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Hepatitis C: Recent Successes and Continuing Challenges in the Development of Improved Treatment Modalities

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Abstract

Dramatic progress is being made toward the development of less-toxic and simpler alternatives to the current standard-of-care therapy for chronic hepatitis C which involves a combination of pegylated interferon (peg-IFN) and ribavirin. Several accessible viral targets have been identified and licensure of the most advanced clinical compounds can be anticipated within the next several years. However, the highly replicative nature of HCV infection, coupled with error-prone viral RNA synthesis and considerable genome diversity, pose extraordinary challenges to drug development. Peg-IFN is likely to remain a mainstay of therapy for the foreseeable future, or until such time that multiple direct-acting antiviral (“STAT-C”) inhibitors are available and shown to provide a sufficiently high barrier to resistance when used in combination.

Hepatitis C virus (HCV) was identified twenty years ago by immunoscreening an expression library for antigens associated with what was then known as post-transfusion non-A, non-B hepatitis. Today, despite greater than 25,000 published studies, HCV continues to exert a substantial disease burden with ~200 million persons chronically infected with the virus worldwide. Classified within the *Flaviviridae* family, HCV is a positive-strand, RNA virus with a genome just under 10 kb in length [1]. There are 6 major genotypes of HCV which differ substantially in nucleotide sequence, and in the amino acid sequence of the single large polyprotein expressed by the virus. This polyprotein is processed into 10 distinct structural and nonstructural proteins, including several potential drug targets (Fig. 1a). The virus has a unique ability to evade host immune responses, and establishes long-term persistent infections in well over 50% of all infected persons; the mechanism(s) responsible for this remain incompletely defined. Importantly, such infections are often associated with progressive liver fibrosis, cirrhosis, and in some individuals, hepatocellular carcinoma.

From a drug discovery perspective, HCV is not an easy target. It is not possible to culture wild-type strains of HCV efficiently in cell culture, and this has impeded the elucidation of the viral life cycle as well as the development of specific antiviral agents. Several important breakthroughs have enabled drug discovery, however, including the development of infectious cDNA clones from which RNA can be transcribed that is infectious in vivo in chimpanzees (the only animal species other than humans susceptible to the virus). The development of RNA replicons in which the segment of the HCV genome encoding the structural and some nonstructural proteins is replaced by a selectable marker (neomycin phosphotransferase gene)

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and translation of the downstream non-structural proteins (NS3-5B) initiated by a heterologous, picornaviral internal ribosome entry site (IRES), has also allowed for the study of HCV RNA replication in cultured cells (typically Huh7 human hepatoma cells) (Fig. 1b) [2]. More recently developed replicons express the entire viral polyprotein, or easily quantifiable reporter enzymes such as firefly luciferase. More recently, it has become possible to generate infectious virus and study the entire virus life cycle in cell culture using an unusual genotype 2a cDNA clone isolated from a Japanese patient with a rare case of fulminant hepatitis C, designated as JFH-1 (“Japanese fulminant hepatitis 1”) [3]. While extraordinarily valuable to the field, this virus unfortunately has limited relevance to drug discovery, as genotype 2a viruses are typically quite responsive to interferon treatment and differ substantially in their genetic sequence from the more problematic genotype 1 viruses. A highly cell-culture adapted genotype 1a genome containing five adaptive mutations (H77-S) has also been shown to produce virus when transfected into Huh7 cells, but does so with only ~1% the efficiency of JFH-1 RNA [4]. Virus produced in vitro by transfection of either JFH-1 or H77S RNA is infectious for the chimpanzee.

Standard-of-care Treatment for Hepatitis C

The current standard treatment for patients infected with HCV is a combination of pegylated interferon- α (peg-IFN) and ribavirin (RBV). While the mechanism by which IFN acts is ill defined, the available evidence suggests that the direct antiviral effects of IFN are likely to outweigh its numerous immunomodulatory actions. An immediate decline in viremia (often described inaccurately as “virus load”) can be observed within hours of the administration of IFN to infected patients, suggesting that IFN induces a shut-off in virus production by infected hepatocytes [5]. This is consistent with IFN induction of a cellular antiviral state, and is associated with the upregulation of numerous IFN-stimulated genes (ISGs) within the liver [6]. Whether more slowly acting immunomodulatory effects also contribute to the success of therapy is unclear. Also unknown is the mechanism of action of RBV in this setting, which remains highly controversial. By itself, RBV has little if any direct antiviral effect, but when combined with peg-IFN it adds considerably to the number of treated patients achieving a sustained viral response (SVR) [7]. Unfortunately, many patients find peg-IFN/RBV difficult to tolerate. Efficacy is also limited, and only ~50% of patients with genotype 1a infections achieve SVR. Thus, there is intense interest in the development of better interferons, or novel small molecule antiviral compounds (specific targeted antiviral therapy for hepatitis C, or STAT-C).

Understanding IFN α failures

Failure to attain an SVR due to a rebound in viremia during or after peg-IFN therapy is common yet poorly understood. Although poor treatment outcomes correlate strongly with a number of host attributes (age, race, extent of fibrosis, obesity, insulin resistance, alcohol consumption), other evidence suggests that there may be true virus resistance to IFN. In particular, treatment outcomes differ significantly among various HCV genotypes, and also according to specific sequence variation in some viral proteins, such as the “interferon sensitivity determining region” (ISDR) of the nonstructural NS5A protein [8]. Recent work has also identified amino acid sequence covariance networks within the polyprotein that are highly predictive of treatment outcome [9].

