Review

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# NS5A inhibitors in the treatment of hepatitis C

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## Summary

Hepatitis C virus infection is a major health problem worldwide and no vaccine has yet been developed against this virus. In addition, currently approved pharmacotherapies achieve suboptimal cure rates and have side effects that result in non-compliance and premature treatment discontinuation. Significant research has been devoted to developing direct-acting antiviral agents that inhibit key viral functions. In particular, several novel drug candidates that inhibit the viral non-structural protein 5A (NS5A) have been demonstrated to possess high potency, pan-genotypic activity, and a high barrier to resistance. Clinical trials using combination therapies containing NS5A inhibitors have reported results that promise high cure rates and raise the possibility of developing interferon-free, all-oral regimens.


## Introduction

Recent estimates indicate that there are more than 120–130 million chronic hepatitis C virus (HCV) carriers worldwide [1], who are at risk of developing cirrhosis and/or hepatocellular carcinoma (primary liver cancer). As many as 4 million persons are thought to be chronically infected in the US [2], 5–10 million in Europe [2], 12 million in India [2], and 1.2 million in Japan (2004 figure) [3]. Most of these individuals are not aware of their infection. The incidence of acute infection in the US has declined from 7.4/100,000 in 1982 to 0.7/100,000 in recent years, primarily due to screening of blood in transfusion centres and improved safety of intravenous drug use [4]. It is estimated that approximately 150,000 new cases occur annually in the United States and in Western Europe, and about 350,000 in Japan. Only 25% of acute cases are symptomatic, but up to 80% of these acute cases progress to chronic infection and liver disease, and up to 20% of chronic infections progress to cirrhosis [2]. Every year, 4–5% of cirrhotic patients develop hepatocellular carcinoma [5]. Despite the decrease in HCV incidence, the number of patients with chronic HCV-related complications is increasing in those aging patients who have been infected for many years, and chronic hepatitis C infection will continue to be a significant cause of premature mortality, causing at least 200,000–300,000 deaths per year worldwide [4].

A number of direct-acting antiviral agents (DAAs) are under development for the treatment of chronic HCV infection. These agents block viral production by directly inhibiting one of several steps of the HCV lifecycle. As shown in Fig. 1, the genomic organization of HCV has been elucidated, and several viral proteins involved in the HCV lifecycle, such as the non-structural (NS) 3/4A serine protease, the NS5B RNA-dependent RNA polymerase (RdRp), and the NS5A protein, have been targeted for drug development [4]. Two NS3/4A protease inhibitors, telaprevir and boceprevir, which inhibit post-translational processing of the HCV polyprotein into individual non-structural proteins, have been approved by the US Food and Drug Administration, the European Medicines Agency, and several other regulatory agencies for the treatment of chronic HCV genotype 1 infection in combination with pegylated interferon (IFN)-α and ribavirin [6,7].

## HCV structure and lifecycle, and physiological role of the NS5A protein

HCV is an enveloped virus with a single-stranded positive RNA genome of approximately 9.6 kb. At the flanking ends of the genome are 2 highly conserved untranslated regions (UTRs). The 5′ UTR is highly structured and contains the internal ribosome entry site (IRES), which is important for the initiation of the cap-independent translation of the polyprotein [8]. The 3′ UTR consists of a short genotype-specific variable region, a tract consisting solely of pyrimidine residues (predominantly uridine) and a conserved 98-nucleotide sequence, known as X region, containing 3 stem-loops [9,10]. The HCV open reading frame is situated between the two UTRs.

After entering the bloodstream, HCV binds to a receptor complex at the surface of its target cells, the hepatocytes. The envelope glycoproteins E1 and E2 are essential for target cell recognition, binding, and internalization [11]. The bound virus

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*Abbreviations:* HCV, hepatitis C virus; DAA, direct acting antiviral; NS, nonstructural; RdRp, RNA-dependent RNA polymerase; IFN, interferon; UTR, untranslated region; IRES, internal ribosome entry site; SVR, sustained virological response; RVR, rapid virologic response; cEVR, complete early virologic response.

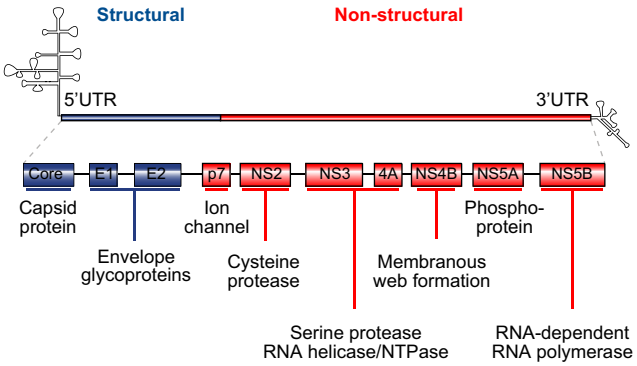


**Fig. 1. Structural organization of HCV RNA and viral proteins.** NS, non-structural; UTR, untranslated region.

then undergoes clathrin-mediated endocytosis [12]. Acidification of the endocytosis vesicle frees the genomic RNA from the nucleocapsid for release into the cytoplasm. Along with host RNA molecules, the viral RNA migrates to the endoplasmic reticulum (ER). Binding of the 40S ribosomal subunit to the HCV IRES produces a stable pre-initiation complex that begins translation of the viral open reading frame to generate an approximately 3000 amino acid polyprotein. Following translation, the polyprotein is cleaved by both cellular and viral proteases to produce at least 10 viral proteins, including structural proteins (core, E1 and E2) and non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) [13,14].

