Hepatitis C virus replication and potential targets for direct-acting agents

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***Abstract*:** We finally stand at the brink of novel, oral, direct-acting antivirals for the treatment of hepatitis C virus (HCV) infection. Basic science research has lead to a greater understanding of the viral life cycle and identified numerous potential targets for therapy. Early compounds were plagued by inconsistent *in vivo* activity and side effects that led to discontinuation of investigational efforts. However, several agents have now progressed to phase 2 human studies and two protease inhibitors have completed enrolment for their phase 3 clinical trials and look promising. Thus, while it appears that protease inhibitors will likely be the next available drugs for the treatment of HCV infection, the quest for additional therapeutic agents will continue. The future of HCV therapy lies in multidrug cocktails of several agents targeted against a variety of targets. In the near future these agents will be added to the current standard therapy consisting of pegylated interferon and ribavirin; however, the ultimate and probably realistic goal will be to develop multidrug oral regiments to replace the need for interferon.

***Keywords*:** HCV replication, new treatment, polymerase inhibitors, protease inhibitors, therapy

Introduction

Hepatitis C infects 170 million people worldwide and 1.6% of the United States population [Armstrong *et al.* 2006; Davis *et al.* 2003; Alter *et al.* 1999; WHO, 1999]. After acute infection, 55% to 85% of patients develop chronic disease. The natural history of chronic hepatitis C varies significantly because of host, viral and environmental factors. Chronic infection leads to cirrhosis in approximately 20% of patients after 20 years of infection [Freeman *et al.* 2001]. Thereafter, other complications including hepatic decompensation (ascites, encephalopathy, variceal hemorrhage, hepatorenal syndrome, or hepatic synthetic dysfunction) and hepatocellular carcinoma ensue at a rate of about 3% per year [Sangiovanni *et al.* 2006; El-Serag, 2004; Serfaty *et al.* 1998; Fattovich *et al.* 1997]. Without liver transplantation, decompensated cirrhosis leads to death in 50–72% of patients after 5 years [Fattovich *et al.* 2002]. As a result of the high prevalence of hepatitis C virus (HCV) infection and resultant complications, HCV is the leading indication for liver transplantation in the United States and the world as a whole [Wasley and Alter, 2000].

Chronic hepatitis C is the only chronic viral infection that can be cured with antiviral therapy. Unlike human immunodeficiency virus and hepatitis B, a sustained virologic response (SVR), defined as HCV-RNA undetectable by a sensitive amplification test 6 months after the completion of therapy, is equivalent to a cure in >99% of cases [Fried *et al.* 2002; Manns *et al.* 2001]. Patients with compensated cirrhosis who achieve an SVR essentially eliminate their subsequent risk of decompensation, may achieve histologic regression, and decrease their risk of hepatocellular carcinoma by two thirds [Bruno *et al.* 2007; Di Bisceglie *et al.* 2007; Camma *et al.* 2004].

The current standard of care for the treatment of HCV infection remains the combination of pegylated interferon and ribavirin [Fried *et al.* 2002; Manns *et al.* 2001]. This therapy eradicates HCV in 40–50% of genotype 1 non-cirrhotic patients and 70–80% of genotype 2 and 3 non-cirrhotic patients. The response to treatment is lower in obese, insulin-resistant, or African-American patients and in those with advanced hepatic fibrosis or high viral loads. Of greatest concern are patients with decompensated cirrhosis and immunosuppressed patients, such as liver transplant recipients, who are rarely able to tolerate full doses of therapy.

