Significantly improved metabolic stability could be realized by adding a fluoro (**39**) or difluoro (**40**) to the P1 ethyl group (Pred CL of 0.20 and <0.17 L/h/kg, respectively), but at the expense of further compromised GT3 potency. A similar trend in metabolic stability improvement and GT3 potency loss was obtained in comparing the P1 vinyl (**41**) to P1 ethyl (**42**) on the 3-ethyl pyrrolidine series. Compound **43**, wherein P1 is truncated to a methyl, had improved metabolic stability vs the vinyl **41**, but lost significant GT3 biochemical potency (~10-fold). Adding two fluorines to the P1 methyl in an attempt to further improve metabolic stability afforded compound **44**, which indeed had substantially improving metabolic stability (Pred CL = 0.20 L/h/kg) while also maintaining the potency of the P1 vinyl containing compound **41**. Thus, the P1 difluoromethyl substituent appeared to be a viable replacement for the reactive vinyl group wherein GT3 potency was maintained and metabolic stability was improved with compound **44**.

Table 4
Impact of P1 (R^1) and P2 (R^2) modifications on GT3 potency and human microsomal stability.



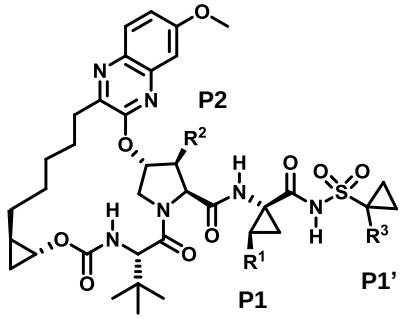
Cpd	R^1	R^2	R^3	GT3 Ki[a] (nM)	GT3a EC_{50}[b] (nM)	Pred CL HLM[c] (L/h/kg)
37	Vinyl	Me	H	0.094	41	0.64
38	Et	Me	H	0.530	188	0.40
39		Me	H	1.3	419	0.20
40		Me	H	0.92	375	<0.17
41	Vinyl	Et	H	0.081	20	0.84
42	Et	Et	H	0.563	272	0.44

43	Me	Et	Me	0.838	109	0.67
44	CF_2H	Et	H	0.043	27	0.20

[a]Ki determined by enzymatic assay using an HCV genotype 3a NS3/4A protein
[b]EC_{50} determined by cell based assay using RLuc cells harboring subgenomic genotype 3a replicon
[c]Pred CL HLM = human metabolic clearance predicted from microsomal stability

After finding the P1 difluoromethyl provided good potency and stability as a vinyl replacement, we sought to understand the effect of P1 groups on preclinical pharmacokinetics. We envisioned combining our NS3/4A PI with sofosbuvir and velpatasvir in an STR, which would require pharmacokinetics consistent with once-daily dosing and a projected daily dose amenable to combination with sofosbuvir (400 mg) and velpatasvir (100 mg) into a single tablet. As the liver is the primary site of HCV replication, we also considered preferential distribution to the liver to be a favorable attribute. The combination of 3-substituted pyrrolidines with P1 modifications had a profound impact on pharmacokinetic properties. While P1 vinyl containing compound **41** had a favorable volume of distribution (**Table 5**, Vss 1.9 L/h/kg) and high liver concentration (20.9 μM at 6 h post dose) in rats, the poor metabolic stability in human microsomes (Pred CL = 0.87 L/h/kg) and high level of protein adduct formation (117 pmol/mg protein) precluded advancement of this compound. Replacement of the P1 vinyl (**41**) with ethyl (**42**) improved the Vss, half-life and liver distribution in rats, but as already noted, P1 ethyl substitution was deleterious to GT3 potency. Much to our dismay, the P1 difluoromethyl group (**44**) that we found to both improve metabolic stability and maintain GT3 potency also came with a detrimental impact to V_{ss} (1.0 L/kg), half-life (0.8 h) and liver concentration (1.2 μM at 6 h) compared to the vinyl comparator **41**. We hypothesized that decreased hydrophobicity surrounding the polar acylsulfonamide motif may contribute to the reduced Vss. While modifications to the R^3 position on P1' would appear a logical position to derivatize, our previous efforts on a related scaffold[44] as well as prior literature[45] highlighted limited tolerance for P1' derivatization and that small hydrophobic groups were generally preferred. Consistent with expectations, P1' analogs wherein R^3 was ethyl, ally, $-CH_2CH_2OH$, $-CH_2CH_2OBn$, or $-CH_2CN$ all lost >3-fold cellular activity when investigating the unsubstituted pyrrolidine core (data not shown). Gratifyingly, R^3 methyl substitution was tolerated for potency on the unsubstituted pyrrolidine core. The P1' methylcyclopropyl substitution was therefore synthesized on our highly optimized scaffold yielding 3-ethyl pyrrolidine (**12)** and 3-methyl pyrrolidine (**11)**, both having potent GT3 cellular potency (23 and 20 nM) and low predicted metabolic clearance (Pred CL of 0.25 and 0.18 L/h/kg). In agreement with our design, addition of a methyl to P1' in compound **12** increased the LogD and improved V_{ss} (1.7 L/kg), MRT (1.4 h) and liver concentration (14.0 μM at 6 h) vs the des-methyl P1' compound **44**. The 3-ethyl pyrrolidine (**12**) proved to have enhanced liver pharmacokinetic properties vs 3-methyl pyrroline (**11**) and was therefore selected for further investigation (liver concentration at 6 h of 3.1 vs 14.0 μM, respectively). Compound **12** was further profiled in protein adduct studies to probe our hypothesis that replacement of the P1 vinyl with a metabolically blocked difluoromethyl would reduce protein adducts. Indeed, compound **12** had a >10-fold reduction in radiolabeled protein adduct formation compared to the vinyl containing comparator **41** (10 vs 117 pmol/mg protein, respectively).

