Ⅲ．　研究成果の刊行に関する一覧表

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書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
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Yasui F. et al. (小原)	Synthetic lipophilic antioxidant BO-653 suppresses HCV replication	***J.Med. Virol.***	85	241-249	2013
Watanabe T. et al. (小原)	Hepatitis C virus kinetics by administration of pegylated interferon-α in human and chimeric mice carrying human hepatocytes with variants of the IL28B gene	***Gut***	In press		2013
Kasama Y. et al. (小原)	Translocase of outer mitochondrial membrane 70 induces interferon response and is impaired by hepatitis C virus NS3	***Virus Res.***	163	405-409	2012
Saito M. et al. (小原)	Hepatitis C virus induces overexpression of 3β-hydroxysterol Δ24-reductase through Sp1	***J. Med. Virol.***	84	733-746	2012
Weng L. et al. (小原)	Detergent-induced activation of the hepatitis C virus genotype 1b RNA polymerase	***Gene***	496	79-84	2012
Konishi H. et al. (小原)	An orally available, small-molecule interferon inhibits hepatitis C virus replication	***Sci. Comm.***	2:259	1-9	2012

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Wang Q. et al. (小原)	PA from an H5N1 highly pathogenic avian influenza virus activates viral transcription and replication, and induces apoptosis and interferon expression	***Virology Journal***	8:9	106-118	2012
Hirata Y. et al. (小原)	Self-enhancement of Hepatitis C Virus Replication by Promotion of Specific Sphingolipid Biosynthesis	***PLoS Pathog.***	8(8)	e1002860	2012
Aoki J. et al. (小原)	Kinetics of Peripheral Hepatitis B Virus-specific CD8+ T Cells in Patients with Onset of Viral Reactivation	***J. Gastroenterology***	In press		2012
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Inoue K. et al. (小原)	Impairment of interferon regulatory factor-1 3 activation by hepatitis C virus core protein basic region 1	***Biochem Biophys Res Commun.***	428(4)	494-499	2012
Sekiguchi S., Ki Kimura K. et al. (小原)	Immunization with a recombinant vaccinia virus that encodes nonstructural proteins of the hepatitis C virus suppresses viral protein levels in mouse liver	***PLoS ONE***	7(12)	e51656	2012
Yoshhida, T. et al. (保富)	Dynamics of cellular immune responses in the acute phase of dengue virus infection.	***Archiv. Virol.***	In press		2013

Karamatsu K. et al. （保富）	Single systemic administration of Ag85B of mycobacteria DNA inhibits allergic airway inflammation in a mouse model of asthma.	***J Asthma Allergy***	5	71-79	2012
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Saito M.et al. (小原恭子)	Hepatitis C virus promotes expression of the 3β-hydroxysterol D24-reductase through Sp1.	***J Med Virol.***	84 (5)	733-46	2012
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Watanabe T,et al (村上)	Hepatitis C virus kinetics by administration of pegylated interferon-l in human and chimeric mice carrying human hepatocytes with variants of the IL28B gene.	***Gut***			2012 Nov 7. [Epub ahead of print]
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Ⅳ．研究成果の刊行物・別刷

Journal of Medical Virology 85:241–249 (2013)

Synthetic Lipophilic Antioxidant BO-653 Suppresses HCV Replication

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The influence of the intracellular redox state on the hepatitis C virus (HCV) life cycle is poorly understood. This study demonstrated the anti-HCV activity of 2,3-dihydro-5-hydroxy-2,2-dipentyl-4,6-di-*tert*-butylbenzofuran (BO-653), a synthetic lipophilic antioxidant, and examined whether BO-653's antioxidant activity is integral to its anti-HCV activity. The anti-HCV activity of BO-653 was investigated in HuH-7 cells bearing an HCV subgenomic replicon (FLR3-1 cells) and in HuH-7 cells infected persistently with HCV (RMT-tri cells). BO-653 inhibition of HCV replication was also compared with that of several hydrophilic and lipophilic antioxidants. BO-653 suppressed HCV replication in FLR3-1 and RMT-tri cells in a concentration-dependent manner. The lipophilic antioxidants had stronger anti-HCV activities than the hydrophilic antioxidants, and BO-653 displayed the strongest anti-HCV activity of all the antioxidants examined. Therefore, the anti-HCV activity of BO-653 was examined in chimeric mice harboring human hepatocytes infected with HCV. The combination treatment of BO-653 and polyethylene glycol-conjugated interferon-α (PEG-IFN) decreased serum HCV RNA titer more than that seen with PEG-IFN alone. These findings suggest that both the lipophilic property and the antioxidant activity of BO-653 play an important role in the inhibition of HCV replication. ***J. Med. Virol. 85:241–249, 2013.*** © 2012 Wiley Periodicals, Inc.

