Review

Development of novel therapies for hepatitis C

Stanley M. Lemon, Jane A. McKeating, Thomas Pietschmann, David N. Frick, Jeffrey S. Glenn, Timothy L. Tellinghuisen, Julian Symons, Phillip A. Furman

Abstract

The current standard of care for the treatment of hepatitis C virus (HCV) infection is a combination of pegylated IFN and ribavirin (Peg-IFN/RBV). Because of the adverse effects associated with both IFN and ribavirin and because Peg-IFN/RBV provides only about a 45–50% sustained virological response (SVR, undetectable HCV RNA for greater than 24 weeks after cessation of therapy) in genotype 1-infected individuals, there is a need for more potent anti-HCV compounds with fewer adverse effects. The twenty-first International Conference on Antiviral Research held in May 2009 in Miami Beach, Florida, featured a special session focused on novel targets for HCV therapy. The session included presentations by world-renowned experts in HCV virology and covered a diverse array of potential targets for the development of new classes of HCV therapies. This review contains concise summaries of discussed topics that included the innate immune response, virus entry, the NS2 protease, the NS3 helicase, NS4B, and NS5A. Each presenter discussed the current knowledge of these targets and provided examples of recent scientific breakthroughs that are enhancing our understanding of these targets. As our understanding of the role of these novel anti-HCV targets increases so will our ability to discover new, more safe and effective anti-HCV therapies.

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(Marcellin and Boyer, 2003). The number of individuals infected with HCV continues to increase and persistently infected persons are at risk of developing cirrhosis and hepatocellular carcinomas. Treatment is limited to the combination of pegylated interferon (Peg-IFN) and ribavirin. Given the severe side effects such as depression, flu-like symptoms, fatigue, and hemolytic anemia, many patients are forced to discontinue therapy.

In recent years there have been significant advances in our understanding of the replication of HCV and the role of viral non-structural proteins (Bartenschlager and Lohmann, 2000; Appel et al., 2006; Brass et al., 2006). HCV has a single-stranded positive sense, 9.5 kb RNA genome that is flanked at each terminus by a 5′- and 3′-non-translated region (NTR) and contains one long open reading frame that encodes a precursor polyprotein of about 3000 amino acids. Translation of the polyprotein is directed by the internal ribosome entry site located within the 5′-NTR. The polyprotein is subsequently processed into both structural (core, envelope 1, envelope 2, and p7) and non-structural (NS2, NS3, NS4A, NS4B, NS5A, NS5B) proteins by cellular and viral proteases. Core protein is a highly basic protein that forms the nucleocapsid. The envelope proteins E1 and E2 are highly glycosylated transmembrane proteins that associate noncovalently to form the viral envelope. Protein p7 is a highly hydrophilic polypeptide that forms hexamers and has been reported to have ion channel activity. NS2 is a cis-acting autoprotease that is essential for viral replication. NS2 catalyzes the cleavage of the polyprotein precursor at the NS2/NS3 junction, and also plays an essential role in virus assembly. NS3 is a bifunctional protein with serine protease activity in the amino terminal one-third, which is responsible for cleavage at the NS3/NS4A, NS4A/NS4B, NS4B/NS5A and NS5A/NS5B sites, and NTPase/helicase activities in the C-terminal two-thirds of the protein. The NS4A polypeptide functions as a co-factor for NS3 protease activity. NS4B was demonstrated to induce specific cellular membrane changes, creating a membranous web that serves as a scaffold for the formation of the viral replication complex. NS5A is a phosphoprotein of multiple functions and is essential for viral replication. A potential role in modulating the IFN response has been suggested for NS5A. NS5B catalyzes the synthesis of both minus-strand and plus-strand RNA.

Major breakthroughs have been made in the development of virus-specific drugs that have demonstrated promising efficacy in reducing viral replication in HCV-infected patients. These include nucleoside and non-nucleoside inhibitors of the NS5B polymerase and inhibitors of the NS3/4A protease (Kwong et al., 2008). More recently inhibitors of NS4B and NS5A have been reported (Einav et al., 2008a,b; Gao et al., 2008; Quinkert et al., 2008). As a part of the twenty-second International Conference on Antiviral Research (ICAR) a mini-symposium was organized to highlight fundamental research aimed at providing an overview of anti-HCV targets other than the NS3 protease and NS5B polymerase. The focus of the symposium was on the innate immune response, viral entry, NS2, the NS3 helicase, NS4B, and NS5A. The invited speakers are renowned for their groundbreaking discoveries that will surely enhance drug discovery and the development of future therapies. In this review, the most relevant aspects of each presentation have been summarized by respective authors with the hope that readers will gain a first-hand description of the event that took place and the future that lies ahead.

2. Innate immune responses in hepatitis C virus infection

A key feature of HCV is its ability to modify the intracellular environment in ways that favor viral replication and promote its long-term persistence within the liver. Its capacity for immune evasion is multi-factorial, and includes disruption of signaling pathways that induce innate antiviral responses, evasion of IFN-mediated effector responses, and poorly understood mechanisms that both delay adaptive T-cell responses and render them insufficient for elimination of HCV. A better understanding of these multiple mechanisms may aid in the design of improved therapeutic interventions.

Among the ten discreet proteins expressed by HCV, the NS3/4A serine protease plays a major role in the ability of the virus to confound early innate immune responses that lead to induction of type I IFN-α/β synthesis and the establishment of a cellular antiviral state. NS3/4A disrupts the viral activation of the transcription factors IFN regulatory factor 3 (IRF-3) and NF-κB by inducing cleavage of two critically important signaling adaptor molecules, TRIF (a.k.a., TICAM-1), and MAVS (a.k.a., IPS-1, VISA, or Cardif) (Li et al., 2005a,b; Meylan et al., 2005). The proteolytic degradation of MAVS by the major HCV protease effectively blocks the induction of IFN synthesis through an intracellular pathway that is initiated upon recognition of viral RNA by the cytosolic DEAD-box helicase, RIG-I, that acts as a sensor for short, cytosolic double- and single-stranded viral RNAs with free 5′ triphosphates (Loo et al., 2006) (Fig. 1A, right). NS3/4A cleavage of TRIF disrupts signaling through a pathway initiated by Toll-like receptor 3 (TLR3) recognition of viral double-stranded RNA within the early endosome compartment (Wang et al., 2009) (Fig. 1A, left). These two pathways converge at the level of the kinases responsible for activation of IRF-3 and NF-κB, latent transcription factors that are dually important for initiation of type I IFN-α/β synthesis (Takeuchi and Akira, 2009).

