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Review

New opportunities in anti-hepatitis C virus drug discovery: Targeting NS4B

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ABSTRACT

Current therapy for chronic hepatitis C virus (HCV) infection constitutes a combination of pegylated interferon alfa-2a or alpha-2b and ribavirin. Although successful for many patient populations, this regimen has numerous limitations, including non-response, relapse, poor tolerability and long duration of treatment. To address these shortcomings, new small molecule agents are advancing in clinical development. Most of the current clinical candidates act by directly inhibiting key enzymes in the viral life-cycle: the NS5B polymerase, or the NS3/4A protease. Less well-studied, the non-structural 4B (NS4B) protein has recently emerged as an alternative target for Direct-acting Antiviral Agents (DAAs). NS4B is a 27-kDa membrane protein that is primarily involved in the formation of membrane vesicles – also named membranous web – used as scaffold for the assembly of the HCV replication complex. In addition, NS4B contains NTPase and RNA binding activities, as well as anti-apoptotic properties. This review summarizes the current understanding of the structure and functions of NS4B, an essential component of the replication machinery of HCV. In this literature and patent review, we report the recent developments in anti-NS4B drug discovery. These advances open the possibility for future combination therapies with other DAAs.

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1. Introduction

1.1. Burden of HCV infection and unmet medical need

Hepatitis C virus (HCV) infection is a serious public health concern that affects 170 million people worldwide (Shepard et al., 2005), for which no vaccine is available. Among those infected, approximately 20–30% develop severe liver disease, such as chronic hepatitis, liver cirrhosis, or hepatocellular carcinoma (Alter and

Abbreviations: HCV, hepatitis C virus; kDa, kilo Dalton; NS4B, non-structural protein 4B; NTPase, nucleoside triphosphatase; ssRNA, single-stranded RNA; DAA, Direct-acting Antiviral Agent; ER, endoplasmic reticulum; GT, genotype; AH, amphipatic helix; ATP, adenosine triphosphate; ADP, adenosine diphosphate; Arg, arginine; Ala, alanine; Cys, cysteine; GFP, green fluorescent protein; IFN, interferon; UTR, un-translated region; TM, trans-membrane; IRES, internal ribosome entry site.

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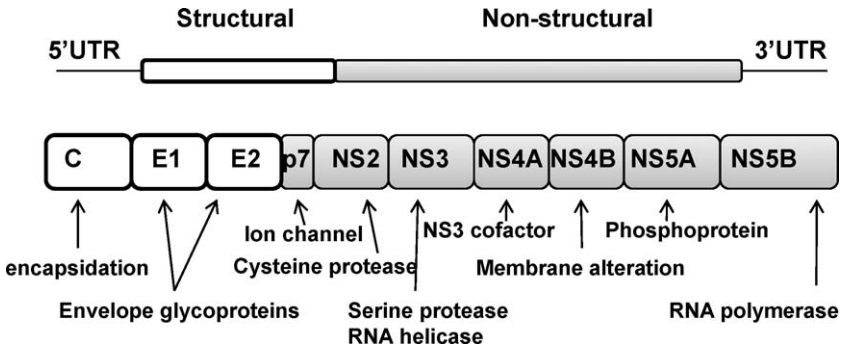


Fig. 1. Organization of the HCV genome. The RNA genome encodes a single open reading frame, flanked by the 5′ and 3′ un-translated regions (UTRs). The polyprotein of approximately 3000 amino acids is processed by host and viral proteases, and results in ten functional structural and non-structural proteins.

Seeff, 2000). The combined use of the nucleoside analog ribavirin and pegylated interferon alpha is the current standard of care. However, success in treatment depends largely on the viral genotype. For instance, the rate of viral clearance upon current standard of care is only ~50% with genotype 1, the most prevalent circulating strain in Western Europe and North America. Additionally, this drug combination has also been associated with severe side effects such as fatigue, nausea and depression, therefore precluding treatment for many individuals (Russo and Fried, 2003; Zeuzem et al., 2000). All of these issues justify the need to develop novel, more efficacious and safer anti-HCV drugs.

1.2. New targets, promising compounds

In recent years there has been significant breakthrough in identifying essential functions within HCV replication that can be directly targeted for antiviral therapy. The HCV RNA genome encodes a polyprotein that is processed into ten smaller polypeptides, including the capsid protein (C), the envelope proteins (E1 and E2), an ion channel (p7), and six nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (Fig. 1). The first proteins to be clinically validated as therapeutic targets for Direct-acting Antivral Agents (DAAs) were the NS3/4A protease and the NS5B polymerase. These two proteins can be inhibited with small molecules (De Francesco and Rice, 2003; Lemon et al., 2010). Similarly to what has been achieved for HIV, the advanced development of protease and polymerase inhibitors brings hope for new treatment options in the next few years. This is exemplified with Telaprevir, and Boceprevir (two NS3/4A protease inhibitors) and RG7128 (a nucleoside NS5B polymerase inhibitor) that have reached advanced clinical trials (Beaulieu, 2009; Kwong et al., 2008). NS3/4A protease and NS5B polymerase are non-structural proteins with well defined enzymatic functions. In addition, there has been a concerted effort to elucidate the specific functions of other non-structural proteins and to determine whether targeting them could lead to novel antiviral therapies. Towards this end and currently in Phase I clinical trials, NS5A-binding molecules have proven themselves to be particularly potent in suppressing HCV replication, both *in vitro* and *in vivo* (Gao et al., 2010; Lemm et al., 2010).

The less well characterized NS4B protein is also emerging as another potentially attractive target for antiviral drug discovery. Although no anti-NS4B molecule has been shown to be efficacious in clinical trials yet, there is an increasing body of evidence from *in vitro* studies that small molecules could suppress HCV replication by altering one of the recently described functions of NS4B. This review provides a comprehensive summary of the biological roles of NS4B within the HCV life cycle. We also report the current public information, from both research articles and patents, related to the inhibition of NS4B for the development of novel DAAs. The implications of using NS4B functional assays to discover and develop novel HCV inhibitors are also discussed.

