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Evolution of HCV NS4B Inhibitors

Giuseppe Manfroni and Rolando Cannalire

Contents

Abstract NS4B has remained for a long time an undisclosed target within the HCV drug discovery programs. However, impressive drug discovery efforts from 2005 to 2016 led to the identification of different chemical classes targeting NS4B as effective anti-HCV agents, and some of them act by impairing AH2-mediated membranous web formation or RNA-binding property. This book chapter aims to discuss research published on NS4B inhibitors focusing on hit identification and hit-to-lead optimization, also with respect to pharmacokinetic properties and structure-activity relationships raised for the different chemical classes taken into account. To date, the only clinical trial conducted with molecules targeting NS4B was focused on clemizole hydrochloride. However, even if NS4B ligands are not currently used in therapy, they can serve in the near future as new weapons to combat resistance to the current therapy.

G. Manfroni (✉) and R. Cannalire
Dipartimento di Scienze Farmaceutiche, Università degli Studi di Perugia, Perugia, Italy
e-mail: giuseppe.manfroni@unipg.it

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1 HCV NS4B Protein and Related Biochemical Assays to Discover New Binders

1.1 Structure and Function of NS4B

The NS4B protein characterization and functions have been already described earlier in this book and highlighted a key contribution of the protein in HCV replication and also cell transformation. However, before presenting the evolution of HCV NS4B inhibitors, a historical reconstruction of studies conducted on this protein deserves further analysis.

Studies on NS4B were first conducted at the end of the twentieth century when its subcellular localization was determined as a first step toward the understanding of its function [1, 2]. Indirect immunofluorescence and green fluorescent protein fusion experiments determined that NS4B is cytoplasmically localized in the perinuclear region where it adopts chicken wire-like and speckled patterns typical of a membrane-associated protein [1–3].

In 2001, Moradpour D. and co-workers published results on the subcellular localization of NS4B employing continuous human cell lines inducibly expressing NS4B, either individually or in the context of the entire HCV polyprotein [4]. In this study it was demonstrated that the majority of the protein was an integral endoplasmic reticulum membrane protein oriented toward the cytoplasm. Although NS4B was computationally predicted as an internal transmembrane protein with four or six domains, authors were unable to experimentally demonstrate the presence of transmembrane or lumenal fragments. These studies revealed that the protein had properties of a cytoplasmically oriented integral membrane protein, and it was observed that 40–70% of NS4B protein sedimented after in vitro transcription-translation in the presence of microsomal membranes.

Later, in 2003, Persson M. and co-workers described more in-depth the cellular location and topology of NS4B [5]. Their studies confirmed previous findings, but researchers also observed new membrane structures visible by immunofluorescence in cells expressing NS4B. The protein was found not only associated to endoplasmic reticulum (ER) but also able to promote rearrangement of intracellular membranes as a result of an intrinsic property. Furthermore, a computer analysis of the protein, conducted on gt 1a, predicted four transmembrane domains (TMDs) with both the C- and the N-terminal tails located in the cytoplasm. The topology organization was further refined through glycosylation studies; observing glycosylation at specific residues of the protein, researchers demonstrated that five TMDs were present instead of the four computationally predicted. Thus, the N-terminal tail of NS4B was thought to be translocated across the ER membrane by a posttranslational

mechanism. Although the dual topology of viral proteins is not unprecedented, for the first time, the work by Persson and colleagues demonstrated this behavior for HCV NS4B.

After the abovementioned key contributions, many scientists have been continuing to improve our understanding of NS4B, thus highlighting structural features and properties of this protein. In 2004, Glenn and co-workers reported on the identification of an N-terminal amphipathic helix (aa 6–29), now called AH1, responsible for NS4B membrane association, correct localization of the HCV replication complex proteins, and RNA elongation [6]. In 2009, two other helical regions (aa 42–66 at N-terminal and aa 229–253 at C-terminal) were successively identified and described by Moradpour and co-workers [7, 8]. The new alpha-helices (initially called AH2 and AH3, which was later called H2) were ascribed as important elements for NS4B membrane association and HCV replication complex formation. To note that the existence of AH1 segment was debated for some years until, in 2014, a key contribution confirmed the presence of an amphipathic α-helix between aa 4–32, with positively charged amino acid residues flanking this structural element and important in membranous web (MW) formation and RNA replication [9].

In summary, NS4B is a predominantly hydrophobic transmembrane protein possessing a multifunctional role within HCV replication. The protein has an N-terminal part (aa 1–69) and a central core with at least four predicted TMDs (aa 70–190) and a C-terminal part (aa 191–261) [4, 5, 8]. The NS4B N-terminus consists of two amphipathic α-helices, AH1 (aa 6–29) and AH2 (aa 42–66), with the last segment which is well conserved in all HCV genotypes (gts) and critical for HCV replication [6, 7, 9]. The N-terminal, oriented on the cytosolic face of ER, seems to cross the membrane yielding the fifth additional TMD also called TMX and promote AH1 translocation into the ER lumen [5]. The N-terminal translocation seems induced by NS4B dimerization/multimerization that promotes lipid vesicle aggregation and in turn seems to play an important role in MW formation for the recruitment of the HCV RNA replication complex [6–8, 10, 11]. Indeed, NS4B plays a structural role in HCV RNA replication complex formation due to the ability to reorganize the intracellular membranes into new membranous structures (e.g., MW) [6, 8, 11]. The NS4B central region harbors a nucleotide-binding motif (NBM) (Walker A motif) located between TM2 and TM3 domains (aa 129–135) with the typical "GXXXXGK" motif of a NTPase [5, 12]. Found in almost all HCV gts, the NBM Walker A permits binding and hydrolysis of GTP and ATP and the synthesis of ATP and AMP from two ADP molecules [13], and it is essential for HCV life cycle, as shown by mutagenesis studies [12]. Although the precise role of the NBM-mediated NTPase activity remains still unclear, it has been proposed that the NS4B GTPase activity plays an important role in cell transformation and tumor formation [14]. The analysis of the secondary structure of NS4B C-terminal reveals two α-helices, named H1 and H2 [8]. The first helix extends from residue 200 to 213 and is highly conserved among HCV gts, while the second is a less conserved twisted amphipathic α-helix composed of aa 229–253 as demonstrated by the 3D NMR structure (PDB code 2KDR) [11, 15, 16]. H2 (originally called AH3) mediates membrane association and it is also involved in the formation of a functional HCV

replication complex [8, 17, 18]. In addition, the C-terminal domain includes arginine-rich motifs at residues 192–193 and 247–248 able to bind the 3′ end of the HCV ss-(−)-RNA, an essential property for efficient in vitro viral replication [19]. NS4B C-terminal has also two palmitoylation sites at two terminal cysteine residues (aa 257 and 261) probably involved in NS4B oligomerization, but the role of C-terminal palmitoylation of NS4B in the HCV life cycle still remains unclear [20].

Attempts to express, purify, and realize membrane-associated NS4B constructs for subsequent studies of the 3D protein structure were conducted by Böckmann and co-workers, but unfortunately these studies did not lead to a definitive structure and thus deserve further investigations [21]. In 2017, Bartenschlager and collaborators reported on the characterization of conserved glycine-zipper motifs within NS4B TM helices 2 and 3 involved in NS4B self-interaction and that contribute significantly to HCV-induced membrane rearrangements, crucial for HCV replication [22]. To date, several functions are attributed to NS4B and can be summarized as follows: (a) recruitment of lipid raft from intracellular membranes, (b) MW formation through a remodeling of ER, (c) effects on HCV RNA translation, (d) modulation of NS5B RdRp activity, and (e) immunomodulation and malignant cell transformation that can in part explain the ability of HCV to facilitate the development of hepatocellular carcinoma [3, 23].

1.2 Screening Assays to Discover NS4B Ligands

The transmembrane nature of NS4B represents a challenge for protein expression and biochemical and structural characterization and has hampered the development of quick screening. Thus, the most prominent approach to discover NS4B ligands entailed the use of a phenotypic approach based on high-throughput screening (HTS) campaigns employing HCV replicons. The protein was successively identified as a target, carrying out genetic validation based on the identification of mutations in HCV genome sequence after compound exposure. All the compounds identified using this approach are discussed in the next section.