Failure to attain an SVR due to a rebound in viremia, and failure to experience significant reductions in viremia at the start of therapy (a true “null response”) may be mechanistically different. The lack of a rapid initial response to IFN therapy correlates remarkably with high level expression of ISGs within the liver prior to treatment, both in patients and in the chimpanzee [6;10;11]. This high level of ISG induction prior to therapy is not associated with lower levels of viremia, suggesting it has little effect on the virus. This counterintuitive

relationship is also reflected in several studies documenting a negative correlation between response to therapy and serum levels of IP-10, a highly induced hepatic ISG. On the other hand, rapid and complete viral responses correlate with a lack of pre-treatment ISG induction in the liver, and substantial increases in intrahepatic ISG transcription upon initiation of therapy [6]. Why some patients lack any pre-treatment ISG response to HCV infection within the liver, while others demonstrate such a response yet fail to have their infection controlled by it, remain fascinating mysteries. Several distinct attributes of the viral-host interaction undoubtedly contribute to these observations. These include the ability of HCV to disrupt multiple signaling pathways by which viral infections typically induce an IFN response, as well as Jak-STAT signaling required for the induction of ISG synthesis by type I IFNs [12-14]. It is possible that continuous stimulation by endogenous IFNs may also contribute to a long-lasting IFN-refractory state within the liver [15].

Novel interferons

There are three members of the IFN λ family: IFN λ 1 (IL29), IFN λ 2 (IL28A) and IFN λ 3 (IL28B) which bind to a receptor comprised of IL28R α and IL10R β [16]. IFN λ 1 exhibits potent antiviral activity against HCV in vitro, with the added advantage of a persistent induction of ISGs without feedback inhibition [17]. There are hopes that these type III IFNs may induce an effective antiviral response in patients who do not respond to standard-of-care therapy. A recent phase Ib trial with pegIFN λ 1 in patients who had relapsed after standard-of-care therapy has demonstrated potent antiviral activity with four weekly doses providing a mean maximum decline of 3.6 log₁₀ in plasma viral RNA [18]. There were no flu-like symptoms or hematological alterations characteristic of IFN α , perhaps reflecting the different tissue distribution of the IL28R α receptor, which is mainly expressed by dendritic cells and cells of epithelial origin. Type III IFN may thus offer a distinct profile from the currently approved type I IFNs, and possibly some therapeutic advantages.

STAT-C Inhibitors

Several of the 10 proteins expressed by HCV possess well-defined enzymatic activities, including NS2 (a *cis*-acting protease), NS3/4A (serine protease-helicase), and NS5B (RNA-dependent RNA polymerase) [1] (Fig. 1a). Of these, the NS3/4A protease and NS5B polymerase have proven to be particularly promising targets for antiviral drug development. The NS3/4A protease, a complex formed by NS3 and NS4A, is responsible for processing the carboxy-terminal two-thirds of the polyprotein, thereby producing the mature nonstructural proteins. The NS3 protein has additional roles in viral RNA replication and virus assembly, typical of the multifunctional nature of many of the proteins expressed by this small RNA virus. However, inhibition of NS3/4A protease activity, because of its role in polyprotein processing, ablates virus replication. The same is also true for inhibitors of the NS5B polymerase.

NS3/4A protease inhibitors

Early efforts to identify compounds that specifically inhibit the serine protease activity of NS3/4A were boosted by X-ray crystallographic studies of the amino-terminal protease domain of NS3, or the full-length NS3 protein, complexed with a peptide sequence representing the NS4A segment that intercalates into the NS3 structure and contributes to the conformation adopted by the mature protease [19]. The maturation of this protein is a matter of interest. First, NS3 is cleaved from the upstream polyprotein sequence (NS2 domain) by a protease activity that acts exclusively *in cis* and resides primarily within the carboxy-terminal residues of NS2. Interestingly, X-ray crystallographic studies suggest that the enzymatic activity responsible for this is a cysteine protease formed by two NS2 molecules [20]. This dimer appears to have two composite active sites, in which catalytic His and Glu residues are contributed by one member of the dimer, and the nucleophilic Cys residue by the other [20]. Thus, two copies of the

polyprotein must be produced prior to scission occurring at the NS2-NS3 juncture, which is a pre-requisite for folding of the amino-terminal domain of NS3, a critical step in maturation of the NS3/4A protease. NS3/4A then cleaves downstream at the NS3/4A junction, but does so slowly, in part to allow for proper folding of the NS4A segment into NS3 prior to processing at the NS3-4A junction [21]. The processing scheme of the HCV polyprotein is thus a highly regulated and tightly orchestrated sequence of events that represents a rich target for therapeutic invention. Interest in the NS2 protease as a potential target is increasing as more has become known of its potential structure and function in recent years, but by far most drug development efforts have focused on the NS3/4A protease.

The crystal structure of the full-length NS3/4A protease revealed the carboxy-terminal NS3 residues to be located within the active site [19]. This is consistent with “product inhibition” of NS3/4A protease activity by short peptide sequences representing the carboxy-terminal NS3 sequence [22]. This was an extraordinarily helpful finding, as the substrate-binding cleft was otherwise found to be unusually broad and shallow, making it a very difficult target for rational structure-based drug design. Peptidomimetic compounds have thus dominated the search for NS3/4A inhibitors, some of which have been modified to produce cyclic compounds by links between side chains. Although no longer in development due to unfortunate cardiotoxicity, the first of these compounds to reach the clinic was a macrocyclic inhibitor, ciluprevir (BILN2061), which caused a mean $\sim 2.5 \log_{10}$ decrease in plasma HCV RNA after only two days of oral therapy [23]. Newer macrocyclic inhibitors, including ITMN-191 and TMC434350, are advancing in the clinic, while two linear ketoamide peptidomimetic inhibitors, telaprevir (VX-950) and boceprevir (SCH503034) are well into phase 2b/3 clinical trials [24]. These compounds have shown variable antiviral effects in clinical trials (perhaps due as much to differences in study design as to intrinsic differences in antiviral activity or PK/PD), but all appear to be on track for continued clinical development.

