Published in final edited form as:

Sci Transl Med. 2010 January 20; 2(15): 15ra6. doi:10.1126/scitranslmed.3000331.

Identification of a class of HCV inhibitors directed against the nonstructural protein NS4B

Nam-Joon Cho¹, Hadas Dvory-Sobol¹, Choongho Lee¹, Sang-Joon Cho², Paul Bryson¹, Marilyn Masek¹, Menashe Elazar¹, Curtis W. Frank³, and Jeffrey S. Glenn^{1,4,+}

¹Division of Gastroenterology and Hepatology, School of Medicine, Stanford University, Stanford, California

²Park Systems Inc., Suwon, South Korea

³Department of Chemical Engineering, Stanford University, Stanford, California

⁴Veterans Administration Medical Center, Palo Alto, California

Abstract

New classes of drugs are needed to combat hepatitis C virus (HCV), an important worldwide cause of liver disease. We describe an activity of a key domain, an amphipathic helix we termed 4BAH2, within a specific HCV nonstructural protein, NS4B. In addition to its proposed role in viral replication, we validate 4BAH2 as essential for HCV genome replication, and identify first generation small molecule inhibitors of 4BAH2 that specifically prevent HCV replication within cells. Detailed mechanistic studies reveal that the inhibitors target 4BAH2 function by either preventing 4BAH2 oligomerization or 4BAH2 membrane association. 4BAH2 inhibitors represent an exciting, additional class of compounds that has potential to effectively treat HCV.

Introduction

Hepatitis C virus (HCV) is an important cause of worldwide chronic liver disease, infecting over 150 million people (1). Current interferon and ribavirin treatment for HCV is quite toxic yet ineffective at curbing disease in many patients (2), highlighting the need for alternative therapies. Understanding of HCV molecular virology has led to the development of NS3 protease and NS5B polymerase inhibitors (3). Although some of these agents have encouraging antiviral activity *in vitro* and *in vivo*, resistance to either of these classes of drugs develops rapidly, precluding their use as monotherapies. Hence, the identification of novel classes of drugs targeting multiple and independent virus-specific functions, similar to pharmacological cocktails used for TB and HIV, is imperative.

The 9.6 kb HCV genome is a positive single-stranded RNA that encodes for a 3010 amino acid protein (4). The immature protein is processed by cellular and viral proteases into structural components of the mature virus and non-structural (NS) proteins that are involved in virus replication (5). All positive strand RNA viruses replicate their genome in intimate association with host intracellular membranes (6–9). Some viruses, such as alphaviruses, exploit the surface of pre-existing vesicular membranes such as endosomes to replicate their genomes (10). Other viruses, like HCV, induce the formation of novel membrane structures that facilitate their membrane-associated RNA replication (11). For HCV, these membrane

⁺To whom correspondence should be addressed: Jeffrey S. Glenn, M.D., Ph.D., ¹Division of Gastroenterology and Hepatology, School of Medicine, Stanford University, CCSR 3115A, 269 Campus Drive, Stanford, CA, 94305-5187; Tel: (650) 725-3373; Fax: (650) 723-3032; jeffrey.glenn@stanford.edu.

structures, termed the membranous web due to their appearance on electron microscopy, consist of aggregates of membrane vesicles and are believed to be derived in part from the endoplasmic reticulum (12,13). One protein, nonstructural protein 4B (NS4B), has been reported to be sufficient for creation of the membranous web (12,13), although the molecular mechanism(s) whereby NS4B might promote membrane rearrangements or vesicle aggregations are largely unknown. NS4B has four predicted transmembrane domains (14–17). An N-terminal amphipathic helix (AH) within NS4B mediates the targeting of the HCV replicase complex components to the apparent sites of replication (14) and an arginine-rich like motif within NS4B binds the 3'-terminus region of the virus negative strand RNA, the presumed template for the initiation of progeny plus-strand RNA genomes (18). Recently, we demonstrated that pharmacologic inhibitors of the NS4B RNA binding activity can be effective at inhibiting genome replication (18), but to date no small molecules capable of inhibiting HCV AH function within NS4B or other viral proteins have been identified.

