JTK-853, a Novel Non-Nucleoside Hepatitis C Virus Polymerase Inhibitor, Demonstrates a High Genetic Barrier to Resistance in vitro

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Key Words

Hepatitis C virus · HCV polymerase inhibitor · Resistance

Abstract

JTK-853 is a novel, non-nucleoside, palm site-binding hepatitis C virus (HCV) polymerase inhibitor that has demonstrated antiviral activity in HCV-infected patients during 3 days of treatment. To estimate the genetic barrier of JTK-853 to resistance in vitro, colony formation assays were conducted using HCV replicon cells (genotypes 1a and 1b). The colony formation assays revealed that the numbers of resistant colonies for JTK-853 were much lower than those for other direct-acting antivirals, including palm site- or thumb pocket-binding non-nucleoside HCV polymerase inhibitors (NNIs), an NS5A inhibitor (NS5Ai), and a protease inhibitor (PI). Furthermore, the numbers of resistant colonies for JTK-853 in combination with the NS5Ai or PI were lower than those for other combinations of NS5Ai + NNI, and NS5Ai + PI. Our findings demonstrate that JTK-853 has a high genetic barrier to resistance, and suggest that its combination therapies will be potent in suppressing the emergence of drug resistance in HCV-infected patients.

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Introduction

Hepatitis C virus (HCV) is a global health concern. Approximately 130 million individuals worldwide are estimated to be infected with HCV [1]. It has been suggested that over 25% of HCV-infected patients develop liver cirrhosis and hepatocellular carcinoma as consequences of chronic infection with HCV [2, 3]. The current standard of care involving treatment with pegylated interferon and ribavirin is not only unsatisfactory for its effectiveness but also unavoidably causes severe side effects. To overcome these problems, many direct-acting antivirals (DAAs) are in clinical trials and some of them (telaprevir and boceprevir) were launched for HCV treatment in 2011. However, when DAAs are used as monotherapies, the emergence of drug-resistant HCV variants is inevitable for DAA-based therapies, and these variants cause viral breakthroughs [4]. Therefore, new DAAs that show a high barrier to resistance are strongly needed for HCV treatment. In some current clinical trials, nucleoside HCV polymerase inhibitors (NIs) appear to show a high barrier to resistance compared with other classes of DAAs [5–7].

The HCV replicon system [8] is useful for drug development, and is widely used for the selection of resistant mutants. In general, when HCV replicon cells are treated with DAAs for over 2 weeks in the presence of Geneticin, DAA-resistant HCV replicon colonies emerge on the surface of the tissue culture plates. Hence, the number of DAA-resistant HCV replicon colonies indicates the genetic barrier to resistance. Therefore, colony formation assays are considered to be useful tools for assessing the genetic barrier of DAAs to resistance [9].

JTK-853 is a novel, non-nucleoside, palm site-binding HCV polymerase inhibitor that has demonstrated effective antiviral activity in HCV replicon cells with EC₅₀ values of 0.38 and 0.035 μ M in genotype 1a H77 and 1b Con1 strains, respectively [10]. Furthermore, JTK-853 showed a viral reduction of more than 1 log in HCV-infected patients during 3 days of treatment [11].

The aim of this in vitro study was to evaluate the emergence of JTK-853 resistance in HCV replicon cells. To achieve this, we conducted colony formation assays of JTK-853 alone or in combination with other DAAs, and compared the genetic barrier to resistance with those of other DAAs, such as other non-nucleoside HCV polymerase inhibitors (NNIs), an NI, an NS5A inhibitor (NS5Ai), and a protease inhibitor (PI).