Nonetheless, some in vitro assays based on biophysical and/or biochemical HTS methods have been applied over the years and include (1) a microfluidic RNA-binding inhibition assay [19], (2) an AH2-mediated lipid vesicle aggregation inhibition assay [24], (3) a quenching fluorescence binding assay [25], and (4) a nontraditional approach based on encoded library technology (ELT) [26].

The microfluidic affinity assay has been developed by Glenn and co-workers with the aim to study the HCV RNA-binding properties of NS4B and, subsequently, to evaluate the capability of small molecules to inhibit the NS4B-RNA complex formation [19]. Interestingly, this assay was advantageously used in a HTS procedure which evaluated 1,280 compounds and led to the identification of 18 potential

hit compounds including clemizole hydrochloride (see Sect. 2.1). In this assay a flow layer containing fluorescently labeled HCV RNA is delivered through a chamber where a static layer formed by immobilized NS4B protein can bind the viral RNA. The increase of unbound RNA is related to the reduced affinity of NS4B for the RNA induced by the inhibitor. Its ability to interfere with the NS4B-RNA binding is expressed as IC_{50}, measured through a method based on mechanical trapping of molecular interactions [19].

Glenn and colleagues have also published a NS4B AH2-mediated lipid vesicle aggregation inhibition assay to evaluate the ability of small molecules to inhibit the NS4B-mediated MW formation, one of the key functions of the viral protein [24]. This assay consists of two consecutive experiments based on different biophysical methods: (1) the fluorescence microscopy and (2) the dynamic light scattering. In the first step, the aggregation of fluorescently labeled synthetic lipid vesicles upon addition of a synthetic AH2 peptide was monitored by fluorescence microscopy, and the intensity of fluorescence was compared in the absence and in the presence of the tested compound. Molecules able to reduce lipid vesicle formation passed to the second screen. Thus, dynamic light scattering measurements of lipid vesicle size were performed in the presence of a compound, and the inhibition activity was definitively confirmed. Finally, the best compounds were further analyzed in transient replication assays showing inhibition of HCV replication in a dose-dependent manner. This approach led to the identification of anguizole and an amiloride analogue as promising starting point for further developments (see Sects. 2.2 and 2.6, respectively). Detailed analysis of the aforementioned compounds revealed that AH2 function can be disrupted by either one of the two mechanisms: inhibition of NS4B AH2 oligomerization or inhibition of the ability of AH2 to associate with membranes.

The quenching fluorescence binding assay is based on measuring fluorescence variations of a recombinant NS4B upon ligand binding, allowing also for the determination of K_D. This assay was exploited by Chunduru and collaborators who identified and patented different anti-HCV compounds targeting NS4B (see Sect. 2.6) [25].

Thompson and co-workers at GlaxoSmithKline have advantageously exploited ELT to screen an unprecedented large collection of small molecules as N4SB binders (see Sect. 2.6) [26]. Several combinatorial libraries were built by conjugating drug-like building blocks with short coding double-strand DNA tags as markers of each chemical library. Split/mix methods were applied to achieve DNA-tagged libraries with wide chemical diversity that were screened by affinity selection on the immobilized NS4B target protein. Bound molecules were first separated from non-bound molecules and then removed by heat elution. After translation of the amplified DNA tagging sequences into reporter protein, chemical libraries containing the NS4B protein binders were indirectly identified. The confirmation of ligands was carried out through the resynthesis of molecules, without the DNA tag, belonging to the identified chemical libraries. Finally, the K_D of each compound was separately determined using a radiolabeled known NS4B ligand in a displacement assay.

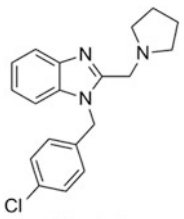
2 HCV NS4B Inhibitors

Until a few years ago, little data was available in the literature about anti-HCV agents targeting NS4B. The last decade has highlighted that NS4B also represents an appealing drug target, thus making this protein one of the last studied targets in HCV drug discovery. Indeed, many hit compounds have been identified by different screening procedures, and hit-to-lead optimization campaigns have been reported. Despite the fact that no drugs acting as NS4B inhibitors have been approved, promising preclinical candidates belonging to different chemical classes have been identified and extensively reviewed [27, 28].

2.1 Clemizole

The old H_1 antihistaminic drug clemizole hydrochloride [29] was one of the first compounds targeting HCV NS4B, identified in 2008 by Glenn and co-workers at Stanford University [19]. In the microfluidic RNA-binding assay, clemizole was shown to be a potent NS4B RNA-binding inhibitor but was a weak inhibitor of HCV replication (Fig. 1) [19, 30]. The discrepancy between biochemical potency and antiviral activity was attributed to low membrane permeability. In addition, NS4B was validated as target of clemizole since two important aa mutations in HCV gt 1b were generated: W55R in AH2 region and R214Q in the cytoplasmic C-terminal segment [19, 30]. Later, clemizole in combination with the first generation of HCV protease inhibitor (i.e., boceprevir or telaprevir) demonstrated a promising synergistic and gt-independent antiviral activity [30].

Successive structure-activity relationship (SAR) campaigns, carried out around clemizole, were not successful, and a number of new analogues possessed undesirable hERG activity [31–34].



Clemizole
IC_{50} (NS4B) = 24 nM
EC_{50} (gt 2a) = 8 μM
EC_{50} (gt 1b) = 23 μM

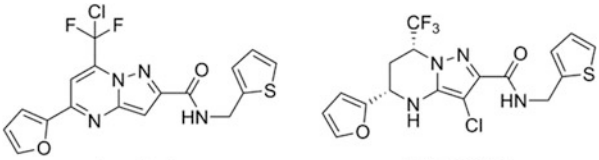
Fig. 1 Structure and activities of clemizole

Thanks to its well-known safety profile, clemizole is the only NS4B inhibitor evaluated into Phase 1B clinical trials in treatment-naïve HCV chronically infected patients (gt 1 and gt 2), but no results have been reported [35].

2.2 *Anguizole and Structurally Related Compounds*

The pyrazolo[1,5-*a*]pyrimidine anguizole (Fig. 2) was discovered in 2005 by Chunduru and colleagues at ViroPharma through the quenching fluorescence binding assay and demonstrated a good NS4B binder [25]. Anguizole was able to reduce HCV protein expression, as determined by an ELISA-based HCV replication assay, without significant toxicity [25, 36]. Later on, Glenn and his team demonstrated that anguizole hampered the interaction of the NS4B-AH2 with lipid vesicles and the lipid vesicle aggregation, thus suggesting a direct binding of the compound to the AH2 region [24, 37]. Furthermore, the molecule was active in a HCV replicon luciferase reporter assay resulting, at that time, in the first NS4B ligand endowed with sub-μM antiviral activity [24]; but the compound was inactive against HCV gt 2a (Fig. 2) [37]. In addition, resistant mutants on NS4B were identified after anguizole treatment of cells carrying the HCV gt 1b replicon, with the H94R mutation being the most common resistance mutation; the F98L and the V105M mutations were also observed in some HCV colonies [37]. Lee and colleagues had also shown that anguizole interfered with (1) NS4B dimerization/multimerization altering the protein subcellular localization and disrupting MW formation and (2) NS4B/NS5A interaction [38].

Successively, a partly saturated analogue of anguizole (compound **5**, called *AP80978*), having 5*S*,7*R* configuration, was reported by Rice and co-workers, at the Rockefeller University, as a sub-μM HCV replication inhibitor targeting NS4B (Fig. 2) [39].