Here we genetically validated an uncharacterized domain within NS4B that is essential for enabling genome replication. This target consists of a second AH—termed 4BAH2—and was found to mediate NS4B oligomerization and lipid vesicle aggregation. The dramatic ability to induce vesicle aggregation also suggested a potential phenotypic readout for a high-throughput screen (HTS) to identify pharmacologic inhibitors of 4BAH2 function. We performed such a high-throughput screen and identified a variety of small molecules capable of inhibiting 4BAH2-mediated lipid vesicle aggregation and HCV RNA replication. Quartz crystal microbalance with dissipation (QCM-D) and atomic force microscopy (AFM) analyses of two of the compounds found to most potently inhibit vesicle aggregation revealed their mechanism of action on 4BAH2 function. These results highlight 4BAH2 as a critical modulator of NS4B function, provide new insight into the molecular mechanism of HCV replication platform assembly, and demonstrate the utility of a novel small molecule anti-HCV strategy.

Results

Amino acids 43 to 65 of NS4B comprise an amphipathic alpha helix (4BAH2)

Secondary structure prediction programs (including DSC, HNNC, SIMPA96, MLRC, SOPM, PHD, and Predator) indicated that amino acids 43 to 65 of NS4B are likely to reside in an alpha helical conformation (see fig. S1). Inspection of this helix revealed it to be amphipathic in nature (Fig. 1A). Because this segment is immediately downstream of another amphipathic helix (14), we defined the former as 4BAH2, and the proximal N-terminal amphipathic helix as 4BAH1 (14). As shown in fig. S2, CD measurements confirmed the helical nature of a synthetic peptide corresponding to 4BAH2. Similar results were recently reported by others (19).

4BAH2 induces vesicle aggregation

Expression of NS4B has been reported to be necessary and sufficient for induction of the membranous web(12,13). Based on initial pilot studies of 4BAH2's interactions with membrane constituents, we hypothesized that 4BAH2 might play a role in membranous web formation. To test this hypothesis, we studied the interaction of 4BAH2 with lipid vesicles composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC). The POPC lipid was selected because phosphatidylcholine is the most abundant class of phospholipid in the endoplasmic reticulum (ER). Dynamic light scattering (DLS) indicated that the extruded POPC vesicles had a relatively uniform size distribution (Fig. 1B). The average POPC vesicle diameter was 49.5 ± 1.4 nm and the relative variance (polydispersity) of the vesicles was 0.118 ± 0.02 . The 4BAH2 peptide was then added to the lipid vesicles, and the reaction

monitored by DLS. A large increase in the average size of the vesicle population was observed, while no such activity was observed with a control amphipathic helical peptide (4BAH1), highlighting the unique, specific, and striking biochemical activity associated with 4BAH2 (Fig. 1C, D).

To determine whether the dramatic increase in size detected by DLS was due to vesicle fusion or vesicle aggregation, we performed transmission electron microscopy on the vesicles before and after addition of 4BAH2 (Fig. 1E, F). Most vesicles appeared to retain their initial size, indicating that they are predominantly organized into large aggregates upon addition of 4BAH2 (Fig. 1F). To further confirm this apparent 4BAH2-induced aggregation of lipid vesicles, we also employed atomic force microscopy to follow the morphological changes associated with the addition of 4BAH2 to lipid vesicles upon interaction with a solid support. We used the hydrophilic SiO₂ substrate (fig. S3A) since it is atomically flat and it is well known that vesicles typically fuse upon interaction with such hydrophilic substrates to make a ~ 5 nm thin bilayer (20). Upon addition of vesicles alone, the flat-appearing, uniform thickness of a ~ 4.5 nm bilayer was observed (fig. S3B). As expected, upon deposition of vesicles in the presence of 4BAH2, we detected massive 4BAH2-induced vesicle aggregates (fig. S3C).

Disruption of 4BAH2's amphipathicity abrogates vesicle aggregation

To determine whether the amphipathic nature of 4BAH2 is necessary for its vesicle-aggregating activity, we generated 4BAH2 peptides (M2, M3, and M4) harboring two to four point mutations that introduced charged amino acids into the hydrophobic face of the 4BAH2 amphipathic helix (M2: A51E, W55D; M3: A51E, W55D, I65D; M4: A51E, W55D, I65D, F53E) (Fig. 2A) and tested their ability to mediate aggregation. Compared to wild-type 4BAH2 (Fig. 2C), point mutants M2, M3, and M4 that disrupted 4BAH2's amphipathic nature completely abrogated its vesicle-aggregating activity (Figs. 2D, E, F). To confirm that these results were not due to conformational changes, we examined the ability of mutant 4BAH2 peptides to retain helicity and found that the loss of amphipathicity appeared to be a key determinant of the 4BAH2 mutants' inability to promote vesicle aggregation (Fig. 2G). Similar results were obtained with AFM (fig. S4). Moreover, the mutations did not appear to alter NS4B stability (fig. S5).