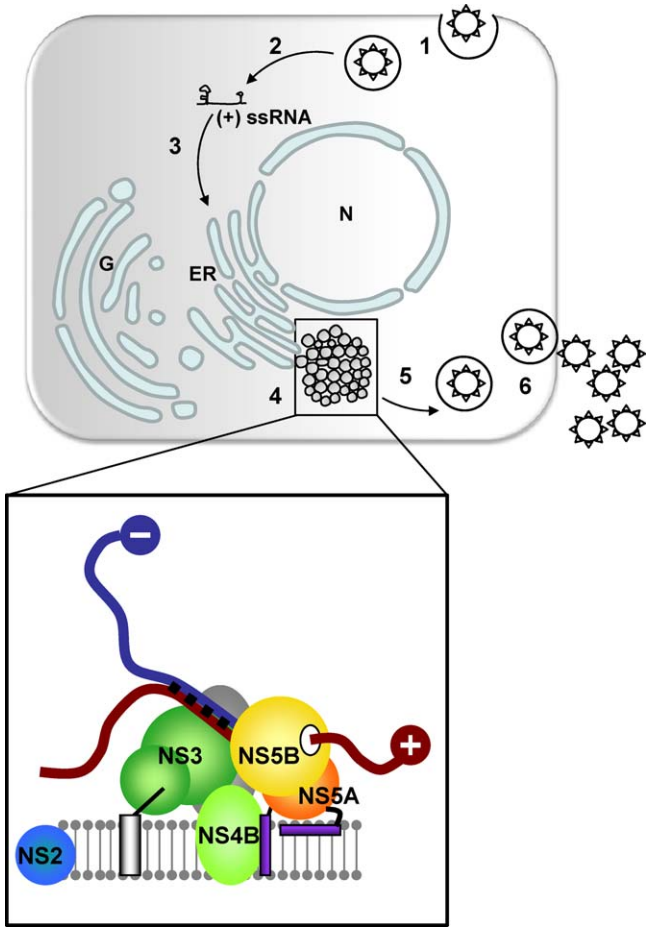


Fig. 2. The HCV life cycle. Following (1) virus attachment and endocytosis, (2) the positive single-stranded RNA ((+) ssRNA) is released by membrane fusion or uncoating. (3) The viral RNA is then recognized by the translation complex through the internal ribosome entry site (IRES). Polyprotein translation and processing take place within the endoplasmic reticulum (ER). (4) Expression of the viral proteins induces lipid rearrangements that support viral RNA synthesis, as well as (5) virion assembly and (6) release. Inset: amplification of viral (+) strand RNA using the (−) strand intermediate occurs through the formation of a replication complex formed by NS5B, the RNA polymerase, as well as the other non-structural proteins.

2. Structural and functional characterization of NS4B

2.1. Structural organization and surface topology

The replication cycle of HCV is depicted in Fig. 2. HCV infects hepatocytes by endocytosis (Dubuisson et al., 2008). After endosomal fusion, the HCV genome made of single stranded RNA (ssRNA)

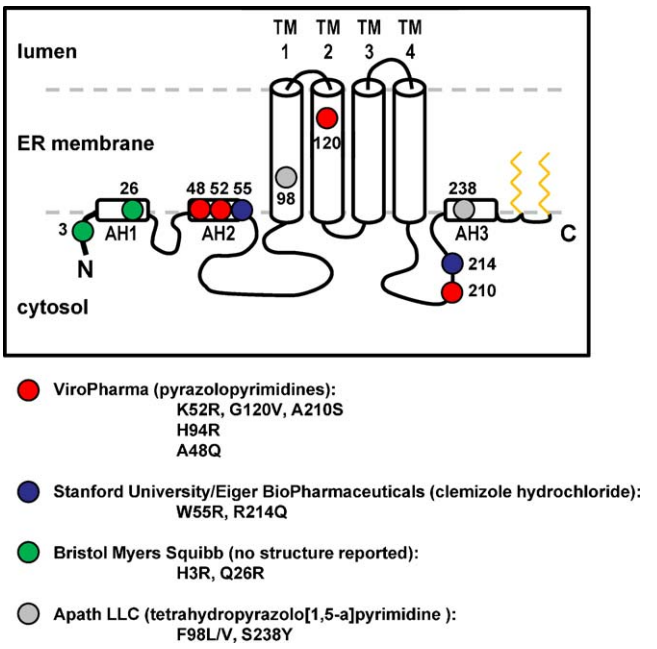


Fig. 3. Surface topology of NS4B and mapping of resistance mutations within NS4B. NS4B is a membrane protein that contains four transmembrane (TM) segments and three amphipatic helices (AH). The two palmitoylation sites are represented in yellow at the C-terminus of the protein. Amino acid changes associated with drug resistance are depicted in circles, with color coding for the different compound classes.

is translated in the cytoplasm to enable the formation of the structural and non-structural proteins (for complete review: Dubuisson, 2007). The non-structural proteins, including NS4B, are associated with the membrane of the ER to form the replication complex that amplifies viral RNA.

NS4B is a hydrophobic protein of 27 kDa, released from the polyprotein precursor after cleavage by the serine protease NS3/4A. During viral replication, NS4B co-localizes with all the other non-structural proteins to the ER in a specific membrane microdomain with lipid raft-like properties (Aizaki et al., 2004; Hugle et al., 2001). When expressed individually, NS4B is also found as an integral part of the ER (Hugle et al., 2001). In the same study it was shown that the majority of the protein is oriented towards the cytoplasm (Fig. 3). Based on computational prediction from primary amino acid structure analysis, NS4B is expected to contain at least 4 trans-membrane domains, of which only two were confirmed experimentally by introducing N-glycosylation sites along the protein sequence (Lundin et al., 2003). Additional anchorage points to the ER membrane are provided by lipid modifications (palmitoylation) on two cysteine residues (cysteines 257 and 261) at the extreme C-terminal end of the protein (Yu et al., 2006). These two residues are believed to facilitate oligomerization of NS4B, as demonstrated by site directed mutagenesis experiments.