KEY WORDS: BO-653; antioxidant activity; chemical structure; HCV replication; chimeric mice

INTRODUCTION

Hepatitis C virus (HCV) causes persistent infection, leading to chronic liver diseases including chronic hepatitis, cirrhosis, and hepatocellular carcinoma. In 2009, the number of patients with HCV infection worldwide was estimated to be 130–170 million [Lavanchy, 2009]. Recent years have seen the development of several promising treatments for patients infected with HCV. The addition of a protease inhibitor (boceprevir or telaprevir) to polyethylene glycol-conjugated interferon-α (PEG-IFN) and ribavirin improved dramatically the sustained virological response rates in treatment-naive patients with genotype 1 infections. However, the sustained virological response rate of triple therapy with a telaprevir-based regimen in null responders treated with PEG-IFN/ribavirin is only 30% [Fontaine and Pol, 2011; Kumada et al., 2012]. There is concern that high-risk groups such as patients with the *IL28B* minor allele (re8099917 SNP; GT/GG), the elderly, or those with fibrosis will be resistant to the triple therapy [Suppiah et al., 2009; Tanaka et al., 2009]. Therefore, new therapeutic strategies are required to treat HCV infection.

Chronic HCV infection is closely associated with oxidative stress. Oxidative stress reflects an imbalance between the production of reactive oxygen species (ROS) and the activity of intracellular antioxidant systems. The cumulative evidence from experimental

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and clinical studies demonstrates that HCV infection causes excessive ROS production and decreased activity of antioxidant enzymes [Kato et al., 2001; Levent et al., 2006]. In addition, previous studies showed that aggravation of oxidative stress in hepatocytes infected with HCV is correlated with the iron overload, while phlebotomy improves oxidative stress markers and liver pathology [Seronello et al., 2007]. Therefore, oxidative stress is a deleterious factor involved in the development of various hepatic diseases ranging from chronic hepatitis to hepatocellular carcinoma. In contrast, the influence of the intracellular redox state on HCV replication is controversial. Exogenous addition of either hydrogen peroxide or unsaturated fatty acid has been shown to induce oxidative stress and inhibit HCV replication in cell culture models [Choi et al., 2004; Huang et al., 2007]. Yano et al. [2007] reported previously that any of several nutrients (including vitamin E, a hydrophobic antioxidant) enhance HCV RNA replication. In contrast, overproduction of the antioxidant enzyme heme oxygenase-1 decreases HCV RNA replication in both full-length and subgenomic replicons [Zhu et al., 2008]. Despite these in vitro results, there have been no reports on the effect of antioxidant or pro-oxidant reagents on the life cycle of HCV in any animal models, such as chimeric mice harboring human hepatocytes infected with HCV.

BO-653 (2,3-dihydro-5-hydroxy-2,2-dipentyl-4,6-di-*tert*-butylbenzofuran), a lipophilic (hydrophobic) antioxidant, was previously a clinical candidate for potential treatment of atherosclerosis and the prevention of post-angioplasty restenosis [Cynshi et al., 1998; Meng, 2003]. This compound is an effective inhibitor of lipid peroxidation and inhibits potently oxidation of lipids such as low-density lipoprotein [Noguchi et al., 1997; Tamura et al., 2003]. The present study examined the anti-HCV activity of BO-653 both in vitro and in vivo, and sought to clarify whether the antioxidant activity of the molecule was integral to the observed anti-HCV activity.