An intact 4BAH2 is required for HCV genome replication

To test the hypothesis that an intact 4BAH2 is essential for viral genome replication, the smallest number of mutations that were sufficient to abrogate 4BAH2's vesicle-aggregating activity in the previous section (i.e., M2: A51E, W55D) were introduced into a bicistronic high efficiency HCV replicon (21) modified so that the HCV internal ribosome entry site (IRES) drives the expression of luciferase, and the non-structural proteins required for replication are expressed from the encephalomyocarditis virus (EMCV) IRES. Transient replication assays were performed on wild-type, mutant, and a negative control replicon harboring a lethal mutation in the NS5B polymerase (amino acids GDD at codons 317–319 of NS5B changed to GND (22)). Disruption of 4BAH2 abrogated genome replication (Fig. 3A). To confirm the dependence of HCV replication on 4BAH2, analogous replicons in which the luciferase gene was replaced with the neomycin phosphotransferase gene were assayed in standard colony formation assays (Fig. 3B) (23). Wild-type replicons yielded numerous colonies, while no colonies resulted upon electroporation of the 4BAH2 mutant replicons (Fig. 3B). Together, these results demonstrate that mutations that impair the vesicle-aggregating activity of 4BAH2 abrogate HCV genome replication.

Small molecules are identified that inhibit 4BAH2 activity

The above results validated the importance of 4BAH2 for HCV genome replication, and elucidated a potential approach to identify pharmacologic inhibitors of 4BAH2 function. To do so, POPC vesicles were fluorescently labeled and vesicle aggregation was monitored by fluorescence microscopy upon addition of 4BAH2. The assay was adapted to a 384-well format (fig. S6) and performed in the presence of compounds available from a small molecule library (24). The presence or absence of aggregates was verified with pattern recognition software in a high-throughput scheme, or by visual inspection (Fig. 4A,B). Although most compounds had no significant effect on the vesicle-aggregating activity of 4BAH2, several inhibited aggregation formation to background levels observed without addition of 4BAH2. These candidate inhibitors, along with selected compounds that displayed no inhibition of lipid vesicle aggregation (negative controls), were further evaluated in a secondary screen in which dynamic light scattering (DLS) assays were performed in the presence of the individual compounds (Fig. 4C). Several of the candidate inhibitors were confirmed to be potent inhibitors of 4BAH2 lipid vesicle-aggregating activity, and we hypothesized that a subset of these, in particular C4 and A2, might similarly inhibit HCV genome replication.

Selected candidate inhibitors display genotype-specific effects on HCV replication

The importance of 4BAH2 to the HCV life cycle is indicated by several lines of genetic evidence. First, a 4BAH2 is conserved across all HCV genotypes and isolates whose sequences are publicly available (fig.S7). This argues strongly for the dependence of productive viral replication *in vivo* on 4BAH2. Second, as shown by the transient replication assay of Figure 3A, an HCV replicon harboring a genetically mutated 4BAH2 was defective in establishing genome replication. Third, similar genetic mutation of 4BAH2 resulted in the inability to maintain genome replication in the longer-term colony formation assays (Fig. 3B). Thus, pharmacologic inhibition of 4BAH2 might be expected to inhibit HCV genome replication.

Transient replication assays using C4 and A2 (fig. S8 for structures) at low to submicromolar concentrations inhibited HCV replication in a dose-dependent manner (Fig. 5A). No significant cellular toxicity was observed under any of these conditions (Fig. 5B), highlighting the specificity of inhibition of HCV replication. The efficacy of C4 and A2 could be assessed on HCV clonal variants (genotype 1b and genotype 2a). We found that both compounds could inhibit genotype 1b (Fig. 5C), but only C4 inhibited genotype 2a replication (Fig. 5D). This suggests a difference in the specificity of the compounds for 4BAH2 on HCV clonal variants.