As alluded to above, the NS3/4A protease targets two cellular proteins for proteolysis (MAVS and TRIF) in addition to the viral polyprotein [25;26]. These are key adaptor proteins in the RIG-I and TLR3-induced signaling pathways involved in virus-activation of interferon synthesis, and their cleavage by NS3/4A prevents interferon responses to virus infection [12; 13]. While inhibitors of the NS3/4A protease also inhibit HCV-induced MAVS and TRIF cleavage, there are as yet no data to suggest that the resulting rescue of endogenous IFN responses contributes to their antiviral effect in infected persons. Recent data suggest that much higher drug concentrations may be required for rescue of RIG-I signaling than for inhibition of viral replication [27]. Thus, the ability of protease inhibitors to prevent the cleavage of these proteins by NS3/4A may not provide any particular advantage over STAT-C compounds that target other viral proteins.

NS5B polymerase inhibitors

Efforts to develop inhibitors of the NS5B polymerase have also benefited from crystallographic studies that have revealed a typical right-handed polymerase conformation with palm, thumb, and finger domains, but with interesting interactions between the finger and thumb domains that result in a fully encircled active site [28]. Recent evidence suggests that NS5B exists in both an open conformation that is likely to be required for elongation, as well as a closed structure that appears to be required for primer-independent initiation of RNA synthesis [29; 30]. A number of small molecule inhibitors of the NS5B polymerase have been identified; these fall into two general types: nucleoside analogs that bind into the active site; and allosteric, non-nucleoside inhibitors (NNI) that bind sites at variable distances from the active site of the polymerase and that comprise different chemical classes, including benzimidazoles, benzothiadiazines, and thiophene 2-carboxylic acids [31;32]. 2'-O-methylcytidine triphosphate is a potent inhibitor of the polymerase that binds into the active site; it acts by

competing with other NTPs as well as by chain termination of nascent RNA strands. In contrast, thiophene 2-carboxylic acid inhibitors of NS5B bind to the base of the thumb domain ~35 Å from the active site [29], while benzothiadiazines and benzimidzoles bind to a distinct site elsewhere in the thumb domain [33]. Unlike nucleoside analogs that bind into the active site and inhibit both initiation and elongation of RNA synthesis, the allosteric inhibitors appear to inhibit only initiation of RNA synthesis, not elongation [29]. They may act by “locking” the polymerase in an “open” conformation that is unable to initiate RNA synthesis. Although the polymerase inhibitors have generally lagged behind the protease inhibitors in their clinical development, they are equally capable of suppressing viremia [34;35].

Inhibitors of other polyprotein enzymatic activities

Although the precise role played by the NS3 helicase in the life cycle of the virus remains a mystery, it is essential for replication of the virus. The helicase forms a structural domain distinct from the NS3 protease and linked to it by a single peptide strand [19]. Its function has been well characterized [36]. To date, however, it has been difficult to identify compounds that inhibit this enzymatic activity and yet are free of significant cellular toxicity, perhaps because of difficulties discriminating between NS3 and numerous cellular DEAD-box helicases. The *cis*-acting NS2 protease represents another possible enzyme target for antiviral drug development. Recent gains in understanding its structure and function, as described above [20], have led to increased interest in discovery of NS2 protease inhibitors.

Non-enzymatic polyprotein targets

Although most antiviral drug development efforts target defined enzymatic activities of the polyprotein, recent data indicate that it is possible to inhibit other essential, non-enzymatic functions of the polyprotein with dramatic results. Several potent inhibitors have been identified in high-throughput, cell-based screens based on HCV replicon amplification that appear to be directed against NS5A, a nonstructural protein with no known enzymatic function, because of their ability to select for resistant viral RNAs with mutations in NS5A. Exactly how these inhibitors function is not known, but recent clinical data obtained with BMS-790052 are especially intriguing, as a single dose of this compound produced a 3.6 log₁₀ decline in viremia with no rebound during a 6 day follow up period [37]. Another potential non-enzymatic target is a recently recognized interaction of the viral RNA with NS4B, a membrane-associated component of the macromolecular viral RNA replicase complex. Clemizole, a generic oral antihistamine, inhibits this interaction and also blocks viral replication with a low micromolar EC₅₀ [38], suggesting that NS4B may also be a useful target for future drug discovery. Unlike the other polyprotein targets described above, however, no crystallographic structure exists for NS4B to guide drug discovery.

Viral Diversity Requires High Barriers to Resistance

Genetic diversity among HCV

Perhaps the greatest barriers to successful HCV therapy are the highly replicative nature of the infection and the capacity of the virus to successfully sustain changes in its nucleotide sequence and thereby become resistant to a small molecule inhibitor. It is estimated that as many as 1×10^{12} virus particles are produced each day in the typical, chronically-infected individual [5]. Coupled with this, the NS5B RNA-dependent RNA polymerase lacks an exonucleolytic proof-reading mechanism and thus has low fidelity. RNA replication is therefore inherently error-prone, with an estimated mutation rate of 10^{-3} to 10^{-5} per nucleotide. Since the genome is only $\sim 10^4$ nucleotides in length, each of the 10^{12} newly synthesized copies of the RNA genome produced each day in the typical patient has a high probability of containing one or more base substitutions. This causes tremendous diversity in the sequence of the virus, which is best described as a “cloud” of individual “quasispecies” sequences in any single infected person.

While many, perhaps most, of these base substitutions are deleterious because of costs they impose on viral fitness, others may allow for escape from a particular virus-neutralizing antibody or cytotoxic T cell clone. Such quasispecies may thus become dominant during persistent infection. Similarly, minor quasispecies variants that are resistant to the antiviral actions of STAT-C compounds will be rapidly selected from the background of sensitive quasispecies and become dominant during therapy.