Anguizole
K_D = 0.622 μM
EC_{50} = 0.63 μM[a]
EC_{50} (gt 1a) = 0.56 μM[b]
EC_{50} (gt 1b) = 0.31 μM[b]
CC_{75} > 100 μM[c]
CC_{50} > 50 μM[d]

5 (AP80978)
EC_{50} (gt 1a) = 0.62 μM
EC_{50} (gt 1b) = 0.63 μM
CC_{50} > 100 μM

Fig. 2 Structure and activities of anguizole and compound **5**. [a] ELISA-based HCV replication assay; [b] HCV replicon luciferase reporter assay. [c] Crystal violet staining-based assay. [d] Cell proliferation reagent WST-1-based assay

Compound **5** was active against gts 1a and 1b but was inactive against gt 2a, and its potency, gt specificity, and resistance profile were very similar to those of anguizole [39].

One of the first examples of a successful hit-to-lead optimization campaign, which led to the identification of a preclinical candidate, was carried out at GlaxoSmithKline on molecules structurally related to anguizole [40–44]. As reported by Shotwell and co-workers in 2012, the project started from the identification of hit imidazo[1,2-*a*]pyridine **6** (Fig. 3), through a cell-based HTS using a HCV gt 1b replicon luciferase reporter assay. It was demonstrated that compound **6** specifically bound to NS4B and induced the production of NS4B-resistant mutants [40]. Interestingly, key mutations were H94N, F98L, and V105M, the same observed for anguizole. Initial modifications on hit **6** entailed the replacement of the C-3 bromine with a chlorine and of the C-5 1*H*-4-pyrazolyl with a 3-furyl, in analogy to anguizole, leading to an equipotent analogue. Optimization of the amide side chain started by replacing the chiral substituted pyrrolidine with a piperidine to yield achiral compounds, while different aromatic and aliphatic heterocyclic rings were explored in place of the 2-thiophenyl ring, which was identified as a main metabolic site by in vitro/in vivo studies.

Among the new compounds, derivative **7** having an oxazolidinone moiety at the amide side chain retained high affinity for NS4B and showed very potent antiviral activity in the low nM range (Fig. 3) [40]. Despite its interesting activity, derivative **7** was still far from desirable pharmacokinetic (PK) properties, being characterized by quick in vivo clearance due to metabolism of oxazolidinone ring [40]. Iterative cycles of optimization focusing on modification of the amide side chain allowed the identification of the piperazinone nucleus as suitable replacement for the piperidinyl oxazolidinone, with *N*-cyclohexyl derivative **8** being a low nM NS4B binder and a good inhibitor of HCV replication (Fig. 3). The presence of the hydrophobic pendant ring was a key feature for obtaining a strong NS4B binding. A 4-hydroxyl substituent was added at the cyclohexyl ring (i.e., **9**, Fig. 3) in an attempt to increase the solubility and to reduce oxidative metabolism on the cyclohexyl ring, observed in compound **8**. Interestingly, compound **9** retained comparable NS4B affinity and HCV replicon activity, with the *anti*-configuration preferred over the corresponding *syn* arrangement. The final round of optimization focused on the C-5 position of the imidazo[1,2-*a*]pyridine nucleus with the aim to replace the metabolically labile furyl ring (data not shown) with different alkyl and/or cycloalkyl groups [40–44]. C-5-Cyclopropyl derivatives, exemplified by compound **10** (known as *GSK8853*), retained the same tight binding to NS4B and comparable anti-HCV activity with respect to parent **9** (Fig. 3) [40–44]. Compound **10** was also characterized by an improved metabolic stability, a better in vivo clearance, and more favorable oral bioavailability in rats and dogs when compared to the direct analogue **9** [40]. Resistance passaging in HCV replicons with compound **10** generated mutants carrying single-point mutations within the NS4B sequence (H94R, F98L, V105M) that were moderately (nearly 30-fold) to highly (nearly 350-fold) resistant.

Due to the good balance between anti-HCV activity and PK properties, compound **10** became the lead within the imidazo[1,2-*a*]pyridine series, and thus its

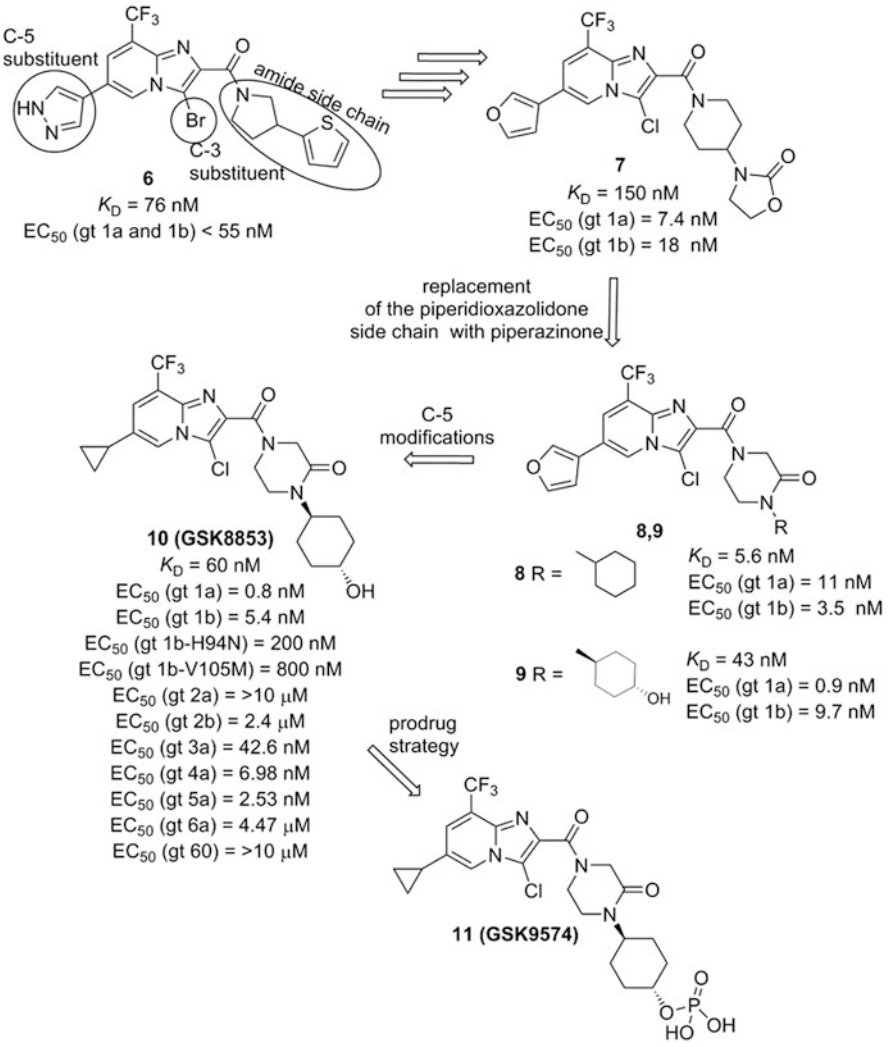


Fig. 3 Structures and activities of representative imidazo[1,2-*a*]pyridines summarizing the chemical optimization process and highlighting the main modification starting from hit **6** to lead **10** and its phosphate prodrug **11**, the first preclinical candidate among the NS4B inhibitors

activity was assessed across a wide panel of different gts showing EC_{50} in the nM range against gts 3a, 4a, and 5a, while it was only a weak inhibitor of gts 2b and 6a. However, compound **10** was inactive against gts 2a and 6o (Fig. 3) [45].

In 2013, Peat A. J. and co-workers reported the in vivo antiviral activity and safety of lead compound **10** [44, 45], but despite improved PK properties, the candidate molecule did not reach an appropriate plasma concentration in rats for preclinical safety studies, due to reprecipitation phenomena. The use of

corresponding phosphate **11** (known as *GSK9574*, in Fig. 3) instead matched the requirements for the in vivo studies, achieving an EC_{90} of 29 nM in PXB mice infected by HCV gt 1a leading to a viral load reduction of 4 log units in a 7-day study [44, 45]. This result provided the first in vivo proof-of-concept that an optimized NS4B inhibitor could be developed into an anti-HCV drug. However, during the 7-day safety study, an adverse cardiovascular event was observed for **10**, and its further development was abandoned [44, 45].