Materials and Methods

Compounds and Reagents

JTK-853 [10, 12] was synthesized at Japan Tobacco Inc., Central Pharmaceutical Research Institute (Osaka, Japan). GS-9190 (palm site NNI) [13], PF-868554 (thumb pocket NNI) [14], PSI-6130 (NI) [15], BMS-790052 (NS5Ai) [16], and TMC435 (PI) [17] were prepared at Japan Tobacco Inc. according to the published protocols. The thumb pocket NNI-A (indole-derivative NNI) [18, 19] was identified at Japan Tobacco Inc. and described elsewhere. VX-222 (thumb pocket NNI) [20, 21] was purchased from Selleck (Houston, Tex., USA). Human serum (HS) was purchased from Gemini Bio-Products (West Sacramento, Calif., USA).

Cells

Huh-7.5 cells [22] were propagated in high-glucose Dulbecco's modified Eagle's medium (Nikken BioMedical Laboratory, Kyoto, Japan) containing 10% fetal bovine serum (Moregate Biotech, Bulimba, Australia), 0.1 mM nonessential amino acids (Invitrogen, Carlsbad, Calif., USA), 100 U/ml penicillin (Invitrogen), 100 μ g/ml streptomycin sulfate (Invitrogen), and 2 mM L-glutamine (Invitrogen).

HCV subgenomic replicon-harboring cells of genotype 1b Con1 and genotype 1a H77 strains [8, 22–24] were used to determine the antiviral activity of JTK-853. The HCV subgenome consisting of NS3 to the 3' untranslated region of the HCV RNA genome was fused with or without luciferase as a reporter gene. All

of the replicon cells harbor a selectable marker, neomycin phosphotransferase, that confers Geneticin resistance. The replicon cells were propagated in a medium containing 0.5–1 mg/ml Geneticin (Invitrogen).

Colony Formation Assay

The HCV subgenomic replicon RNAs (4 μ g) of genotype 1a H77 and 1b Con1 strains [8, 22] were individually transfected into Huh-7.5 cells (4×10^6) using a GenePulser (Bio-Rad, Hercules, Calif.) at 960 μ F and 270 V. The cells were then seeded in 6-well plates at a density of 1×10^5 cells/well. Four hours after transfection, the culture medium was removed and replaced with medium containing JTK-853 or other DAAs [prepared in dimethyl sulfoxide (DMSO) and added to a final concentration of 0.1% (v/v) DMSO], 600 μ g/ml Geneticin, and 40% HS. For treatment with JTK-853 or DAA alone, each compound was added at $1 \times$ or $1/3 \times 90\%$ effective concentration (EC₉₀) in the presence of 40% HS [4]. For treatment with JTK-853 in combination with another DAA, the compounds were added at $1/4 \times$ EC₉₀ in the presence of 40% HS. The DAA-containing medium was replaced twice weekly. After treatment of the cells for 2–3 weeks, the colonies that emerged in the presence of the DAAs were visualized with crystal violet [1% (v/v) in methanol]. The lysis buffer was prepared as 1:1 mixture of ethanol and the Buffer RLT included in RNeasy RNA extraction kit (Qiagen, Venlo, Netherlands) for measurement of colony number. The colonies were lysed by the lysis buffer, and the resistant colonies were quantified as a measurement of optical density (OD) 595 nm. For the determination of cytotoxicity of JTK-853 or other DAAs, the Huh-7.5 cells were treated with JTK-853 or other DAAs for 2 weeks. The thumb pocket NNI-B and NS5Ai were added at 100 μ M and 100 nM, respectively. JTK-853 and other DAAs were added at 10 μ M. JTK-853- or DAA-containing medium was changed twice a week. Two weeks after the culture, the cells were stained with crystal violet [1% (v/v) in methanol], and then lysed by the lysis buffer. The cytotoxicity was determined as a measurement of OD 595 nm of the cell lysates.