Table 5
R^1, R^2 and R^3 effect on drug metabolism and pharmacokinetic properties



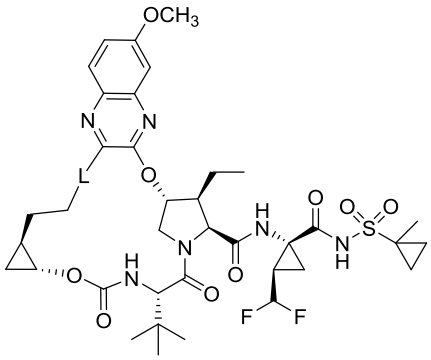
Cpd	R^1	R^2	R^3	GT3a EC_{50} (nM)	LogD	Pred CL HLM[a] (L/h/kg)	Protein adducts (pmol equivalent /mg protein)	Rat CL (L/h/kg)	Rat Vss (L/kg)	Rat $t_{1/2}$ (h)	Rat F (%)	Rat [liver] @6h (μM)
41	Vinyl	Et	H	20	4.1	0.84	117	0.88	1.9	2.2		20.9
42	Et	Et	H	272	4.6	0.44		0.67	3.1	5.0	100	30.2
44	CF_2H	Et	H	27	3.5	0.20		2.0	1.0	0.8	36	1.2
12	CF_2H	Et	Me	23	3.8	0.25	10	1.3	1.7	1.4	31	14.0
11	CF_2H	Me	Me	20	3.5	0.18		2.1	2.2	1.3	100	3.1

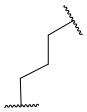
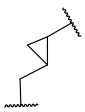
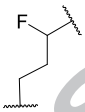
[a]Pred CL HLM = predicted clearance from human liver microsomes