MATERIALS AND METHODS

Chemicals

BO-653 (molecular weight [MW], 388.6) was a gift of the Chugai Pharmaceutical company (Tokyo, Japan). Probucol [4,4′-(isopropylidenedithio)bis(2,6-di-*tert*-butylphenol)] was purchased from Wako Pure Chemical Industries (Osaka, Japan). *N*-acetyl cysteine and ascorbic acid (vitamin C) were obtained from Sigma–Aldrich (St. Louis, MO). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and D-α-tocopherol (vitamin E) were obtained from Calbiochem (San Diego, CA) and MP Biomedical LLC (Solon, OH), respectively.

Viruses and Cells

Patients provided written informed consent prior to blood sample collection.

HuH-7 cells harboring a HCV subgenomic replicon (FLR3-1 cells; genotype 1b, Con-1 strain) were maintained at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium GlutaMAX-I (DMEM-GlutaMax I; Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS) and 0.5 mg/ml G418 [Inoue et al., 2007].

HuH-7 cells infected persistently with HCV (RMT-tri cells; genotype 1a) were generated in the laboratory as described below and were maintained in DMEM containing 10% FCS, nonessential amino acids, 10 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], and 0.4% glucose. Complementary DNA (cDNA) of the full-genome HCV (nucleotides 1–9,598; GenBank accession number AB520610) was prepared from the serum of a patient with acute hepatitis infected with HCV genotype 1a [Inoue et al., 2007]. The resulting serum harbored HCV at a titer of $10^{8.6}$ copies/ml, as detected by a quantitative real-time polymerase chain reaction (qRT-PCR) as described previously [Takeuchi et al., 1999]. The sequence of the final cDNA construct was determined from a consensus of more than 10 clones, and was subcloned under the control of a T7 promoter (pHCV-RMT). The construct of HCV subgenomic replicon was generated from pHCV-RMT. HCV subgenomic RNA was transcribed using T7 RNA polymerase and the MEGAscript in vitro transcription kit (Ambion, Austin, TX) according to the manufacturer's instructions. The resulting synthetic RNA of the HCV subgenomic replicon was transfected into HuH-7 cells by electroporation. Following transfection, the HCV subgenomic replicon-bearing HuH-7 cells was established. Total RNA was extracted by the acid guanidinium–phenol–chloroform method from a sample of the HCV subgenomic replicon-bearing HuH-7 cells and reverse transcribed. Sequence of the resulting cDNA has three nonsynonymous substitutions compared to that of the original HCV subgenomic replicon. Next, three nonsynonymous substitutions were inserted into the original full-length HCV sequence to enhance the replication rate in HuH-7 cells. Full-length RNA was also transcribed as described above. The resulting synthetic RNA of full-length HCV was transfected into HuH-7 cells by electroporation. Following transfection, the HCV RNA level in the transfected cells was measured on a weekly basis, revealing persistent infection at a level of $10^{6.5}$–$10^{6.8}$ copies/μg total cellular RNA over the course of 50 days. Finally, the transfected cell line was designated as RMT-tri.

Analysis of Anti-HCV Effect of BO-653 in FLR3-1 Cells

The anti-HCV activity of BO-653 in FLR3-1 cells was measured by inhibiting luciferase activity [Inoue et al., 2007]. In brief, FLR3-1 cells were seeded at 4 × 10^3 cells/well in 96-well white plates. After 24 hr, the culture medium was replaced with fresh medium containing various concentrations of BO-653

(12–1,000 μM). The culture medium containing 1% MeOH was used as the negative control. After 72 hr incubation, the luciferase activity of the cells was measured using the Bright-Glo luciferase assay (Promega, Madison, WI) according to the manufacturer's instructions.

Analysis of Anti-HCV Effect of BO-653 in RMT-Tri Cells

RMT-tri cells were seeded at 2.5 × 10^4 cells/well in 24-well plates. After 24 hr, the culture medium was replaced with fresh medium containing various concentrations of BO-653 (12–1,000 μM). The culture medium containing 1% MeOH was used as the negative control. After 72 hr incubation, the cell monolayer was harvested by adding 400 μl of 5 M guanidine-isocyanate solution containing 5.6 μl of 2-mercaptoethanol. The total RNA was extracted as above; HCV RNA was quantified by qRT-PCR.

