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Propagation of hepatitis C virus infection: Elucidating targets for therapeutic intervention

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The hepatitis C virus affects 2–3% of the world's population. The inability to efficiently propagate the virus in cell-culture greatly impaired the study of the viral life cycle. Recent advances have facilitated extensive study of viral entry, translation, proteolysis, and replication that will foster intelligent drug design for this infection, a major cause of cirrhosis and hepatocellular carcinoma worldwide.

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Introduction

The hepatitis C virus (HCV), most commonly via injection drug use or blood transfusion, after a generally asymptomatic acute infection, persists in 50–75% of exposed subjects. Persistent infection is associated with chronic hepatitis, which progresses to cirrhosis at a rate of approximately 10% per decade [1]. Current first-line therapy for chronic hepatitis C combines two non-HCV specific medications, interferon-alpha (IFNα) injections and oral ribavirin, a nucleoside analogue. Sustained virological response (SVR), the absence of detectable viremia in peripheral blood 6 months after discontinuation of a 24–48 week course of IFNα/ribavirin therapy, occurs in fewer than half of patients infected with the most common genotype of HCV (genotype 1) [2,3]. IFNα/ribavirin is associated with significant treatment-related fatigue, depression, hemolytic anemia and other serious side effects. Thus, unsatisfactory response rates, poor tolerability and frequent adverse events have stirred strong interest in specific antiviral therapies, the development of which has been recently hastened by advances in *in vitro* techniques to study HCV viral replication.

The virus

The hepatitis C virus (HCV) is a highly mutable, hepatotropic, enveloped 9.5 kB single-stranded RNA virus of the Flaviviridae family that can infect humans and chimpanzees. HCV has high sequence heterogeneity, with at least 6 distinct genotypes and at least 50 subtypes distributed worldwide [4]. Within individual subjects, HCV exists as a swarm of closely related variants (quasispecies); this variation results from a highly error prone RNA polymerase that lacks proofreading capacity. While most mutations are deleterious, a small percentage of random mutations confer upon nascent viral strains a selective replication advantage, which fosters continual viral adaptation to the unique and dynamic environment provided by the host, a process that plays a major role in drug resistance under conditions of sub-maximal viral suppression.

Hepatitis C, like all viruses, must be capable of entering target cells, transcribing and/or translating the genetic material to produce structural and nonstructural components for new virion formation, replicating the genetic material to new include in nascent virions, packaging the genetic material into the virion structure, and releasing daughter viruses (Fig. 1). Characterization of the critical steps of