It is important to realize that most potential double- and many triple-nucleotide substitutions are likely to present in the quasispecies swarm prior to the initiation of therapy. This was vividly demonstrated in a recent study that examined treatment-naïve patients for preexisting resistance mutations for 27 developmental STAT-C antivirals targeting NS3 and NS5B (protease inhibitors, nucleosides and NNIs). The results revealed that 44.4% of genotype 1b patients have at least one identifiable mutation at sites associated with resistance and 2.7% had such mutations in both NS3 and NS5B [39]. An even greater frequency of such mutations would undoubtedly be found with “deeper” sequencing methods. As discussed above, genetic diversity may also contribute to IFN resistance. However, compared with small molecule STAT-C agents, true viral resistance to IFN is probably limited by the diversity of antiviral mechanisms associated with the IFN-induced antiviral state, a number of which target key cellular processes such as protein kinase R-mediated effects on translation. However, with respect to STAT-C therapy, this capacity for genetic diversity will demand an exceptionally high barrier to resistance if efforts to treat HCV infection are to succeed. Direct-acting, STAT-C antivirals will likely need to be used in triple drug combinations, with peg-IFN, or with a particular compound to which resistance is not easily attained.

In addition to the genetic diversity evident among the viral quasispecies present in any single infected person, there are much larger genetic differences in the sequences of the 6 major HCV genotypes. These are associated with major differences in the susceptibility of these genotypes to candidate antiviral compounds, particularly allosteric NS5B inhibitors and NS3/4A protease inhibitors. Broader cross-genotype antiviral activity is preserved with nucleoside analogs because of the conservation of structure at the polymerase active-site. Although genotype 1 viruses predominate among individuals with significant liver disease in most countries, other genotypes also cause disease and genotype differences in STAT-C activity are likely to limit the future clinical utility of many of these compounds.

Resistance to STAT-C inhibitors

Among issues relevant to the development of STAT-C drug resistance are the frequency of a particular drug-resistant genetic variant within the quasispecies prior to treatment, the degree of drug resistance that the variation confers, the replication capacity (“fitness”) of the variant virus, and the potency and bioavailability of the compound itself. Although quasispecies distributions are undoubtedly less complex in cell-based replicon systems compared to chronically-infected patients, it is nevertheless possible to select and thereby identify specific resistance mutations in vitro. This has been done for a number of protease and polymerase inhibitors. Importantly, the results of these studies typically mirror later findings from clinical trials.

Because all known NS3/4A protease inhibitors bind to the catalytic site of the enzyme, they generally share at least one resistance mutation in common with other protease inhibitors. Such mutations typically include changes at R155, A156 and D168. A156T, R155Q and D168V substitutions confer 357-, 24- and 144-fold increases in the EC₅₀ of BILN2061 respectively [40]. Resistance to another macrocyclic inhibitor, ITMN-191 (RO5190591), is also conferred by substitutions at D168 as well as A156S/V, F43S, Q41R and S138T. Interestingly, decreased sensitivity to ITMN-191 has also been associated with substitutions outside the NS3 protease domain, within the helicase domain (S489L) and in the NS4A protease co-factor (V23A). A

structurally different compound, telaprevir, shows a distinct drug resistance profile that has been well characterized in patient samples from clinical trials [41;42]. There is little resistance conferred by BILN2061-resistance mutations at D168, while the telaprevir-resistant A156S is fully sensitive to BILN2061. Nevertheless, R155 substitutions and A156T or V mutations cause resistance to both inhibitors.

NS5B inhibitors show very different resistance profiles [31;43;44]. As discussed above, NNIs bind to several distinct sites on the surface of the polymerase, while nucleoside analogs bind into the active site. There are substantial differences in the resistance profiles of these compounds, and relatively little cross-resistance amongst them. While a complete discussion is beyond the scope of this review, it is important to note that resistance is less easily gained to nucleoside analogs than to NNIs [45]. In one clinical study, resistance was not observed after a total of 14 days of monotherapy [35]. This is likely to reflect the need for conservation of the active site structure, and may translate into therapeutic advantages for nucleosides.

As described above, many mutations that allow for resistance to STAT-C antivirals also cause substantial loss of viral replication competence, or “fitness”. The impact of resistance mutations on viral fitness can be assessed in vitro by determining how they affect the ability of replicon RNAs to amplify in cell culture. Losses in viral fitness often can be explained by negative effects on enzymatic activity. For example, an NS3 mutation that commonly causes resistance to protease inhibitors, A156T, sharply decreases the activity of the enzyme [42]. However, resistance mutations do not always cause significant loss of replication capacity in vitro. Results reported from replicon studies also show some inconsistencies, and are not always predictive of clinical results. Clinical data remain sparse, but early results with telaprevir suggest that viral mutants emerging on therapy with high level drug resistance and concomitant substantial loss of fitness were associated with lower levels of rebound viremia than mutants with NS3 substitutions causing lower levels of drug-resistance and less impact on viral fitness [41]. Second-site compensatory mutations may also partially correct defects in fitness resulting from primary resistance mutations [46].