In addition to the trans-membrane domains, several N- and C-terminal amphipatic helices have been identified. Elazar et al. (2003) was the first to report membrane association at amino acid residues 6–29, although these findings could not be reproduced in a subsequent study (Gouttenoire et al., 2009a). Two other helical regions have recently been identified: AH2 between positions 42 and 66, and AH3 between positions 229 and 253 (Gouttenoire et al., 2009a,b) (Fig. 3). In each case, alanine substitution of the conserved residues on the hydrophobic helix side abrogated membrane association and disrupted the formation of a functional replication complex.

2.2. Biological functions of NS4B in HCV replication

Early on, cytoplasm alterations in hepatic tissues from HCV-infected chimpanzees were documented (Pfeifer et al., 1980). Such alterations are caused by the presence of an intracellular membranous web during HCV replication, which can be detected by electron microscopy (Egger et al., 2002; Gosert et al., 2003). These membranes consist of aggregates of membrane vesicles that are believed to be derived in part from the ER. Expression of NS4B alone is sufficient to induce these morphological changes (Egger et al., 2002; Lundin et al., 2003), and it is now recognized that one of the main roles of NS4B is to reorganize intracellular membranes into distinct membranous structures at the site of viral replication inside infected cells. Thus, NS4B has been proposed to play a structural role in RNA replication by serving as the scaffold for replication complex assembly (for complete review: Gouttenoire et al., 2010). Membrane alteration is a commonly observed feature among plus-strand RNA viruses, and there have been speculations that it provides an expandable membrane source for virion formation, in addition to preventing the activation of host defense (Aizaki et al., 2004; Miller and Krijnse-Locker, 2008).

It is well recognized that, in cell culture experiments, adaptive mutations located in NS4B can increase RNA replication (Elazar et al., 2004; Lindstrom et al., 2006; Lohmann et al., 2003; Lundin et al., 2003). Moreover, it has recently been shown that NS4B can also modulate the production of infectious particles, therefore pointing towards a role of the protein in virus assembly and release (Jones et al., 2009).

NS4B also contains an ATP/GTPase function. The first report of a nucleotide-binding motif appears in a patent issued in 1999 (Delvecchio et al., 1999). The authors report an ATP-binding consensus sequence G*x*x*G*x*G*K (Walker A motif, where *x* indicates any amino acid) contained within amino acids 1712–1972 of the HCV polyprotein, that corresponds to the NS4B region. This motif, together with another downstream D*xx*G region, is perfectly conserved across all genotypes of HCV and cannot be modified without impairing HCV RNA replication (Einav et al., 2004). The described conserved regions are responsible for the hydrolysis of ATP to ADP, as well as GTP to GDP (Delvecchio et al., 1999; Einav et al., 2004). Overall, the slow intrinsic conversion rates for both substrates suggest that the NS4B NTPase function might require interaction with a protein partner to reach full activation (Thompson et al., 2009). Recently, Einav et al. (2008a) reported that NS4B also specifically recognizes the 3′ terminus of the negative strand of the HCV RNA genome. The authors employed a microfluidic affinity device to measure RNA binding. They identified key arginines at the C-terminal region of NS4B (positions 192–193 and 247–248) that are required for RNA binding and HCV replication. Double mutations from Arg-Arg to Ala-Ala at any of these two positions reduced the binding affinity of NS4B to RNA. While novel and exciting, the precise role of this newly discovered function for NS4B in viral replication remains to be further elucidated. Notably, the biochemical functions that were described are reminiscent of the protein 2C from the picornavirus family, a protein that has been associated with cellular membrane rearrangements (Samuilova et al., 2004, 2006).

Although less well supported, NS4B has been associated with a number of additional functions. This includes cell transformation, a function that has been proposed to provide a mechanism for the development of hepatocellular carcinoma (Park et al., 2000). This anti-apoptotic effect has been recently linked to the NTPase activity of NS4B (Einav et al., 2008b). Also, NS4B has been shown to interact with NS5B, that might modulate the RNA polymerase activity of the latter protein (Piccininni et al., 2002). Finally, NS4B can adopt an oligomerization state (Yu et al., 2006). The main oligomerization determinants were mapped at the N-terminus of the protein,

Table 1
Compounds identified as NS4B binding molecules.