Cleavage of both MAVS and TRIF has been demonstrated in vitro in cells infected with cell culture-derived HCV (Fig. 1B), and this has been shown to lead to disruption of both RIG-1 and TLR3 signaling, blocking the induction of IFN-β promoter activity normally observed when cells are infected with Sendai virus or stimulated with poly-(I:C), a double-stranded RNA surrogate (Loo et al., 2006; Wang et al., 2009). Both TLR3-mediated signaling and RIG-1 signaling are initially activated by infection, indicating that both RIG-1 and TLR3 sense HCV RNAs, and both function to partially restrict HCV replication in vitro. However, as the abundance of the NS3/4A protease accumulates within the cell, both signaling pathways are disrupted by HCV infection (Loo et al., 2006; Wang et al., 2009). The blockade of these pathways is likely to be important to the successful infection of the liver by HCV, as signaling through either pathway normally contributes to the induction of a cellular antiviral state. In both cases, the cleavage of the adaptor protein can be reversed by treatment of the cells with inhibitors of the NS3/4A protease (Loo et al., 2006; Wang et al., 2009). These data indicate that the NS3/4A serine protease has a dual role in the viral replication cycle, including both an essential role in processing of the polyprotein, and an accessory role in modifying the intracellular environment to limit the induction of IFN responses.

It is not clear, however, how disruption of TLR3 and RIG-1 signaling contributes to the long-term persistence of HCV infection in chronic hepatitis C. Both immune evasion mechanisms are likely to be important, but MAVS proteolysis is also efficiently induced by infection of cells with hepatitis A virus (HAV), a picornavirus that is incapable of causing long-term persistent infection or chronic hepatitis. In this case, the 3ABC precursor of the HAV 3C(pro) cysteine protease co-localizes with and cleaves MAVS where it is resident on the mitochondrial outer membrane. This disrupts activation of IRF-3 through a pathogen recognition receptor pathway that runs parallel to the RIG-1 pathway, and that is initiated by sensing of viral dsRNA by the cytosolic DEAD-box helicase, MDA5 (Yang et al., 2007). The 3ABC cleavage of MAVS requires both the protease activity of 3C(pro) and a transmembrane domain in 3A that specifically directs 3ABC to mitochondria, thereby highlighting the importance of mitochondria in the innate response to hepatitis virus infections (Yang et al., 2007). However, these results also suggest that, while...
blockade of RIG-I signaling by NS3/4A may be necessary for HCV persistence, it is not by itself sufficient since it is also observed with HAV. The same may be said for the disruption of TLR3 signaling that occurs as a result of NS3/4A-mediated cleavage of TRIF, as recent evidence suggests that this signaling pathway is also effectively disrupted by HAV infection (L. Qu and S.M. Lemon, unpublished data). However, the blockade of TLR3 signaling may be more important, because this pathway contributes to T-cell cross-priming, the induction of adaptive T-cell responses, and the expression of type II IFN-γ (Negishi et al., 2008).

It has been suggested that restoration of IFN signaling pathways by antiviral inhibitors of the NS3/4A protease may contribute to the clinical effectiveness of these compounds. Consistent with this, recent studies have confirmed the potential of NS3/4A inhibitors to reverse the RIG-I signaling defect in replicon cells, while this was not possible with a potent allosteric inhibitor of the NS5B RNA-dependent RNA polymerase (Liang et al., 2008). However, these studies also indicate that pharmacologic rescue of RIG-I signaling is achieved in vitro in HCV replicon cells or in cells infected with HCV only at concentrations of antiviral compounds far in excess of those expected to be achieved in vivo. In addition, the RIG-I signaling defect persists in such cells even when antiviral concentrations that appear to fully prevent NS3/4A-mediated proteolysis of MAVS (Liang et al., 2008). Taken together, these data suggest that antiviral reversal of HCV-mediated RIG-I blockade may only be achieved in the clinic by exceptionally potent protease inhibitors that accumulate to high concentrations within the hepatocyte. Collectively, the data suggest that protease inhibitors are unlikely to possess an intrinsic therapeutic advantage over other classes of antiviral compounds that target alternative steps in the viral replication cycle. Any antiviral that limits HCV protein expression will eventually reverse HCV-mediated blockade of innate immune signaling pathways.

Several studies have documented abundant expression of numerous IFN-stimulated genes (ISGs) in HCV-infected chimpanzees and in many (but not all) HCV-infected persons, prior to therapy (Bigger et al., 2004; Sarasin-Filipowicz et al., 2008). This suggests that HCV infection is capable of producing a robust IFN response in many patients, despite the capacity of NS3/4A to block virus stimulation of both TLR3 and RIG-I signaling leading to activation of IRF-3. The origin of this apparent IFN response remains obscure, but it is likely to be the plasmacytoid dendritic cell (pDC). pDCs represent the dominant cell type responsible for IFN responses in many virus infections, and in these cells IFN synthesis is activated via a TRIF- and MAVS-independent pathway initiated by TLR7 recognition of viral RNA. Much remains to be learned about the role of this innate immune signaling pathway in the pathogenesis of HCV.