Sequence Analysis

Total RNA was extracted from the JTK-853- and DAA-resistant colonies using an RNeasy RNA Extraction Kit (Qiagen) and the HCV replicon cDNA was reverse-transcribed with a SuperScript III First-Strand Synthesis System (Invitrogen). The NS5B, NS5A, and NS3 genes were amplified by PCR with TaKaRa LA Taq or PrimeSTAR MAX (TaKaRa, Ohtsu, Japan). The primers used for the reverse transcription were 5'-AGAGCCCGAGT-GTTTACCC-3' for genotype 1a H77 and 5'-TGGAGTGTT-TAGCTCCCCGT-3' for genotype 1b Con1. The primers used for PCR of H77 were as follows: NS5B, 5'-GGCCGACACGGAA-GATG-3' (forward) and 5'-AGAGCCCGAGTGTTTACCC-3' (reverse); NS5A, 5'-CCTTTGAAAAACACGATAATACC-3' (forward) and 5'-TGACGGCAGCTGTAAAAGCC-3' (reverse), and NS3, 5'-GGCAGTGCAATGGATGAAC-3' (forward) and 5'-CTTCCTCTACGGATAGCAAG-3' (reverse). The primers used for PCR of Con1 were as follows: NS5B, 5'-GTGAGGAC-GTCGTCTGCTGC-3' (forward) and 5'-TGGAGTGTTTAGCT CCCCCGT-3' (reverse); NS5A, 5'-CCTTTGAAAAACACGATAA TACC-3' (forward) and 5'-CCAGGATGTTAAACAGGAGGG-3' (reverse), and NS3: 5'-CCCTCCTGTTTAAACATCCTGG-3' (forward) and 5'-TTCGCCTTCATCTCCTTAG-3' (reverse).

Table 1. In vitro antiviral activity of JTK-853 in the presence of 40% HS

Direct-acting antivirals	EC ₉₀	
	1a (H77)	1b (Con1)
JTK-853, μM	6.5 $\pm$ 0.5	0.34 $\pm$ 0.05
GS-9190, μM	2.4 $\pm$ 0.5	0.30 $\pm$ 0.04
NNI-A, μM	0.22 $\pm$ 0.04	0.42 $\pm$ 0.01
PF-868554, μM	15 $\pm$ 4	6.4 $\pm$ 0.5
VX-222, μM	0.59 $\pm$ 0.05	0.31 $\pm$ 0.03
PSI-6130, μM	6.3 $\pm$ 0.3	6.8 $\pm$ 0.4
BMS-790052, nM	1.5 $\pm$ 0.2	0.45 $\pm$ 0.03
TMC435, μM	0.42 $\pm$ 0.04	0.17 $\pm$ 0.03

Data represent mean $\pm$ standard error from four independent experiments.

Results

Antiviral Activity of JTK-853 in vitro in the Presence of 40% HS

Prior to performing the colony formation assays, we determined the antiviral activity of JTK-853 in the presence of 40% HS. This supplementation of 40% HS in the culture is thought to be almost equivalent to the physiological concentrations in human blood [10]. When JTK-853 was incubated with the replicon cells for 48 h, it showed antiviral activity against genotype 1a H77 and 1b Con1 replicon cells with EC₉₀ values of 6.5 $\pm$ 0.5 and 0.34 $\pm$ 0.05 μM (mean $\pm$ standard error), respectively (table 1). The EC₉₀ values of the other DAAs are also shown in table 1.

Colony Formation Assays for JTK-853 in a Single Treatment

Prior to performing the colony formation assay to demonstrate that JTK-853 and DAAs did not show cytotoxicity, the compounds were added to the parental Huh-7.5 cells without Geneticin for 2 weeks. The thumb pocket NNI-B and NS5Ai were added at 100 μM and 100 nM, respectively. The JTK-853 and other DAAs were added at 10 μM . The results revealed no Huh-7.5 cell death was apparent at these concentrations in 2-week culture (fig. 1e). Therefore, the cell death observed in replicon cells in the presence of the DAAs with Geneticin would result from antiviral activity of the DAAs and not through cytotoxicity. Then, we conducted the colony formation assay to evaluate the emergence of drug resistance.

