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Rapid Communication

The hepatitis C virus NS5A inhibitor (BMS-790052) alters the subcellular localization of the NS5A non-structural viral protein

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ABSTRACT

The hepatitis C virus (HCV) non-structural (NS) 5A protein plays an essential role in the replication of the viral RNA by the membrane-associated replication complex (RC). Recently, a putative NS5A inhibitor, BMS-790052, exhibited the highest potency of any known anti-HCV compound in inhibiting HCV replication in vitro and showed a promising clinical effect in HCV-infected patients. The precise mechanism of action for this new class of potential anti-HCV therapeutics, however, is still unclear. In order to gain further insight into its mode of action, we sought to test the hypothesis that the antiviral effect of BMS-790052 might be mediated by interfering with the functional assembly of the HCV RC. We observed that BMS-790052 indeed altered the subcellular localization and biochemical fractionation of NS5A. Taken together, our data suggest that NS5A inhibitors such as BMS-790052 can suppress viral genome replication by altering the proper localization of NS5A into functional RCs.

Introduction

Hepatitis C virus (HCV) is an important human viral pathogen infecting more than 170 million people worldwide (Shepard et al., 2005). HCV infection is responsible for the development of chronic liver diseases such as liver cirrhosis and hepatocellular carcinoma (Alter et al., 1999; Di Bisceglie, 2000). Current standard of care for HCV infection using PEGylated interferon-α and ribavirin has significant toxicity and its efficacy is suboptimal for many patients (Liang et al., 2000; Zeuzem et al., 2000), emphasizing an urgent need to develop alternative anti-HCV therapeutics.

HCV is a positive strand RNA virus and the only member of the *Hepacivirus* genus of the *Flaviviridae* family. The HCV genome is composed of a ~9.6 kb long single-stranded RNA, which encodes a polyprotein of ~3000 amino acids. This viral polyprotein undergoes proteolytic cleavage by host and virally encoded proteases to yield more than 10 different viral proteins (Grakoui et al., 1993a; Grakoui et al., 1993b). Among those viral proteins, structural viral proteins such as E1, E2, and core serve as components of the mature virus particle, whereas non-structural (NS) viral proteins such as NS3, NS4A, NS4B, NS5A, and NS5B serve as components of a functional replication complex (RC) that replicates the viral genome but are not packaged into mature virus particles (Blight et al., 2000; Lohmann et al., 1999; Moradpour et al., 2007).

HCV replicates its RNA genome in association with membranes that are derived in part from the endoplasmic reticulum (ER). Precisely how this RC is assembled and maintained, however, remains largely unknown. The NS5A protein is thought to play an essential role in the assembly of the viral RC although its exact molecular functions needed for this process are still poorly characterized (Hijikata et al., 1993; Moradpour et al., 1998). While enzymatic activities encoded in other HCV non-structural proteins such as NS3 (protease) and NS5B (RNA polymerase) (Manns et al., 2007) have enabled the development of anti-HCV therapeutics against those targets, the lack of an enzymatic activity described for NS5A has made the latter a more challenging target against which to design specific anti-HCV drugs.

Recently, however, using a cell-based replicon screen a new class of anti-HCV compounds was identified that appear to target NS5A (Lemm et al., 2010). Surprisingly, one member of this class, BMS-790052, showed the highest in vitro potency of any known anti-HCV compound with a picomolar range of half-maximum effective concentration (EC_{50}) against HCV replicons from various genotypes. In addition, this compound exhibited a very potent clinical effect on patients chronically infected with HCV in a phase I clinical trial (Gao

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et al., 2010). Analysis of mutations resistant to NS5A inhibitors identified the first 76 amino acids from NS5A as important determinants for a replicon's susceptibility to NS5A inhibitors (Lemm et al., 2010). Although BMS-790052 and related compounds were reported to bind to NS5A from cell extracts and be associated with decreased NS5A hyperphosphorylation, a correlate of genome replication (Gao et al., 2010; Lemm et al., 2010; Neddermann et al., 2004), the apparent Kd for NS5A binding appears to be significantly different than the EC_{50} for inhibition of viral replication leaving BMS-790052's precise mechanism of action unclear.

In this report, we sought to test the hypotheses that BMS-790052 might exert its powerful anti-HCV effect in part by disrupting the proper assembly of functional RCs or by direct inhibition of RCs. We used morphologic and biochemical fractionation assays to show that BMS-790052 alters the subcellular localization of NS5A protein without affecting its expression level. We also determined that BMS-790052 has no activity in assays for either in vitro replication activity of pre-assembled RCs or NS5A self-dimerization. Taken together, our data suggest that NS5A inhibitors like BMS-790052 suppress viral genome replication by altering the subcellular localization of NS5A, thereby preventing the assembly of NS5A into functional RCs.

Results

BMS-790052 blocks HCV genome replication

The NS5A inhibitors with a thiazolidinone core structure including BMS-858 and BMS-824 were originally identified by a cell-based high-throughput HCV/bovine viral diarrhea virus (BVDV) replicon screen (Lemm et al., 2010). They were further optimized by studying their structure and activity relationship to yield BMS-790052 (Fig. 1A). 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 therapeutic index (CC_{50}/EC_{50}) (>100,000), demonstrating its high specificity against HCV and low toxicity to host cells (Gao et al., 2010).