Prevention of drug resistance

While the selection of resistant viruses during monotherapy with STAT-C compounds is virtually certain, this risk can be reduced by using these drugs in combination with IFN, or potentially other STAT-C compounds that lack the potential for cross-resistance. In vitro studies have shown that compounds targeting different viral enzymes (for example, the NS3/4A protease and NS5B polymerase) do not show cross-resistance and can be used in combination successfully. Nonetheless, it is possible to select double-resistant mutants in vitro, as has been done for BILN2061 and the benzothiadiazine polymerase inhibitor, A782759. The frequency of double mutants was significantly lower than that of mutants resistant to either drug individually, and the double mutants were also highly unfit [47]. In fact, when replicon cells were passed for 16 days in the presence of both compounds (without G418 selection) there was a $>7 \log_{10}$ reduction in cellular abundance of the replicon RNA. Combinations of two other NNIs (A848837 and A837093) with the NS3/4A inhibitors BILN2061 or boceprevir were synergistic and eliminated replicon RNAs with long-term treatment, while monotherapy led to the selection of resistant replicons. These in vitro studies are promising indicators of the success of future combinations of STAT-C inhibitors. In addition to combinations of protease and polymerase inhibitors, it may also prove possible to use combinations of different NNIs and/or nucleoside inhibitors of the NS5B polymerase, since these compounds show little cross-resistance. As indicated above, nucleoside analogs may be especially useful components of future combination STAT-C therapies since they appear less likely to select for drug resistance [45].

Because of the potential for rapid selection of STAT-C resistance, peg-IFN (and possibly RBV) will remain important components of therapy for the foreseeable future. While the Food and Drug Administration generally requires that candidate STAT-C agents demonstrate clinical antiviral activity as monotherapy, the duration of such phase 1 studies is kept as short as possible to prevent the selection of drug-resistant variants in study subjects. Longer-term studies generally involve combinations of STAT-C compounds with peg-IFN to prevent drug resistance. The optimal timing and manner of initiating such combination therapy remains uncertain, as the use of such combinations in peg-IFN null responders constitutes in essence STAT-C monotherapy with all its attendant risks of drug resistance. Nonetheless, recent large clinical studies involving the combination of peg-IFN, RBV and telaprevir have shown substantial improvements in SVR [24], reinforcing the potential for STAT-C compounds used in combination with the current standard-of-care peg-IFN and RBV regimen. The ultimate goal, however, is the development of STAT-C combinations that obviate the need for peg-IFN and its associated toxicities. A clinical study now being undertaken by Roche that involves the combination of a potent protease inhibitor (RO5190591/ITMN-121) and nucleoside analog (R05024048) in treatment-naïve patients represents an important step toward this goal, and will help to define the mutational barrier required for ultimate therapeutic success.

Targets possessing an intrinsically high barrier to resistance

Alternatives to STAT-C therapy that may provide a greater barrier to resistance include drugs targeting host functions essential for viral replication. The first of these to emerge were the cyclophilin inhibitors [48]. Yet, in vitro studies have already identified specific viral mutations that result in resistance to these inhibitors [49]. Other potential cellular targets include four distinct host proteins that are utilized sequentially for attachment and cell entry by the virus, and host enzymes responsible for protein geranyl geranylation that are required for viral RNA replication [50]. Yet another attractive target is the cellular micro-RNA, miR-122, which interacts directly with two sites within the 5' nontranslated segment of the viral RNA and is required for efficient replication of HCV [51]. Therapies based on the locked nucleic acid platform are being developed that sequester miR-122, and data from HCV studies should be forthcoming soon. The challenge, of course, with all host-directed inhibitors, will be to ensure there is no toxicity when such compounds are administered for extended periods.

Reference List

1. Lemon, SM.; Walker, C.; Alter, MJ.; Yi, M. Hepatitis C viruses. In: Knipe, D.; Howley, P.; Griffin, DE.; Martin, MA.; Lamb, RA.; Roizman, B.; Straus, SE., editors. *Fields Virology*. Vol. 5th. Philadelphia: Lippincott Williams & Wilkins; 2007. p. 1253-1304.
2. Lohmann V, Korner F, Koch J, Herian U, Theilmann L, Bartenschlager R. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* 1999;285:110–113. [PubMed: 10390360]
3. Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, Murthy K, Habermann A, Krauslich HG, Mizokami M, Bartenschlager R, Liang TJ. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nature Med* 2005;11:791–796. [PubMed: 15951748]
4. Yi M, Villanueva RA, Thomas DL, Wakita T, Lemon SM. Production of infectious genotype 1a hepatitis C virus (Hutchinson strain) in cultured human hepatoma cells. *Proc Natl Acad Sci USA* 2006;103:2310–2315. [PubMed: 16461899]
- **5. Neumann AU, Lam NP, Dahari H, Gretch DR, Wiley TE, Layden TJ, Perelson AS. Hepatitis C viral dynamics in vivo and the antiviral efficacy of interferon-alpha therapy. *Science* 1998;282:103–107. [PubMed: 9756471] The authors modeled decreases in circulating viral RNA in patients receiving therapy with IFN, and in so doing provided novel insights into both the dynamics of HCV replication in infected persons as well as the mechanism of IFN action.
- **6. Sarasin-Filipowicz M, Oakeley EJ, Duong FH, Christen V, Terracciano L, Filipowicz W, Heim MH. Interferon signaling and treatment outcome in chronic hepatitis C. *Proc Natl Acad Sci USA* 2008;105:7034–7039. [PubMed: 18467494] By examining gene expression in paired liver biopsies,

the authors demonstrate novel associations of pre- and post-treatment induction of IFN-stimulated genes within the liver with the outcome of peg-IFN treatment.