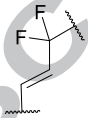
With compound **12** we had improved GT3 potency by introduction of the 3-ethyl pyrrolidine core, increased metabolic stability and decreased radiolabeled protein adduct formation by replacing the P1 vinyl with a difluoromethyl group, and improved the Vss and liver concentration at 6 h by introduction of the P1' methyl. To realize our goal of a low dose once-daily administered compound amenable to an STR with sofosbuvir and velpatasvir, we next focused on further improving the predicted human metabolic stability and GT3 potency. A human microsomal Met ID study was performed on compound **12** wherein three distinct hydroxylated metabolites were observed on the P2 to P4 macrocycle fragment. We hypothesized the benzylic methylene connected to the 3-position of the quinoxaline would be the most readily oxidized position. We were further concerned that if this position were to be hydroxylated a quinone-methide could be formed, based on the position of the methoxy group on the P2 quinoxaline, and might be another source of protein adduct formation. Therefore, a series of metabolic blocks at this position were synthesized to determine if additional gains in metabolic stability could be realized. Upon binding to the protease, this benzylic methylene is also positioned directly above the hydrophobic alkyl portion of R155 so there was also the potential for van der Waals interactions with this amino acid. The two cyclopropyl diastereomer compounds **45** and **46** had slightly improved microsomal metabolic stability consistent with blocking of benzylic methylene oxidation, but both compounds lost significant potency in both biochemical (>25 fold) and cellular assays (>5 fold). The benzylic monofluoro substituted diastereomeric mixture (**47**) maintained GT3 potency and also had a minor improvement in human microsomal stability. Benzylic difluorination of the P2 quinoxaline with an adjacent olefin (a consequence of the ring closing metathesis reaction) afforded compound **48**. While compound **48** had an approximate 4-fold improvement in GT3 potency vs the unsubstituted comparator **12** (5.3 vs 23 nM, respectively), the human microsomal metabolic stability was unchanged (Pred CL = 0.25 L/h/kg). To mitigate concerns the macrocyclic olefin may contribute to metabolic instability and potentially generate reactive metabolites, the olefin was reduced to the corresponding alkylene, affording compound **36**. Indeed, compound **36** had improved metabolic stability in human microsomes (Pred CL <0.17 L/h/kg), which was further supported by a very low Pred Cl of <0.07 L/h/kg in cryopreserved human hepatocytes. Radiolabeled protein adducts were also reduced to <1 pmol equivalent/mg protein for compound **36**, supporting a low risk of clinical ALT elevation and hepatotoxicity. This decrease in adduct formation is

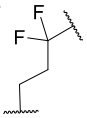
consistent with the possibility of quinone-methide formation if benzylic oxidation were to occur on compound **12**. Compound **36** was also potent in both GT3 biochemical (63 pM) and cellular (6.1 nM) assays. The improved GT3 potency for **36** compared to **12** may result from a hydrophobic interaction between the difluoro substitution on the macrocycle and the alkyl portion of R155, as highlighted in the co-crystal structure (**Figure 3**).

Table 6
Macrocycle modifications (L) to improve human microsomal stability and GT3 potency



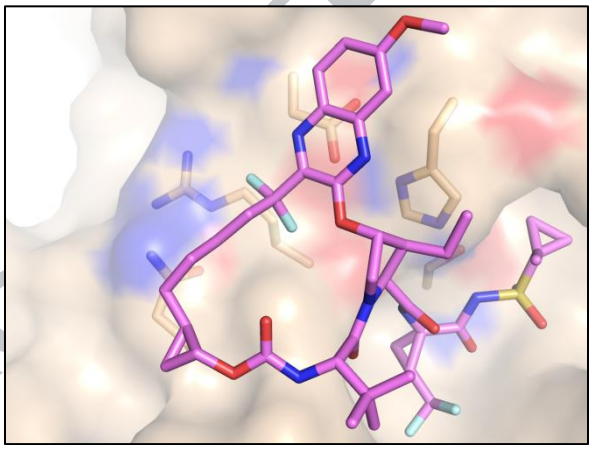








	12	45[d]	46[d]	47	48	36
GT 3 K_i[a] (nM)	0.067	1.7	9.4	0.13	0.053	0.063
GT 3a EC_{50}[b] (nM)	23	128	829	35	5.3	6.1
Pred CL HLM[c] (L/h/kg)	0.25	0.21	0.23	0.23	0.25	<0.17

[a]Ki determined by enzymatic assay using an HCV genotype 3a NS3/4A protein
[b]EC_{50} determined by cell based assay using RLuc cells harboring subgenomic genotype 3a replicon
[c]Pred CL HLM = predicted clearance from human liver microsomes
[d]Cyclopropyl diastereomers synthesized from trans olefin. Absolute stereochemistry for cyclopropyl diasteromers **45** and **46** not determined.



Compound 36
GS-9857
voxilaprevir

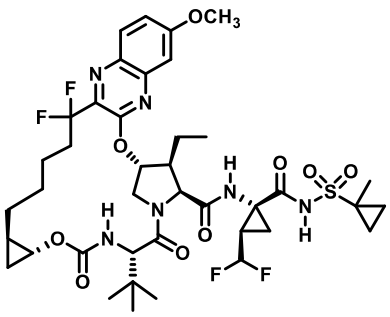


Figure 3. Difluoromethylene/R155 interaction in crystal structure of compound **36** bound to GT3 surrogate (GT1 D168Q) NS3/4A protease (PDB code: 6NZT)