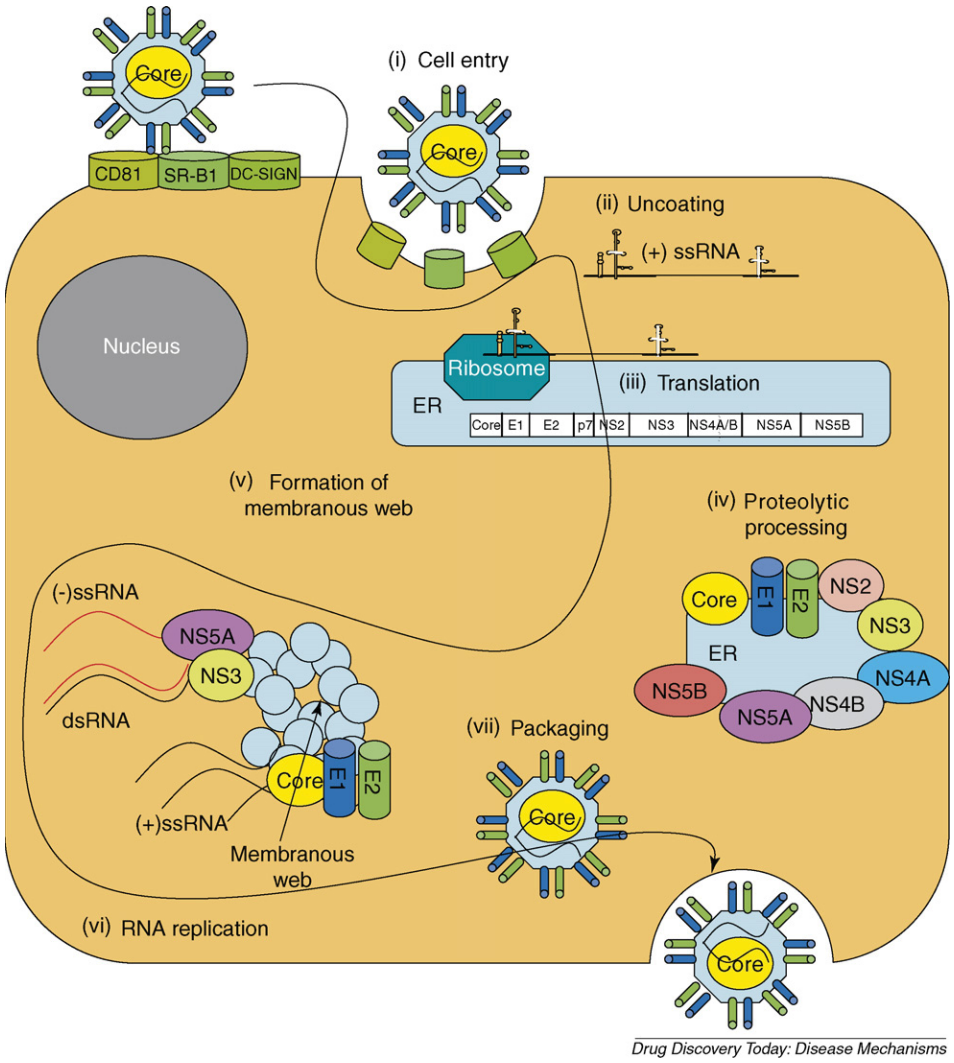

Figure 1. Hepatitis C viral lifecycle. (i) Cell entry is mediated by E1 and E2 glycoprotein interacting with cellular receptors such as CD81 and HDL scavenger receptor type B1, which triggers receptor-mediated endocytosis. (ii) The virus uncoats, leaving positive-strand RNA in cytoplasm. (iii) RNA interacts with ribosome on endoplasmic reticulum (ER) via internal ribosome entry site (IRES) leading to translation of large polyprotein. (iv) Host and viral enzymes cleave the polyprotein into individual proteins. (v) Viral proteins lead to organization of membranous web (replication complex). (vi) NS5B and NS3 helicase along with host factors catalyze transcription of negative-strand RNA template, then replication of positive-strand copies with temporarily double-strand RNA intermediate.

grow wild-type HCV in culture and the lack of widely available small animal models to study infection *in vivo*. Most investigation of HCV protein function and replication has resulted from use of recombinant viral constructs that express HCV proteins under the control of promoters, internal ribosome entry sites (IRES), or nonstructural proteins borrowed from unrelated viruses. These constructs (replicons, retroviral pseudoparticles, chimeric viruses) recapitulated some but not all steps of the viral life cycle *in vitro*, but often required specific adaptive mutations and/or highly adapted host cell lines (reviewed in [5,6]). In 2005, JFH1, an infectious clone of HCV capable of replication in human hepatoma cell lines and chimpanzee hepatocytes [7], was characterized. Combined, the various *in vitro* model systems have markedly advanced clinical development of a variety of specific inhibitors targeting critical steps in viral replication (Table 1).