7. McHutchison JG, Gordon SC, Schiff ER, Shiffman ML, Lee WM, Rustgi VK, Goodman ZD, Ling MH, Cort S, Albrecht JK. Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. Hepatitis Interventional Therapy Group. *N Engl J Med* 1998;339:1485–1492. [PubMed: 9819446]
8. Chayama K, Tsubota A, Kobayashi M, Okamoto K, Hashimoto M, Miyano Y, Koike H, Koida I, Arase Y, Saitoh S, Suzuki Y, Murashima N, Ikeda K, Kumada H. Pretreatment virus load and multiple amino acid substitutions in the interferon sensitivity-determining region predict the outcome of interferon treatment in patients with chronic genotype 1b hepatitis C virus infection. *Hepatology* 1997;25:745–749. [PubMed: 9049229]
9. Aurora R, Donlin MJ, Cannon NA, Tavis JE. Genome-wide hepatitis C virus amino acid covariance networks can predict response to antiviral therapy in humans. *J Clin Invest* 2009;119:225–236. [PubMed: 19104147]
10. Chen L, Borozan I, Feld J, Sun J, Tannis LL, Coltescu C, Heathcote J, Edwards AM, McGilvray ID. Hepatic gene expression discriminates responders and nonresponders in treatment of chronic hepatitis C viral infection. *Gastroenterology* 2005;128:1437–1444. [PubMed: 15887125]
11. Lanford RE, Guerra B, Bigger CB, Lee H, Chavez D, Brasky KM. Lack of response to exogenous interferon-alpha in the liver of chimpanzees chronically infected with hepatitis C virus. *Hepatology* 2007;46:999–1008. [PubMed: 17668868]
12. Wang N, Liang Y, Devaraj S, Wang J, Lemon SM, Li K. Toll-like receptor 3 mediates establishment of an antiviral state against hepatitis C virus in hepatoma cells. *J Virol*. 2009
13. Loo YM, Owen DM, Li K, Erickson AL, Johnson CL, Fish P, Carney DS, Wang T, Ishida H, Yoneyama M, Fujita T, Saito T, Lee WM, Hagedorn CH, Lau DT, Weinman SA, Lemon SM, Gale M Jr. Viral and therapeutic control of interferon beta promoter stimulator 1 during hepatitis C virus infection. *Proc Natl Acad Sci USA* 2006;103:6001–6006. [PubMed: 16585524]
14. Duong FH, Filipowicz M, Tripodi M, La Monica N, Heim MH. Hepatitis C virus inhibits interferon signaling through up-regulation of protein phosphatase 2A. *Gastro* 2004;126:263–277.
15. Filipowicz M, Wang X, Yan M, Duong FH, Poli V, Hilton DJ, Zhang DE, Heim MH. Interferon alpha induces long-lasting refractoriness of JAK-STAT signaling in the mouse liver through induction of USP18/UBP43. *Mol Cell Biol*. 2009
16. Ank N, West H, Paludan SR. IFN-lambda: novel antiviral cytokines. *J Interferon Cytokine Res* 2006;26:373–379. [PubMed: 16734557]
- *17. Marcello T, Grakoui A, Barba-Spaeth G, Machlin ES, Kotenko SV, MacDonald MR, Rice CM. Interferons alpha and lambda inhibit hepatitis C virus replication with distinct signal transduction and gene regulation kinetics. *Gastroenterology* 2006;131:1887–1898. [PubMed: 17087946]The authors describe in vitro studies suggesting that IFN λ may have unique therapeutic potential.
18. Shiffman M, Lawitz E, Zaman A, Vierling JM, Yoffe B, Freeman J, Gray T, Hausman D, Muir A. Peg-IFN-[lambda]: Antiviral activity and safety profile in a 4-week phase 1b study in relapsed genotype 1 hepatitis C infection. *J Hepatol* 2009;50:S327.
- *19. Yao N, Reichert P, Taremi SS, Prosise WW, Weber PC. Molecular views of viral polyprotein processing revealed by the crystal structure of the hepatitis C virus bifunctional protease-helicase. *Structure Fold Des* 1999;7:1353–1363. [PubMed: 10574797]This manuscript describes important structural aspects of one of the most promising HCV targets, the NS3/4A protease and its associated helicase domain.
- *20. Lorenz IC, Marcotrigiano J, Dentzer TG, Rice CM. Structure of the catalytic domain of the hepatitis C virus NS2-3 protease. *Nature* 2006;442:831–835. [PubMed: 16862121]This manuscript describes a recently developed model of the unique dimeric structure of the NS2 protease of HCV.
21. Wang W, Lahser FC, Yi M, Wright-Minogue J, Xia E, Weber PC, Lemon SM, Malcolm BA. Conserved C-terminal threonine of hepatitis C virus NS3 regulates autoproteolysis and prevents product inhibition. *J Virol* 2004;78:700–709. [PubMed: 14694101]
22. Steinkuhler C, Biasiol G, Brunetti M, Urbani A, Koch U, Cortese R, Pessi A, De Francesco R. Product inhibition of the hepatitis C virus NS3 protease. *Biochemistry* 1998;37:8899–8905. [PubMed: 9636031]