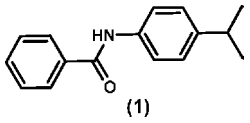
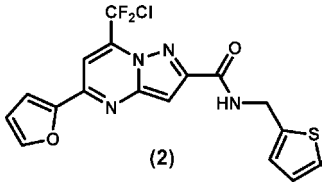
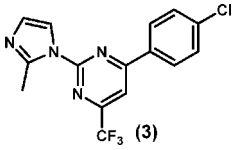
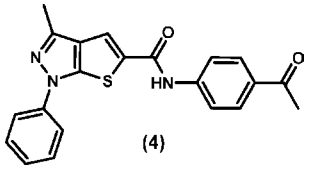
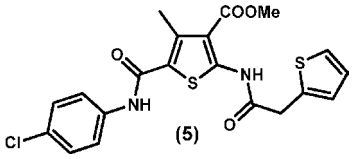
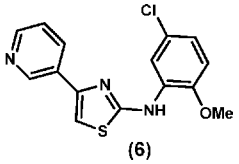
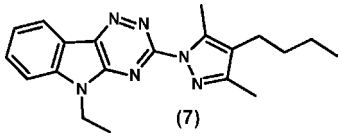
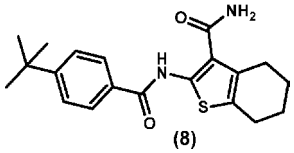
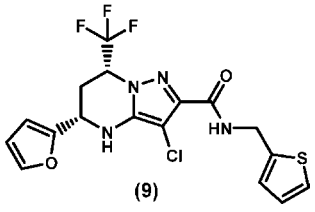
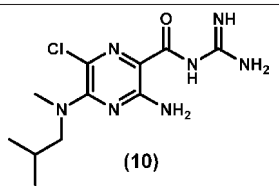
Patent #	Compound class (Example#, Table# in patent)	Structure	EC_{50} (μM)	CC_{75} (μM)
ViroPharma US 2007/0269420	Phenyl benzamide (Example 4, Table 1)	 (1)	2.8	100
	Pyrazolo pyrimidines (Example 3, Table 2)	 (2)	0.63	>100
	Trifluoromethyl pyrimidine (Example 1, Table 3)	 (3)	2.2	>100
	Thienopyrazole (Example 1, Table 4)	 (4)	3.2	50
	Aminothiophenes (Example 1, Table 5)	 (5)	0.7	75
	Phenylthiazolylamines (Example 1, Table 6)	 (6)	1.5	50
	Triazinoindoles (Example 1, Table 7)	 (7)	1.13	20 (CC_{50})
	Tetrahydrobenzothiophenes (Example 1, Table 8)	 (8)	0.87	n/a
Apath, LLC WO 2010/096115	Tetrahydropyrazolopyrimidine-2-carboxamides: 6 compounds specifically claimed (#AP 80978)	 (9)	0.45	100 (CC_{50})

Table 2
Compounds identified as inhibitors of vesicle formation.

Patent #	Compound class (Example# in patent)	Structure	EC_{50} (μM)	CC_{50} (μM)
Stanford University WO 2010/039195;	Amiloride analogs 38 compounds claimed (Compound 10 specifically claimed as C4)	 (10)	3	>5

within the AH2 helix region (Welker et al., 2010). In addition, the C-terminal palmitoylation also contributes to this function through Cys-261, which might be critical for HCV genome replication (Yu et al., 2006).

3. NS4B as an emerging target for HCV therapy

3.1. The pyrazolopyrimidines and other ViroPharma Inc. compounds

One of the first reports of an inhibitor of NS4B comes from a patent issued by ViroPharma Inc. (Chunduru et al., 2007). Using an NS4B-expressing cell line, they identified small molecules inhibiting the anti-apoptotic effect of NS4B. Compound selectivity was measured by the capacity to induce cell-death only in cells expressing NS4B, and binding to the target was confirmed by quenching of intrinsic fluorescence from tryptophan residues contained within the purified NS4B. Using this method, the authors identified eight chemical families or compound classes that were able to inhibit HCV replication. Representative examples from each compound class (Compounds 1–8) are shown in Table 1 These compounds were evaluated to bind to NS4B with a K_d in the low micromolar to sub-micromolar range. Compound 7 from the triazino indole series was used for further target validation. Huh7 hepatic cells containing genotype 1b HCV replicon were passaged seven times in the presence of 5 μM of Compound 7. The resulting cells were ten-fold less susceptible to the compound while a control cell line passaged in parallel in the absence of compound remained fully susceptible to compound inhibition. Furthermore, the mutations that resulted were located in NS4B (K52R, G120 V, A210S), while no amino acid changes were observed in the NS5B region. These mutations were not reintroduced back into the wild-type replicon backbone to confirm the observed phenotype. Among the other chemical families, the pyrazolopyrimidine and the aminothiophene series appear to be the most attractive with sub-micromolar binding affinities, significant replicon activity and good safety window.

The pyrazolopyrimidine (Compound 2, Table 1) was further studied by Genelabs (now part of GSK) in collaboration with Stanford University (Bryson et al., 2010). Using an NS4B-GFP fusion construct expressed in Huh7.5 cells, Compound 2 was shown to alter the sub-cellular distribution of NS4B. It was reported to have a replicon EC_{50} of 0.3 and 0.6 μM in genotype 1b and 1a, respectively. More importantly, its role as an inhibitor of NS4B function was supported through resistance studies. Genotype 1b replicon cells were grown in the presence of Compound 2 at concentrations up to 5 μM, and the most common mutation selected under drug pressure was H94R within NS4B. The H94R mutation, when reintroduced by mutagenesis into the wild-type NS4B replicon construct, conferred a 37-fold potency loss to Compound 2. In another publication by the Stanford group, the mechanism of action of Compound 2 was further defined to disrupt the interaction between helix AH2 and the membrane surface (Cho et al., 2010). In this article, a series of mutations conferring resistance against Compound 2 were selected by passaging cells containing HCV replicon in the presence of the drug. Another resistance-associated mutation at position 48 (A48Q) which mapped to the second amphipatic alpha helix (AH2) is reported (Fig. 3). Interestingly, Compound 2 was not as potent against genotype 2a (EC_{50} >50 μM) as it was against 1b (EC_{50} = 0.3 μM), suggesting it interacts with non-conserved residues in NS4B.

Recently, new compounds related to the pyrazolopyrimidines series (Compound 9, Table 1) were discovered by Apath LLC as anti-HCV NS4B inhibitors (Slomczynska et al., 2010). The Apath patent claims analogs of the tetrahydropyrazolo[1,5-a]pyrimidine scaffold which results from a partial saturation of ViroPharma's pyrazolopyrimidine core. The patent exemplifies 6 compounds; Compound 9 (AP 80978) was the most potent with 1b replicon EC_{50} of 0.45 μM; it had an EC_{50} <2 μM against the genotype 1a replicon. Resistance selection experiments identified a change at position 98 (F98L/V) and 238 (S238Y) that were associated with a loss of sensitivity to Compound 9. However, only the phenotype of the mutation F98L/V was confirmed by site directed mutagenesis. Although Compound 9 was not active against genotype 2a, chimeric replicons containing the genotype 1b con-1 sequence inserted into a 2a background at positions 53–218 regained sensitivity to the drug.