Genome-wide studies have thus far failed to identify any polymorphisms in the genes that encode host proteins involved in the RIG-I or TLR3 signaling pathways that are associated with either the response to therapy with Peg-IFN and ribavirin, or the initial outcome of HCV infection (clearance or persistence of virus). In contrast, such polymorphisms have been identified within the IFN-λ3 (a.k.a., IL-28B) gene and shown to have a very strong influence on the success or failure of Peg-IFN and ribavirin therapy (Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009). Whether this will be shown to be the case with small molecule antiviral therapies is unknown at this point. However, it seems very likely that polymorphisms in the IFN-λ3 gene will influence the outcome of therapy with direct acting antiviral agents, as they are also strongly associated with the success or failure of the endogenous host response to spontaneously eliminate HCV following initial infection with the virus (Thomas et al., 2009). Type III IFNs (IFN-λ) are induced through similar pathways as type I IFN-α/ß, and induce a similar array of ISGs in cells bearing specific type III IFN receptors. However, type III IFN receptors are unique, and are thought to have a relatively restricted distribution compared to type I IFN receptors. Type III receptors are predominantly expressed on epithelial cells, and are present on hepatocytes. However, a mechanistic understanding of
the role of IFN-λ3 in HCV infection and the response to therapy is lacking, and is certain to be the focus of many research groups in the field.

The multiple evasive maneuvers adopted by HCV in an effort to escape innate immune surveillance provide a compelling argument for the importance of the host IFN response in virus defense within the liver. However, these mechanisms of immune evasion are also likely to significantly impact the strength of later adaptive immune responses. While well beyond the scope of this discussion, cytokines and costimulatory molecules induced via innate immune recognition of the virus play key roles in T-cell activation and in the development of antigen-specific T- and B-cell responses (Iwasaki and Medzhitov, 2010). Thus, the successful evasion of innate immune responses early in the course of acute infection could be a critical factor in determining persistence vs. clearance of the virus.

3. The complexities of hepatitis C virus entry

Viruses initiate infection by attaching to host cell molecules or receptors and blocking particle internalization provides an attractive target for therapeutic intervention. HCV is an enveloped positive-stranded RNA virus that encodes two glycoproteins (gps), E1 and E2, both of which are essential for particle entry. The observation that retroviral pseudoparticles bearing HCV E1E2 gps (HCVpp) infect hepatocytes and hepatoma derived cell lines suggest that virus–receptor interactions may in part define HCV tropism for the liver. The recent discovery that some strains of HCV can replicate in cell culture (HCVcc) and release infectious particles (reviewed in Timpe and McKeating, 2008) and Occludin (OCLN) (Benedito et al., 2009; Liu et al., 2009; Ploss et al., 2009, submitted for publication). Critical factors defining HCV entry (Fig. 2). Two additional Claudin family members, CLDN6 and CLDN9, have been reported to confer HCV entry (Meertens et al., 2008; Zheng et al., 2007). CD81 and SR-BI interact with HCV E1E2 gps, suggesting an initial role in mediating high affinity virus attachment to the cell. In contrast, there is minimal data to support an interaction of the tight junction proteins with HCV that may reflect the multi-step nature of HCV entry or an indirect role for these proteins in the internalization process.

Since expression of all four molecules is not uniquely restricted to hepatocytes in the liver, receptor activity may be defined by higher order interactions between these proteins mediating HCV particle trafficking and internalization. Imaging techniques have been developed that take advantage of fluorescence resonance energy transfer (FRET) to study protein–protein interactions. AcGFP and DsRed tagged forms of CD81 and CLDN1 co-localize and FRET occurs between the tagged co-receptors, consistent with the formation of a CD81–CLDN1 co-receptor complex (Harris et al., 2008). Several independent reports have demonstrated CD81 association with CLDN1 using a variety of imaging and biochemical techniques (Cukierman et al., 2009; Kovalenko et al., 2007; Yang et al., 2008). To ascertain the role of CD81–CLDN1 complexes in HCV entry, we investigated the relationship between various members of the CLDN family and CD81 in the CLDN1 null 293T human embryonal renal cell line. Only CLDNs 1, 6, 9 and 12 co-localized with CD81 in a defined organization at the plasma membrane, consistent with a 1:1 molar relationship or stoichiometry. FRET studies confirmed interaction(s) between CLDN1, 6 and 9 and CD81, suggesting a relationship between CLDN association with CD81 and HCV receptor activity. Importantly, mutation of residues 32 and 48 in the CLDN1 first extracellular loop (EC1) ablated association with CD81 and viral receptor activity (Harris et al., 2008, submitted for publication). Furthermore, mutation of the same residues in the receptor inactive CLDN7 molecule allowed CLDN7-M32I/K48E to associate with CD81 and facilitated viral entry into 293T cells, demonstrating an essential role of CLDN1–CD81 complexes in HCV internalization.

Many tissues in the body contain polarized cells and hepatocytes are known to be highly polarized with tight junctions regulating the paracellular transit of solutes (Fig. 3). To address the role of polarization in HCV entry, we utilized the well characterized colorectal adenocarcinoma Caco-2 cell line and reported that disrupting epithelial barrier formation increased HCV entry, suggesting that TJs impose a physical barrier and restrict viral access to receptors (Mee et al., 2008). However, hepatocytes are multipolar and do not possess the columnar morphology of simple epithelial cells, with at least two basal surfaces facing the circulation and a branched network of grooves between adjacent cells constituting the apical or bile canalicular (BC) surface (Wang and Boyer, 2004) (Fig. 3). Thus, simple epithelial cell systems may fail to recapitulate the more complex hepatic polarity that HCV encounters in the liver.