HCV variants did not impair the ability of 4BAH2 to induce vesicle aggregation (Fig. 5C–D). Addition of either C4 or A2 inhibited vesicle aggregation induced by 4BAH2 of genotype 1b (Fig. 5C). However, only addition of C4, but not A2, abrogated the ability of 4BAH2 of genotype 2a to induce vesicle aggregation (Fig. 5D). These results parallel the inhibitory effects of the compounds on replication of the respective genotypes (Figs. 5A), and highlight the specificity of the two compounds for 4BAH2.

A variety of mutations have been identified that confer phenotypic resistance to pharmacologic inhibition of 4BAH2. As expected, these mutations map to NS4B, including A48Q, which is a single amino acid change within 4BAH2 that increases the EC₅₀ for A2 by 3 to 4 fold (fig. S9).

We envisage at least two possible mechanisms whereby 4BAH2-induced lipid vesicle aggregation can be inhibited: 1) preventing oligomerization of 4BAH2 peptides, and/or 2) disrupting the ability of 4BAH2 to interact with lipid vesicles (Fig. 6). To tease apart the

mechanisms by which C4 and A2 compounds inhibited aggregation, we performed atomic force microscopy (AFM) to quantitatively determine surface topology and particle sizes of 4BAH2 oligomers (Fig. 7A–D, fig. S10), and quartz crystal microbalance-dissipation (QCM-D) to assess membrane association (Fig. 7E, F). The combined AFM and QCM-D data suggest that C4 acts primarily via disruption of 4BAH2 oligomerization (Fig. 7C), whereas the predominant effect of A2 is to prevent interaction of 4BAH2 with membranes (Fig. 7D, E). In particular, there is prominent self-oligomerization of 4BAH2 peptides in the absence of inhibitor (Fig. 7B) whereas self-oligomerization is dramatically inhibited in the presence of C4 (Fig. 7C). The extent of inhibition was as great as that achieved by mutations in 4BAH2 that blocked 4BAH2 oligomerization (fig. S11). In contrast, addition of A2 had a minimal effect on the ability of 4BAH2 to oligomerize (Fig. 7D) but completely prevented genotype 1b 4BAH2 membrane association (Fig. 7E, fig. S12). Again, the effect of A2 on 4BAH2 was limited to a genotype 1b target, with no significant inhibition of genotype 2a 4BAH2 membrane association (Fig. 7F, fig. S12). C4 had a minor effect on the membrane association of 4BAH2 of either genotype (Figs. 7E,F). The net effect of either C4 or A2, however, is to abrogate 4BAH2-mediated vesicle aggregation, a function that appears to be critical for the formation of the membranous web replication platform (fig. S13) and membrane-associated HCV RNA genome replication.

Discussion

The limitations of current therapies for hepatitis C and the requirement for treatment cocktails to thwart the rapid development of multi-drug resistance, highlight the need for new classes of HCV drugs. Here we tested a new target within the HCV NS4B protein, a conserved amphipathic helix (AH) essential for viral genome replication. This AH, termed 4BAH2, displays a potential for self-oligomerization as well as the ability to promote the aggregation of lipid vesicles into macromolecular assemblies resembling key features of membranous webs—the HCV intracellular replication platform (12,13). Furthermore, we used 4BAH2 vesicle aggregation-promoting activity to screen for candidate pharmacologic inhibitors. We showed that several such inhibitors could also alter HCV genome replication in a dose-dependent fashion. Moreover, the specificity of compounds for a particular HCV genotype could be predicted by their ability to inhibit 4BAH2 function based on AFM, QCM-D, and DLS assays. Detailed analysis of two compounds revealed that 4BAH2 function can be disrupted by either one of two mechanisms: inhibition of 4BAH2 oligomerization, or inhibition of the ability of 4BAH2 to associate with membranes. These results reveal a biochemical activity that may constitute a critical component of the mechanism of HCV's replication platform assembly, and identify an anti-HCV strategy distinct from, and complementary to, other classes of drugs in development for hepatitis C.

Oligomerization of NS4B has been reported by others (25), but we found that specific point mutations within 4BAH2 that disrupted its amphipathic (Fig. 2A), but not helical (Fig. 2G), nature impaired the ability to oligomerize (fig. S14). Further, as shown in Figures 1 and 2, 4BAH2 induces dramatic aggregation of lipid vesicles, and defines a heretofore unreported function within NS4B.