3.2. Inhibition of vesicle formation by an amiloride analog

One of the functions of NS4B is to cause membrane aggregation to form the so-called membranous web. Helix AH2 expressed alone also induces lipid vesicle aggregation that can be monitored by fluorescence microscopy. Furthermore, mutations within AH2 (A51E and W55D, 1b replicon) abrogate HCV replication, thus suggesting that anti-HCV small molecules might be able to interfere with the AH2 function. An assay was therefore developed by Dr. Glenn's group at Stanford University to screen for compounds inhibiting AH2-mediated vesicle formation (Cho et al., 2010). The authors identified a class of pyrazine compounds as inhibitors of vesicle formation, whose activity was confirmed by dynamic light scattering measurements. This class of compounds was further claimed in a patent application (Glenn et al., 2010), in which 38 compounds are claimed and exemplified with a specific claim to Compound 10 (C4), shown in Table 2 Compound 10 is an amiloride analog (3-amino-N-carbamimidoyl-6-chloro-5-(isobutyl(methyl)amino)pyrazine-2-carboxylate) that is active against both genotype 1b and 2a, with an EC_{50} of ~3 μM (Cho et al., 2010).

3.3. The discovery of clemizole hydrochloride (Table 3, Compound 11)

If the interaction between NS4B and the HCV RNA is essential for virus replication, preventing this interaction would constitute a novel and potentially attractive avenue for drug discovery. With this objective in mind, the microfluidic RNA binding assay described earlier was used to screen a restricted library of pharma-

Table 3
Compounds identified as RNA binding inhibitors.

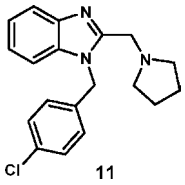
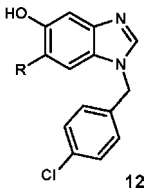
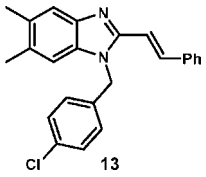
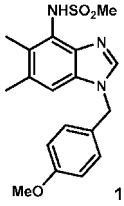
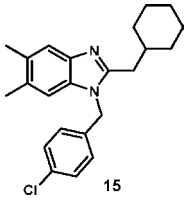
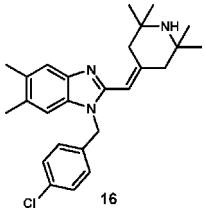
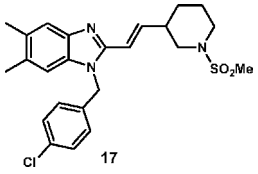
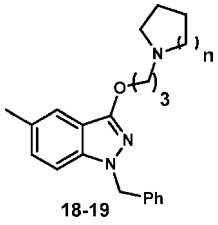
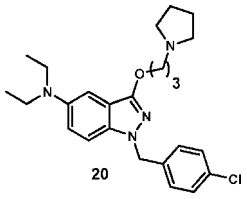
Patent #	Compound class (Example# in patent)	Structure	EC_{50} (μM)	CC_{50} (μM)
Stanford University US 2010/0028299	Clemizole	 11	>20 (GT 2a)	>20
	Clemizole analog Compound 60 (R=H) Compound 62 (R=OH)	 12	<5 (GT 2a)	>5
	Clemizole analog Compound 145	 13		
	Clemizole analog Compound 106	 14		
Stanford University WO 2010/107739	Clemizole analog Compound EBP468	 15	8	>25
	Clemizole analog Compound EBP871	 16	2.7	na
	Clemizole analog Compound EBP550	 17	9.3	>25
Stanford University US 2010/0015093	Indazole series Compound 193 ($n=1$) Compound 199 ($n=2$)	 18-19	<5 (GT 2a)	>5

Table 3 (*Continued*)

Patent #	Compound class (Example# in patent)	Structure	EC_{50} (μM)	CC_{50} (μM)
Stanford University WO 2010/107742	Indazole series Compound EBP534	 20	3.3	>25

cologically active compounds against a purified NS4B-GFP fusion protein (Einav et al., 2008a). One of these small molecules, clemizole hydrochloride (Compound 11, Table 3), was able to inhibit RNA binding with an IC_{50} of 24 nM, that translated to an EC_{50} of 8 μM against the infectious virus of genotype 2a (JFH1). Interestingly, clemizole hydrochloride might not be active against genotype-1 viruses (EC_{50} >20 μM) (Choong et al., 2010a; Einav et al., 2010c). Prolonged passaging of cells harboring the HCV replicon in the presence of clemizole hydrochloride resulted in the selection of resistant clones containing mutations located either at the 3′ UTR or within the NS4B gene. When introduced to the replicon system, mutations W55R and R214Q conferred a 2.2- and 5-fold resistance to clemizole hydrochloride, respectively (Fig. 3). Surprisingly, these mutations increased binding affinity between NS4B and the 3′-terminal RNA sequence. Clemizole hydrochloride is a well-characterized H1 histamine receptor antagonist that was introduced to the US market in the late 1950s as Reactrol/Allercur. The molecule has a wide safety margin, and was therefore taken forward in 2009 into Phase 1B clinical trials in HCV-infected patients. The first ongoing open-label study consists of four weeks of oral treatment with 100 mg BID of clemizole hydrochloride administered immediately prior to the initiation of treatment with standard of care therapy (PEG-IFN + ribavirin) in treatment-naïve subjects chronically infected with HCV genotype 1 or 2.