The limitations associated with compound **10**, such as low solubility, decreased activity against NS4B mutants (H94N, F98L, V105M), and the cardiovascular toxicity, prompted researchers at GlaxoSmithKline to engage a strategy based on a isosteric replacement of the imidazo[1,2-*a*]pyridine core with the pyrazolo[1,5-*a*] pyridine, as exemplified by derivatives **12–15** (Fig. 4) [44, 46]. Overall, the pyrazolopyridines possessed (1) acceptable physicochemical properties (measured LogD = 4.1 for **12**), (2) improved NS4B binding affinity, and (3) antiviral activity against wild-type and stable H94N NS4B mutant replicons. To note, the presence of a [3.1.0]bicyclohexane at the piperazinone nitrogen provided the sub-nM anti-HCV

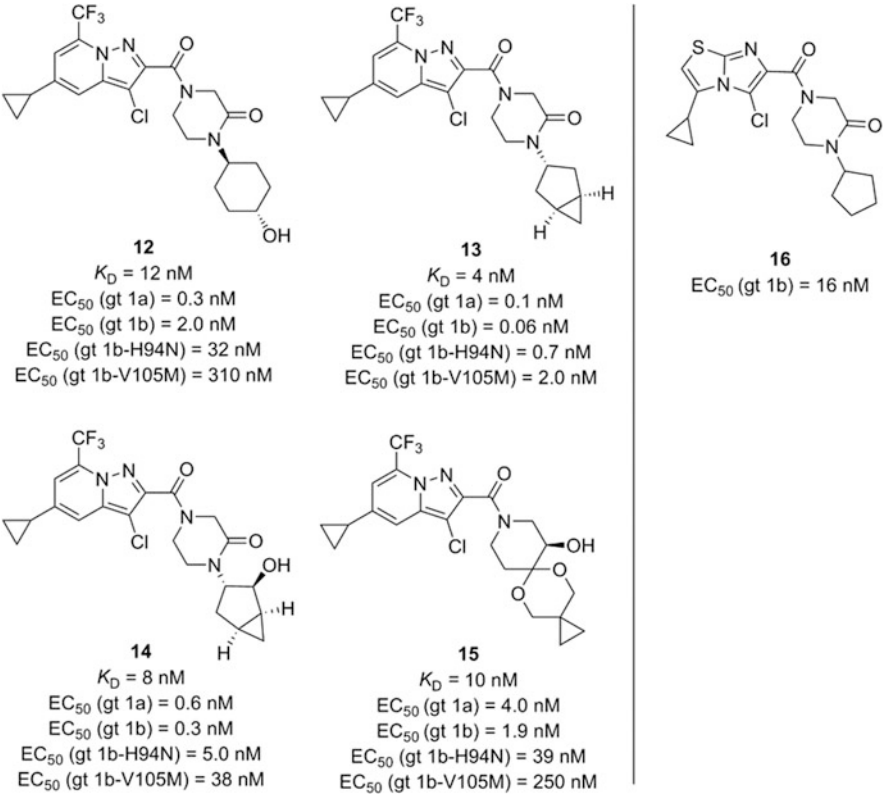


Fig. 4 Structures and activities of representative pyrazolopyridines **12–15** (left) and imidazo[2,1-*b*] thiazole **16** (right)

activity of derivative **13**, highly potent also against the H94N- and V105M-resistant replicons. The dextrorotatory hydroxyl derivative (*S*,*S*,*S*,*S*) **14** was designed in an attempt to reduce the lipophilicity (measured LogDs = 4.6 vs. 6.4, for **14** and **13,** respectively) and simultaneously to improve metabolic stability (in rat clearance of **14** and **13** were 25 and 70 mL min^{-1} kg^{-1}, respectively). Indeed, as shown for imidazopyridines, the insertion of a hydroxyl group reduced metabolism of **14**, compared to its parent analogue **13** without affecting anti-HCV activity. Interestingly, the [3.1.0]bicyclohexanol isomers of **14** bound to NS4B with a similar nM affinity but showed different anti-HCV activity especially against H94N- and V105M-resistant replicon, thus indicating the *S,S,S,S* configuration was the most favored.

Despite an increase in lipophilicity, compound **14** (LogD = 4.6) showed an increased solubility in biorelevant medium in comparison to imidazopyridine lead **10** (LogD = 3.1) [44]. Furthermore, the increased hydrophobicity was commeasured to a higher binding affinity for the target protein. In vitro studies showed also that derivative **14** did not inhibit CYP450 isoforms, and in vivo PK investigation indicated a low-to-moderate clearance across different species, high oral bioavailability, and high plasma concentration [44]. Based on the impressive anti-HCV activity and its very promising in vivo PK profile, compound **14** proceeded into 7-day preclinical safety studies in mice without the need of a prodrug [44], but no results have been reported.

Yu and collaborators, at the Sichuan University, pursued a scaffold hopping approach replacing the imidazo[1,2-*a*]pyridine with the imidazo[2,1-*b*]thiazole nucleus as exemplified by derivative **16** (Fig. 4) [47]. The validation of NS4B as the target was demonstrated by evaluation of compound **16** in an array of known resistant replicons of NS4B as well as of NS3/4A, NS5A, and NS5B. The compound retained the same order of activity in all the replicons with the exception of H94R, F98C, and V105M NS4B mutants. Interesting results were obtained when hit compound **16** was evaluated in association with different DAAs in HCV gt 1b replicon observing synergistic effect with simeprevir, daclatasvir, and sofosbuvir, and an additive effect was demonstrated with clemizole [47].

2.3 6-(Indol-2-yl)pyridine-3-sulfonamides and Related Compounds

Chen and his research team, at PTC Therapeutics, reported a novel anti-HCV chemotype based on the indole core and exemplified by weak HCV gt 1b inhibitor **17** identified through a cell-based HTS (Fig. 5) [48]. At that time, no studies to elucidate the molecular target of the molecule were reported. Chemical optimization based on (1) the replacement of the unsuitable 3-nitro with a cyano group, (2) the shifting of the *para*-methoxyphenyl to the indole C-2 position, and (3) the ethylation of the nitrogen led to more potent derivative **18** (Fig. 5) [48].

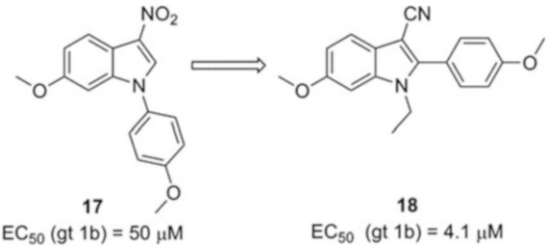


Fig. 5 HTS-derived indole **17** and initial optimization to inhibitor **18**

Then, systematic investigation of different substituents at *N*-1, C-5, and C-6 and at the C-2 phenyl ring provided sub-μM HCV replication inhibitors with SI > 130 as exemplified by representative derivatives **19–24** (Fig. 6) [48]. They are characterized by the presence of an alkylated sulfonamide at the C-2 phenyl ring, in place of the *para*-methoxy group, small linear or cyclic alkyls at the indole nitrogen, and mainly lipophilic fluorinated substituents at C-6 position. Additional SAR information indicated that small alkyls at the sulfonamide moiety were preferred over larger substituents, while polar groups at the C-6 position of the indole core or removal of the substituents were not tolerated. Furthermore, C-5 as well as C-5/C-6 di-substitution gave less active compounds, with the active C-5 fluorine derivative **24** representing an interesting exception (Fig. 6). Also the reverse sulfonamide **25** retained the same potency in the sub-μM range (Fig. 6) [48]. Again, NS4B was still not recognized as the target for this chemical class albeit compound **22**, used as chemical probe, was shown to be inactive against the most-exploited HCV proteins (i.e., NS5B polymerase and NS3/4A protease) [48].