Characterization and inhibition of HCV entry into permissive host cells

Unlike HIV infection in which the cellular receptor for the viral envelope proteins is well characterized and has spawned development of fusion inhibitors, the mechanism by which HCV enters target cells remains incompletely characterized. The circulating virion includes the a nucleocapsid consisting of the HCV Core protein and positive-strand ssRNA surrounded by an envelope, which consists of a lipid bilayer derived from the previous host cell's membrane and HCV envelope glycoproteins E1 and E2. E1 and E2 form heterogeneous mixtures of

Table 1. Viral and host cellular protein targets for specific inhibitor therapy[a]

	Therapy against target	Stage of development	Advantages and/or disadvantages	Who is working on the target (group and institute or company)	Refs
NS3/4 protease	Peptidomimetic	Phase II	Potent as monotherapy– 3–4 $\log_{10}$ drop; viral escape; strain-specificity	Vertex Schering-Plough Gilead Intermune Others	[25,26]
NS5B polymerase	Nucleotide analogue Non-nucleotide inhibitors (benzimidazole, benzothiadiazine, thiophene caroboxylic acids)	Phase III	Low potency as monotherapy? Adverse effects? Viral escape	Idenix Roche XTL Pharmasset Viropharma Others	[30,31]
NS3 helicase	Modified benzimidazoles, ribavirin esters, tropolone analogues	Phase I?	Unknown effects *in vivo*	Vertex	[33,34]
IRES[b]	Antisense shRNA siRNA Ribozyme Aptamers Peptide-nucleic acids Other small molecule	Phase II	Delivery mechanism Toxicity? Potentially more resistant to viral escape due to conservation of IRES Cross-genotype activity	Viral Genomix AVI Biopharma Others	[18,19,23]
Cell entry (CD81, SR-B1[c], DC-SIGN[d])	Blocking antibody (endogenous, exogenous) Small molecules	Phase I	Strain specificity Viral escape	Innogenetics XTL Others	[13]
Particle formation (lipids, glycoproteins)	Alpha-glucosidase inhibitors Prenylation inhibitors Sphingolipidation blockers	Pre-clinical	Unknown	Migenix	[36,38–41]

[a] Published safety and efficacy data are very limited—advantages/disadvantages are highly speculative.
[b] Internal ribosome entry site.
[c] HDL scavenger receptor type B1.
[d] Dendritic cell-specific ICAM3 grabbing nonintegrin.

mediate viral entry. In cell culture systems, mainly utilizing viral pseudoparticles and more recently the JFH1 clone, viral entry occurs via low-pH-dependent, clathrin-mediated receptor-mediated endocytosis [8]. The E2 glycoprotein mediates binding to cellular receptors such as CD81, a tetraspannin receptor expressed on hepatocytes among other cell types, and the HDL receptor scavenger receptor class B type 1 (SR-B1), blockade of which inhibits E2-mediated viral entry *in vitro* [9,10]. Other putative co-receptors including the LDL-receptor, C-type lectins, L-SIGN and DC-SIGN [11], may be involved. Recent data identifies a conserved GWLAGLFY motif at position 436 of E2 as the critical residues required for CD81-dependent viral entry [12]. The accessibility of virus-cell surface interactions allows consideration for antibody-based, peptidomimetic or small molecule approaches to disrupt early stages of cellular infection.

Some preliminary success has been reported with envelope protein-including vaccines [13] (INN-0101, Innogenetics, Gent, Belgium, http://www.innogenetics.com/) and with Rehovot, Israel, http://www.xtlbio.com/) to augment clearance of circulating viral particles and/or inhibit cellular re-infection. A polyclonal immune globulin is also in development (Civacir, Nabi Pharmaceuticals, Boca Raton, FL USA, http://www.nabi.com/). At this time, data regarding specific small molecule inhibitors of E2-CD81 or other envelope-cell receptor binding have yet to be published.