We investigated the genetic barrier of JTK-853 to resistance in HCV replicons of genotype 1a H77 and 1b

Con1 strains. To avoid the influence of preexisting drug-resistant variants, the DAAs were applied for treatment at just 4 h after transfection of the HCV replicon RNAs into Huh-7.5 cells. The cells were treated with the palm site NNIs JTK-853 and GS-9190 at 1 $\times$ EC₉₀ (H77) or 1/3 $\times$ EC₉₀ (Con1) in the presence of 40% HS and 600 $\mu\text{g}/\text{ml}$ Geneticin. After 14–17 days of culture, the JTK-853- and GS-9190-resistant colonies were visualized by crystal violet staining. As shown in figure 1a, JTK-853 suppressed the drug-resistant colony formation in the genotype 1a replicon cells, and the numbers of JTK-853-resistant colonies were much lower than those of GS-9190-resistant colonies for both genotypes. The dyed DAA-resistant colonies with crystal violet were lysed with the lysis buffer, and the OD value of the lysate was determined in order to quantify the emergence of the DAA-resistant colonies. The OD value of JTK-853-resistant colonies was 2.8% of the DMSO control in the genotype 1a replicon cells, while that of GS-9190-resistant colonies was 71% as shown in figure 1a and table 2. In a similar fashion, JTK-853 suppressed the drug-resistant colony formation in the genotype 1b replicon cells as shown in figure 1b and table 2. Sequence analyses revealed an amino acid substitution of L466V/I in NS5B in the JTK-853-resistant colonies, while Y448H in NS5B was detected in the GS-9190-resistant colonies. We previously demonstrated that L466V confers resistance to JTK-853 with more than 20-fold reduced susceptibility in vitro [10], which is almost consistent with the colony formation assay results. On the other hand, Y448H is known to be a resistance mutation for GS-9190 [13, 25]. Without Geneticin selection, no cell death was apparent after treatment with JTK-853 and the DAAs, suggesting that the colonies consisted of cells harboring drug-resistant replicons. These findings demonstrate that JTK-853 shows a higher genetic barrier to resistance than the other palm site-binding NNI GS-9190 in genotypes 1a and 1b HCV.

We also compared the genetic barrier of JTK-853 with those of other classes of DAAs using colony formation assays. After transfection of replicon RNA, the cells were treated with JTK-853, NNI-A, PF-868554, VX-222, PSI-6130, BMS-790052, or TMC435 at 1/3 $\times$ EC₉₀ for 13–17 days. The DAA-resistant colonies for genotype 1a H77 are shown in figure 1c and table 2. The OD value of JTK-853-resistant colonies was 0.2 and 2.4% of the control, which was much lower than those of the other classes of NNIs, i.e. NNI-A, PF-868554, and VX-222. JTK-853 also showed a reduced number of resistant colonies compared with the NS5Ai BMS-790052, while the numbers of JTK-

Fig. 1. Resistance barriers of JTK-853 for HCV genotype 1a H77 and 1b Con1 in a single treatment. The JTK-853- or DAA-containing medium was changed twice a week. **a** Genetic barriers of JTK-853 and GS-9190 for H77. JTK-853 and GS-9190 were treated at $1 \times EC_{90}$ for 17 days. **b** Genetic barriers of JTK-853 and GS-9190 for Con1. JTK-853 and GS-9190 were treated at $1/3 \times EC_{90}$ for 14 days. **c, d** Genetic barriers of JTK-853, NNI-A, PF-868554, VX-222, PSI-6130, BMS-790052, and TMC435 for Con1. JTK-853 and the other DAAs were treated at $1/3 \times EC_{90}$ for 17 days. Representative images from two experiments are shown.

