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Report on Investigational Drugs

Discovery of Hepatitis C Virus NS5A Inhibitors as a New Class of Anti-HCV Therapy

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Chronic hepatitis C virus (HCV) infection is responsible for development of liver cirrhosis and hepatocellular carcinoma. In addition to PEGylated interferon-α, ribavirin, and HCV NS3 protease inhibitors, recently identified HCV NS5A inhibitors such as BMS-790052 showed a great promise in clinical trials as another new class of direct-acting anti-HCV therapeutics with a distinct mechanism of action. This clinical proof-of-concept study with NS5A inhibitors demonstrated that small molecules targeting a viral protein without any known enzymatic activity can also have profound antiviral effects. In conclusion, NS5A inhibitors will serve as a valuable component of future therapy for HCV patients.

INTRODUCTION ON HEPATITIS C VIRUS

More than 170 million people are estimated to be infected with Hepatitis C virus (HCV) worldwide (Shepard et al., 2005). Individuals who are chronically infected with HCV are at high risk of developing various liver diseases including hepatitis, liver cirrhosis, and hepatocellular carcinoma (Alter et al., 1999). Current standard of care for HCV infection mainly relies on a combination of PEGylated interferon-α and ribavirin. However, this combination therapy has showed significant toxicity and poor efficacy for many HCV patients (Liang et al., 2000; Zeuzem et al., 2000). Therefore, development of alternative anti-HCV therapeutics is urgently needed.

HCV is a positive strand RNA virus and the only member of the *Hepacivirus* genus of the *Flaviviridae* family. The ~9.6 kb long single-stranded viral RNA genome expresses a single polyprotein consisting of ~3000 amino acids, which undergoes proteolytic cleavage with the help of host and virally-encoded proteases. This results in production of up to 10 different viral proteins (Grakoui et al., 1993a, 1993b). Among those individually expressed viral proteins, structural viral proteins such as E1, E2, and core are incorporated as structural components into the mature virus particle, whereas non-structural (NS) viral proteins such as NS3 (protease/helicase), NS4A, NS4B, NS5A, and NS5B (RNA polymerase) serve as key components of the "replication complex", which is necessary for replicating the viral RNA genome (Fig. 1) (Lohmann et al., 1999; Blight et al., 2000; Moradpour et al., 2007).

HCV RNA GENOME REPLICATION AS ANTI-VIRAL TARGET

HCV replicates its RNA genome in association with endoplasmic reticulum (ER)-derived membranes. Since viral RNA replication is an indispensable step in the HCV life cycle, pharmacological disruption of viral RNA replication has been regarded as an ideal antiviral strategy. Each individual nonstructural viral protein plays a key role in the assembly and maintenance of the replication complex and thus could be a potential

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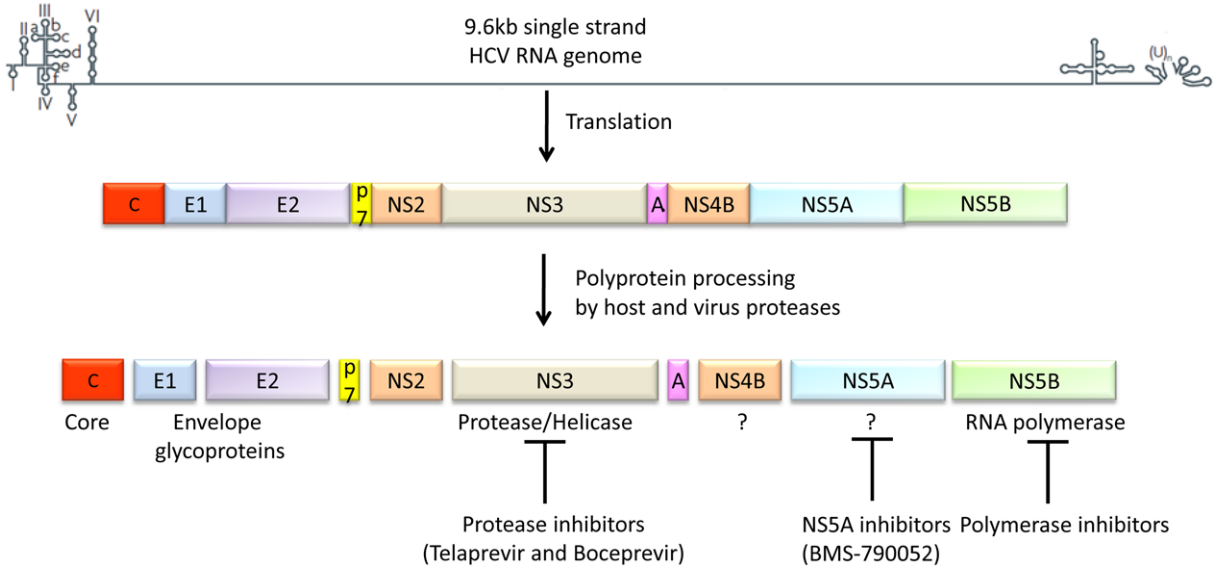

