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Development and characterization of a replicon-based phenotypic assay for assessing HCV NS4B from clinical isolates

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ABSTRACT

The hepatitis C virus (HCV) NS4B inhibitors have shown potent inhibition of HCV replication in vitro. To assess the effect of viral diversity on the susceptibility to NS4B inhibitors, genotype (GT)-specific GT1a and GT1b replicon shuttle vectors were designed and created for cloning HCV NS4B genes from clinical isolates. For the GT1b NS4B shuttle vector, the S2204I adaptive mutation was introduced in NS5A to improve replication due to the replacement of the K1846T adaptive mutation in NS4B with NS4B from the clinical isolates. In addition to the adaptive mutations, a newly identified Huh-7 cell line, Huh-7-1C, which is highly permissive for both GT1a and GT1b replication, was used to further enhance the replication levels. HCV NS4B gene from clinical isolates was amplified and inserted into the corresponding GT1a and GT1b modified lab strain chimeric replicons. GT1a and GT1b chimeric replicons expressing diverse NS4B genes from corresponding subtypes of clinical isolates replicated at highly efficient levels for phenotypic analysis. Due to natural variation in their amino acid residues in NS4B, these isolates displayed varying drug susceptibilities to an NS4B inhibitor. In mixed populations with wild-type, the sensitivity of resistance detection of NS4B resistant mutants H94R and V105M was between 20% and 80%. The chimeric shuttle vectors can be used to characterize the activity of antiviral drugs targeting NS4B from diverse natural clinical isolates and aid in the development of novel compounds against HCV NS4B.

1. Introduction

Hepatitis C virus (HCV) is among the leading causes of persistent chronic liver disease and liver transplants worldwide. The recent approval of two direct antiviral agents (DAA), the HCV NS3 protease inhibitors telaprevir and boceprevir, for treatment of HCV in combination with peglyated interferon and ribavirin, has improved the sustained virologic response (SVR) rates to 60–70% vs. 40–50% SVR rates in pegylated interferon (peginterferon alfa) and ribavirin treated patients with HCV genotype 1 (Bacon et al., 2011; Jacobson et al., 2011; Sherman et al., 2011). As more DAA combinations are being tested in clinical trials to further improve clinical outcome, overcoming viral resistance remains a challenge. HCV, an RNA virus, has an error-prone polymerase which causes accumulation of nucleotide substitutions within the genomes during replication, resulting in a highly diverse population of quasispecies in infected individuals (Chayama and Hayes, 2011). Among patient quasispecies, preexisting drug-resistant variants are present as minor populations, but can quickly become dominant during antiviral treatment, resulting in treatment failure (Farci, 2011). Polymorphisms, which represent natural variations in HCV sequence, can be associated with resistance and can lead to reduced viral response to antiviral treatment. Polymorphisms affecting drug response have been identified in the NS3 protease (Ferreon et al., 2005; Zhao et al., 2012) and NS5A proteins of HCV (El-Shamy et al., 2008).

The most advanced HCV targets have been the NS3/4A serine protease and NS5B RNA polymerase, with recent advances made to target the non-enzymatic NS5A protein (Park et al., 2009). Another protein of interest is the HCV NS4B. NS4B is a hydrophobic transmembrane protein localized in the endoplasmic reticulum (ER) (Aizaki et al., 2004). It is a key enzyme required for RNA replication and for the assembly of other non-structural proteins in membrane-associated replication complexes (Blight, 2011; Paul et al., 2011; Einav et al., 2004; Gouttenoire et al., 2010). Disrupting NS4B function represents an attractive anti-HCV strategy (Zheng et al., 2005). Biochemical assays and in vitro replicon resistance assays have identified compounds from a pyrazolopyrimidine series that specifically target NS4B as well as key resistance mutations in NS4B, such as H94R and V105M/L that confer resistance to these NS4B inhibitors (Bryson et al., 2010). The residue 94 in NS4B is highly polymorphic, amino acid variations such as histadine (H), serine (S) and asparagine (N) have been observed in clinical isolates (EU HCV database). The frequency of naturally occurring H94R across genotypes is rare. The residue 105 in NS4B is mostly conserved with valine (V). Leucine (L) at 105 has been observed in small percent of clinical isolates (EU HCV database). Other

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antiviral agents effective in disrupting NS4B are amiloride analogues, clemizole hydrochloride, and clemizole-related indazole series (Dvory-Sobol et al., 2010; Rai and Deval, 2011).

HCV subgenomic replicons provide a valuable tool in measuring antiviral effects on replication and compound susceptibility, however, only a few replicating GT1a, GT1b, and GT2a reference lab strains are available. The spectrum of antiviral activity of inhibitors can be better understood by measuring activity against naturally occurring HCV clinical isolates using a phenotypic assay. Phenotypic assays for the NS3 protease, NS5A, and NS5B genes from HCV isolates have been developed by modifying existing replicon strains. However, NS4B phenotypic assays have not been described and are needed for evaluation of NS4B inhibitors.