The majority of immortalized hepatic cell lines and primary hepatocytes de-differentiate in culture and fail to demonstrate a complex polarized phenotype. Several studies have reported that human HepG2 hepatoblastoma cells polarize in culture, forming apical cysts that are analogous to BC in the liver. We have uti-
lized the HepG2 cell line to investigate the effect(s) of hepatic polarity on HCV entry. Tight junction strands encircle the apical region and comprise multiple transmembrane (CLDNs, OCLN, and junctional adhesion molecule JAM) (Fig. 4), scaffolding and signaling proteins (reviewed in Paris et al., 2008). We recently reported CLDN1 expression at basolateral and apical hepatocyte membranes in normal liver tissue (Reynolds et al., 2008) and in polarized HepG2 cells, with an enrichment at tight junction-associated apical sites (Mee et al., 2009b). Treatment of polarized HepG2 cells with the Protein Kinase A antagonist (Rp-8-Br-cAMPS) (Farquhar et al., 2008) reduced CLDN1 expression at the basal membrane and inhibited HCV entry. In contrast, disruption of HepG2 polarization with the phorbol ester (PMA) induced a re-localization of CLDN1, ZO-1 and OCLN from TJs and promoted HCV entry, suggesting that non-junctional pools of CLDN1 and OCLN may play a role in HCV entry. Importantly, CD81–CLDN1 co-receptor complexes were only observed at the basolateral membrane and were undetectable at tight junctions (Harris et al., 2008, submitted for publication).

Since HCV enters the liver via the sinusoidal blood the virus will encounter receptors expressed on the sinusoidal or basal surface of the hepatocyte. The essential role of CLDN1–CD81 complexes in viral entry and their localization at the basolateral surface of polarized hepatoma cells, supports a model where virus-receptor engagement at the basal membrane initiates particle internalization (Mee et al., 2009b). The role of OCLN in HCV entry into polarized cells is poorly defined and is hindered by the lack of antibodies to extracellular expressed OCLN epitopes. Recent data from our laboratory demonstrates that vascular endothelial growth factor treatment of polarized HepG2 cells increases the basolateral pools of OCLN, concomitant reduction in polarity and increased HCV entry (Mee et al., 2009a). At present it is unknown whether any of the viral receptors, including CD81 and CLDN1, are endocytosed.

**Fig. 4.** Claudin oligomerisation during tight junction strand formation. CLDNs exhibit a similar topology to tetraspanins and have the capacity to associate with themselves (homophilic interactions) and other CLDNs (heterophilic interactions) both within a cell and between apposing cells via interactions between their extracellular loops. CLDN polymerization is critical for the establishment of membranous tight junction strands, however, additional transmembrane (occludin, tricellulin, junctional adhesion molecule) and scaffolding proteins are required for the spatial and functional organization of tight junctions in polarized epithelia.
with HCV and further research on the trafficking and endocytic routing of receptor complexes and virus particles in polarized hepatocytes is required to fully appreciate the complex entry process of HCV.

Targeting viral receptors can be accomplished by various methods, including the design of small molecules that bind to proteins and prevent interaction(s) with HCV. Efforts are currently underway to screen for small molecule inhibitors of E2–CD81 interaction (Ziegler et al., 2009; Holzer et al., 2008). While the crystal structure of soluble CD81 large extracellular loop (sCD81, LEL) (Kitadokoro et al., 2001) is available to aid the design of small molecules targeting the CD81–E2 interface, studies from our laboratory to identify CD81 residues that interact with HCV demonstrate that mutations in sCD81 which ablate viral interaction had negligible effect(s) when engineered in the native molecule, suggesting that sCD81 may not be an accurate mimic for the native molecule and a poor model for the screening of inhibitors (Flint et al., 2006). Recent studies to express full-length CD81 in yeast provide a promising tool for structure-function studies of CD81 and its interaction(s) with HCV (Jamshad et al., 2008). Further studies are required to ascertain whether CD81 or CD81–CLDN1 complexes will yield as targets for therapeutic intervention without any cellular toxicity.

Developing therapeutics targeting SR-BI may be complicated given its central role in reverse cholesterol transport, the mechanism by which the body disposes of cholesterol. Loss of this function would undoubtedly have serious side effects such as coronary heart disease. Thus, any therapy targeting SR-BI would need to inhibit interaction(s) with HCV while maintaining its physiological role. Recent reports suggest that the small molecule entry inhibitor ITX5061 targets SR-BI and we eagerly await the outcome of current clinical trials (McKelvy et al., 2009). Similar concerns arise over targeting CLDN1 and OCLN, which have a critical role in tight junction formation and in the maintenance of cell polarity. A recent study reported that antibodies targeting extracellular epitopes of CLDN1 inhibit HCV infection and had minimal effect(s) on HepG2 polarity or tight junction function (Krieger et al., 2010). Reports that HCV can infect polarized and non-polarized Caco-2 cells, suggest that CLDN1 does not need to be recruited into a functional tight junction to allow viral entry (Mee et al., 2008). Therefore, it may be possible to target non-junctional pools of CLDN1 without disrupting tight junctions, but the feasibility of this approach remains to be determined. A greater understanding of the mechanisms of HCV entry will be required before receptor-specific therapeutics are fully realized.

4. Targeting hepatitis C virus NS2—an unusual protease with multiple functions

HCV research has striven to unravel the structure and function of viral proteins, the role of cellular co-factors for replication and searched for ways to exploit this knowledge for the development of novel therapeutic options to cure infected patients. These efforts have not only yielded remarkable progress in our understanding of the mechanisms of virus replication but also propelled a number of drug candidates inhibiting key viral enzymes like the NS5B RNA dependent RNA polymerase or the principal viral protease, NS3, into clinical testing. Despite promising first experiences, side effects, genotype-specific differences in efficacy and drug resistance attenuate optimism and fuel development of additional therapeutics directed against different viral or host targets and operating with novel mode of action. Among the multiple viral therapy targets, the NS2 protease has been neglected at least in part due to limited knowledge about its function and precise mode of catalysis. However, a number of recent advances now highlight this protein as unique enzyme and drug target with multiple functions.