- *23. Lamarre D, Anderson PC, Bailey M, Beaulieu P, Bolger G, Bonneau P, Bos M, Cameron DR, Cartier M, Cordingley MG, Faucher AM, Goudreau N, Kawai SH, Kukulj G, Lagace L, LaPlante SR, Narjes H, Poupert MA, Rancourt J, Sentjens RE, St George R, Simoneau B, Steinmann G, Thibeault D, Tsantrizos YS, Weldon SM, Yong CL, Llinas-Brunet M. An NS3 protease inhibitor with antiviral effects in humans infected with hepatitis C virus. *Nature* 2003;426:186–189. [PubMed: 14578911] The first report of a STAT-C agent used in humans, this study demonstrated strong antiviral activity of BILN2061 and provided important proof-of-concept for HCV protease inhibitor therapy.
- **24. Hezode C, Forestier N, Dusheiko G, Ferenci P, Pol S, Goeser T, Bronowicki JP, Bourliere M, Gharakhanian S, Bengtsson L, McNair L, George S, Kieffer T, Kwong A, Kauffman RS, Alam J, Pawlotsky JM, Zeuzem S. Telaprevir and peginterferon with or without ribavirin for chronic HCV infection. *N Engl J Med* 2009;360:1839–1850. [PubMed: 19403903] This recent report of a phase 2 study shows that the addition of a STAT-C agent (the NS3/4A protease inhibitor, telaprevir) to standard-of-care therapy can increase the frequency of sustained viral responses.
25. Li K, Foy E, Ferreon JC, Nakamura M, Ferreon ACM, Ikeda M, Ray SC, Gale M Jr, Lemon SM. Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the TLR3 adaptor protein TRIF. *Proc Natl Acad Sci USA* 2005;102:2992–2997. [PubMed: 15710891]
26. Meylan E, Curran J, Hofmann K, Moradpour D, Binder M, Bartenschlager R, Tschoop J. Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. *Nature* 2005;437:1167–1172. [PubMed: 16177806]
- *27. Liang Y, Ishida H, Lenz O, Lin TI, Nyanguile O, Simmen K, Pyles RB, Bourne N, Yi M, Li K, Lemon SM. Antiviral suppression vs restoration of RIG-I signaling by hepatitis C protease and polymerase inhibitors. *Gastroenterology* 2008;135:1710–1718. [PubMed: 18725224] This in vitro study demonstrated that substantially higher concentrations of the NS3/4A inhibitor TMC435350 were required to rescue virus-imposed defects in interferon signaling than to inhibit polyprotein processing and growth of virus.
28. Lesburg CA, Cable MB, Ferrari E, Hong Z, Mannarino AF, Weber PC. Crystal structure of the RNA-dependent RNA polymerase from hepatitis C virus reveals a fully encircled active site. *Nat Struct Biol* 1999;6:937–943. [PubMed: 10504728]
29. Biswal BK, Cherney MM, Wang M, Chan L, Yannopoulos CG, Bilimoria D, Nicolas O, Bedard J, James MN. Crystal structures of the RNA-dependent RNA polymerase genotype 2a of hepatitis C virus reveal two conformations and suggest mechanisms of inhibition by non-nucleoside inhibitors. *J Biol Chem* 2005;280:18202–18210. [PubMed: 15746101]
30. Chinnaswamy S, Yarbrough I, Palaninathan S, Kumar CT, Vijayaraghavan V, Demeler B, Lemon SM, Sacchettini JC, Kao CC. A locking mechanism regulates RNA synthesis and host protein interaction by the hepatitis C virus polymerase. *J Biol Chem* 2008;283:20535–20546. [PubMed: 18442978]
- *31. Hang JQ, Yang Y, Harris SF, Leveque V, Whittington HJ, Rajyaguru S, Ao-Ieong G, McCown MF, Wong A, Giannetti AM, Le PS, Talamas F, Cammack N, Najera I, Klumpp K. Slow binding inhibition and mechanism of resistance of non-nucleoside polymerase inhibitors of hepatitis C virus. *J Biol Chem* 2009;284:15517–15529. [PubMed: 19246450] This study demonstrates that the magnitude of reduction of NNI binding affinity for the NS5B proteins with various resistance mutations in the palm and thumb-binding sites correlates with drug resistance in polymerase assays and cell-based replicon assays.
32. Carroll SS, Olsen DB. Nucleoside analog inhibitors of hepatitis C virus replication. *Infect Disord Drug Targets* 2006;6:17–29. [PubMed: 16787301]
33. Tomei L, Altamura S, Bartholomew L, Bisbocci M, Bailey C, Bosserman M, Cellucci A, Forte E, Incitti I, Orsatti L, Koch U, De Francesco R, Olsen DB, Carroll SS, Migliaccio G. Characterization of the inhibition of hepatitis C virus RNA replication by nonnucleosides. *J Virol* 2004;78:938–946. [PubMed: 14694125]
- **34. Carroll SS, Ludmerer S, Handt L, Koeplinger K, Zhang NR, Graham D, Davies ME, MacCoss M, Hazuda D, Olsen DB. Robust antiviral efficacy upon administration of a nucleoside analog to hepatitis C virus-infected chimpanzees. *Antimicrob Agents Chemother* 2009;53:926–934. [PubMed: 19075052] This paper demonstrates the potential of nucleoside analogs to exert very powerful antiviral effects in vivo in a unique animal model of hepatitis C.