Compound **36** demonstrated potent antiviral activity across genotype 1-6a cellular replicon EC_{50} assays (1.5-6.6 nM), with less than a 2-fold shift between GT1a (3.9 nM) and GT3 (6.1 nM) (**Table 7**). Our optimization strategy invoking interactions with the conserved catalytic triad also proved beneficial

against some common GT1 RASs including Q80K, R155K, and D168E, with potency shifts of less than two-fold (**Table 7**)[46]. As is common with HCV NS3/4A PIs, a high shift in potency was observed with the A156T variant as this amino acid is positioned below the core pyrrolidine motif present in nearly all approved NS3/4A PIs. Despite a high level of protein binding across species (>99%), **36** had a V_{ss} of 0.80-0.94 L/kg and the distribution of **36** into liver was high with liver to plasma ratios of >50-fold across rat, dog and cynomolgus monkey (**Table 8**). Additional *in vitro* assays were performed to assess the potential for preferential distribution to the liver in humans. Rapid time-dependent uptake of compound **36** into cryopreserved human hepatocytes as well as organic-anion-transporting polypeptide (OATP) 1B1/1B3 over-expressing Chinese hamster ovary (CHO) cells was also observed, supporting an expectation for preferential liver distribution of compound **36** in humans. Compound **36**, now known as voxilaprevir (GS-9857, VOX), was selected for development due to its potent antiviral activity across genotypes, improved activity against common GT1 RASs, decreased risk of hepatotoxicity, projected long half-life in humans, and potential for combination in an STR.

Table 7
Compound **36** pan-genotypic potency and activity against GT1 NS3/4A RAS

	Genotype (wild-type)								RAS			
	1a	1b	2a	2b	3a	4a	5a	6a	1a Q80K	1a R155K	1b D168E	1a/1b A156T
EC_{50}[a] (nM)	3.9	3.3	3.7	6.6	6.1	4.9	1.9	1.5	3.1	2.7	4.0	>500

[a]EC_{50} determined by cell based assays using RLuc cells harboring subgenomic genotype replicons of the corresponding genotype

Table 8
Drug metabolism and pharmacokinetic profile of compound **36**

	In vitro		*In vivo*[a,b]				
	% Free plasma	Pred CL* (L/h/kg)	CL (L/h/kg)	V_{ss} (L/kg)	$t_{½}$ (hr)	F / F_a (%)	Liver:plasma ratio, @2h
Rat	0.42	0.39	0.82	0.84	0.93	83 / 100	>500x
Dog	0.28	0.17	0.19	0.94	4.2	27 / 30	55x
Monkey	0.91	1.01	0.89	0.80	2.1	10 / 23	100x
Human	0.46	<0.07					

[a]Intravenous administration in rats at 1.0 mg/kg, dogs at 0.5 mg/kg, and in cyno at 0.5 mg/kg
[b]Oral administration in rats at 3.0 mg/kg, dogs at 1.0 mg/kg, and in cynomolgus monkey at 1.0 mg/kg
*Pred CL = predicted clearance from liver microsomes of rat, dog and monkey, and from hepatocytes from human
F = bioavailability
F_a = fraction absorbed[47]

Voxilaprevir was advanced into a Phase 1 double-blind, placebo-controlled, single-and multiple-dose study to evaluate the safety and pharmacokinetics at 30, 100 and 300 mg. Overall, voxilaprevir was safe and well tolerated at all doses with no clinically significant abnormalities. Consistent with preclinical optimization efforts focused on high metabolic stability, voxilaprevir had long median steady-state half-

lives of 28 to 41 h, supporting once-daily dosing. Dose-proportional exposure was observed over the dose range tested after single- or multiple-dose administration (**Figure 4**). In a Phase 1b trial evaluating voxilaprevir as monotherapy in GT1-4 HCV infected patients, potent antiviral activity was observed with median maximal viral load reductions of >3 $\log_{10}$ IU/mL at the 100-mg dose across all genotypes[48]. Therefore, the 100-mg dose of voxilaprevir was selected for further evaluation in combination with sofosbuvir (400 mg) and velpatasvir (100 mg) in Phase 2 and 3 clinical studies.