The hit-to-lead optimization process of indoles proceeded in a joint program between PTC Therapeutics and Merck starting from derivative **25** characterized by low solubility and metabolic liability [49]. Furthermore, in vivo production of inactive *N*-sulfonamido dealkylated metabolite was observed when the compound was orally administered in rats. At first, the C-2 phenyl ring was replaced by nitrogen-containing heteroaryl rings, and the 6-(indol-2-yl)pyridine-3-sulfonamide **26** showed the most potent replicon activity coupled with improved in vitro metabolic stability, as demonstrated by human liver microsomal (HLM) clearance evaluation (Fig. 7). Then, more in-depth SAR exploration indicated that the combination of 6-difluoromethoxy/1-cyclobutyl as in compound **27** or 6-cyclopropyl/1-cyclobutyl as in compound **28** granted a good balance between potency and metabolic stability (Fig. 7). The metabolic improvement of these compounds was achieved introducing lipophilic electron-withdrawing groups on the alkyl chain of the sulfonamide moiety to suppress oxidative aminosulfonyl *N*-dealkylation.

Accordingly, compound **29** was characterized by a very good metabolic stability and a potent low nM anti-HCV activity and used as chemical probe to demonstrate the mechanism of action for the whole compound class (Fig. 7) [49]. In fact, NS4B was determined to be the molecular target for the 6-(indol-2-yl)pyridine-3-

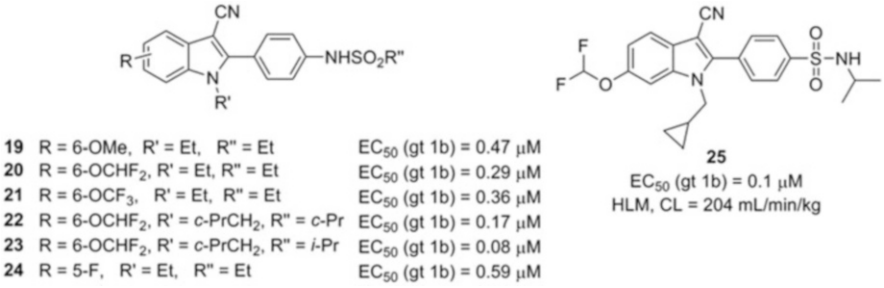


Fig. 6 Structures and activities of representative indoles **19**–**25** summarizing the chemical optimization process

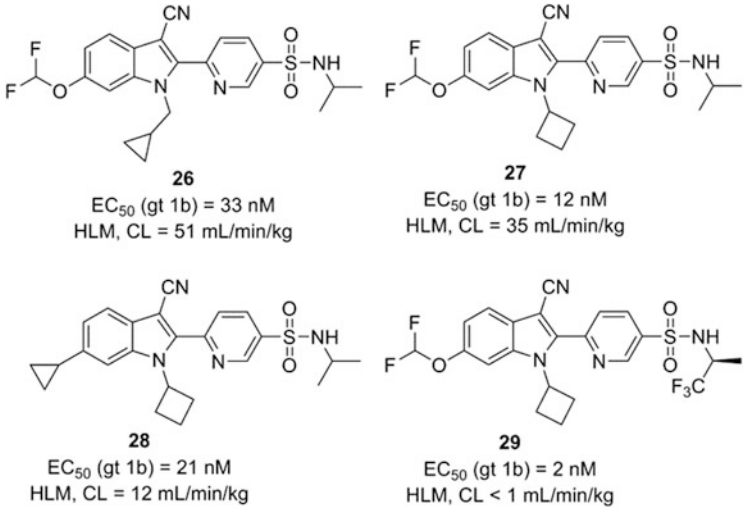


Fig. 7 Structures and activities of representative 6-(indol-2-yl)pyridine-3-sulfonamides

sulfonamide class, employing the replicon assay in which mutations in the NS4B sequence were induced after treatment with compound **29**. The most frequent mutation was a F98L substitution that produced a significant loss in activity (>70-fold). Interestingly, this mutation is localized in the TM1 domain of NS4B, the same region involved in generation of escape mutants identified for anguizole and related compounds.

Successive medicinal chemistry efforts pointed toward a further improvement of the physicochemical/PK properties of derivative **29** [50]. Chemical modifications focused on the benzene ring of the indole core and the *N*-1 substituent. It was observed that the contemporary presence of a fluorine at C-5 and of small lipophilic alkyls at C-6 reduced oxidative metabolism on the indole benzene ring and that the introduction of aryl groups at the *N*-1 position improved oral bioavailability in rats.

The synthesis of analogues containing different combinations of these chemical modifications was pursued leading to a new set of compounds having the 5-F/6-alkyl di-substitution and the pyrimidinyl moiety as *N*-1 aryl substituent, as exemplified by derivatives **30**, **31**, and **32** (*PTC725*) (Fig. 8), and showing high anti-HCV potency, good PK properties, and great metabolic stability.

6-Ethyl derivative **32** emerged as the most promising lead due to its excellent potency and favorable balance in PK properties [50]. Compound **32** was characterized by comparable low nM potency against gt 1a and 1b and a high degree of selectivity; however, it showed significantly less activity against HCV gt 2a. Selection of resistant gt 1b replicons revealed substitutions in NS4B sequence, especially H94R, F98L, and V105M. Retrospectively, the low potency against gt 2a can be explained by the presence of L98 naturally expressed in the wild-type gt 2a. Worthy of note, the lead **32** showed a low nM activity also against HCV gt 3a [51]. Interestingly, combination of compound **32** with boceprevir or VX-222 (non-nucleoside NS5B inhibitor) resulted in an additive or a synergistic effect, respectively, against HCV gt 1b replicon [50]. After oral administration, lead compound **32** showed good PK properties in rats and dogs, but a poor bioavailability was observed in monkeys [50]. Due to its excellent safety profile, it has been advanced into preclinical development, but no further data has been reported.

In another report, a related series of azaindoles has been reported (Fig. 9) [52]. In comparison with the 6-(indol-2-yl)pyridine-3-sulfonamide series, a slight decrease in activity was observed for the 4- or 5-azaindoles (e.g., **33** and **34**, respectively),

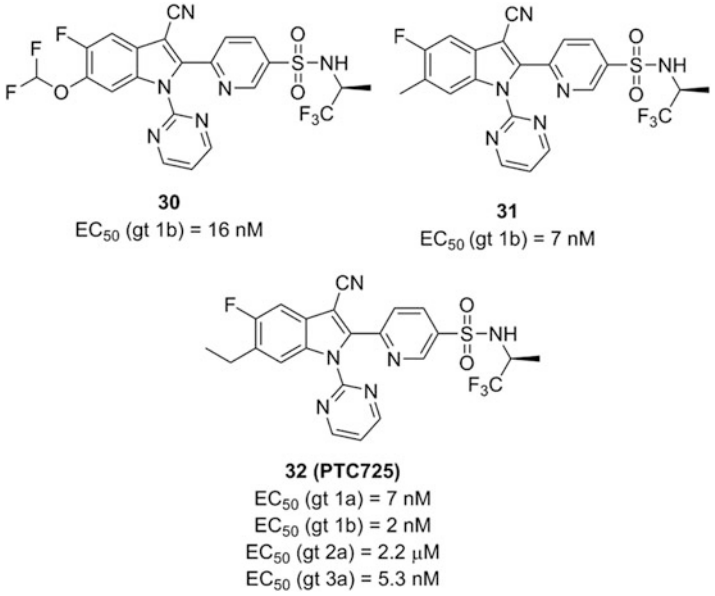


Fig. 8 Structures and activities of representative 6-(indol-2-yl)pyridine-3-sulfonamides summarizing the chemical optimization process leading to preclinical candidate **32** (PCT725)

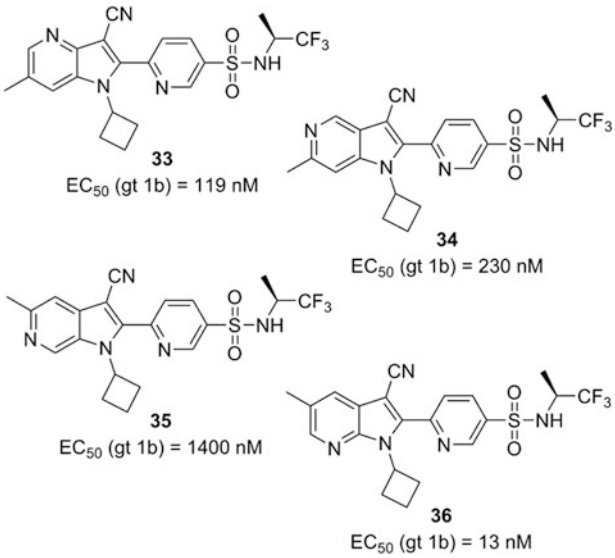


Fig. 9 Structures and activities of 6-(azaindol-2-yl)pyridine-3-sulfonamides

while the 6-azaindole subseries (e.g., **35**) showed a drop in potency. Conversely, potent derivatives, exemplified by compound **36**, were obtained by placing the nitrogen atom at position 7.