Genome translation

Once HCV has entered a target cell, translation of the viral positive strand RNA proceeds using host cell machinery. The 9.6 kB RNA genome encodes a single open reading frame (ORF) flanked by 5′ and 3′ non-coding regions. Three hundred thirty nucleotides of the 5′ untranslated region (and beginning of the core protein encoding nucleotides) of the virus contain the internal ribosome entry site (IRES), a highly structured domain critical for initiation of HCV polyprotein translation. The HCV IRES contains three stem-loops (domains II–IV) and a pseudoknot [14]. Translation mediated

RNA, ATP-dependent scanning, and multiple canonical initiation factors (reviewed in [15]). The human La autoantigen appears required for the interaction of the IRES and the 40S ribosome subunit [16]. Due to constraints on stem-loop base-pairing, only a minority of nucleotides can be substituted without impairment IRES functionality and thus the region is highly conserved among HCV subtypes [17]. These constraints similarly make the IRES an attractive target for therapeutics.

Initial approaches to inhibit IRES function have focused on antisense oligonucleotides to block the interaction of the 5′ IRES with the 40S ribosome [18] and two agents are currently in phase I/II trials (VGX-410C, Viral Genomix Inc. Blue Bell, PA, USA, http://www.viralgenomix.com/; AVI-4065, AVI Biopharma, Portland, OR, USA, http://www.avibio.com/). ISIS 14803 (ISIS Pharmaceuticals, Carlsbad, CA, USA, http://www.isispharm.com/) showed some promise but questions regarding toxicity in early development [19] and was suspended. Other RNA-based IRES inhibitors such as RNAi, shRNA, ribozymes, aptamers, and peptide nucleic acids that disrupt IRES function have been shown to inhibit *in vitro* replication of HCV RNA [20,21]. A caveat to this approach is that there is some evidence that HCV core protein interferes with siRNA activity [22]. A novel competitive La peptide variant [23] has been shown *in vitro* to impair HCV replication via IRES inhibition and may be a candidate for further development.

Protein processing

After IRES engagement with ribosomes, viral RNA is translated as a single long polyprotein which requires proteolytic cleavage (Fig. 2). Host cell signal peptidases cleave at the Core/E1, E1/E2, E2/p7 and p7/NS2 junctions. The Core protein forms the viral nucleocapsid which localizes replicated RNA via a highly conserved RNA binding domain, with the budding envelope studded with E1 and E2 heterodimers. The p7 protein encodes an ion channel critical for viral infectivity. The NS2/NS3 cleavage is catalyzed by NS2, a transmembrane zinc-dependent protease, a step critical to efficient function of the NS3 protein; NS2 may also regulate certain cellular factors involved with apoptosis and fibrosis. The remaining cleavages, those at NS3/NS4, NS4A/NS4B, NS4B/NS5A, and NS5A/NS5B, are catalyzed by the NS3 using NS4A as a cofactor, termed the NS3/4A serine protease. Additionally, NS3 has a separate NTP-dependent RNA helicase domain. NS4A is a cofactor for the NS3 protease and recent data indicate that the NS3/4A protease blocks signaling pathways that trigger NF-kB nuclear translocation and interferon-response factor pathways by cleaving an adaptor protein necessary for RIG-I signaling, called Cardif (also known as MAVS, IPS-1 and VISA) [24]. The role of NS4B is unclear, but it may along with NS4A regulate viral translation and replication. The NS5A protein contains a PKR binding region and may modulate sensitivity to interferon-alpha and NS5B is the viral RNA-dependent RNA polymerase.

As described, the NS3 protein has three functional domains: an autoprotease that cleaves the NS2/3 junction using NS2 as a cofactor, an RNA helicase domain and the NS3/4A protease. The NS3/4A serine protease in addition to its proteolytic function also appears to regulate the NS3 helicase domain. Due to the critical role that the NS3/4 protease plays in various aspects of viral replication, development of a specific inhibitor has been a therapeutic priority. BILN 2061 (Boehringer Ingelheim GmbH, Ingelheim, Germany, http://www.boehringer-ingelheim.com/), the first of these inhibitors to enter clinical trials achieved 2–3 $\log_{10}$ reductions in HCV RNA titers [25] in genotype 1 infections. Both *in vitro* and *in vivo* BILN 2061 was significantly less effective