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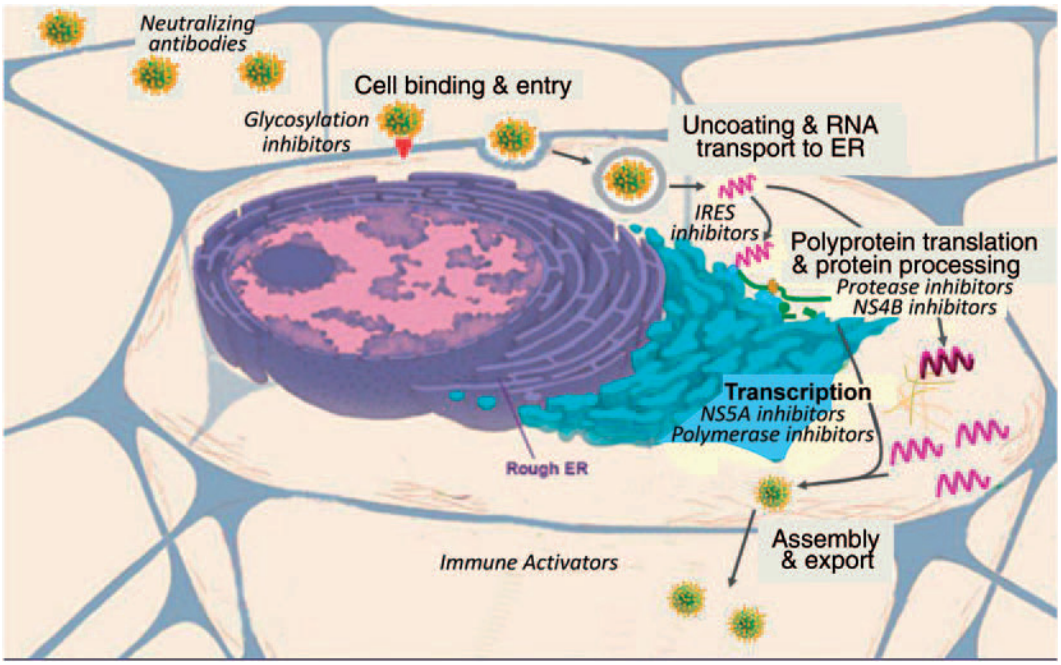

Figure 1. A graphic depiction of the viral life cycle with the potential antiviral targets listed. NS, nonstructural proteins; ER, endoplasmic reticulum; IRES, internal ribosome entry side.

Fortunately, we are entering a new era of HCV therapy. A greater understanding of the HCV replication machinery has led to a large list of potential targets for therapy (Figure 1). One such target is the nonstructural 3 (NS3) viral protease, an enzyme that is critical to post-translational processing of the viral polyprotein. Drugs that effectively inhibit this enzyme are in the final stages of clinical trials, and it appears that the combination of a protease inhibitor with pegylated interferon and ribavirin will significantly improve the SVR rate, perhaps even with a shorter duration of treatment. In addition, these medications will allow us to retreat previous relapsers and nonresponders to pegylated interferon and ribavirin with some success. However, while such drugs will be a tremendous addition to our therapeutic armamentarium, it is important to recognize that they will not eradicate infection in all patients and barriers to using interferon or ribavirin in many patients will still be a problem.

This article will review the most promising potential targets for new direct-acting antiviral agents (DAA). Drugs for some of these targets are well along in clinical development while others are only hypothetical and supported by *in vitro* studies (Table 1). It is important for the reader to understand that the high replication and nucleic acid substitution rate of HCV will likely lead to rapid emergence of viral drug resistance if a single one of these replicative steps is targeted. Thus, the future of HCV therapy lies in the combination of multiple agents against different targets such as receptor binding, cell entry, viral transcription, translation, polyprotein processing, particle assembly and export of virus progeny. These DAAs might also be combined with non-specific antiviral agents such as those that enhance the endogenous immune response to the virus, e.g. interferons or therapeutic vaccines, or neutralize extracellular virus, e.g. hyperimmune globulins. The goal, of course, is to seek combinations that will both increase efficacy and improve tolerability.

Early inhibitors: receptor binding and cell entry

Entry of HCV into the hepatocyte involves a series of sequential interactions with soluble and cell surface host factors, and remains incompletely understood. Plasma-derived HCV is complexed with low-density and very-low-density lipoproteins (LDL and VLDL), which probably facilitates the initial attraction and concentration of virus on the cell surface via interaction with the low-density lipoprotein receptor (LDL-R) [Andre *et al.* 2005]. The highly glycosylated viral envelope proteins E1 and E2

Table 1. Direct-acting agents to treat hepatitis C in phase 2 and 3 clinical trials.