A further evolution around the 6-(indol-2-yl)pyridine-3-sulfonamides led to carboxamide analogues (**37–40**) endowed with broader genotypic anti-HCV activity (Fig. 10) [53]. In particular, compounds **38–40** derived from **37**, which was identified as a byproduct during the optimization of lead **32** wherein the indole 3-cyano group was hydrolyzed to a carboxyamide functionality. Biological testing highlighted a reduced but still interesting activity for compound **37**, and then several derivatives were synthesized and tested against HCV gts 1a, 1b, 2a, and 3a. The SAR around the new indole nucleus was thus reevaluated, taking into account that the cyano group may be advantageously replaced by an amide function. However, for the design of new derivatives, the 5-F in combination with a small alkyl or a fluorinated substituent at the C-6 position of the indole was retained in order to reduce the oxidative glutathione conjugation, as learned from the previous SAR studies. Unlike the 3-cyano derivatives, in 3-carboxamides, a cycloalkyl N-1 substituent was favored instead of an aryl group, and substituted phenyl was preferred over a pyridine at the C-2 indole position. As a result, 2-(4-sulfonamidophenyl)-indole 3-carboxamides **38–40** showed the best balance of activity across the gts used, with EC_{50} values ranging from sub-nM to low nM, including gt 2a, and thus serving as a new promising starting point for the identification of NS4B inhibitors with broad anti-HCV gt activity (Fig. 10).

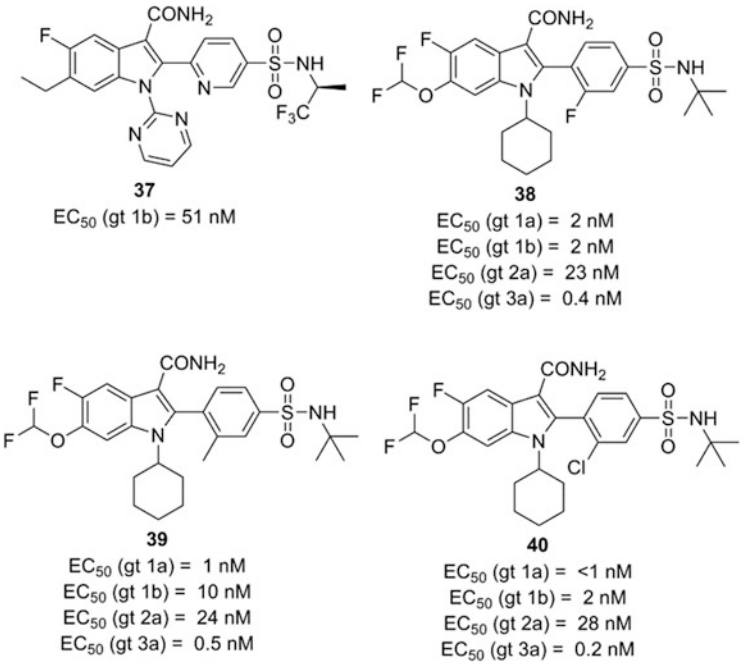


Fig. 10 Structures and activities of representative 2-(4-sulfonamidophenyl)-indole 3-carboxamides

2.4 Piperazinone Derivatives

Kakarla and co-workers, at Pharmasset, reported a new anti-HCV chemotype targeting NS4B, based on the piperazinone scaffold [54]. Exploiting a cell-based HTS (HCV gt 1b replicon luciferase reporter assay) of their in-house library, piperazinone **41** was identified as a promising and selective anti-HCV agent (Fig. 11). Generation of NS4B mutant replicons (residues 90 and 98 in protein sequence) revealed its mode of action. The *S,S* configuration at C-3 and C-6 piperazinone stereocenters and the *trans-R,R* configuration of the (2-phenylcyclopropyl)carbonyl side chain of the starting hit were critical since other modifications were not tolerated.

Preliminary SAR investigation around hit **41** indicated that the endocyclic unsubstituted amide of the piperazinone scaffold was a key pharmacophoric element, and therefore, N-alkylation or carbonyl reduction was detrimental [54–56]. Simplification of the side chain was attempted replacing the chiral cyclopropyl bridge with olefinic or aromatic/heteroaromatic linkers. In particular, a *trans* double bond (**42**) and an isoxazole ring (**43**) were good replacements for the cyclopropyl bridge (Fig. 11); indeed, a *cis* version of **42** or other aryl or heteroaryl groups instead

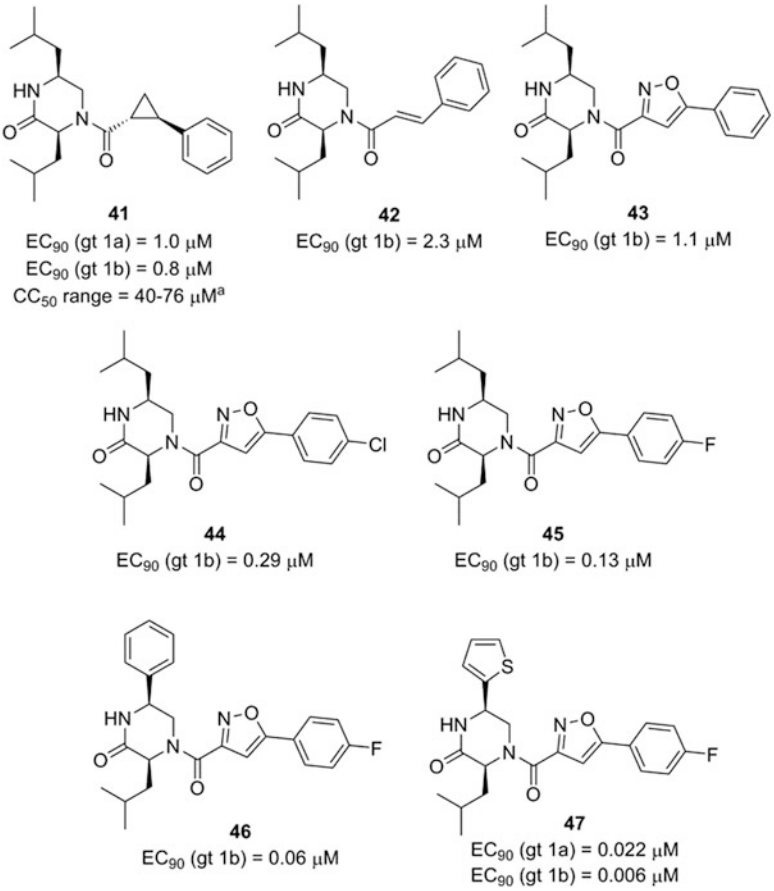


Fig. 11 Structures and activities of representative piperazinones from the HTS-derived hit **41** to compound **47**, the most potent within the series. [a]Range values from four different cell lines (Huh7, HepG2, BxPC3, and CEM)

of the isoxazole abolished the anti-HCV activity. Starting from piperazinones cinnamide **42** and isoxazolylamide **43**, two sets of analogues carrying different substituents at the phenyl side chain ring were prepared. The best results were observed with the isoxazolylamide subseries, as exemplified by the potent *p*-chloro, the *p*-fluorophenyl-substituted derivatives **44** and **45** endowed with sub-μM activity (Fig. 11).