In contrast to NS3, which cleaves the HCV polyprotein at four different positions and in addition interferes with induction of innate immune responses by processing two cellular adaptor proteins, NS2 catalyzes a single vital cleavage at the NS2/NS3 boundary which is essential for RNA replication. The protein consists of a hydrophobic N-terminal domain likely encompassing three transmembrane helices and a C-terminal cytoplasmic domain. Recently, the crystal structure of the latter domain which is sufficient for catalysis was solved by Lorenz et al. (2006) revealing remarkable structural features and shedding light on the mode of catalysis: NS2 is a dimeric cysteine protease with no known cellular homologue. Importantly, the active site of the enzyme is composed of both monomers with a histidine and a glutamate residue contributed by one and the nucleophilic cysteine provided by the other monomer (Fig. 5). This architecture has important functional implications, raises intriguing regulatory possibilities and may define ways how to interfere with enzyme function: Initially NS2/NS3 cleavage was thought to proceed in cis via a unimolecular reaction. The finding that dimerization is crucial for catalysis suggests that a certain concentration of NS2 is necessary for cleavage and in turn for production of active replicase complexes and initiation of de novo RNA synthesis. It has been speculated that as a consequence RNA replication may be delayed facilitating accumulation of sufficient NS3–4A complexes to antagonize induction of type I IFNs by the host cell. However, in terms of drug development this requirement may be an additional Achilles heel of the protein: On one hand, a delayed enzyme kinetic may facilitate access of small molecules for inhibition of the protease. On the other hand, compounds that prevent dimerization and thus interfere with enzyme activity may be an additional means to block virus replication.

Along similar lines, a crucial interplay between NS2 and NS3 is emerging as a possible toehold for development of inhibitors. It has been known for years that NS2/3 cleavage requires at least the N-terminal 181 residues of NS3, which encompasses the NS3 serine protease domain as well as a structural Zn$^{2+}$ binding site that is important for function of both enzymes. Schregel et al. (2009) dissected the mechanistic details of this requirement and thereby identified two independent domains within this portion of NS3 which cooperate with NS2 during catalysis. While these authors noted that NS2 with as little as two residues of NS3 has basal activity clearly demonstrating that NS2 itself is a bona fide protease, the two NS3 subdomains dramatically enhanced catalysis. Although the reason for this complex regulation of NS2–NS3 protease activity is not fully understood, this may serve as a “timer” that coordinates protease activity with folding of NS2 and NS3 thus ensuring cleavage precisely at the right time for all downstream events of the viral replication cycle. Similar to interference with NS2 dimerization prevention of the NS2–NS3 interaction may be a therapeutic means to disturb NS2–NS3 processing and in turn virus replication.

The recent development of fully permissive tissue culture infection systems for HCV cleared the way for discovery of a second and unexpected function of NS2. It has been questioned for a long time why the virus encodes a protease solely for the release of itself from the polyprotein. New findings obtained with the JFH1–1-based infection model now implicate NS2 as crucial co-factor for virus assembly (Fig. 6) (Jirasko et al., 2008; Jones et al., 2007). Although the precise role of NS2 during virus production is unclear, genetic evidence suggests that this function may involve interactions with viral structural proteins (C, E1 or E2) or p7 via the N-terminal portion of NS2 and possibly interact with NS3 through the C-terminal part of the protein. Besides this, NS2 also cooperates with cellular factors. More specifically, Ciesek et al. (2009) noted that NS2 function relies on cyclophilin A which via its cis-trans prolyl isomerase activity may modulate NS2 protein folding and in turn function. Additional intriguing findings link NS2 with regulation of apoptosis and inhibition of gene expression. These functions may be
Fig. 5. Schematic representation of the HCV genome and architecture of the dimeric NS2 protease domain. Composite active sites of NS2 consisting of residues derived from each monomer (blue and red) are highlighted by a dashed box. Polyprotein cleavages conducted by viral and cellular enzymes are indicated. NS2 protease structure was kindly provided by Ivo Lorenz, Joseph Marcotrigiano and Charles M. Rice. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Fig. 6. Full-length NS2 protein is crucial for production of infectious HCV particles. (A) Schematic representation of constructs used to dissect NS2 function in virus production. HCV replication was uncoupled from NS2 protease activity by insertion of an internal ribosome entry site of the encephalomyocarditis virus (EMCV IRES). The putative membrane topology and the features of individual deletion mutants of NS2 are depicted below. (B) Assessment of HCV replication and release of HCV particles from Huh7.5 cells transfected with the given constructs as determined by using a core-specific ELISA. (C) Infectivity released into the culture fluid of transfected cells determined using a limiting dilution infection assay. (D) Comparison of intracellular and extracellular infectivity 48 h post transfection. Intracellular infectivity was determined in cell lysates prepared by three cycles of freeze and thaw. The black bar in panels (B) to (D) represents the background of the assays (For additional details compare (Jirasko et al., 2008).)
crucial for establishment of a chronic infection in two ways: First, by preventing apoptosis, NS2 may permit survival of host cells and thus ongoing replication. Second, interference with cytokines like for instance IFN-β may dampen immune responses thus facilitating viral immune evasion.

Together these recent advances have much refined our understanding of NS2, the second crucial viral protease, and have highlighted intriguing novel facets and molecular details of this versatile protein. In addition these advances point towards possible modes of therapeutic intervention with NS2 function(s). In particular the availability of the crystal structure of the catalytic domain of NS2 and novel model systems for dissection of protein function should not only facilitate deeper insights in the multiple function of NS2 but also help development and evaluation of NS2-specific antivirals.

5. New methods to identify and analyze HCV helicase inhibitors

Great progress has been made over the last decade in understanding the multifunctional NS3 protease/helicase encoded by the hepatitis C virus (HCV), and the NS3 protease activity is the likely target for the next generation of HCV antivirals. In contrast, the NS3 helicase function remains one of the most undeveloped targets for anti-HCV agents. The NS3 helicase separates duplex RNA (Kim et al., 1995), separates duplex DNA (Tai et al., 1996), and displaces nucleic acid-bound proteins (Morris et al., 2002) in reactions that are fueled by ATP hydrolysis (Suzich et al., 1993). The helicase portion of NS3 is a Y-shaped molecule with several ligand-binding regions (Fig. 7). ATP likely binds in the cleft separating two RecA-like motor domains, and one strand of RNA binds in the cleft separating the two N-terminal motor domains from the C-terminal helicase domain (Kim et al., 1998). A beta loop extends from one motor domain to separate duplex nucleic acids (Lam et al., 2003), and a second single-stranded RNA strand might bind in the cleft separating the helicase from the protease portion of the enzyme (Frick et al., 2004).