Our data suggest that 4BAH2-induced vesicle aggregation is also important for NS4B's role in the HCV life cycle. HCV replication is believed to occur in association with the membranous web (12,13). Ectopic expression of NS4B alone has been reported to be sufficient for inducing membranous web formation (12). The relevant mechanism(s) whereby NS4B induces membranous webs are poorly understood. Although likely to involve multiple factors including ones supplied by the host cell, the 4BAH2 vesicle-aggregating activity reported here provides an attractive mechanism to account for some of the key elements of the previously described membranous web.

The mutation analysis of Figure 3 represents a critical first step in the targeted development of new potential HCV therapeutics. To efficiently translate such knowledge into new classes of HCV drugs, however, target-specific assays must be developed, and understanding the mechanism of action of candidate inhibitors is needed.

One class of drugs currently in advanced clinical development for hepatitis C is the NS5B polymerase inhibitors, where inhibition of NS5B function can be achieved by targeting NS5B—at both the active site as well as at several epitopes distinct from the active site (26). Similarly, our study of how C4 and A2 inhibit 4BAH2 function suggests that the 4BAH2 class of inhibitors can inhibit HCV RNA genome replication, as well as a common target by an alternative mechanism, namely by disrupting oligomerization or membrane association (see Fig. 6). Moreover, the above oligomeric model of 4BAH2 suggests the potential for transdominant inhibition of 4BAH2 function.

Materials and Methods

Peptides

Peptides corresponding to the wild-type sequence of 4BAH2, as found in genotypes 1b (WRTLEAFWAKHMWNFISGIQYLA) and, 2a (WPKVEQFWARHMWNFISGIQYLA) were commercially synthesized (Anaspec Corporation, San Jose, CA, USA). For negative controls, three peptides harboring mutations in 4BAH2 (genotype 1b) were also synthesized. The sequences of the three different mutant peptides can be found in Supplementary Information.

Small Unilamellar Vesicle Preparation

Detailed methods are included in the Supplementary Material.

Plasmids

Detailed methods are included in the Supplementary Material.

Circular Dichroism

Detailed methods are included in the Supplementary Material.

Quartz Crystal Microbalance-Dissipation (QCM-D)

Detailed methods are included in the Supplementary Material.

Dynamic Light Scattering

Detailed methods are included in the Supplementary Material.

Western Blot Analysis

Detailed methods are included in the Supplementary Material.

Production of NS4B-expressing lentivirus and isolation of stable NS4B-expressing clone

Detailed methods are included in the Supplementary Material.

Atomic Force Microscopy

Detailed methods are included in the Supplementary Material.

High Throughput Screen (HTS)

In order to screen for compounds that inhibit 4BAH2-mediated aggregation of nano-size vesicles, we performed a high-content imaging, high-throughput screen (HTS) (fig. S6 for schematic). The assay was based on the ability of the 4BAH2 peptide to induce large-scale aggregation of fluorescently-labeled vesicles that are readily detected by fluorescent microscopy. The fluorescent lipid vesicles were prepared as described above for small unilamellar vesicles, except for the addition of 0.05% Texas Red-DHPE (molar ratio with POPC) during preparation of the lipid films.

A Caliper Life Sciences Sciclone ALH3000 liquid handler integrated system (Stanford University High-Throughput Bioscience Center (HTBC)) was used to accommodate 384-tip manifolds, enabling it to rapidly pipet volumes into 384-well microplates. The Z8 module that contains eight independent syringe-based pipets, allowing liquid transfers with integrating a V&P Scientific 384 Pin Tool that is capable of 100 nL range transfers, was used (see Suppl. Methods for sequence details).

Transmission Electron Microscopy (TEM)

Samples were fixed in 4% glutaraldehyde (Electron Microscopy Sciences, USA) and 2% OsO₄, dehydrated in a series of ethanol washes, and infiltrated with EMBED-812 resin (Electron Microscopy Sciences) essentially as previously described (27). Center sections were stained in saturated uranyl acetate and 0.2% lead citrate. Samples were observed in a JEOL 1230 TEM at 80kV and images were taken using a Gatan multiscan 791 digital camera (see Suppl. Methods for additional details).