Class	Drug name	Drug Company
Entry and RNA-Binding Inhibitors		
Neutralizing Antibodies	Civacir	NABI Pharmaceuticals
Glycosylation Inhibitors	Celgosivir	Migenix, Inc.
	*UT-231B	Unither Pharmaceuticals
IRES Inhibitors	*Heptazyme	Ribozyme Pharmaceuticals, Inc.
	*ISIS-14803	ISIS Pharmaceuticals
	VGX-410C	VGX Pharmaceuticals
Protease Inhibitors		
NS2 Inhibitors	none	
NS3 Inhibitors	Telaprevir	Vertex Pharmaceuticals
	Boceprevir	Schering-Plough Corporation
	ITMN-191	Intermune, Inc.
	TCM435	Tibotec Pharmaceuticals Limited
	MK-7009	Merck
	BMS-650032	Bristol-Myers Squibb
	BMS-791325	Bristol-Myers Squibb
NS4A Inhibitors	*ACH-806	Achillion Pharmaceuticals Inc.
RNA Transcription		
NS4B Inhibitors	none	
Helicase Inhibitors	none	
Cyclophilin A Inhibitors	Debio-025	DebioPharm Group
	NIM-811	Novartis
NS5B Polymerase Inhibitors	R7128	Pharmasset, Inc. and Roche
	*R1626	Roche
	*NM283	Idenix Pharmaceuticals, Inc.
	*HCV-796	ViroPharm, Inc.
	VCH-759	Vertex Pharmaceuticals
	PF-868554	Pfizer
	IDX184	Idenix Pharmaceuticals, Inc.
	GS 9190	Gilead
NS5A Inhibitors	BMS790052	Bristol-Myers Squibb
Other		
TLR-9 Agonists	*CPG 10101	Pfizer
NKT Cell Agonist	KRN7000	Kyowa Hakko Kirin Company, Limited
Other	Nitazoxanide	Romark Laboratories L.C.

Data available at: www.clinicaltrials.gov and www.hcvdrugs.com. NS, nonstructural; IRES, internal ribosome entry site; TLR-9, toll-like receptor-9.
*Future investigation of these compounds has been aborted.

conformationally attach to glycosamioglycans at the hepatocyte cell surface and to the c-type lectins DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin; CD209) and L-SIGN (DC-SIGNr; liver and lymph node specific; CD209L) on neighboring dendritic cells and liver sinusoidal cells [Cormier *et al.* 2004a]. E2 then sequentially attaches to the tetraspanin CD81 and scavenger receptor class B type 1 (SR-B1) to form a receptor complex that utilizes the tight junction claudin proteins, particularly CLDN1, for internalization [Zeisel *et al.* 2007; Cormier *et al.* 2004b; Pileri *et al.* 1998]. Occludin (OCLN), a tight junction protein, is also essential for cell entry but its exact role remains to be defined [Lanford *et al.* 2009; Ploss *et al.* 2009; Evans *et al.* 2007]. Interestingly, EWI-2wint, a small protein that is associated with CD81 in several cell lines and efficiently blocks HCV entry, is absent in hepatocytes and this probably explains the selective susceptibility of hepatocytes to HCV infection [Schuster and Baumert, 2009; Rocha-Perugini *et al.* 2008].

The role of the humoral immune response in controlling HCV infection is not clear. Monoclonal anti-CD81 antibodies have been utilized to effectively block HCV infection of mice with humanized livers [Lanford *et al.* 2009]. However, they do not affect HCV infection after it has been established. Monoclonal and polyclonal antibodies with HCV envelope neutralizing capacity have been tested in humans at the time of liver transplant but have been ineffective in preventing reinfection of the donor liver; however, this

remains a potential perioperative strategy during transplantation [Davis *et al.* 2005]. The currently available antibodies' lack of efficacy may be attributed to the heterogeneity of the virus, its association with apolipoproteins, or other factors.