Fig. 1. Genomic structure of hepatitis C virus. The single-stranded viral RNA genome expresses a single polyprotein, which undergoes a polyprotein processing with the help of host and virus proteases. This leads to production of 10 different viral proteins. Structural viral proteins such as E1, E2, and core are incorporated as structural components into the mature virus particle, whereas non-structural (NS) viral proteins such as NS3 (protease/helicase), NS4A, NS4B, NS5A, and NS5B (RNA polymerase) serve as key components of the RNA genome replication complex. Protease and polymerase inhibitors target the enzymatic activities of NS3 protease and NS5B polymerase, respectively, whereas NS5A inhibitors such as BMS-790052 disturb the proper subcellular localization of NS5A proteins, thereby preventing the assembly of NS5A into functional replication complexes.

antiviral target. Due to their well-characterized enzymatic activities, NS3 (protease) and NS5B (RNA polymerase) have been the target of most of efforts to discover new anti-HCV therapeutics (Fig. 1). Numerous small molecules with a specific inhibitory activity against the viral protease or polymerase have been identified. These direct-acting antiviral (DAA) molecules include NS3 protease inhibitors such as Telaprevir (Fig. 2A) and Boceprevir (Fig. 2B), which have been approved by FDA recently, and NS5B polymerase inhibitors, which are in various stages of clinical development (Asselah et al., 2009; Gane et al., 2010; Pawlotsky, 2011). Very promising results have been obtained with a combination therapy of NS3 protease or NS5B polymerase inhibitors plus PEGylated interferon-α and ribavirin for patients chronically infected with HCV (Zeuzem et al., 2010; Marcellin et al., 2011).

DISCOVERY OF NS5A INHIBITORS

In spite of successful progression of NS3 protease and NS5B polymerase inhibitors, it is still necessary to develop a new class of DDAs targeting non-traditional viral proteins with a different mechanism of action. A combination therapy using different kinds of DDAs with distinct mode of actions is likely to be the ideal therapy for HCV infection because it may achieve synergistic potency with a low risk of development of multi-drug resistance. From this perspective, one of non-structural (NS) viral proteins, NS5A has received much attention as another promising anti-HCV target due to its multiple roles in the assembly and the maintenance of the viral replication complex, the morphogenesis of the new viral particle, and the resistance against interferon *in vivo* (Hijikata et al., 1993; Moradpour et al., 1998). However, the lack of an enzymatic activity described for NS5A has made this viral protein a more challenging target against which to design specific anti-HCV drugs. Recently, however, a group of HCV inhibitors with a thiazolidinone core structure, including BMS-858 and BMS-824, were identified by a cell-based random high-throughput HCV replicon screen (Lemm et al., 2010). Interestingly, sequence profiles of mutant viruses resistant to these new inhibitors and drug sensitive domain mapping studies using chimeric viruses strongly suggested that all of these new anti-HCV compounds specifically target NS5A for their antiviral activities (Lemm et al., 2010). These NS5A inhibitors were then further optimized through structure-activity relationship analysis to yield BMS-790052 (Fig. 2C). This compound is the most potent HCV replication inhibitor described to date, with reported *in vitro* EC_{50} values of 9 and 71 pM against HCV genotypes **1b** and **2a**, respectively. BMS-790052 also exhibited a high

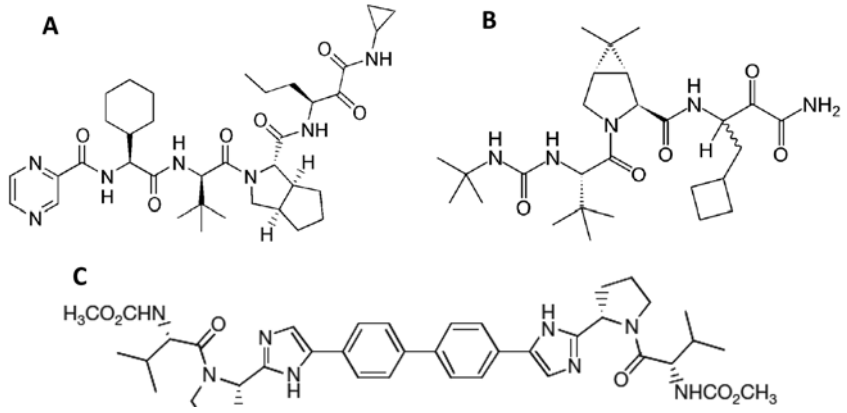

Fig. 2. Chemical structures of NS3 protease inhibitors, Telaprevir (**A**) and Boceprevir (**B**) and NS5A inhibitor, BMS-790052 (**C**)