853-resistant colonies were as small as those for the NI PSI-6130 and PI TMC435. Sequence analyses revealed an emergence of amino acid substitution of M414T in NS5B of JTK-853-resistant colonies, while A499T, I482V, and L419M in NS5B of NNI-A-, PF-868554-, and VX-222-resistant colonies, respectively. In addition, the amino acid substitutions of P32S in NS5A in BMS-790052-resistant colonies and

V36A in NS3 in TMC435-resistant colonies were identified, respectively. These mutations are known to be resistance mutations to the corresponding DAAs [10, 13, 14, 16, 21, 26, 27]. These findings demonstrate that JTK-853 shows a higher genetic barrier to resistance than the thumb pocket-binding NNIs and NS5Ai, and shows a high genetic barrier that is as potent as those of the NI and PI.

Fig. 1. e The cytotoxicity of JTK-853 or other DAAs. The Huh-7.5 cells were treated with JTK-853 or other DAAs for 2 weeks. The thumb pocket NNI-B and NS5Ai were added at 100 μ M and 100 nM, respectively. JTK-853 and other DAAs were added at 10 μ M. The cells were dyed with crystal violet [1% (v/v) in methanol].

Table 2. In vitro barrier of JTK-853 to the resistance

Direct-acting antivirals	1a (H77)			1b (Con1)		
	concentrations ^a	OD 595 nm (% of DMSO control) ^b	amino acid substitutions ^c	concentrations ^a	OD 595 nm (% of DMSO control) ^b	resistant mutations ^c
JTK-853	1 $\times$ EC ₉₀	2.8	L466I	1/3 $\times$ EC ₉₀	7.6	L466V
GS-9190	1 $\times$ EC ₉₀	71	Y448H	1/3 $\times$ EC ₉₀	13	Y448H
JTK-853	1/3 $\times$ EC ₉₀	0.2	M414T	1/3 $\times$ EC ₉₀	2.4	M414L
NNI-A	1/3 $\times$ EC ₉₀	3.8	A499T	1/3 $\times$ EC ₉₀	1.1	— ^d
PF-868554	1/3 $\times$ EC ₉₀	85	I482V	1/3 $\times$ EC ₉₀	40	I482V
VX-222	1/3 $\times$ EC ₉₀	12	L419M	1/3 $\times$ EC ₉₀	17	— ^e
PSI-6130	1/3 $\times$ EC ₉₀	1.4	— ^d	1/3 $\times$ EC ₉₀	1.3	— ^d
BMS-790052	1/3 $\times$ EC ₉₀	7.3	P32S	1/3 $\times$ EC ₉₀	20	L31V
TMC435	1/3 $\times$ EC ₉₀	0.5	V36A	1/3 $\times$ EC ₉₀	14	D168E

^a Concentrations used for colony formation assay.

^b Data represent relative values of the drug-resistant colonies to DMSO control for colony formation assay.

^c Amino acid substitutions known to the resistant mutations corresponding to the DAAs.

^d Sequence analysis was not performed because of small number of colonies.

^e Amino acid substitutions known to the resistant mutations were not detected.

The DAA-resistant colonies for genotype 1b Con1 are shown in figure 1d and table 2. The number of JTK-853-resistant colonies was much lower than those of the other DAAs, such as PF-868554, VX-222, BMS-790052, and TMC435, but slightly higher than those of PSI-6130 and NNI-A. Amino acid substitutions of M414L in NS5B, I482V in NS5B, L31V in NS5A, and D168E in NS3

were identified in the JTK-853-, PF-868554-, BMS-790052-, and TMC435-resistant colonies, respectively. These mutations are known to confer resistance to the corresponding DAAs [10, 14, 16, 17]. These data demonstrate that JTK-853 also shows a higher genetic barrier to resistance than the DAAs for genotype 1b HCV.

Fig. 2. Resistance barriers of JTK-853 in combination with BMS-790052 or TMC435. HCV replicons of genotype 1a H77 strain were treated at $1/4 \times EC_{90}$ for 19 days. The JTK-853- or DAA-containing medium was changed twice a week. Representative images from two experiments are shown.

Thus, these findings demonstrate that JTK-853 also shows a higher genetic barrier to resistance than some different classes of DAAs for genotype 1a and 1b HCV.