Another approach to blocking cellular infection is to inhibit binding and processing of HCV via the cell surface receptor proteins involved in cell entry. Glycosylation inhibitors may alter the structure of cell surface glycosaminoglycans thereby decreasing or eliminating viral concentration at the cell surface [Pawlotsky *et al.* 2007]. MX-3256 (Celgosivir; Migenix Inc.) is an oral alpha-glucosidase I inhibitor that acts through host-directed glycosylation to prevent proper folding of the HCV envelope. In preclinical studies, celgosivir demonstrated strong synergy with pegylated interferon plus ribavirin, but a phase IIa monotherapy study did not show any reduction in HCV-RNA [Kaita *et al.* 2007; Yoshida *et al.* 2006]. Unfortunately, a 12-week study of another glycosylation inhibitor, UT-231B (Unither Pharmaceuticals), in HCV-infected patients who previously failed interferon-based therapy also failed to reduce virus levels. Despite these early setbacks, this remains an interesting target for future therapies.

In addition, the lectin cyanovirin-N interacts with HCV envelope glycoproteins and blocks the association between E2 and CD81 [Helle *et al.* 2006]. Therefore, targeting the cell surface and/or viral glycans could be a promising approach to antiviral therapy.

Virus uncoating, HCV-RNA release and attachment to the endoplasmic reticulum

The HCV and receptor complex fuses with the hepatocyte cell membrane and undergoes clatharin-mediated endocytosis. Acidification of the vesicle leads to fusion of the viral and vesicle membranes resulting in uncoating and release of the viral RNA into the cytoplasm. The presence of viral RNA in the cytosol has pathogenic and potential therapeutic implications.

The cell recognizes the presence of single-strand RNA (ssRNA) in the cytosol as abnormal and initiates a pathogen-associated molecular pattern (PAMP)-associated response via toll-like receptors [Sen, 2001]. This process involves activation of retinoic acid inducible gene-1 (RIG-1) and Toll-IL-1 receptor domain containing adaptor-inducing interferon-beta (TRIF) that under normal circumstances leads to cellular production of type 1 interferons and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) mediated apoptosis [Saito and Gale, 2008]. However, HCV is capable of down-regulating this step of the innate immune response. The HCV NS3/4 protease cleaves and inactivates the RIG-1 adaptor protein IFN-beta promoter stimulator-1 (IPS-1) and TRIF itself thereby blocking downstream activation of the interferon regulatory genes [Zhu *et al.* 2007; Foy *et al.* 2005; Li *et al.* 2005]. Therefore, NS3/4 inhibition (see later), in addition to its direct effect on viral polyprotein processing, has the potential to restore the RIG-1 and TRIF pathways of innate immunity.

The cytosolic viral ssRNA is also a vulnerable potential target for therapeutic oligonucleotides such as antisense nucleotides (small non-coding strands of RNA that hybridize and inactivate mRNA) or ribozymes (RNA molecules that catalyze cleavage of a target RNA). However, these agents require an absolutely conserved target in an otherwise very heterogeneous virus in order to have their effect. The internal ribosomal entry site (IRES) at the 5' end of the viral RNA is that highly conserved target. IRES is the landing pad that directs the positive strand HCV-RNA to the endoplasmic reticulum (ER) for protein translation. Thus, inhibition of attachment of IRES to both cellular and viral proteins by oligonucleotides could effectively inhibit HCV replication. While several class-specific problems with oligonucleotides such as drug delivery, instability, proinflammatory effects, and other unintended 'off-target' side effects have been partially overcome by modifications of the compounds, all candidate drugs to date have been plagued by safety concerns. Development of Heptazyme (Ribozyme Pharmaceuticals, Inc.), a ribozyme that cleaved IRES, was stopped secondary to toxicity. ISIS-14803 (ISIS Pharmaceuticals), a 20 base pair antisense oligodeoxynucleotide, led to a 1.2 to 1.7 log decline in HCV RNA when given as monotherapy three times a week for 4 weeks in three out of 28 patients; however, asymptomatic liver function test abnormalities also occurred in five treated patients [McHutchison *et al.* 2006]. VGX-410C (VGX Pharmaceuticals) was a small molecule that also targeted HCV IRES binding. It appeared to be safe in phase 2 clinical trials, but was not effective. Despite these early setbacks, this approach

remains intriguing and warrants further exploration.