SAR exploration continued by replacing the isobutyl group at the C-6 position of the piperazinone core with either different hydrophobic moieties (acyclic, branched, cyclic, and saturated/unsaturated alkyls) or more polar alkyl and aryl substituents, having H-bond forming properties [54–56]. In general, acyclic and cyclic alkyls were tolerated, while the addition of polar functionalities caused a decrease in antiviral potency. On the contrary, the insertion of unsaturated systems increased

potency as showed by the phenyl analogue **46** and other C-6 heteroaryl analogues, exemplified by compound **47** having a 2-thiophene as C-6 substituent (Fig. 11). Indeed, this compound showed the highest potency in the replicon assay. In addition, derivative **47** showed good activity against HCV gt 1a but was inactive against HCV gt 2. Due to their lack of broad genotype (gt) coverage, the anti-HCV piperazinones targeting NS4B were abandoned by Pharmasset and did not progress into further preclinical development [54].

2.5 2-Oxadiazoloquinoline Derivatives

A series of NS4B inhibitors based on the 2-oxadiazoloquinoline scaffold has been reported by Phillips and collaborators at Gilead Sciences [57]. Noteworthy, the hit-to-lead development of the 2-oxadiazoloquinolines led to derivatives characterized by potent pan-genotypic anti-HCV activity. The initial hit **48** was already endowed with excellent and selective activity against HCV gt 1b, but it was inactive against HCV gt 2a and characterized by high lipophilicity (Fig. 12). Thereby, the optimization strategy adopted aimed at reducing lipophilicity and obtaining anti-HCV gt 2a activity. Modification focused on the replacement of the two phenyl groups at each end of the molecule with more polar and less planar substituents. Thus, the phenyl ring at the aminoxadiazole moiety was replaced by several cycloalkyl ethers with the methylene-1,3-dioxolane moiety emerging as the best one. On the other hand, alkyl ethers (e.g., trifluoroethyloxy) at C-6 position of quinoline core proved to be effective replacements of the C-6-phenyl group. Moreover the C-8-trifluoromethyl on the quinoline nucleus was replaced by a *tert*-butyl group (Fig. 12). As a consequence of these medicinal chemistry efforts, derivative **49** was obtained and compared to **48**, and it showed (1) reduced lipophilicity (LogD = 3.9), (2) significant improvement in anti-HCV gt 2a activity, and (3) a fourfold increase of the potency in gt 1b replicon. As a consequence, compound **49** was submitted to a NS4B binding assay (scintillation proximity assay using a recombinant HCV NS4B gt 1b) and showed a K_D of 31 nM (Fig. 12).

The classical bioisosteric replacement of the oxygen with a NH group in the C-6 substituent further enhanced the anti-HCV activity, as exemplified by compound **50** (Fig. 12). Starting from this latter compound, the methylene-1,3-dioxolane group was replaced by different hydroxy cycloalkyl rings, furnishing potent compounds against both HCV gt 1b and 2a. Indeed, compound **51** and **52** having a hydroxycyclobutyl and hydroxycyclohexyl, respectively, resulted in an impressive increase in the anti-HCV activity against both gt 1b and gt 2a (Fig. 12). Being the most potent derivative, compound **52** was also evaluated in a panel of HCV replicons to assess the activity across a broad range of gts as well as against 1b-resistant mutants displaying high potency against all the replicons included in the study. Finally, PK studies in rats indicated lead **52** as a promising preclinical drug candidate in terms of half-life and oral bioavailability [57]. Overall, among the

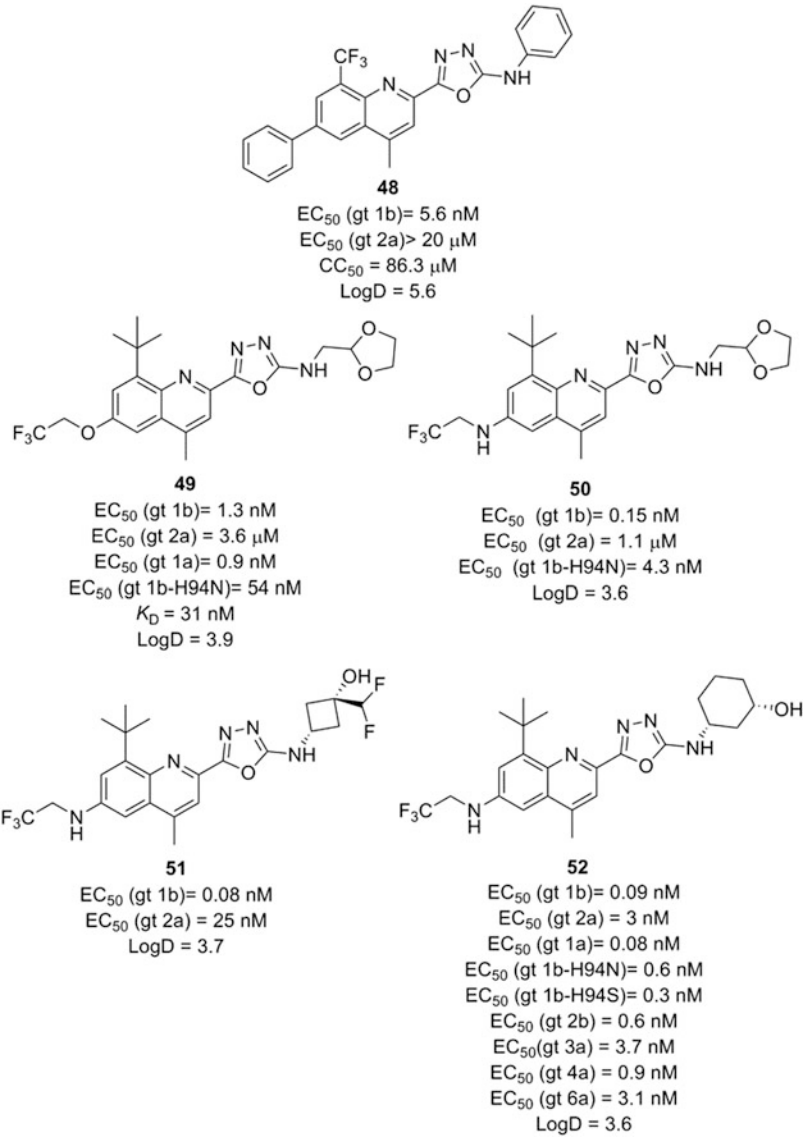


Fig. 12 Structures and activities of representative 2-oxadiazoloquinolines summarizing the chemical optimization process from the initial hit **48** to pan-genotypic anti-HCV lead **52**

NS4B binders, **52** can be considered to be the most potent and have the broadest anti-HCV activity reported so far.

2.6 Other NS4B Binders

Other NS4B ligands have been reported in literature without any information regarding a systematic chemical optimization.

For example, the quenching fluorescence binding assay was exploited by Chunduru and colleagues to identify not only anguizole but also compounds **53**–**59** (Fig. 13) [25]. Albeit these compounds bound NS4B with low μM affinity, only for the triazinoindole derivative **58** were mutations in NS4B sequence (K52R, G120V, A210S) generated [25].

Through the AH2-mediated lipid vesicle aggregation inhibition assay, Gleen and co-workers identified a series of amiloride analogues, exemplified by compound **60**, which were able to inhibit HCV replication in both gt 1b and gt 2a without showing significant cytotoxicity (Fig. 14) [24, 58].