The role of the helicase in HCV replication is not entirely clear, but HCV RNA without a functioning helicase cannot replicate in cells (Lam and Frick, 2006), and HCV with a defective NS3 ATPase function is not infectious (Kolykhulov et al., 2000). Similarly, cellular HCV RNA replication can be blocked with RNA aptamers, antibodies, or small molecules that target the NS3 helicase (reviewed in Frick, 2007). It is therefore somewhat puzzling why so few helicase inhibitors have progressed into clinical trials, especially considering that atomic structures of the HCV helicase and high-throughput helicase assays have been available for more than a decade. In addition to the small molecule inhibitors that have been recently reviewed elsewhere (Frick, 2007; Belon and Frick, 2009), a few HCV helicase inhibitors have been reported this year (Table 1). Although this latest generation of compounds all inhibit HCV RNA replication in cells, selectivity and toxicity is still an issue. The one exception is the acridone derivative Compound 23, which is potent against HCV replicons (EC\textsubscript{50} = 3 μM) and relatively non-toxic (CC\textsubscript{50} > 50 μM). Compound 23 does not inhibit the HCV NS5B polymerase but it inhibits NS3 catalyzed DNA unwinding with an IC\textsubscript{50} of 110 μM (Manfoni et al., 2009). The more potent new HCV helicase inhibitors are also quite toxic. For example, Compound 4, a novel rationally designed inhibitor that might occupy the known NS3RNA-binding cleft, inhibits HCV replicons with an EC\textsubscript{50} of 9 μM but is relatively toxic to cells with a CC\textsubscript{50} of 30 μM (Kandil et al., 2009). Compound 21, which is the most selective of a series of amidinoanthracelines, inhibits HCV replicons with an EC\textsubscript{50} of 0.13 μM but is toxic to cells at a CC\textsubscript{50} of 4.3 μM (Krawczyk et al., 2009). Compound 12, which was derived by fragment based analysis of the helicase inhibitor Soluble BlueHT and lead optimization of triphenylmethane derivatives, inhibits HCV replicons with an EC\textsubscript{50} of 2.7 μM and but is toxic to cells at a CC\textsubscript{50} of 10 μM (Chen et al., 2009).

One explanation for the relative toxicity of HCV helicase inhibitors is that past screens might not have utilized ideal assays. The simplest method to monitor HCV helicase activity in a high-throughput format is to measure nucleic acid-stimulated ATP hydrolysis using one of the common commercially available kits (Funk et al., 2004). The problem with relying on such assays is that the HCV helicase ATP-binding site is virtually identical to the ATP-binding sites of related cellular motor proteins, and many of the hits in such screens might also bind those proteins. Directly monitoring HCV helicase activity is preferable, but such assays are more difficult, primarily because of the reversible nature of the unwinding reaction. Typical assays monitoring DNA or RNA unwinding require the addition of agents to capture the single-stranded products to prevent their annealing. These product traps are typically ssDNA-binding proteins or complementary DNA, and thus, any hits in such assays might target the action of these traps rather than the helicase itself. Another problem with many high-throughput helicase assays is that they usually monitor the emergence of a fluorescent product. Typically, a fluorescently labeled oligonucleotide is annealed to an oligonucleotide that quenches the fluorophore, and fluorescence increases in these assays when the quenching strand is displaced by the helicase (Boguszewska-Chachulska et al., 2004; Tani et al., 2009). In such assays, any compound that also quenches the fluorescence of the chosen fluorophore would be identified as a hit even if it has no effect on helicase action. In addition any DNA-binding compounds would act as potent inhibitors, but such compounds would also likely be toxic to cells.

We recently developed a new helicase assay suitable for high-throughput screening that monitors the displacement of a molecular beacon bound to DNA or RNA. This molecular beacon based helicase assay (MBHA) is essentially irreversible because the reaction products form hairpins. Another advantage to the MBHA is that it monitors a decrease in substrate fluorescence, and thus any compound that acts simply by quenching substrate fluorescence can be quickly identified (Belon and Frick, 2008). We have used the MBHA to analyze a series of compounds that were pre-

![Image](http://www.cgl.ucsf.edu/chimera/) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)
Recently reported HCV helicase inhibitors.

Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Reference</th>
<th>Helicase (IC50)</th>
<th>Antiviral (EC50)</th>
<th>Toxicity (CC50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound 4</td>
<td>(Kandil et al., 2009)</td>
<td>0.26 μM</td>
<td>9 μM</td>
<td>30 μM</td>
</tr>
<tr>
<td>Compound 23</td>
<td>(Manfroni et al., 2009)</td>
<td>110 μM</td>
<td>3 μM</td>
<td>&gt;50 μM</td>
</tr>
<tr>
<td>Compound 21</td>
<td>(Krawczyk et al., 2009)</td>
<td>0.51 μM</td>
<td>0.13 μM</td>
<td>4.3 μM</td>
</tr>
<tr>
<td>Compound 12</td>
<td>(Chen et al., 2009)</td>
<td>110 μM</td>
<td>2.7 μM</td>
<td>10 μM</td>
</tr>
</tbody>
</table>

a The concentration required to reduce observed rates of HCV helicase catalyzed DNA unwinding by 50%.

b The concentration leading to a 50% reduction in the amount of HCV replicons in cells.

c The concentration that inhibits the proliferation of exponentially growing cells by 50%.