Polyprotein translation and protein processing

Once the viral RNA attaches to the ER, a single large polyprotein is translated. This polyprotein is then co- and post-translationally processed by host and viral proteases into at least 11 viral proteins. Two host cellular pepsidases are required for cleaving HCV structural proteins. These are not targets for HCV therapy as they are essential for cellular function. NS2 complexes with NS3 and zinc to form a cystine protease. This complex autocatalytically cleaves NS2 from NS3, and is then degraded by the proteasome. To date, no inhibitors of the NS2 protease have entered clinical development.

In contrast, the NS3 protease has been the primary focus of recent drug development. NS3 complexes with NS4A and acts as a serine protease to cleave the polyprotein at the NS3-NS4A, NS4A-NS4B, NS4B-NS5A, and NS5A-NS5B sites. The NS3-NS4A complex's catalytic triad lies adjacent to a shallow substrate binding area that has made design of potent inhibitors challenging. It is this active site that is the target for drugs that are currently in clinical trials. Telaprevir (VX-950; Vertex Pharmaceuticals) and boceprevir (SCH503034; Schering-Plough Corporation) are both in phase 3 clinical trials. While these agents are potent inhibitors of HCV replication, monotherapy leads to rapid selection of drug-resistant strains of the virus, typically within days [Kieffer *et al.* 2007]. Therefore, effective treatment, where each drug is dosed three times per day, requires combination with other agents which means that all current studies include pegylated interferon and ribavirin. This significantly reduces the likelihood of drug resistance, but the requirement for frequent dosing with these first-generation agents may impede compliance and increase the chance of resistance outside the setting of clinical trials.

Telaprevir administered during the first 12 weeks of a 24 week course of pegylated interferon and ribavirin in previously untreated genotype-1-infected patients resulted in an SVR in 61% compared with 41% in those treated with pegylated interferon and ribavirin alone for 48 weeks (standard treatment) [McHutchison *et al.* 2008]. A similar trial in Europe achieved an SVR in 68% with the 24-week triple-drug regimen compared to 48% with standard treatment [Dusheiko *et al.* 2008]. A lower dose or elimination of ribavirin resulted in a lower chance of response. Thus, it appears that, at least for the time being, both pegylated interferon and ribavirin continue to be essential components of treatment. Telaprevir in combination with pegylated interferon and ribavirin is also effective in retreating genotype 1 patients who had relapsed or failed to respond to a prior course of pegylated interferon and ribavirin. The previously described 24 week regimen achieved an SVR in 69% of prior relapsers and 39% of previous non-responders [Manns *et al.* 2009b]. Extension of the total length of treatment to 48 weeks did not appear to improve the response to retreatment. Efficacy and side effects are both increased with triple drug therapy. Rash is most common, but rarely leads to drug discontinuation. Pruritus, nausea, diarrhea and rectal discomfort are also more common.

Twenty-eight weeks of therapy with Boceprevir, given orally three times a day, in combination with peginterferon and ribavirin, led to an SVR rate of 55% in previously untreated genotype 1-infected patients [Kwo *et al.* 2008]. To address concerns about resistance, a second group of patients was treated with a 4-week lead-in phase of pegylated interferon and ribavirin to reduce HCV-RNA levels before the introduction of boceprevir; however, the SVR rate remained unchanged indicating that this is unnecessary. Unlike telaprevir, boceprevir treated patients may benefit from longer therapy. Patients treated with 4 weeks of pegylated interferon and ribavirin were then treated with either 44 weeks of pegylated interferon and ribavirin (38% SVR), 24 weeks of boceprevir, pegylated interferon and ribavirin (56% SVR), or 44 weeks of triple therapy (75% SVR) [Kwo *et al.* 2009]. Side effects with boceprevir include headache, gastrointestinal complaints and anemia that may limit the ability to maintain the ribavirin dose.

ITMN-191 (Intermune, Inc.), another protease inhibitor, has been used in combination with pegylated interferon and ribavirin for 14 days to achieve an undetectable HCV-RNA in 71% of treated patients [Kamal and Nasser, 2008]. ITMN-191 is active against HCV strains that are resistant to telaprevir and boceprevir. This drug is being studied in combination with pegylated interferon and ribavirin, as well as in combination with a polymerase inhibitor (R7128; Pharmasset, Inc. and Roche) without either

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