Before embarking on our mechanism of action studies for BMS-790052, we sought to confirm its specificity and potency against HCV genome replication. First, we examined its effect on transient HCV replication. Huh7 cells transiently transfected with luciferase-linked subgenomic replicon RNA (Blight et al., 2000; Elazar et al., 2003; Tscherne et al., 2006) were treated with increasing doses of BMS-790052. As expected, BMS-790052 displayed a very potent inhibitory activity against transient HCV replication with an EC_{50} value of 1 pM against genotype 1b replicon and 18 pM against genotype 2a J6/JFH replicon, respectively (Supplementary Fig. 1). Assessment of antiviral potency in the stable genotype 1b subgenomic replicon (Bart79I; Elazar et al., 2003) was based on the detection of NS5A protein content and yielded an EC_{50} of 15 pM (Fig. 1B), a value comparable to the prior reported EC_{50} in the stable Con1 genotype 1b HCV replicon system (Gao et al., 2010). In contrast to Con1, the Bart79I replicon variant contains an adaptive mutation in the NS5A coding sequence, which impairs hyperphosphorylation of NS5A (Blight et al., 2000). While treatment of Con1 replicon cells with BMS-790052 resulted in a reduced concentration of the intracellular hyperphosphorylated form of NS5A as previously reported (Lemm et al., 2010), this effect could not be observed in Bart79I replicon cells (Fig. 1C); yet BMS-790052 remained a highly potent inhibitor of HCV replication in these cells, suggesting that inhibition of hyperphosphorylation may not be a sole mechanism of action for NS5A inhibitors. Taken together, these data confirmed that BMS-790052 is a potent inhibitor of both transient as well as stable HCV genome replication.

BMS-790052 alters the subcellular localization of NS5A

Having confirmed the extraordinary potency of BMS-790052 against HCV replication, we next sought to study its mechanism of action. Since NS5A plays an essential role in the assembly of the viral RC, we hypothesized that BMS-790052 disrupts NS5A function(s) important for this assembly process. To test this hypothesis, we used a vaccinia virus-based HCV replicase assembly system to express and assemble NS proteins into RCs in the presence or absence of BMS-790052. In this system, the HCV non-structural proteins NS2 to NS5B are constitutively expressed via a T7 RNA polymerase promoter, with a vaccinia virus recombinant that provides a source of T7 RNA polymerase. This allows the study of HCV RC assembly without interference from HCV replication inhibition.

First, we examined the effect of BMS-790052 on the subcellular localization pattern of NS5A proteins using immunofluorescence analysis. As shown in Fig. 2Ai, when NS5A protein was expressed in

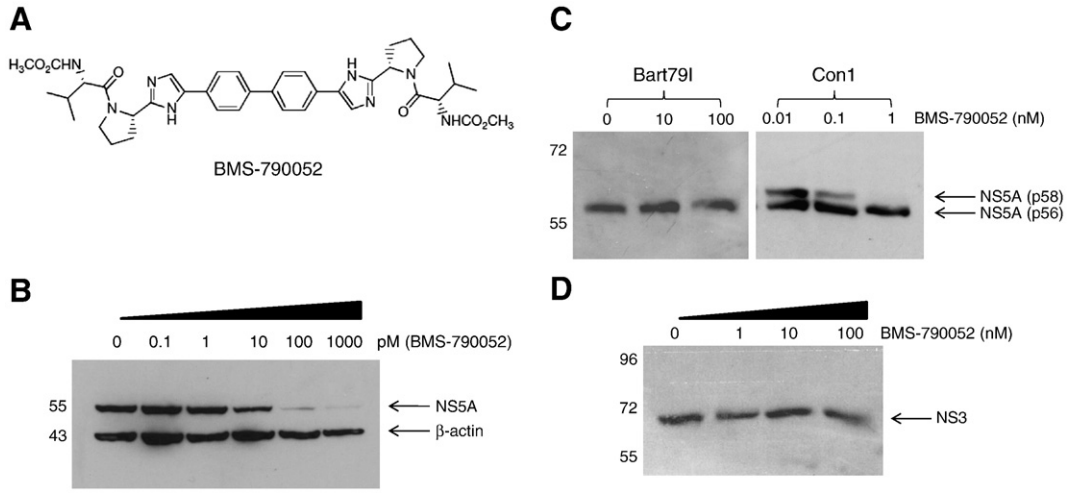

Fig. 1. BMS-790052 blocks HCV genome replication. (A) Structure of BMS-790052. (B) Effect of BMS-790052 on stable replication of a HCV replicon derived from genotype 1b. Huh7 cells harboring replicating subgenomic replicons derived from genotype 1b were treated with 0, 0.1, 1, 10, 100, or 1000 pM of BMS-790052 for 3 days. A total cell lysate from cells was prepared and an equal amount of each cell lysate was separated by SDS–PAGE. Levels of NS5A and β-actin protein expression were examined by Western blot analysis using monoclonal anti-NS5A and anti-β-actin antibodies. (C) Huh7.5 cells were infected with a vaccinia virus expressing a T7 RNA polymerase and then transfected with either Bart79I or Bartman (Con1) plasmid construct containing the NS viral proteins downstream of a T7 promoter. Transfected cells were incubated with 0.1% of DMSO or 0.01, 0.1, 1, 10, or 100 nM of BMS-790052 for 9 h. A total cell lysate was prepared and an equal amount of each cell lysate was separated by SDS–PAGE. Levels of NS5A protein expression were examined by Western blot analysis using a monoclonal anti-NS5A antibody. (D) All procedures were performed as in (C) except that levels of NS3 protein expression were examined by Western blot analysis using a monoclonal anti-NS3 antibody.