6. New targets within HCV NS4B

NS4B is a protein often described as having an “unknown function,” but it has recently emerged as a critical player in the viral replication cycle (Sklan and Glenn, 2006). Like other positive strand RNA viruses, HCV replicates its genome in intimate association with host cell intracellular membrane structures. In the case of HCV, the latter appear to be novel structures induced by HCV and termed the membranous web due to their appearance on electron microscopy (Egger et al., 2002; Gosert et al., 2002). The membranous web appears to be the platform upon which HCV replication occurs and NS4B has been shown to be necessary and sufficient to induce the formation of these structures (Fig. 8). The molecular mechanism(s) employed by NS4B to induce the membranous web remains unknown, although several functional domains that might contribute to this process have been identified in NS4B. These include a nucleotide binding and NTPase activity (Einav et al., 2004, 2008b; Thompson et al., 2009) and an N-terminal amphipathic helix (Elazar et al., 2004)—which have been genetically validated as essential for viral RNA replication, and thus represent candidate new anti-HCV targets. Because NS4B induces the membranous replication platform, and has been shown to bring together the other non-structural protein components of the replicase complex to these apparent sites of replication, we hypothesized that NS4B might also bind the viral RNA.

We discovered that NS4B does indeed have RNA binding activity. Because NS4B has at least four transmembrane domain segments, its purification in large quantities when expressed in bacteria (which lack an endoplasmic reticulum) proved to be somewhat challenging. To more fully study its RNA binding properties, we turned to an in vitro translation system supplemented with mammalian microsomal membranes where we expected a significant amount of the expressed NS4B to adopt its proper transmembrane topology. Because the amounts of protein typically produced in such systems tend to be low, however, we analyzed RNA-protein binding using a microfluidics based format, which can afford accurate measurements with nanoliter protein consumption.

NS4B was found to bind its RNA target with an apparent Kd of ~3 nM. NS4B displayed specificity for a specific segment of HCV RNA—namely the 3’ terminus of the negative RNA strand, the presumed region where progeny plus-strand genomes are initiated (Einav et al., 2008b). To characterize the determinants of RNA binding within NS4B, pairs of highly conserved positively charged amino acids, representing candidate so-called arginine-rich like motifs, were mutated to alanine. Such mutations were shown to partially or completely abrogate NS4B RNA binding. Moreover, introduction of these same mutations into HCV replicons resulted in similar partial or complete abrogation of RNA genome replication, thus genetically validating the RNA binding activity as a potential antiviral target (Einav et al., 2008b).

A small molecule screen for inhibitors of NS4B RNA binding identified clemizole hydrochloride as a relatively potent inhibitor of the NS4B: RNA interaction, with an IC50 of ~24 nM. Moreover, clemizole was found to inhibit HCV replication in vitro (EC50 = 8 μM) at non-cytotoxic concentrations. Clemizole-resistant mutants of HCV were isolated over a period of several months. Sequencing of the genomes harbored in resistant cells revealed mutations mapping to either NS4B or the 3’ terminus of the negative RNA strand, although they appear to yield virus with impaired replication fitness (Einav et al., 2008b). In addition, a derivative of clemizole that was more potent at inhibiting NS4B RNA binding exhibited greater antiviral activity. These results thus provide both further genetic and pharmacologic validation of clemizole’s mechanism of action. Finally, clemizole was found to be highly synergistic with NS3 protease inhibitors in advanced clinical development, with the degree of...
synergy being among the highest observed for any combination of antivirals (Einav et al., submitted for publication).

Of note, clemizole was widely used in patients with great tolerability (albeit for a completely different indication based on its originally intended H1 antihistamine activity) in the 1950s and 1960s, although it is not currently marketed in the U.S. Because of the unique bipartite nature of its HCV target (consisting of two viral components—the NS4B protein and the 3′-negative terminus RNA—that must interact with each other) there may be a reduced mutational space available for the NS4B to mutate to escape inhibition by clemizole—because NS4B still must be able to maintain the interaction with its partner RNA ligand.

Taken together, these results suggest that the NS4B RNA binding activity joins a growing collection of candidate targets within NS4B, and that clemizole in particular may represent an exciting potential component of future anti-HCV cocktails.

7. The hepatitis C virus NS5A protein; new functions and new mysteries

Despite decades of intensive study, mysteries abound about the basic aspects of the HCV lifecycle, hindering our understanding of HCV persistent infections and viral pathogenesis. In the infected cell many RNA directed processes must occur to maintain and spread an infection. Viral genomic RNA is constantly being replicated, serving as template for translation, and being packaged into new virions; processes that cannot occur simultaneously without functional conflicts. Little is known about the regulation of these events. The HCV encoded NS5A phosphoprotein has been proposed as a regulator of HCV replication cycle events for years, but the details have remained enigmatic.

Previous data from our lab and others have begun to decipher functional information about the essential role of NS5A in RNA replication, with most replication phenotypes mapping to the first of the three NS5A domains (domain I) (Fig. 9). Despite these advances, the role of the remaining two domains (domains II and III) in the HCV lifecycle remained enigmatic. We generated a series of deletions spanning all of domains II and III and demonstrated that domain II contains several residues involved in RNA replication where as domain III is dispensable for this process (Tellinghuisen et al., 2008b). Small RNA viruses rarely encode things that are not important to at least some aspect of the viral replication cycle, and indeed, domain III of HCV contains a number of conserved sequences suggesting an active maintenance of this region. We therefore decided to assess the importance of domain III in other aspects of the HCV replication cycle, focusing on the HCV infectious cell culture system (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). We generated a series of deletions in the context of the genotype 2a J6/JFH-1 infectious clone, and assessed the ability of viral genomes bearing these deletions to replicate and produce infectious virions. This effort identified a 15 amino acid deletion, designated deletion B (Fig. 9), which had no effect on HCV RNA replication, but completely blocked infectious virus production (Tellinghuisen et al., 2008a). Our initial characterization of this mutant indicated that it did not merely lead to the trapping of infectivity inside the host secretory pathway or lead
to the release of empty or otherwise defective virus particles. Deletion B clearly affected an early event in the HCV virion production pathway.