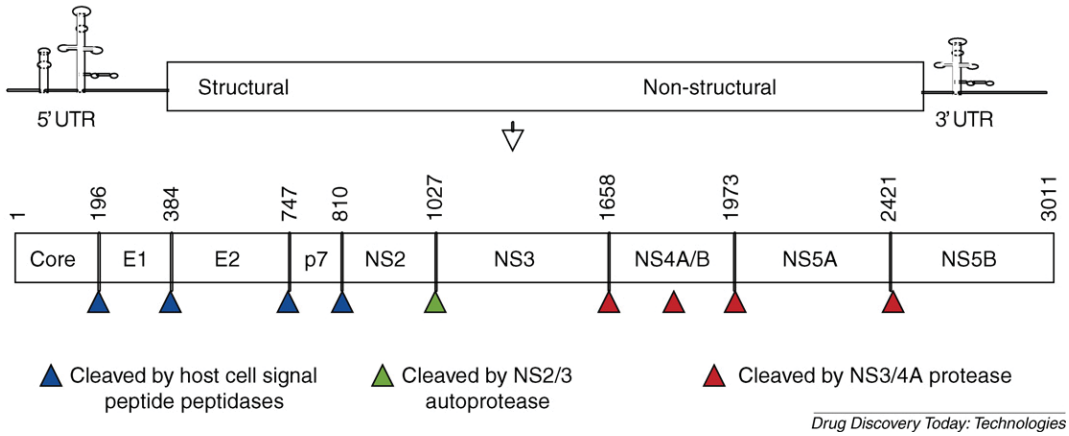

Figure 2. Hepatitis C viral translation and proteolysis. Translation and proteolysis of the HCV polyprotein. 5′ UTR (untranslated region) contains the internal ribosome entry site, which facilitates interaction of positive-strand RNA with the 40S ribosome resulting in translation of a single polyprotein of 3011 amino acids. Numerals indicate amino acid positions for subsequent cleavages. Blue triangles indicate cleavages catalyzed by host cell signal peptide peptidases. The green triangle shows cleavage site of NS2/3 autoprotease. Red triangles indicate cleavage sites for the NS3/4A protease.

against genotype non-1 NS3/4A proteases. Development of BILN 2061 was suspended due to cardiotoxicity in non-human primates given the drug. VX-950 (Vertex Pharmaceuticals, Cambridge MA USA, [26], http://www.vpharm.com/) and SCH 503034 (Schering-Plough, Kenilworth NJ USA, http://www.schering-plough.com/) are currently in phase II and at least two more candidates (GS 3192/ACH806, Gilead, Foster City CA USA, http://www.gilead.com/; ITMN 191, Intermune, Brisbane CA, USA, http://www.intermune.com/) are in phase I testing with a host of other candidate drugs nearing clinical testing. Published safety, tolerability, and efficacy data for this class of drugs are limited, but VX-950 administered orally three times daily led to median 3 $\log_{10}$ reductions in HCV RNA after 3 days and 4.4 $\log_{10}$ reductions after 14 days [26] in a phase II trial.

Genomic replication

The primary goal of the viral proteins is to coordinate the replication of viral RNA and the packaging new RNA strands into nascent structural units that can be extruded. As a positive-strand RNA virus, RNA replication depends on the creation of a negative-strand RNA template, from which new positive-strands can be duplicated. RNA replication occurs in the cytoplasm facilitated by the formation of a membrane-associated complex involving all of the nonstructural viral proteins as well as host factors [27]. Two viral enzymes play the dominant roles in this process: the NS5B RNA, the RNA-dependent RNA polymerase and the NS3 helicase.