An ELT screening approach using immobilized NS4B was pursued by Thompson and collaborators at GlaxoSmithKline [26]. For this study, 28 libraries containing one million to eight billion compounds were screened, and two families of NS4B binders were identified. The first was dominated by the bipiperidyl-triazine scaffold and the other by the spiro-diazaundecane pyrimidine core. Authors focused on the

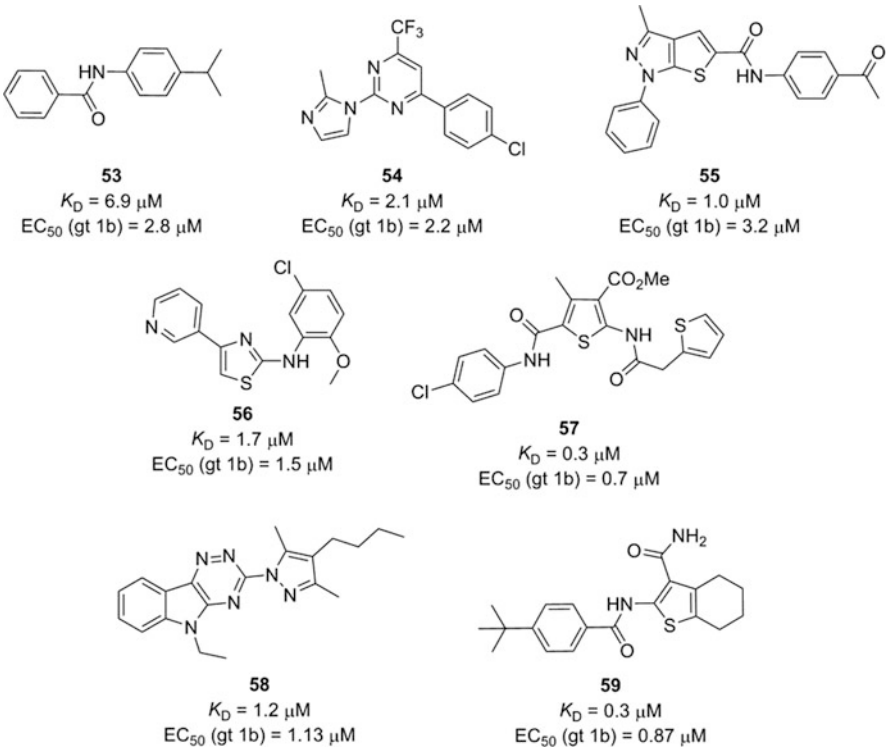


Fig. 13 Structures and activities of compounds **53**–**59**

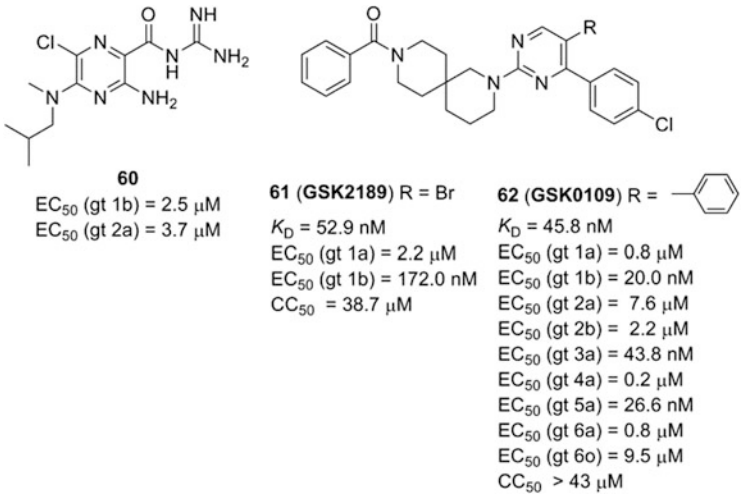


Fig. 14 Structures and activities of amiloride **60** and of ELT-derived compounds **61** and **62**

latter compound class identifying compound **61** (*GSK2189*, Fig. 14) resulting the most potent ELT-derived hit able to bind NS4B with high affinity. Moreover, the hit compound exerted good potency against HCV gt 1b, while moderate to weak activity in gts 1a and 2a was observed, respectively.

Early optimization focused on obtaining a wide gt coverage led to derivative **62** (*GSK0109*, Fig. 14) endowed with a good biological profile against gts 1a and 2a. Interestingly, compound **62** was found to be a potent HCV replicon inhibitor of gts 3a and 5a, a good inhibitor of gts 4a and 6a, and a modest inhibitor of gts 2b and 6o. Mutational studies highlighted that the activity of compound **62** was sensitive to F98L alteration, while it was still active in replicons carrying H94R and V105M mutations.

3 Final Overview and Future Directions

Over the years, the NS4B function and structure have been investigated, and new anti-HCV agents targeting this viral protein have been reported. However, the NS4B membranous nature hampered its purification and crystallization; thus the lack of structural data on NS4B did not allow structure-based drug design programs.

Nonetheless, some biochemical assays have been developed to identify NS4B ligands able to block either the RNA-binding activity (microfluidic affinity assay) or

the MW formation (AH2-mediated lipid vesicle aggregation inhibition assay). Also the innovative ELT approach has been exploited toward identification of NS4B binders. However, most of the promising compounds described so far were derived from hits identified through HCV replicon-based HTS, followed by genetic validation of NS4B as target. The information available about mutation sites are depicted in Fig. 15 [27].

The currently available direct-acting antiviral combinations for chronic HCV treatment are definitely breakthroughs in the field of life sciences. Indeed, these new therapies are able to cure the infection (SVR > 90%), but sooner or later, these therapeutic regimens will have to deal with the development of drug resistance, thus making necessary the use of new DAAs. To note that, the new drugs are less effective against HCV gt 3, while some NS4B binders (i.e., **32**, **37**, and especially **52**) proved to be efficacious also against this gt. In this context, the development of drugs targeting NS4B is certainly of great interest.

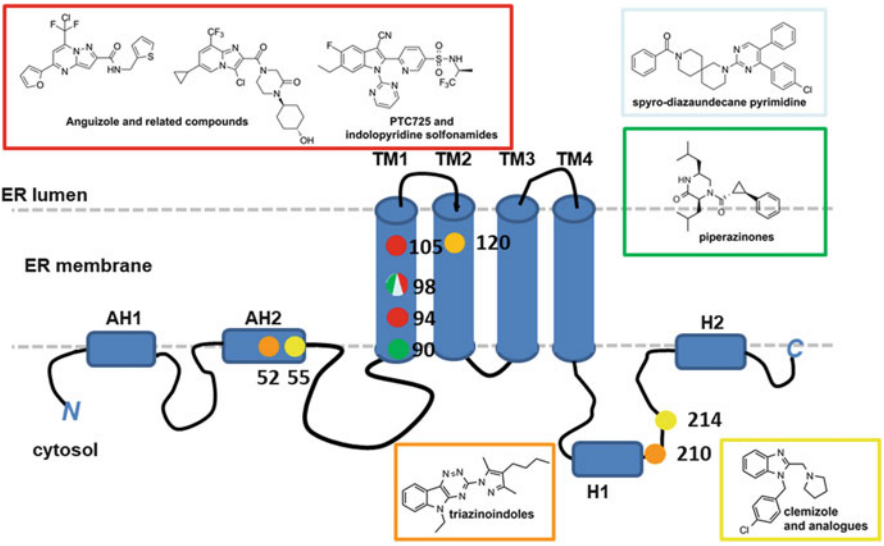


Fig. 15 Schematic representation of NS4B structure showing the mutation sites that confer drug resistance to representative ligands. Amino acid changes associated with resistance are depicted in color-coded circles according to the different ligand classes: (1) H94R, F98L, and V105M mutations at the TM1 segment confer resistance to anguizole and related compounds as well as to other structurally unrelated chemical families (i.e., indolopyridine sulfonamides and piperazinones); (2) the single mutation F98L was observed for the spiro-diazaundecane pyrimidines; (3) W55R mutation within the AH2 amphipathic α-helix and R214Q within the cytoplasmic C-terminal segment of NS4B confer resistance to clemizole and analogues; (4) the K52R replacement at the AH2 region, G120V in the TM2 segment, and A210S within the *C*-terminal segment are responsible of the resistance for triazinoindole derivatives (reused with permission and free of charge from Ref. [27])

Compliance with Ethical Standards

Conflict of Interest Giuseppe Manfroni declares that he has no conflict of interest. Rolando Cannalire declares that he has no conflict of interest.

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

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