Further genetic mapping of deletion B indicated that a single serine residue within the deletion, serine 457, was responsible for the production of infectivity. This residue lies in a predicted casein kinase II (CKII) consensus phosphorylation motif, leading us to generate phosphomimetic mutations at this position. Alteration of serine 457 to alanine had no effect on RNA replication, but eliminated the release of infectivity, recapitulating the phenotype observed for deletion B. Mimicking phosphorylation by substituting serine 457 for an aspartic acid residue in the virus lowered RNA replication efficiency slightly, and had no effect on the release of infectivity. These data suggested serine 457 might function as a phosphorylation dependent signal regulating the switch from RNA replication to virion production, but they did little to demonstrate if this residue was actually phosphorylated by CKII. Using a cell permeable specific inhibitor of CKII, DMAT, we were able to inhibit infectivity release by more than 90% at concentrations near the published IC$_{50}$ of DMAT for CKII, with no effect on cell viability or RNA replication. CKII silencing by siRNA had no effect on cell viability or HCV RNA replication, but again led to a significant inhibition of infectivity release. Although chemical and genetic inhibition of CKII activity disrupted infectivity release of wild type J6/JFH-1 virus, this was not the case for the serine 457 to aspartic acid mutation that genetically mimics phosphorylation. We have also shown that peptides corresponding to the wild type sequence of this region of NS5A can be phosphorylated in vitro by purified human CKII, while peptides containing the serine 457 to alanine mutation cannot. Analysis of cell lysates by Western blotting with anti-NS5A antibodies suggests that mutations of serine 457 alter the phosphoform distribution of NS5A, favoring the accumulation of hypophosphorylated NS5A at the expense of the hyperphosphorylated form of the protein. We are currently investigating the phosphorylation state of serine 457 in infected cells using mass spectrometry based approaches, with a goal of determining if this residue is modified in vivo.

With a fair understanding of the signal that controls the switch between replication and assembly, one can begin to investigate how this might work in an infected cell. HCV replication occurs on modified membranes derived from the endoplasmic reticulum termed the membranous web, and early assembly events take place on viral nucleocapsid (C) coated cytoplasmic lipid droplets (LD) (Miyanari et al., 2007); distinct organelles in the infected cell. It is known that NS5A, and possibly other viral replicase proteins, localize to LDs, and this appears to be essential for virus production (Miyanari et al., 2007; Appel et al., 2008; Tellinghuisen et al., 2008a; Ma et al., 2008). Deletion B, and analogous mutations, have no defect in localizing to LDs, but appear to have lost the ability to co-localize with the viral C protein on LDs, thereby short circuiting an early step in virion biogenesis (Appel et al., 2008; Tellinghuisen et al., 2008a). It is increasingly clear that mutations that stabilize replication events tend to inhibit virion production, indicating a close relationship between these processes (Ma et al., 2008). A modification of the model originally proposed by Evans et al. (2004) in which NS5A is maintained in the functional replicase via association with host factors and loses this association upon phosphorylation, is an attractive model to explain these assembly results (Fig. 10). It is intriguing to imagine the association of NS5A with replicase (Fig. 10X) and assembly specific (Fig. 10Y) host proteins in a manner dependent on phosphorylation as a means to regulate the switch between these events. We have initiated the search for these host factors using proteomic and high-throughput genetic screening approaches and have begun to identify NS5A binding proteins that are hallmarks of NS5A activity in replication and assembly. What these factors will tell us about the role of NS5A as a regulator of the viral lifecycle is not known, but it is likely that exciting new answers ... and tantalizing new questions await.

8. Challenges for pursuing HCV therapies

There is a clear need for improved strategies for the treatment of HCV infection that leads to a complete cure of all infected patients and many challenges remain to be addressed. A shift
away from IFN-based therapy would constitute a major breakthrough. Genotype-dependent virological response is well known for Peg-IFN/RBV with genotypes 1 and 4 being less responsive than genotypes 2 and 3. The activity of non-nucleoside inhibitors of NS5B and NS3 protease inhibitors also has been found to vary across genotypes and subtypes (Qi et al., 2009; Cubero et al., 2008; Carroll and Olsen, 2006; Kwong et al., 2008). Therefore, because of the genetic heterogeneity of HCV, future compounds should display activity against a broad range of HCV genotypes and subtypes thereby increasing the SVR rate and/or decreasing the duration of treatment.

Because NS5B is devoid of a proof reading mechanism, HCV RNA replication is a highly error prone process that ultimately gives rise to a population of virus with a diversified genome. A consequence of this high mutation rate is that drug resistant polymorphisms existing as minor populations could become the dominant subtype under drug selection pressure. Therefore, a major challenge will be to develop drugs and strategies that minimize the emergence of drug-selected resistant mutations. This suggests that combination therapy aimed at several viral and/or host immune targets will probably become the future approach to treatment to reduce or eliminate drug resistance. This will require identifying and developing drugs that have a high barrier to resistance as well as combinations of small molecules that target different therapeutic areas to prevent and manage resistance. Experience with HIV suggests that combination of two or more small molecules would be required to control infection and prevent the emergence of drug resistant variants. Drug combinations will likely be tailored to the individual patient, based on baseline parameters, including genotype, and viral kinetics during therapy. An unanswered question is whether combinations of small molecules will be sufficient to cure patients or will a cure require the stimulation of the host immune response by immunomodulators or therapeutic vaccines.

Finally, as long as PEG-IFN/ribavirin remains the standard of care, it will be important to develop strategies for treating patients that are difficult to treat or previously have failed Peg-IFN/RBV therapy including patients of African-Americans, patients that fail to respond to Peg-IFN/RBV (non-responders), patients with severe fibrosis or cirrhosis, and HCV infected patients co-infected with HIV. For these patients new therapies and therapeutic approaches are needed.

References


Einav, S., Ivers-Skol, H., Czesnuk, E., Glenn, J.S. The Hepatitis C Virus (HCV) NS5B RNA binding inhibitor, clemizole, is highly synergistic with HCV protease inhibitors submitted for publication.