Comparison of Anti-HCV Activity of Lipophilic and Hydrophilic Antioxidants

The anti-HCV activity of various antioxidants, including hydrophilic and lipophilic compounds, was compared in FLR3-1 cells. BO-653, α-tocopherol, and probucol were used as lipophilic antioxidants; *N*-acetyl cysteine, ascorbic acid, and trolox were used as hydrophilic antioxidants. The anti-HCV activities of these compounds were determined by luciferase assays as described above.

Cytotoxicity Testing

Simultaneously with the luciferase assays, the cell viability was measured by using a WST-8 cell counting kit (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions.

Western Blot Analysis

FLR3-1 cells were treated with BO-653 as described above. After 96 hr, the cells were lysed with lysis buffer (protease inhibitor cocktail [Complete, Roche Diagnostics, IN] formulated according to the manufacturer's instructions in 10 mM Tris [pH 7.4], 150 mM NaCl, 1% sodium dodecyl sulfate (SDS), 0.5% Nonidet P-40). The cell lysates were resolved by SDS–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. After incubation with a blocking buffer consisting of 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 [TBS-T], the membranes were incubated with rabbit polyclonal anti-NS3 antibody (R212 clone) and goat anti-rabbit IgG horseradish peroxidase (HRP)-conjugated IgG (GE Healthcare, Little Chalfont, Buckinghamshire, UK) as the primary and secondary antibodies, respectively. Labeling was visualized using the Immobilon Western system (Millipore, Billerica, MA). To provide a loading control, β-actin was detected using mouse anti-β-actin monoclonal antibody (Sigma-Aldrich) and sheep anti-mouse IgG HRP-conjugated IgG (GE Healthcare) as the primary and secondary antibodies, respectively.

Immunofluorescent Staining

FLR3-1 cells treated with 111 μM BO-653 for 96 hr were probed with the primary antibody (anti-NS3) after blocking with TNB blocking buffer (PerkinElmer, Waltham, MA). An anti-rabbit IgG Alexa-Fluor 488 conjugate (Invitrogen, Grand Island, NY) was then applied as the secondary antibody.

Measurement of Antioxidant Activity of BO-653, α-Tocopherol, and Probucol in Lipid Peroxidation

Oxidation of methyl linoleate (10 mM) was carried out at 37°C under air in acetonitrile solution by adding 0.2 mM AMVN (2,2′-azobis-2,4-dimethylvaleronitrile) as a radical initiator in the presence of various concentrations of BO-653, α-tocopherol, and probucol. These antioxidants were added at concentrations of 0.2–20 μM to the reaction mixture; after 60 min incubation at 37°C, the reactions were stopped by chilling on ice.

The levels of lipid peroxidation were determined by the ferrous oxidation-xylenol orange (FOX) method [Nourooz-Zadeh et al., 1994]. In brief, completed methyl linoleate oxidation reactions were diluted 10-fold with MeOH containing 4.4 mM 2,6-di-*tert*-butyl-4-methylphenol (BHT). Each diluted solution (1.8 ml) was mixed with 0.1 ml of 2 mM xylenol orange solution (in 250 mM H_2SO_4) and 0.1 ml of 5 mM ferrous chloride solution (in 250 mM H_2SO_4). The mixture was incubated at room temperature for 60 min, at which point the absorbance at 570 nm was measured using a UV/visible light spectrophotometer. Cumene hydroperoxide was used to generate a standard curve for lipid hydroperoxidation.