Clemizole analogs with improved anti-NS4B activity have also been claimed and exemplified in a patent (Einav et al., 2010b). In this patent, several clemizole analogs inhibit HCV replication with EC_{50} (genotype 2a) below 5 μM. It was noted that the pyrrolidinomethyl substitution at the 2-position of the benzimidazole ring (which is apparently required for the H1 antagonism property of clemizole) is not important for its anti-NS4B activity (Table 3, Compounds 12–14). Furthermore, the 4-chlorobenzyl substituent is the most exemplified at the 1-position of the benzimidazole. Analogs of clemizole were further claimed in a recent patent by the Stanford group (Choong et al., 2010a). In this patent, selected compounds are reported with genotype 1b replicon potency of 1.8–12 μM, a clear improvement over clemizole. Furthermore, the hERG activity of some of these compounds was tracked since clemizole-like compounds contain an aromatic core and a pendant tertiary amine that can block hERG K+ channel (Cavalli et al., 2002). Selected compounds from this patent are shown in Table 3; Compound 15 with a 1b replicon EC_{50} of 8 μM is clean in the hERG assay (IC_{50} >10 μM). Compounds 16 and 17 incorporate a piperidine ring whose basicity is masked either sterically or through derivatization as a sulfonamide–strategies commonly used to improve the hERG profile of leads.

3.4. The clemizole-related indazole series

Additional clemizole-related molecules with improved *in vitro* potency have recently emerged. A first patent describes an indazole core replacing the benzimidazole of the clemizole family of compounds (Einav et al., 2010a). Compounds 18–19 (Table 3) are selected compounds from this patent that are claimed and exemplified. A second follow-up patent covering additional indazole compounds reports low micromolar activity in the genotype 1b replicon assay with a good safety window as assessed by cell viability (Choong et al., 2010b). The hERG IC_{50} for selected compounds is reported; these compounds lack a window between antiviral potency and hERG activity. An example (Compound 20) shown in Table 3 has 1b replicon EC_{50} of 3.3 μM and a hERG IC_{50} of 2.8 μM.

3.5. Other efforts in finding anti-HCV compounds targeting NS4B

In 2008, scientists from Genelabs presented their NS4B program at Cambridge Health Institute Conference (Roberts, 2008). Lead compounds identified through a replicon screen were inactive when tested against NS5B polymerase and NS3/4A protease. Resistance screening with selected compounds identified mutated amino acid changes that occur in a well-defined region of NS4B that were subsequently confirmed to decrease compound potency in a transient HCV replicon assay. Optimization of this series of compounds by the Genelabs group has led to compounds that are potent – EC_{50} <50 nM in replicon assays using genotype 1b and 1a – and have antiviral activity in combination with agents currently approved or under clinical development. While specific structures were not revealed, the pharmacokinetic profile and biological characterization of this class of compounds offered hopes for clinical development.

Similarly, Bristol Myers Squibb (BMS) recently reported the result of a HCV replicon screening campaign that led to the discovery of new molecules with EC_{50} values against HCV genotype 1a and 1b replicons of around or below 1 μM, respectively (Sheaffer et al., 2008). Mutations selected by drug pressure mapped to the N-terminal region of NS4B (H3R and Q26R), and provide evidence for target identification (Fig. 3). Interestingly, the BMS group observed cellular rearrangements of lipid droplets in cells incubated with their compounds, implying that cellular determinants are also involved in the mechanism of action of these inhibitors.

4. Conclusion: current and future developments

In recent years, there has been tremendous progress made in understanding the complex role of NS4B in the replication cycle of HCV. As we described, the identification of unique functions such as vesicle aggregation, cell transformation, NTPase activity, and RNA binding has helped in developing new assays to screen for potential inhibitors. In the absence of clinical proof of concept, it is still difficult at this point to assess the most "druggable" functions of NS4B. For example, although the NTPase activity of NS4B has been described now for over 10 years, it is still not clear whether a small molecule could specifically inhibit this catalytic activity, and how such inhibition would translate at the level of virus replication. On the other hand, the membranous web formation seems like a tractable function to target for the discovery of new NS4B inhibitors, at least based on the

convergence of assays and methods reported so far that would be amenable to drug discovery. Using the assays we described, a number of publications and patents report on the inhibition of NS4B with small molecules, the majority of which constitute early pre-clinical studies. Therefore, the presented molecules can be considered as early leads and do not contain to this point all of the drug-like properties required for successful clinical development.

The furthest advanced anti-NS4B molecule is clemizole hydrochloride, an old drug that has previously been clinically approved as an antihistamine. It will be interesting to monitor the clinical progression of clemizole hydrochloride in the currently ongoing Phase Ib study. Preliminary data show that HCV infected patients might positively respond to clemizole hydrochloride when added to the current standard of care (Choong et al., 2010a). *In vitro* studies also predict that clemizole hydrochloride could be synergistic with protease inhibitors, and additive with interferon, ribavirin, nucleoside and non-nucleoside NS5B polymerase inhibitors (Einav et al., 2010c). If clemizole hydrochloride or any other anti-NS4B compound further demonstrates efficacy in human clinical trials, evaluating their combination with other clinical candidates will considerably broaden the potential treatment options for HCV-infected patients.

Conflict of interest

The authors are both employed by Alios BioPharma.

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References

Aizaki, H., Lee, K.J., Sung, V.M., Ishiko, H., Lai, M.M., 2004. Characterization of the hepatitis C virus RNA replication complex associated with lipid rafts. Virology 324, 450–461.

Alter, H.J., Seeff, L.B., 2000. Recovery, persistence, and sequelae in hepatitis C virus infection: a perspective on long-term outcome. Semin. Liver Dis. 20, 17–35.

