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Elimination of HCV via a non-ISG-mediated mechanism by vaniprevir and BMS-788329 combination therapy in human hepatocyte chimeric mice

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ABSTRACT

We previously reported that interferon (IFN)-free direct-acting antiviral combination treatment succeeded in eradicating genotype 1b hepatitis C virus (HCV) in human hepatocyte chimeric mice. In this study, we examined the effect of vaniprevir (MK7009, NS3/4A protease inhibitor) and BMS-788329 (NS5A inhibitor) combination treatment on HCV genotype 1b and the expression of IFN-stimulated genes (ISGs) using a subgenomic replicon system and the same animal model. Combination treatment with vaniprevir and BMS-788329 significantly reduced HCV replication compared to vaniprevir monotherapy in HCV replicon cells (Huh7/Rep-Feo cells). HCV genotype 1b-infected human hepatocyte chimeric mice were treated with vaniprevir alone or in combination with BMS-788329 for four weeks. Vaniprevir monotherapy reduced serum HCV RNA titers in mice, but viral breakthrough was observed in mice with high HCV titers. Ultra-deep sequence analysis revealed a predominant replacement by drug-resistant substitutions at 168 in HCV NS3 region in these mice. Conversely, in mice with low HCV titers, HCV was eradicated by vaniprevir monotherapy without viral breakthrough. In contrast to monotherapy, combination treatment with vaniprevir and BMS-788329 succeeded in completely eradicating HCV regardless of serum viral titer. IFN-alpha treatment significantly increased ISG expression; however, vaniprevir and BMS-788329 combination treatment caused no increase in ISG expression both in cultured cells and in mouse livers. Therefore, combination treatment with vaniprevir and BMS-788329 eliminated HCV via a non-ISG-mediated mechanism. This oral treatment might offer an alternative DAA combination therapy for patients with chronic hepatitis C.

1. Introduction

Hepatitis C virus (HCV) infection is a major cause of chronic liver diseases, such as cirrhosis and hepatocellular carcinoma (Kiyosawa et al., 1990; Niederau et al., 1998). The goals of antiviral treatment in HCV-infected patients are to eradicate the virus and to decrease disease-related hepatic mortality. Recently, a number of new direct-acting antivirals (DAA) that selectively inhibit HCV proteins such as nonstructural protein (NS) 3/4A protease and NS5A and NS5B polymerase activity are approved in many part of the world.

Combinations of two DAAs may overcome interferon (IFN) nonresponsiveness in null responders by increasing antiviral activity and reducing the risk of developing resistance-associated variants (Soriano and Gallego, 2013). Combination therapy of the NS5A inhibitor daclatasvir (DCV) and the NS3/4A protease inhibitor asunaprevir (ASV), the only DAA combination therapy approved for genotype 1HCV-infected patients in Japan, resulted in a sustained virological response (SVR) rate of 87% (Kumada et al., 2014).

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Moreover, 8 weeks of combination therapy with nucleotide polymerase inhibitor sofosbuvir and the NS5A inhibitor ledipasvir in treatment-naïve genotype 1 patients was sufficient to achieve an SVR rate of 99% (Afdhal et al., 2014; Mizokami et al., 2015).

The immunodeficient urokinase-type plasminogen activator (uPA) mouse enables mouse livers to be repopulated with human hepatocytes that can then be infected with HCV (Mercer et al., 2001). This animal model is useful for evaluating anti-HCV drugs such as PEG-IFN and DAAs (Hiraga et al., 2011; Kneteman et al., 2006; Ohara et al., 2011; Shi et al., 2013). Using this animal model, we recently described the successful elimination of HCV genotype 1b by treatment with the combination of BMS-788329, a close analog of DCV, and the NS3/4A protease inhibitor BMS-605339 (Ohara et al., 2011; Shi et al., 2013).

Vaniprevir (MK-7009) is a macrocyclic HCV NS3/4A protease inhibitor with potent antiviral efficacy. A clinical study showed that 48 weeks of vaniprevir plus PEG-IFN and RBV combination treatment led to SVR in 82% of patients with genotype-1HCV infection (Lawitz et al., 2013; Liverton et al., 2010; Manns et al., 2012; Olsen et al., 2011).

In the present study we investigated the anti-HCV effects of vaniprevir and BMS-788329 and examined intrahepatic expression of IFN-stimulated genes (ISGs) using uPA/SCID mice transplanted with human hepatocytes.

2. Materials and methods

2.1. Cell culture

Cells supporting replication of the genotype 1b-derived subgenomic HCV replicon, Huh7/Rep-Feo cells (Ohara et al., 2011), were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Invitrogen Life Technology, Carlsbad, CA) containing 10% fetal bovine serum in the presence of G418 (400 μg/ml; Geneticin, Invitrogen, Carlsbad, CA). Cells were seeded onto 24-well plates and incubated for 48 h with or without 1.5 nM of either vaniprevir alone or in combination with 0.01 nM of BMS-788329. Cells were treated with 5 IU/ml of IFN-alpha (Dainippon Sumitomo Pharma Co., Tokyo, Japan) for comparison.