Colony Formation Assays of JTK-853 in Combination with DAAs

Next, we conducted colony formation assays of JTK-853 in combination with DAAs for genotype 1a HCV. After transfection of the replicon RNA, the cells were treated with JTK-853 in combination with BMS-790052 or TMC435 at $1/4 \times EC_{90}$, respectively. As shown in figure 2, the numbers of resistant colonies after treatment with JTK-853 in combination with BMS-790052 or TMC435 were lower than those after treatment with JTK-853 alone. Moreover, the number of resistant colonies after treatment with JTK-853 in combination with BMS-790052 was lower than those after treatment with BMS-790052 in combination with GS-9190 or TMC435, while JTK-853 suppressed the emergence of drug-resistant colonies as potently as GS-9190 in combination with TMC435. Amino acid substitutions of Q41R in NS3 and M28V in NS5A were identified in the resistant colonies treated with TMC435 + BMS-790052 and GS-9190 + BMS-790052, respectively. Regarding the treatments with JTK-853 + BMS-790052, JTK-853 + TMC435, and GS-9190 + TMC435, sequence analyses could not be performed because of the very low numbers of resistant colonies. These findings demonstrate that JTK-853 shows

a higher genetic barrier to resistance than the other palm site NNI, GS-9190, even in combination with an NS5Ai (BMS-790052) or a PI (TMC435).

Discussion

In vitro selection studies and colony formation assays are thought to be useful for estimating resistance mutations and genetic barriers to resistance in clinical studies [16, 20, 28–30]. Actually, a high genetic barrier of an NI to resistance in vitro well reflects a low frequency of viral breakthrough or rebound during treatment of HCV-infected patients with the NI. On the other hand, NNI, NS5Ai, and PI showed low genetic barriers in both clinical and in vitro studies [30]. Therefore, the colony formation assay of JTK-853 is useful for estimating the genetic barrier of JTK-853 to resistance. Although NNIs are generally considered to show low genetic barriers to resistance, JTK-853 showed a high genetic barrier to resistance in vitro. The number of JTK-853-resistant colonies was much lower than those of the other NNIs. In addition, Y448H, a persistent mutation, was not observed in the JTK-853-resistant colonies. Therefore, JTK-853 appeared to show a high genetic barrier, which differs greatly from the other NNIs.

To identify more potent regimens for antiviral activity and higher genetic barriers, combinations of two or more DAAs have been evaluated in clinical studies [31–33].

Combination treatments showed more potent antiviral effects than single treatments, but it remains unclear which combinations are effective for high genetic barriers in clinical studies. In fact, treatment with BMS-790052 (NS5Ai) in combination with BMS-650032 (PI) in HCV-infected patients frequently showed viral rebound during the treatment [31]. Consistently, drug-resistant colonies emerged in combination treatments with BMS-790052 (NS5Ai) + GS-9190 (NNI) and BMS-790052 + TMC435 (PI) in our in vitro colony formation assays. Interestingly, JTK-853 in combination with BMS-790052 or TMC435 completely suppressed the emergence of drug-resistant colonies in our study, and a high genetic barrier of JTK-853 was more distinct in the combination treatments than in the single treatments. Taken together, JTK-853 will show a high genetic barrier in clinical studies, especially in combination treatments.

Recently, we reported the co-crystal structure of JTK-853 with HCV polymerase [10]. The binding mode of JTK-853 to HCV polymerase is characteristic and clearly

distinct from that of other palm site-binding HCV polymerase inhibitors in the aspect of binding to the β -hairpin region and palm I site of HCV polymerase [14, 18]. This unique binding mode of JTK-853 possibly leads to the high genetic barrier.

Collectively, this study has demonstrated that JTK-853 shows a higher genetic barrier to resistance than the other NNIs, PI, and NS5Ai examined. JTK-853 exhibits a promising profile with its unique ability to inhibit the emergence of viral resistance.

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