Beaulieu, P.L., 2009. Recent advances in the development of NS5B polymerase inhibitors for the treatment of hepatitis C virus infection. Expert Opin. Ther. Pat. 19, 145–164.

Bryson, P.D., Cho, N.J., Einav, S., Lee, C., Tai, V., Bechtel, J., Sivaraja, M., Roberts, C., Schmitz, U., Glenn, J.S., 2010. A small molecule inhibits HCV replication and alters NS4B's subcellular distribution. Antiviral Res..

Cavalli, A., Poluzzi, E., De Ponti, F., Recanatini, M., 2002. Toward a pharmacophore for drugs inducing the long QT syndrome: insights from a CoMFA study of HERG K(+) channel blockers. J. Med. Chem. 45, 3844–3853.

Cho, N.J., Dvory-Sobol, H., Lee, C., Cho, S.J., Bryson, P., Masek, M., Elazar, M., Frank, C.W., Glenn, J.S., 2010. Identification of a class of HCV inhibitors directed against the nonstructural protein NS4B. Sci. Transl. Med. 2, 15ra6.

Choong, I.C., Cory, D., Glenn, J.S., Yang, W., 2010. WO2010/107739.

Choong, I.C., Glenn, J.S., Yang, W., 2010. WO2010/107742.

Chunduru, S.K., Benatatos, C.A., Nitz, T.J., Bailey, T.R., Inventors, 2007. US/2007/0269420.

De Francesco, R., Rice, C.M., 2003. New therapies on the horizon for hepatitis C: are we close? Clin. Liver Dis. 7, 211–242, xi.

Delvecchio, A., Zhong, W., Inventors, 1999. WO/1999/001582.

Dubuisson, J., 2007. Hepatitis C virus proteins. World J. Gastroenterol. 13, 2406–2415.

Dubuisson, J., Helle, F., Cocquerel, L., 2008. Early steps of the hepatitis C virus life cycle. Cell Microbiol. 10, 821–827.

Egger, D., Wolk, B., Gosert, R., Bianchi, L., Blum, H.E., Moradpour, D., Bienz, K., 2002. Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. J. Virol. 76, 5974–5984.

Einav, S., Elazar, M., Danieli, T., Glenn, J.S., 2004. A nucleotide binding motif in hepatitis C virus (HCV) NS4B mediates HCV RNA replication. J. Virol. 78, 11288–11295.

Einav, S., Gerber, D., Bryson, P.D., Sklan, E.H., Elazar, M., Maerkl, S.J., Glenn, J.S., Quake, S.R., 2008a. Discovery of a hepatitis C target and its pharmacological inhibitors by microfluidic affinity analysis. Nat. Biotechnol. 26, 1019–1027.

Einav, S., Glenn, J.S., Yang, W., Dvory-Sobol, H., Choong, I.C., McDowell, R., Inventors, 2010. US/2010/0015093.

Einav, S., Glenn, J.S., Yang, W., Dvory-Sobol, H., Choong, I.C., McDowell, R., Inventors, 2010. US/2010/0028299.

Einav, S., Sklan, E.H., Moon, H.M., Gehrig, E., Liu, P., Hao, Y., Lowe, A.W., Glenn, J.S., 2008b. The nucleotide binding motif of hepatitis C virus NS4B can mediate cellular transformation and tumor formation without Ha-ras co-transfection. Hepatology 47, 827–835.

Einav, S., Sobol, H.D., Gehrig, E., Glenn, J.S., 2010c. The hepatitis C virus (HCV) NS4B RNA binding inhibitor clemizole is highly synergistic with HCV protease inhibitors. J. Infect. Dis. 202, 65–74.

Elazar, M., Cheong, K.H., Liu, P., Greenberg, H.B., Rice, C.M., Glenn, J.S., 2003. Amphipathic helix-dependent localization of NS5A mediates hepatitis C virus RNA replication. J. Virol. 77, 6055–6061.

Elazar, M., Liu, P., Rice, C.M., Glenn, J.S., 2004. An N-terminal amphipathic helix in hepatitis C virus (HCV) NS4B mediates membrane association, correct localization of replication complex proteins, and HCV RNA replication. J. Virol. 78, 11393–11400.

Gao, M., Nettles, R.E., Belema, M., Snyder, L.B., Nguyen, V.N., Fridell, R.A., Serrano-Wu, M.H., Langley, D.R., Sun, J.H., O'Boyle 2nd., D.R., et al., 2010. Chemical genetics strategy identifies an HCV NS5A inhibitor with a potent clinical effect. Nature 465, 96–100.

Glenn, J.S., Cho, N.-J., Yang, W., Inventors, 2010. WO/2010/039195.

Gosert, R., Egger, D., Lohmann, V., Bartenschlager, R., Blum, H.E., Bienz, K., Moradpour, D., 2003. Identification of the hepatitis C virus RNA replication complex in Huh-7 cells harboring subgenomic replicons. J. Virol. 77, 5487–5492.

Gouttenoire, J., Castet, V., Montserret, R., Arora, N., Raussens, V., Ruysschaert, J.M., Diesis, E., Blum, H.E., Penin, F., Moradpour, D., 2009a. Identification of a novel determinant for membrane association in hepatitis C virus nonstructural protein 4B. J. Virol. 83, 6257–6268.

Gouttenoire, J., Montserret, R., Kennel, A., Penin, F., Moradpour, D., 2009b. An amphipathic alpha-helix at the C terminus of hepatitis C virus nonstructural protein 4B mediates membrane association. J. Virol. 83, 11378–11384.

Gouttenoire, J., Penin, F., Moradpour, D., 2010. Hepatitis C virus nonstructural protein 4B: a journey into unexplored territory. Rev. Med. Virol. 20, 117–129.