Pharmacokinetics of BO-653 in uPA/SCID Mice Harboring Human Hepatocytes

Chimeric uPA/SCID mice harboring human hepatocytes were purchased from PhoenixBio (Hiroshima, Japan). All animal experiments were approved by the Ethics Committee of Tokyo Metropolitan Institute of Medical Science and were performed in accordance with the guidelines of the Animal Experimental Committee of Tokyo Metropolitan Institute of Medical Science. Two chimeric mice were administered BO-653 (at 800 or 2,000 mg/kg in 3% gum arabic solution) by single oral gavage. At 24 hr after administration, blood was collected and the plasma concentration of BO-653 was measured by high-performance liquid chromatography (HPLC). Aliquots of plasma (100 μl) were mixed with 50 μl MeOH containing 10 mM ascorbic acid and 100 μl acetonitrile containing 30 μg/ml MeO-BO-653 as the internal control. The mixtures were centrifuged at 9,100*g* for 5 min, and the resulting supernatants of 30 μl each were separated using an octadecyl column (Capcell Pak C18 UG120, 3 μm, 4.6 mm × 50 mm; Shiseido, Tokyo, Japan) at 30°C, a detection wavelength of 300 nm, and an eluent (acetonitrile) flow rate of 1.0 ml/min.

Treatment of HCV-Infected Chimeric Mice With BO-653 and/or PEG-IFN

Chimeric mice also were used as an in vivo model of persistent HCV infection, as described previously [Inoue et al., 2007]. uPA/SCID mice were engrafted with human hepatocytes; 6 weeks later, the chimeric mice were infected by intravenous (IV) injection with patient serum containing 10^6 copies of HCV genotype 1b (HCR6; GenBank accession no. AY045702). By 4 weeks after infection, the HCV RNA levels reached a plateau of 10^6–10^7 copies/ml of mouse serum. To determine anti-HCV activity of BO-653 in the early phase of the treatment, the chimeric mice ($n = 2$–5 per group) infected with HCV were given once-daily oral gavage with 2,000 mg/kg BO-653 in 3% gum arabic, and/or twice weekly subcutaneous injection with 30 μg/kg PEG-IFNα-2a (Chugai Pharmaceutical) as shown in Table I. Body weights were monitored daily, and blood for serum was collected prior to the start of treatment (Day-1) and once weekly thereafter (Days 8 and 14). Following the terminal bleed, animals were sacrificed and liver specimens were collected.

Quantitation of HCV RNA by qRT-PCR

After completion of the treatment, total RNA was purified from the serum and liver specimens by the acid guanidinium-phenol-chloroform method and qRT-PCR was used to quantify HCV RNA from the RNA samples corresponding to 1 μl serum and about 5 mm^3 of liver.

Quantitation of Serum Human Albumin

The human albumin concentration in the blood of chimeric mice was measured in 2-μl serum samples by using an Alb-II kit (Eiken Chemical, Tokyo, Japan) according to the manufacturer's instructions.

Statistical Analysis

Data are presented as mean ± standard deviations (SDs). Statistical analysis was performed by using either Student's *t*-test or ANOVA, followed by Tukey's test or Dunnett's test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Inhibitory Effect of BO-653 on HCV Replication In Vitro

The anti-HCV activity of BO-653 (Fig. 1A) was investigated in cells harboring HCV subgenomic replicons (FLR3-1 cells). BO-653 suppressed the replication of HCV subgenomic replicons in a concentration-dependent manner (Fig. 1B). The half-maximal inhibitory concentration (IC_{50}) of BO-653 in FLR3-1 cells was 36.0 μM. In contrast, no cytotoxicity was observed with up to 1,000 μM of BO-653 in FLR3-1 cells (Fig. 1B). Western blotting and immunofluorescent staining of FLR3-1 cells demonstrated that the level of HCV NS3 protein, but not that of β-actin, was reduced as the concentrations of BO-653 increased (Fig. 1C and D). A similar trend was seen in RMT-tri cells for the replication of full-genome HCV genotype 1a (Fig. 1E).

Comparison of Anti-HCV Activity of Lipophilic Antioxidants

The antioxidant activity of BO-653 has been compared previously with that of probucol and α-tocopherol [Cynshi et al., 1998]. Therefore, the anti-HCV activity of these three lipophilic antioxidants was compared in FLR3-1 cells. At a concentration >37 μM, BO-653 exhibited stronger inhibitory effects against HCV replication than did the two other compounds (Fig. 2A). In addition, the antioxidant activity of these compounds was determined by an in vitro lipid peroxidation system. BO-653 had the strongest antioxidant activity against lipid peroxidation in this in vitro assay (Fig. 2C).