It has been demonstrated that replication of the GT1a H77 strain of HCV is attenuated compared to the GT1b Con1 strain in both Huh-7 Lunet cells and Huh-7.5 cells (Blight et al., 2003). Consequently, GT1b Con-1 or 1b-N replicons as backbones were used for the replicon-based phenotypic assays for GT1a and GT1b NS3 protease, NS5A, and NS5B genes (Middleton et al., 2007; Qi et al., 2009; Tripathi et al., 2007; Le Pogam et al., 2008). In these phenotypic assays, chimeric GT1b replicons carrying NS3 protease, NS5A, or NS5B from both GT1a and GT1b produced robust replication for drug susceptibility testing. However, this strategy will likely present a challenge for a GT1a NS4B phenotypic assay, as a lethal phenotype in transient replication assays was observed for a GT1b Con1 chimeric replicon containing the NS4B gene from H77 in place of the Con1 NS4B sequence (Paredes and Blight, 2008). To overcome these issues, we have developed subtype specific replicon shuttle vectors for cloning NS4B genes from quasispecies pools of clinical isolates and have used it to investigate how the genetic heterogeneity of NS4B affects HCV replication and determine the sensitivity of patient derived NS4B clinical isolates to HCV inhibitors using a replicon-based transient system.

2. Methods and materials

2.1. Compounds

The imidazo [1,2-a] pyridine NS4B inhibitor GS-546288 and the protease inhibitor GS-9451 used as a control were made by Gilead Sciences. 10 mM stocks were prepared and stored at −20 °C. All compounds were solubilized in DMSO.

2.2. Clinical isolates

Twenty-three HCV NS4B clinical isolates (twelve GT1a and eleven GT1b) were obtained from serum samples of untreated HCV infected individuals.

2.3. Cell lines

Huh-7-1C cell line is a clone of Huh7-Lunet, which was isolated and identified by Gilead from drug resistance replicons cured with multiple, distinct classes of HCV inhibitors (Butkiewicz et al., 2000). The transfected replicon cells were plated in Dulbeccos's modified Eagle's medium (DMEM) with GlutaMAX-I (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT), 1 U/mL penicillin (Invitrogen), 1 μg/mL streptomycin (Invitrogen), and 0.1 mM nonessential amino acids (Invitrogen).

2.4. Design and construction of subtype specific NS4B shuttle vectors

The parental replicon vector genotype GT1b Con1, designated as GT1b PiRluc, was used for the construction of the GT1b NS4B shuttle vector as depicted in Fig. 1A (Friebe et al., 2001). GT1b PiRluc has three adaptive mutations, two in NS3 (E1202G + T1280I) and one in NS4B (K1846T). The parental replicon vector GT1a H77, designated as GT1a PiRluc, was used for the construction of the GT1a NS4B shuttle vector. GT1a PiRluc has five adaptive mutations, two in the NS3 (Q1067R + V1655I), one in the NS4A (K1691R), two in the NS5A (K2040R + S2204I). The firefly luciferase reporter in these vectors was replaced with renilla luciferase, which results in higher luciferase reading.

To create the GT1b Con1 NS4B shuttle vector, three unique restriction sites (XbaI, BsrGI and ClaI) were introduced into the parental GT1b PiRluc vector. XbaI was introduced in NS3 helicase, 3 amino acid upstream from the NS3–NS4A junction. BsrGI was introduced in NS5A, 11 amino acid downstream from the NS4B–NS5A junction. Both the restriction sites did not change amino acid sequence. ClaI was introduced at the 3′ end of NS4A, seven amino acids upstream from the NS4A-4B junction and caused a conservative amino acid from phenylalanine (Phe) to isoleucine (Ile). The pre-existing BsrGI site in NS3 at position 3019, was removed prior to addition of BsrGI in NS5A. To generate a replication-defective shuttle vector, another XbaI site was introduced at position 5363

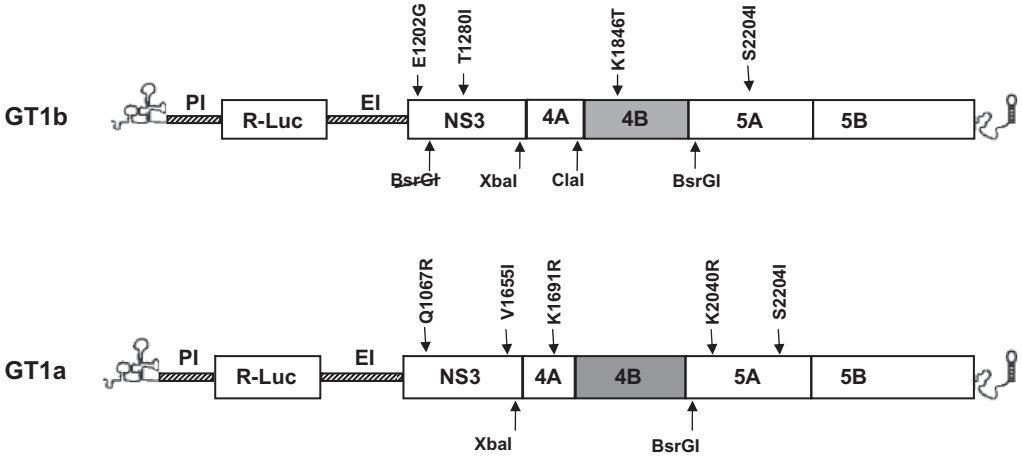

Fig. 1A. HCV replicons from reference strains genotype 1b Con1 or genotype 1a H77 were modified to create NS4B shuttle vector. Cloning sites and adaptive mutations are indicated.