Colony Formation Assays

5 µg of in vitro-transcribed wild type and mutant Bart79I RNAs were mixed with 6×10^6 Huh7 cells in RNase-free PBS (Biowhitaker) and transferred into a 2 mm-diameter gap cuvette (BTX, San Diego, CA). Electroporation was performed using a BTX model 830 electroporator, essentially as previously described (23). The electroporation condition was as follows: 680 V, five periods of 99 µs at 500 ms intervals. The electroporated cells were diluted in 30 ml of cell culture medium. 300 µl of cells were transferred to 10 cm tissue culture dishes. At 24 hours post electroporation, cells were supplemented with untransfected feeder Huh7 cells to a final density of 10^6 cells per plate. After an additional 24 hours, the medium was supplemented with G418 to a final concentration of 750 µg per ml. This selection medium was replaced once every three days for three weeks. Following selection, the plates were washed with PBS, incubated in 1 % crystal violet in 20 % ethanol for 5 min, and washed five times with water. Colonies on triplicate plates results were counted and expressed as colonies per microgram electroporated RNA.

Transient Replication Assays

10 µg of in vitro-transcribed wild type or mutant Bart79I-Luc RNAs were electroporated into Huh7 cells (23) as described above. The electroporated cells were diluted in 40 ml of cell culture medium. 2 ml of cells were aliquoted in 6 well tissue culture plates. Firefly luciferase activities were measured at 8, 48, 96, and 144 hours post electroporation by using a firefly luciferase kit from Promega.

Antiviral Assays of Compounds

In vitro transcribed wild-type FL-J6/JFH-5'C19Luc2Aubi and Bart79I-luc RNAs were electroporated into Huh7.5 cells, essentially as previously described (23). Cells were seeded in 96-well plates. After 24 hours, individual test compounds (e.g. C4 and A2) were added to the cells and media changes were performed daily with fresh aliquots of the same

compounds. After 72 (for genotype 2a) or 120 (for genotype 1b) hours of treatment, cells were incubated for 2 hours at 37°C in the presence of 10 % Alamar Blue reagent (TREK Diagnostic Systems) to assess cytotoxicity, followed by lysis and luciferase assays to assess for viral genome replication. Signal was normalized relative to untreated samples or samples grown in the presence of the corresponding concentration of DMSO. Experiments were repeated three times, each time with 4 replicates. (See Suppl. Methods for additional details).

Infection and Transfection Expression

Infection and transfection was performed essentially as previously described (23). Briefly, a vaccinia virus that expresses the T7 RNA polymerase was used to infect Huh-7 cells at a multiplicity of infection (MOI) of 10. Following 45-minutes incubation at 37°C, the cells were washed twice with Optimem (Invitrogen) and subjected to transfection with pcDNA3.1-NS4B wild type or pcDNA3.1-NS4B-AH2 (M2) mutant. The cells were supplemented with growth media and incubated for 6 hours at 37°C, with ~80–90 % of cells expressing the desired proteins confirmed by immunofluorescence analysis using a rabbit polyclonal anti-NS4B antibody.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by a Burroughs Wellcome Fund Clinical Scientist Award in Translational Research (to JSG), NIH RO1 DK066793, and the Stanford University SPARK Program. We also wish to thank the Stanford University High-Throughput Bioscience Center and Digestive Disease Center's Molecular Core. NJC is a recipient of an American Liver Foundation Postdoctoral Fellowship Award and a Global Roche Postdoctoral Fellowship. We wish to thank Dr. John McLauchlan, MRC Virology Unit, Institute of Virology, Glasgow G11 5JR, UK for a gift of the anti-NS4B polyclonal antibody and Harry Greenberg, Peter Sarnow, and Richard Zare for kindly reading and thoughtful comments on the manuscript. A patent related to this work has been filed by Stanford University. NJC, HDS, CHL, and JSG have an equity interest in Eiger BioPharmaceuticals, Inc. JSG is also a consultant to Eiger BioPharmaceuticals, Inc.