Hugle, T., Fehrmann, F., Bieck, E., Kohara, M., Krausslich, H.G., Rice, C.M., Blum, H.E., Moradpour, D., 2001. The hepatitis C virus nonstructural protein 4B is an integral endoplasmic reticulum membrane protein. Virology 284, 70–81.

Jones, D.M., Patel, A.H., Targett-Adams, P., McLauchlan, J., 2009. The hepatitis C virus NS4B protein can trans-complement viral RNA replication and modulates production of infectious virus. J. Virol. 83, 2163–2177.

Kwong, A.D., McNair, L., Jacobson, I., George, S., 2008. Recent progress in the development of selected hepatitis C virus NS3.4A protease and NS5B polymerase inhibitors. Curr. Opin. Pharmacol. 8, 522–531.

Lemm, J.A., O'Boyle 2nd., D., Liu, M., Nower, P.T., Colonno, R., Deshpande, M.S., Snyder, L.B., Martin, S.W., St Laurent, D.R., Serrano-Wu, M.H., et al., 2010. Identification of hepatitis C virus NS5A inhibitors. J. Virol. 84, 482–491.

Lemon, S.M., McKeating, J.A., Pietschmann, T., Frick, D.N., Glenn, J.S., Tellinghuisen, T.L., Symons, J., Furman, P.A., 2010. Development of novel therapies for hepatitis C. Antiviral Res. 86, 79–92.

Lindstrom, H., Lundin, M., Haggstrom, S., Persson, M.A., 2006. Mutations of the Hepatitis C virus protein NS4B on either side of the ER membrane affect the efficiency of subgenomic replicons. Virus Res. 121, 169–178.

Lohmann, V., Hoffmann, S., Herian, U., Penin, F., Bartenschlager, R., 2003. Viral and cellular determinants of hepatitis C virus RNA replication in cell culture. J. Virol. 77, 3007–3019.

Lundin, M., Monne, M., Widell, A., Von Heijne, G., Persson, M.A., 2003. Topology of the membrane-associated hepatitis C virus protein NS4B. J. Virol. 77, 5428–5438.

Miller, S., Krijnse-Locker, J., 2008. Modification of intracellular membrane structures for virus replication. Nat. Rev. Microbiol. 6, 363–374.

Park, J.S., Yang, J.M., Min, M.K., 2000. Hepatitis C virus nonstructural protein NS4B transforms NIH3T3 cells in cooperation with the Ha-ras oncogene. Biochem. Biophys. Res. Commun. 267, 581–587.

Pfeifer, U., Thomssen, R., Legler, K., Bottcher, U., Gerlich, W., Weinmann, E., Klinge, O., 1980. Experimental non-A, non-B hepatitis: four types of cytoplasmic alteration in hepatocytes of infected chimpanzees. Virchows Arch. B: Cell Pathol. Incl. Mol. Pathol. 33, 233–243.

Piccininni, S., Varaklioti, A., Nardelli, M., Dave, B., Raney, K.D., McCarthy, J.E., 2002. Modulation of the hepatitis C virus RNA-dependent RNA polymerase activity by the non-structural (NS) 3 helicase and the NS4B membrane protein. J. Biol. Chem. 277, 45670–45679.

Roberts, C., 2008. Targeting HCV NS4b function: a new approach to anti-HCV activity. In: Cambridge Health Institute Conference: HCV Drug Discovery , April 28–29, http://www.healthtech.com/Conferences_Overview.aspx?id=72032.

Russo, M.W., Fried, M.W., 2003. Side effects of therapy for chronic hepatitis C. Gastroenterology 124, 1711–1719.

Samuilova, O., Krogerus, C., Fabrichniy, I., Hyypia, T., 2006. ATP hydrolysis and AMP kinase activities of nonstructural protein 2C of human parechovirus 1. J. Virol. 80, 1053–1058.

Samuilova, O., Krogerus, C., Poyry, T., Hyypia, T., 2004. Specific interaction between human parechovirus nonstructural 2A protein and viral RNA. J. Biol. Chem. 279, 37822–37831.

Sheaffer, A.K., Lee, M.S., Chaniewski, L.S., Beaulieu, D., Prack, M., Agler, M., McPhee, F., 2008. Resistance to a novel HCV replication inhibitor maps to amino acid changes within the NS4B sequence. In: 15th International Symposium on Hepatitis C Virus and Related Viruses , (Abstract # 307).

Shepard, C.W., Finelli, L., Alter, M.J., 2005. Global epidemiology of hepatitis C virus infection. Lancet Infect. Dis. 5, 558–567.
Slomczynska, U., Olivo, P., Beattie, J., Starkey, G., Noueiry, A., Roth, R., Inventors, 2010. WO/2010/096115.
Thompson, A.A., Zou, A., Yan, J., Duggal, R., Hao, W., Molina, D., Cronin, C.N., Wells, P.A., 2009. Biochemical characterization of recombinant hepatitis C virus nonstructural protein 4B: evidence for ATP/GTP hydrolysis and adenylate kinase activity. Biochemistry 48, 906–916.
Welker, M.W., Welsch, C., Meyer, A., Antes, I., Albrecht, M., Forestier, N., Kronenberger, B., Lengauer, T., Piiper, A., Zeuzem, S., et al., 2010. Dimerization of the hepatitis C virus nonstructural protein 4B depends on the integrity of an aminoterminal basic leucine zipper. Protein Sci. 19, 1327–1336.
Yu, G.Y., Lee, K.J., Gao, L., Lai, M.M., 2006. Palmitoylation and polymerization of hepatitis C virus NS4B protein. J. Virol. 80, 6013–6023.
Zeuzem, S., Feinman, S.V., Rasenack, J., Heathcote, E.J., Lai, M.Y., Gane, E., O'Grady, J., Reichen, J., Diago, M., Lin, A., et al., 2000. Peginterferon alfa-2a in patients with chronic hepatitis C. N. Engl. J. Med. 343, 1666–1672.