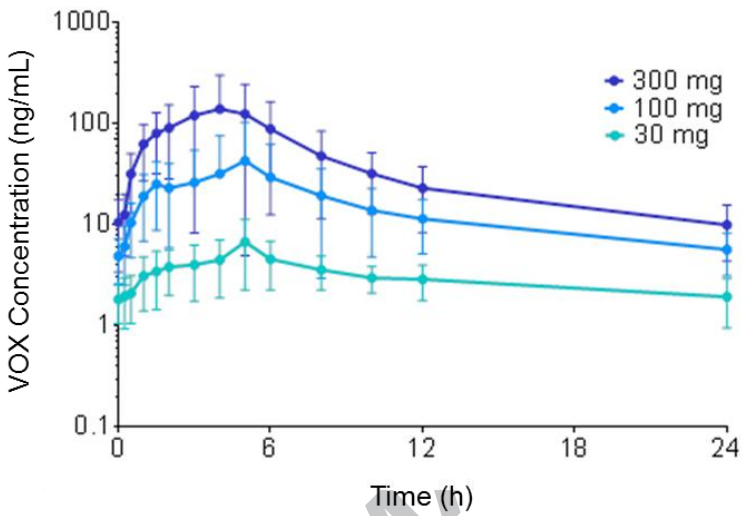


Figure 4. Voxilaprevir (VOX) PK in healthy volunteers after seventh daily dose.

The main goals of the clinical development program investigating voxilaprevir in combination with sofosbuvir and velpatasvir (SOF/VEL/VOX) were to 1) demonstrate that SOF/VEL/VOX is a highly effective and safe therapy for the most difficult-to-cure patients who had previously failed DAA-based regimens and 2) assess the possibility of shortening treatment duration for DAA-naïve patients. Two Phase 3 studies (POLARIS-1 and POLARIS-4) were conducted to assess the antiviral efficacy, safety, and tolerability of SOF/VEL/VOX administered for 12 weeks in DAA-experienced patients with and without compensated cirrhosis. POLARIS-1 was a placebo controlled study investigating SOF/VEL/VOX in NS5A inhibitor experienced patients harboring genotype 1 to 6 chronic HCV infection. POLARIS-4 was an open-label study assessing SOF/VEL/VOX and SOF/VEL in GT1-4 DAA-experienced patients who had not previously received a NS5A inhibitor. In the combined POLARIS-1 and POLARIS-4 studies, the overall SVR rate was 97% in DAA-experienced patients treated with SOF/VEL/VOX, and high SVR rates were observed irrespective of genotype, prior DAA regimen, or presence of pre-existing RASs[49]. To assess whether the addition of VOX to the SOF/VEL regimen would allow shortening treatment duration for DAA-naïve patients, the POLARIS-2 and POLARIS-3 studies were conducted. POLARIS-2 and POLARIS-3 were open-label studies which assessed the antiviral efficacy, safety, and tolerability of 8 weeks of SOF/VEL/VOX compared with 12 weeks of SOF/VEL in DAA- naïve patients with chronic HCV infection. Overall, 611 DAA- naïve patients with and without compensated cirrhosis were treated with 8 weeks of SOF/VEL/VOX and the overall SVR12 rate was 95%[50]. Treatment-emergent resistance following treatment with SOF/VEL/VOX was uncommon in both the DAA-experienced and DAA-naïve populations consistent with the regimen having a high barrier to resistance. In the combined Phase 3 studies, over 1000 GT 1-6 HCV-infected patients were treated with SOF/VEL/VOX as a once-daily STR. No treatment-related serious adverse events were reported. Gratifyingly, and consistent with the preclinical optimization efforts of voxilaprevir directed at reducing reactive metabolites as described herein, no safety signal of hepatotoxicity was observed.

In summary, discovery efforts focused on forming favorable binding interactions with the conserved catalytic triad of the HCV NS3/4A protease resulted in compounds with improved genotype 3 potency. Additional modifications to the macrocycle resulted in further improved potency. To mitigate risk of clinical hepatotoxicity associated with ALT elevations, distinct structural modifications were introduced that drove protein adduct formation to the lower limit of detection (<1 pmol equivalent/mg protein). Consistent with this strategy, hepatotoxicity was not been observed in Phase 3 clinical trials with voxilaprevir involving over 1000 patients. The potent pan-genotypic activity, safety, and low once-daily dose has enabled voxilaprevir to be combined with sofosbuvir and velpatasvir into a once daily STR treatment for HCV infection known as Vosevi®, approved mid-2017 in the both the US and Europe. Vosevi® represents a treatment option for some of the most difficult to cure patient populations who have failed previous DAA containing regimens.

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