therapeutic index (CC_{50}/EC_{50}) (>100,000), demonstrating its high specificity against HCV and low toxicity to host cells (Gao et al., 2010). In a phase I clinical trial in patients chronically infected with HCV, administration of a single 100-mg dose of BMS-790052 was able to result in a 3.3 log reduction in mean viral load as measured 24 h post-dose. Surprisingly, this antiviral effect was sustained for an additional 120 h in two patients infected with genotype 1b virus (Gao et al., 2011). This clinical proof-of-concept study with NS5A inhibitors indicates that small molecules targeting a non-traditional viral protein without any known enzymatic activity can also have profound antiviral effects in HCV-infected subjects. Phase II clinical studies combining BMS-790052 with the NS3 protease inhibitor BMS-650032 are ongoing and interim results have shown that this combination therapy alone or with PEGylated interferon-α and ribavirin results in undetectable HCV RNA through 12 weeks of therapy in HCV genotype 1 null responders (Lok et al., 2010), further emphasizing the power of a cocktail regimen using multiple anti-HCV drugs with distinct mode of actions.

MECHANISM OF ACTION FOR NS5A INHIBITORS

In regard to their mechanism of action, analysis of mutations resistant to NS5A inhibitors identified the first 76 amino acids from NS5A as important determinants for a HCV replicon's susceptibility to NS5A inhibitors (Lemm et al., 2010). In particular, an Y93H mutation within NS5A was identified to be one of the most common mutations, conferring a 20 to 1000-fold resistance to BMS-790052 and related compounds, depending on HCV genotype (Fridell et al., 2010; Gao et al., 2010). Interestingly, high resolution structural studies of NS5A protein reveal that the protein forms a dimer via contacts near the N-terminus, and the Y93H resistance mutation lies at the interface between two NS5A proteins (Tellinghuisen et al., 2005). This raises the possibility that BMS-790052 might inhibit HCV replication by interfering with self-dimerization of NS5A proteins.

Immobilized BMS-790052 or related compounds were reported to be able to precipitate NS5A from cell extracts, and treatment of HCV replicon cells with such compounds was associated with decreased NS5A hyperphosphorylation (Neddermann et al., 2004; Gao et al., 2010; Lemm et al., 2010). Two pieces of important evidence were published recently regarding the direct action of NS5A inhibitors on molecular functions of NS5A protein. First, Lee et al. showed that BMS-790052 alters the subcellular localization of NS5A protein from large foci to diffuse patterns with minimal effects on other NS proteins by using morphologic and biochemical fractionation assays (Lee et al., 2011). In this study, the Y93H mutation on NS5A confers resistance to BMS-790052 by preventing its ability to alter the subcellular localization of NS5A protein. Second, researchers at Pfizer demonstrated that NS5A was redistributed from the endoplasmic reticulum to lipid droplets by treatment of NS5A inhibitors (Targett-Adams et al., 2011). These data strongly suggest that NS5A inhibitors like BMS-790052 suppress viral genome replication by altering the subcellular localization of NS5A protein specifically, thereby preventing the assembly of NS5A into functional replication complexes rather than acting on pre-formed complexes (Targett-Adams et al., 2011).

CONCLUSION

In conclusion, a new class of anti-HCV therapeutics

targeting NS5A protein has been discovered. Clinical proof-of-concept validation of these NS5A inhibitors will help us to expand our ability to search for a new anti-viral target without an enzymatic activity in other clinically important viral diseases. Together with NS3 protease and NS5B polymerase inhibitors, NS5A inhibitors offer considerable promise as a valuable component of future therapy for HCV patients.

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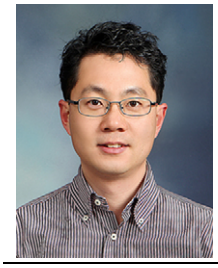

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Main Research Area

Hepatitis C Virus (HCV)-induced Liver Diseases
Human Papillomavirus (HPV)-induced Cervical Cancer
Replication of Viral RNA or DNA Genomes
Development and Mechanism of Action Study of Antiviral Drugs