Reference

1. Shepard CW, Finelli L, Alter MJ. *Lancet Infect Dis* 2005 Sep;5:558. [PubMed: 16122679]
2. Manns MP, et al. *Nat Rev Drug Discov* 2007 Dec;6:991. [PubMed: 18049473]
3. Soriano V, Peters MG, Zeuzem S. *Clin Infect Dis* 2009 Feb 1;48:313. [PubMed: 19123867]
4. Lindenbach BD, Rice CM. *Nature* 2005 Aug 18;436:933. [PubMed: 16107832]
5. Moradpour D, Penin F, Rice CM. *Nat Rev Microbiol* 2007 Jun;5:453. [PubMed: 17487147]
6. Bienz K, Egger D, Rasser Y, Bossart W. *Virology* 1980 Jan 30;100:390. 1980. [PubMed: 6243433]
7. Chu PW, Westaway EG. *Arch Virol* 1992;125
8. Lazarus LH, Barzilai R. *Journal of General Virology* 1974 May;23:213. 1974. [PubMed: 4364882]
9. Rice, CM. *Fields Virology*. Fields, BN.; Knipe, DM.; Howley, PM., editors. Philadelphia: Lippincott-Raven Publications; 1996. p. 931-959.
10. Froshauer S, Kartenbeck J, Helenius A. *Journal of Cell Biology* 1988 Dec;107:2075. 1988. [PubMed: 2904446]
11. Suhy DA, Giddings TH, Kirkegaard K. *J Virol* 2000;74:8953. [PubMed: 10982339]
12. Egger D, et al. *J Virol* 2002 Jun;76:5974. [PubMed: 12021330]
13. Gosert R, et al. *Hepatology* 2002;36:212A.
14. Elazar M, Liu P, Rice CM, Glenn JS. *J Virol* 2004 Oct;78:11393. [PubMed: 15452261]
15. Hugle T, et al. *Virology* 2001 May 25;284:70. [PubMed: 11352669]

16. Lundin M, Lindstrom H, Gronwall C, Persson MA. J Gen Virol 2006 Nov;87:3263. [PubMed: 17030859]
17. Lundin M, Monne M, Widell A, Von Heijne G, Persson MA. J Virol 2003 May;77:5428. [PubMed: 12692244]
18. Einav S, et al. Nat Biotechnol 2008 Sep;26:1019. [PubMed: 18758449]
19. Gouttenoire J, et al. J Virol 2009 Jun;83:6257. [PubMed: 19357161]
20. Ohki S. J Theor Biol 1969 Apr;23:158. [PubMed: 5798214]
21. Blight KJ, Kolykhalov AA, Rice CM. Science 2000 Dec 8;290:1972. [PubMed: 11110665]
22. Sklan EH, et al. J Virol 2007 Oct;81:11096. [PubMed: 17686842]
23. Elazar M, et al. J Virol 2003 May;77:6055. [PubMed: 12719597]
24. Stanford High-Throughput Bioscience Center. <http://htbc.stanford.edu>.
25. Yu GY, Lee KJ, Gao L, Lai MM. J Virol 2006 Jun;80:6013. [PubMed: 16731940]
26. Beaulieu PL. Expert Opin Ther Pat 2009 Feb;19:145. [PubMed: 19441916]
27. Ushiki T. Tanpakushitsu Kakusan Koso 2004 Aug;49:1714. [PubMed: 15377005]

Figure 1. An amphipathic alpha helical segment of NS4B, 4BAH2, promotes large-scale vesicle aggregation

(A) Helix net diagram of amino acids 43–65 of NS4B that comprise 4BAH2 (depicted in the N-terminal to C-terminal direction from bottom to top). Hydrophobic portions are indicated in green. Size distribution of lipid vesicles in the (B) absence or (C) presence of 4BAH2.

(D) DLS of lipid vesicles with a control amphipathic helical peptide (4BAH1). The red bars represent the histogram of size distribution. Note the x-axis scale is separated into two linear size ranges to directly compare the vesicle size distribution upon addition of wild-type 4BAH2 versus 4BAH1. Transmission electron microscopy (TEM) images (E) before and (F) after addition of 4BAH2. Note that the inset image of Fig. 1F has the same level of magnification as Fig. 1E for direct comparison.

Figure 2. Disruption of 4BAH2 amphipathicity abrogates vesicle aggregation

(A) Helix net diagrams (depicted in the N-terminal to C-terminal from bottom to top) of amino acids 43–65 of NS4B, which comprises 4BAH2, representing point mutations indicated in red. POPC lipid vesicle size distribution was measured by DLS in the (B) absence or (C) presence of 4BAH2, or in the presence of mutant versions of 4BAH2 harboring (D) two point mutations, 4BAH2 (M2) (A51E, W55D), (E) three point mutations, 4BAH2 (M3) (A51E, W55D, I65D), or (F) four point mutations, 4BAH2 (M4) (A51E, W55D, I65D, F53E). Note the x-axis scale is separated into two linear size ranges to directly compare the average vesicle size distribution upon addition of the wild type, but not mutant, 4BAH2 peptides. (G) Far-UV circular dichroism (CD) spectra of the 4BAH2 mutants.