2.2. Luciferase assay

After 48 h of treatment with vaniprevir, BMS-788329 or IFN, HCV RNA replication level was quantified by internal luciferase assay. Luciferase activities were quantified using a luminometer (Lumat LB9501; Promega, Madison, WI) and the Bright-Glo Luciferase Assay System (Promega).

2.3. WST assay

Cell viability was determined using Cell counting kit-8 (Dojindo Laboratories., Kumamoto, Japan), according to the instructions provided by the manufacturer.

2.4. Quantitation of HCV RNA and ISG mRNA

RNA was extracted from cellular lysate, mouse serum and liver samples by SepaGene RV-R (EIDIA Co., LTD., Tokyo, Japan), and dissolved in 8.8 μl RNase-free water. Extracted RNA was reverse transcribed using random primer (Takara Bio Inc., Shiga, Japan) and M-MLV reverse transcriptase (ReverTra Ace, TOYOBO Co., LTD., Osaka, Japan) in 20 μl reaction mixture according to the instructions provided by the manufacturer. Nested polymerase chain reaction (PCR) and HCV quantitation by Light Cycler (Roche Diagnostics K.K., Tokyo, Japan) were performed as previously described (Ohara et al., 2011; Shi et al., 2013). Quantitation of human ISGs (Myxovirus resistance protein A [MxA], (2′-5′ oligoadenylate synthetase [OAS] and RNA-dependent protein kinase [PKR]) was performed using TaqMan® Gene Expression Master Mix (Applied Biosystems, Foster City, CA, USA) and TaqMan Gene Expression Assay primer and probe sets (PE Applied Biosystems, Foster City, CA) as previously reported (Abe et al., 2011). ISG mRNA expression levels were expressed relative to the endogenous RNA levels of the human housekeeping reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.5. Animal treatment

Generation of the $uPA^{+/+}/SCID^{+/+}$ mice and transplantation of human hepatocytes was performed as described previously (Tateno et al., 2004). All mice were transplanted with frozen human hepatocytes obtained from the same donor. All animal protocols described in this study were performed in accordance with the guidelines of the local committee for animal experiments, and all animals received humane care. Infection, extraction of serum samples and sacrifice were performed under anesthesia. Mouse serum concentration of HSA, which is correlated with the repopulation index (Tateno et al., 2004), was measured as described previously (Tsuge et al., 2005). Eight weeks after hepatocyte transplantation, mice were injected intravenously with 50 μl of HCV-positive human serum samples. Mouse serum samples were obtained at one or two week intervals after HCV infection and HCV RNA levels were measured.

2.6. Human serum samples

Human serum containing a high titer of genotype 1b HCV (2.2×10^6 copies/ml) was obtained from a patient with chronic hepatitis who had given written informed consent to participate in the study. Serum samples were divided into small aliquots and stored in liquid nitrogen until use. The study protocol conforms to the ethical guidelines of the Declaration of Helsinki and was approved a prior by the institutional review committee.

2.7. Treatment of HCV-infected mice with anti-HCV inhibitors

Eight weeks after HCV infection when the mice developed stable viremia, mice were twice daily perorally administered 0, 50 or 100 mg/kg body weight of vaniprevir (MK-7009; Merck Sharp & Dohme Corp., NJ). Mice receiving combination therapy were administered 100 mg/kg body weight of vaniprevir and 20 mg/kg body weight of BMS-788329 (Bristol-Meyers Squibb, NY) once per day for four weeks. Mice were sacrificed four hours after the last administration of combination treatment at either 4 (the end of treatment) or 15 weeks (11 weeks after cessation of the treatment), and serum HCV RNA titers and ISG expression in livers were analyzed. To analyze the expression of intrahepatic ISG in IFN-treated mice, mice were treated with 1000 IU/g/day of IFN-alpha for 4 weeks, then, sacrificed four hours after the last IFN injection.

2.8. Ultra-deep sequence analysis of NS3 aa168 substitutions

The frequencies of NS3 aa168 was determined by ultra-deep sequencing. The NS3 region in the HCV genome was amplified using NS3-V3717F (5′-GGC AGC TCG GAC CTT TAC TTG GT-3′; nucleotides 3717-3739) and NS3-3950R (5′-AGT TTC CAT AGA CTC AAC GGG-3′; nucleotides 3950-3930). The PCR products were purified using AMPure XP beads (Beckman Coulter, Brea, CA), and its distributions were measured using Agilent Bioanalyzer 2100 Plat form. An Adaptor-ligated library was prepared using an Ion Xpress Plus Fragment Library Kit (Life technologies, Carlsbad, CA) and purified subsequently. Sequence analysis was performed using Ion PGM™