35. Roberts SK, Cooksley G, Dore GJ, Robson R, Shaw D, Berns H, Hill G, Klumpp K, Najera I, Washington C. Robust antiviral activity of R1626, a novel nucleoside analog: a randomized, placebo-controlled study in patients with chronic hepatitis C. *Hepatology* 2008;48:398–406. [PubMed: 18553458]
36. Dumont S, Cheng W, Serebrov V, Beran RK, Tinoco I Jr, Pyle AM, Bustamante C. RNA translocation and unwinding mechanism of HCV NS3 helicase and its coordination by ATP. *Nature* 2006;439:105–108. [PubMed: 16397502]
37. Nettles RE, Chien C, Chung E, Persson A, Gao M, Belema M, Meanwell N, DeMicco M, Marbury T, Goldwater R, Northrup PG, Combis J, Kraft WK, Charlon M, Lopez-Talavera JC, Grasela DM. BMS-790052 is a first-in-class potent hepatitis C virus (HCV) NS5A inhibitor for patients with chronic HCV infection: results from a proof-of-concept study. *Hepatology* 2008;48:LB12.
38. Einav S, Gerber D, Bryson PD, Sklan EH, Elazar M, Maerkl SJ, Glenn JS, Quake SR. Discovery of a hepatitis C target and its pharmacological inhibitors by microfluidic affinity analysis. *Nat Biotechnol* 2008;26:1019–1027. [PubMed: 18758449]
- *39. Gaudieri S, Rauch A, Pfafferoth K, Barnes E, Cheng W, McCaughan G, Shackel N, Jeffrey GP, Mollison L, Baker R, Furrer H, Gunthard HF, Freitas E, Humphreys I, Klenerman P, Mallal S, James I, Roberts S, Nolan D, Lucas M. Hepatitis C virus drug resistance and immune-driven adaptations: relevance to new antiviral therapy. *Hepatology* 2009;49:1069–1082. [PubMed: 19263475] This clinical study demonstrates that sequence polymorphisms conferring resistance to STAT-C compounds are commonly present in circulating viral quasispecies prior to the initiation of therapy.
40. Lu L, Pilot-Matias TJ, Stewart KD, Randolph JT, Pithawalla R, He W, Huang PP, Klein LL, Mo H, Molla A. Mutations conferring resistance to a potent hepatitis C virus serine protease inhibitor in vitro. *Antimicrob Agents Chemother* 2004;48:2260–2266. [PubMed: 15155230]
- *41. Sarrazin C, Kieffer TL, Bartels D, Hanzelka B, Muh U, Welker M, Wincheringer D, Zhou Y, Chu HM, Lin C, Weegink C, Reesink H, Zeuzem S, Kwong AD. Dynamic hepatitis C virus genotypic and phenotypic changes in patients treated with the protease inhibitor telaprevir. *Gastroenterology* 2007;132:1767–1777. [PubMed: 17484874] The authors examine the dynamics of drug-resistant virus selected during treatment with an NS3/4A inhibitor, and demonstrate that changes in the frequency of mutations after the end of dosing occur in an inverse fashion with respect to in vivo viral fitness and drug resistance.
42. Lin C, Gates CA, Rao BG, Brennan DL, Fulghum JR, Luong YP, Frantz JD, Lin K, Ma S, Wei YY, Perni RB, Kwong AD. In vitro studies of cross-resistance mutations against two hepatitis C virus serine protease inhibitors, VX-950 and BILN 2061. *J Biol Chem* 2005;280:36784–36791. [PubMed: 16087668]
43. Le PS, Kang H, Harris SF, Leveque V, Giannetti AM, Ali S, Jiang WR, Rajyaguru S, Tavares G, Oshiro C, Hendricks T, Klumpp K, Symons J, Browner MF, Cammack N, Najera I. Selection and characterization of replicon variants dually resistant to thumb- and palm-binding nonnucleoside polymerase inhibitors of the hepatitis C virus. *J Virol* 2006;80:6146–6154. [PubMed: 16731953]
44. Le PS, Jiang WR, Leveque V, Rajyaguru S, Ma H, Kang H, Jiang S, Singer M, Ali S, Klumpp K, Smith D, Symons J, Cammack N, Najera I. In vitro selected Con1 subgenomic replicons resistant to 2'-C-Methyl-Cytidine or to R1479 show lack of cross resistance. *Virology*. 2006
- **45. McCown MF, Rajyaguru S, Le PS, Ali S, Jiang WR, Kang H, Symons J, Cammack N, Najera I. The hepatitis C virus replicon presents a higher barrier to resistance to nucleoside analogs than to nonnucleoside polymerase or protease inhibitors. *Antimicrob Agents Chemother* 2008;52:1604–1612. [PubMed: 18285474] The authors provide in vitro data that support clinical observations suggesting that nucleoside analogs may be less prone to select resistant viruses than NNIs, or inhibitors of the NS3/4A protease.
46. McCown MF, Rajyaguru S, Kular S, Cammack N, Najera I. GT-1a or GT-1b subtype-specific resistance profiles for hepatitis C virus inhibitors telaprevir and HCV-796. *Antimicrob Agents Chemother* 2009;53:2129–2132. [PubMed: 19273674]
- *47. Mo H, Lu L, Pilot-Matias T, Pithawalla R, Mondal R, Masse S, Dekhtyar T, Ng T, Koev G, Stoll V, Stewart KD, Pratt J, Donner P, Rockway T, Maring C, Molla A. Mutations conferring resistance to a hepatitis C virus (HCV) RNA-dependent RNA polymerase inhibitor alone or in combination with an HCV serine protease inhibitor in vitro. *Antimicrob Agents Chemother* 2005;49:4305–4314.

[PubMed: 16189112]The authors describe an important series of in vitro experiments using combinations of STAT-C compounds targeting different HCV functions.

48. Flisiak R, Feinman SV, Jablkowski M, Horban A, Kryczka W, Pawlowska M, Heathcote JE, Mazzella G, Vandelli C, Nicolas-Metral V, Groscurin P, Liz JS, Scalfaro P, Porchet H, Crabbe R. The cyclophilin inhibitor Debio 025 combined with PEG IFNalpha2a significantly reduces viral load in treatment-naive hepatitis C patients. *Hepatology* 2009;49:1460–1468. [PubMed: 19353740]
49. Robida JM, Nelson HB, Liu Z, Tang H. Characterization of hepatitis C virus subgenomic replicon resistance to cyclosporine in vitro. *J Virol* 2007;81:5829–5840. [PubMed: 17376913]
50. Ploss A, Evans MJ, Gaysinskaya VA, Panis M, You H, de Jong YP, Rice CM. Human occludin is a hepatitis C virus entry factor required for infection of mouse cells. *Nature* 2009;457:882–886. [PubMed: 19182773]
51. Jopling CL, Yi M, Lancaster AM, Lemon SM, Sarnow P. Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA. *Science* 2005;309:1577–1581. [PubMed: 16141076]

Figure 1.

Organization of the HCV genome. **(a)** Organization of the single-stranded, positive-sense RNA genome of HCV. The polyprotein coding region (shown as a box) is flanked by short noncoding regulatory sequences. Potential antiviral targets are identified. **(b)** Organization of selectable subgenomic (top) and full-genome length HCV RNA replicons that are capable of autonomous amplification when transfected into Huh7 cells, and that are typically used in drug discovery research.