The NS5B polymerase is a right hand structured polymerase with thumb, palm and finger domains and a completely encircled active site [28], for which both nucleotide and non-nucleoside inhibitors have been identified. Nucleotide analogs compete with nucleotide binding at the polymerase active site and can cause chain termination upon incorporation into the RNA molecules [29]. Non-nucleoside inhibitors of NS5B RNA synthesis activity mainly consist of a heterogeneous group of benzimidazole, benzothiadiazine, and thiophene carboxylic-acid-based compounds that bind to three non-overlapping binding sites on NS5B [30] and work primarily by allosterically inhibiting formation of replicase complexes. There are at least five RNA polymerase inhibitors in phase I–III human clinical testing at present: NM283 (Idenix, Cambridge MA, USA, http://www.idenix.com/), R1626 (Roche, Nutley NJ USA, http://www.roche.com/), XTL-2125 (XTL, Rehovot, Israel), PSI-6130 (Pharmasset Inc., Princeton, NJ USA http://www.pharmasset.com/) and HCV-796 (Viropharma, Exton PA USA, http://www.viropharma.com/). Currently, safety and tolerability data are limited, but preliminary results from NM283, a prodrug of 2′-C-methylcytidine, indicate a fairly modest reductions in HCV RNA titers (<1 $\log_{10}$ decrease) for monotherapy but virologic response rates similar to interferon/ribavirin when used in combination

The NS3 helicase enzymatically unwinds duplex RNA structures by disrupting the hydrogen bonds that keep the two strands together, an activity that requires hydrolysis of nucleoside triphosphate (NTP). Potential specific inhibitors of NTPase/helicases theoretically could function by inhibiting NTP binding or NTPase activity, uncoupling NTP hydrolysis from the unwinding reaction, competing with dsRNA binding and/or by blocking translocation of the NTPase/helicase along the dsRNA and thus blocking propagation of unwinding [32]. Peptide libraries have identified aminophenylbenzimidazole moieties [32], imidazo[4,5-d]pyridazine nucleosides, halogenated benzimidazoles, halogenated benzotriazoles, 5′-*O*-(4-fluorosulphonylbenzoyl)-esters of ribavirin [33], and bromo- and morpholinomethyl-analogues of tropolone [34] as potential inhibitors of the NS3 helicase.

Inhibitors of viral particle formation

In order for new virions to be completed, nascent positive strand HCV RNA must be packaged into structural units. The replication of HCV RNA occurs in membrane-associated complexes, and the structural units are assembled closely associated with membranes. This membrane association depends on glycosylation of proteins and lipophilic protein modifications, processes that could be considered for therapeutic interference.

The importance of lipids and HCV was first suggested by the interaction of HCV with the LDL receptor and/or HDL scavenger receptor B1. Evidence now suggests that HMG-coA reductase inhibitors (fluvastatin > atorvastatin, lovastatin > simvastatin) do indeed have anti-HCV activity [35], but surprisingly that this inhibition more likely occurs at the level of viral replication, not at the level of cell entry, and that these effects are reversed by the addition of cholesterol biosynthesis metabolites [35–37]. Studies in HCV replicon systems further demonstrate that HCV replication is intricately controlled by fatty acid and cholesterol metabolism; HCV RNA replication is inhibited *in vitro* with polyunsaturated fatty acids and acetyl-coA carboxylase inhibitors [37]. In culture, the combination of interferon-alpha and fluvastatin exhibited strong synergistic inhibitory effects on HCV RNA replication suggesting that fluvastatin in particular, but potentially other statins, could be potentially useful as an adjunct to interferon-alpha [35].

An offshoot of cholesterol metabolism, the prenylation pathway, also appears to be important in viral particle assembly for the unrelated hepatitis D virus [38,39] and the findings from the HCV replicon system also suggest dependence of HCV particle production on a prenylated substrate [36]. Geranylgeranyl diphosphate is derived from farnesyl diphosphate itself a product of the cholesterol biosynthesis pathway (Fig. 3). Farnesylation and geranylgeranylation (together termed prenylation) are catalyzed by specific enzymes (far-

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