Viral replication (i.e., the synthesis of new positive RNA genomes that may also serve as messenger RNAs for viral protein synthesis) is catalyzed by the viral RdRp, or NS5B protein. A negative-strand intermediate of replication is initially produced, which then serves as a template for the synthesis of numerous positive strands. The NS5A viral protein has been shown to play an important role in the regulation of replication. In addition, host cell proteins, such as cyclophilin A, act as necessary co-factors of HCV replication through their interactions with both NS5A and the RdRp in the replication complex [15,16].

The non-structural NS5A protein bears pleiotropic functions, including roles in viral replication and assembly, and complex interactions with cellular functions. The latter include inhibition of apoptosis and promotion of tumorigenesis, both of which may play a role in the triggering of the hepatocarcinogenic process [17–20]. The protein is comprised of approximately 447 amino acids and localizes to ER-derived membranes. It basally exists in phosphorylated (p56) and hyperphosphorylated (p58) forms that are implicated in different functions [21–23]. Its cytoplasmic moiety contains 3 domains, of which Domain I is the most conserved [24]. The mechanism by which NS5A regulates replication regardless of the HCV genotype is still unclear [25]. Considerable information has been gathered on its molecular interactions and role in the viral lifecycle. NS5A and the RdRp directly interact, both *in vivo* and *in vitro* [26]. *In vitro*, this interaction stimulates RdRp-catalyzed synthesis of the negative RNA strand [27]. It was shown that all 3 domains of NS5A bind to RNA [9]. The interactions of Domain I with the polypyrimidine tract of 3′ UTR suggest it may affect the efficiency of RNA replication by the RdRp; however, these results also suggested the binding of RdRp and NS5A to RNA are mutually exclusive. In addition, Domain II of NS5A interacts with cyclophilin A, a host cell protein required for replication, and this interaction is vital for RNA binding [28]. NS5A also plays a role in viral packaging and assembly. Domain III appears to be essential for this function [29,30]. This may be due, at least in part, to NS5A recruiting apolipoprotein E, a component of the HCV production process [29,31]. Indeed, inhibiting apolipoprotein E expression results in marked reduction of infectious particle production without affecting viral entry and replication [31].

## NS5A inhibitor mechanism of action

Several viral proteins have generated interest as potential targets for specific inhibitory drugs. In addition to the two NS3/4A protease inhibitors already approved for clinical use, numerous other protease inhibitors are being developed as well as inhibitors of viral replication, including nucleoside/nucleotide analogue inhibitors of HCV RdRp, non-nucleoside inhibitors of RdRp, cyclophilin inhibitors, and NS5A inhibitors.

Because of its critical involvement in viral replication and assembly [32], NS5A has been identified as a target for viral inhibition, leading to development of therapeutic agents. In HCV replicon-containing cells, inhibition of NS5A, but not other HCV proteins, resulted in redistribution of NS5A from the ER to lipid droplets. NS5A-targeting agents did not cause similar alterations in the localization of other HCV-encoded proteins, and the transfer of NS5A to lipid droplets coincided with the onset of inhibition of replication [33]. Inhibition of NS5A at picomolar concentrations has been associated with significant reductions in HCV RNA levels in cell culture-based models, which makes these agents among the most potent antiviral molecules yet developed [34–36]. NS5A inhibitors have pan-genotypic activity, i.e., they suppress replication of all HCV genotypes, but their antiviral effectiveness against genotypes other than 1 may vary from one molecule to another [35]. Use of multiple DAAs including an NS5A inhibitor in replicon systems in cell culture has resulted in additive/synergistic inhibition of viral production and an increased barrier to resistance [37].

The exact mechanism of antiviral action of NS5A inhibitors is unknown. Available evidence suggests that they have multiple effects, which contribute to their potency [32]. One putative mechanism is the inhibition of hyperphosphorylation. Phosphorylation of NS5A seems required for viral production [38], but the relative roles of the phosphorylated and hyperphosphorylated forms are unclear, and conflicting results have been reported suggesting that reduced hyperphosphorylation may either enhance or reduce replication [21,39]. It is thought that a tightly regulated control of phosphorylation *vs.* hyperphosphorylation is required for efficient viral function. It was also shown that NS5A acts in two different pathways in RNA replication, and one of them likely requires hyperphosphorylation [23]. However, other mechanisms may also play a role. For instance, NS5A inhibitors alter the subcellular localization of NS5A, which may cause faulty viral assembly [33,40].

## Resistance to NS5A inhibitors

HCV displays a large degree of genomic variability, resulting in its quasispecies distribution [41]. Variants that confer resistance to NS5A inhibitors pre-exist within HCV quasispecies populations in the absence of any previous exposure to these drugs.