Figure 3. An intact 4BAH2 domain of NS4B is required for HCV RNA genome replication
(A) Luciferase reporter-linked transient HCV replication assays were performed on Huh7 cells harboring wild type (Bart79I-luc-WT), 4BAH2 mutant (Bart79I-luc-4B-AH2 (M2)), or negative control polymerase minus mutant (Bart79I-luc-Pol-) luciferase harboring HCV replicon RNAs. **(B)** Colony formation assays of wild-type Huh7 cells harboring (Bart79I-WT), 4BAH2 mutant (Bart79I-NS4B-AH2 (M2)), or negative control polymerase minus mutant (Bart79I-Pol-) HCV replicon RNAs containing the neomycinphosphotransferase gene. Surviving colonies were stained with crystal violet. Representative plates, left panel. Average (+/- standard deviation) number of colonies per microgram electroporated RNA, right panel.

Figure 4. Small molecule compounds are identified that can inhibit 4BAH2-induced vesicle aggregation

(A) Fluorescence microscopy of 4BAH2-induced aggregation of lipid vesicles (10X). Top panels: POPC alone, POPC with 4BAH2. Bottom panels: POPC with 4BAH2 and the A2 or C4 compounds. (B) The presence or absence of aggregates was analyzed in a high throughput fashion using pattern recognition software based on total granule area of vesicle aggregation induced by 4BAH2. (C) DLS assay (left panel) on selected candidate molecules identified to be positive in the high throughput screening assay (and controls). Right panel: compounds assayed. CZ (clemizole (18)), H8, and H10 were included as negative controls.

Figure 5. HCV genome replication is inhibited by small molecule inhibitors of 4BAH2 function in a genotype-specific manner

Huh7.5 cells harboring genotype 1b replicon RNA (Bart79ILuc), or full-length genotype 2a HCV RNA (J6/JFH Luc) were treated with various concentrations of C4 or A2 and assayed for (A) viral replication by luciferase activity and (B) cell viability. DLS assays of 4BAH2 induced vesicle aggregation were performed with (C) genotype 1b 4BAH2 or (D) genotype 2a 4BAH2 in the absence (control) or presence of either C4 or A2. Note the x-axis scale is separated into two linear size ranges to directly compare the average vesicle size distribution in presence or absence of compounds.

Figure 6. 4BAH2 self-oligomerization and induction of lipid vesicle aggregation can be prevented by hydrophilic or hydrophobic dissociation

4BAH2 self-oligomerization is driven by hydrophobic interactions (top panel). Lipid vesicle aggregation is promoted by the interaction of the hydrophilic portions of 4BAH2 peptides with vesicles and the hydrophobic parts with each other (middle panel). Drug candidates inhibit 4BAH2-mediated lipid vesicle aggregation by either hydrophilic dissociation or hydrophobic dissociation (middle panel). In the case of hydrophilic dissociation, the candidate drug (green/yellow molecules) interacts with the hydrophilic portion of 4BAH2 so that it inhibits 4BAH2 association with charged lipid head groups. For hydrophobic dissociation, different classes of drugs (schematized in yellow) interact with the

hydrophobic portion of 4BAH2 to prevent peptide oligomerization, leading to abrogation of vesicle aggregation but not vesicle association of 4BAH2.

Figure 7. Small molecule inhibitors exhibit different effects on 4BAH2 inhibition

AFM images were taken of bare SiO_x substrate (A) and 4BAH2 aggregates on SiO₂ substrate following adsorption in the absence of compounds (B), or in the presence of C4 (C) or A2 (D). Original scan size of 5 μm × 5 μm (see fig. S10 for quantitative sample height analysis with linescans at the level of two different arrowheads (red and green), and magnification at 5X of the indicated boxed areas). Binding mass of genotype 1b 4BAH2 (E) or genotype 2a 4BAH2 (F) to a SiO_x-supported POPC lipid bilayer platform in the absence or presence of A2 or C4, as determined by QCM-D monitoring (fig. S12).