Comparison of Anti-HCV Activity of Hydrophilic and Lipophilic Antioxidants

The anti-HCV activities of some representative antioxidants were investigated further (Fig. 3A). As noted above, lipophilic antioxidants exhibited anti-HCV activity in cell culture; however, hydrophilic antioxidants (*N*-acetyl cysteine, ascorbic acid, and trolox) did not inhibit the replication of HCV subgenomic replicons at comparable concentrations. None of the

TABLE I. Schedule of Blood Collection and Drug Administration for Chimeric Mice Infected With HCV

	Day															
	−1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Collection of blood	B									B						B
BO-653		BO	BO	BO	BO	BO	BO	BO	BO	BO	BO	BO	BO	BO	BO	
PEG-IFN		I			I				I			I				
BO-653 + PEG-IFN		BO/I	BO	BO	BO/I	BO	BO	BO	BO/I	BO	BO	BO/I	BO	BO	BO	

B, sampling of blood; BO, orally administrated BO-653 (2,000 mg/kg); I, subcutaneous injection of PEG-IFN (30 μg/kg).

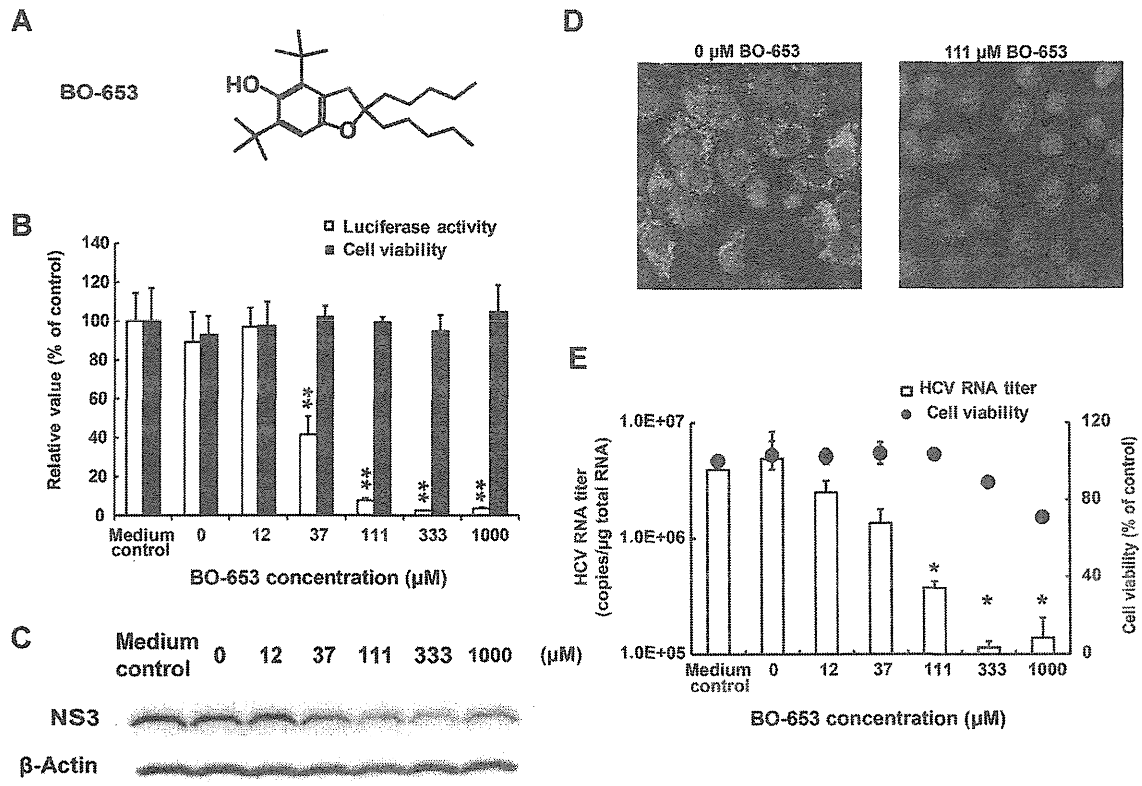

Fig. 1. In vitro anti-HCV activity of BO-653. **A**: The chemical structure of BO-653. **B**: The inhibitory effect of BO-653 on the replication of HCV subgenomic replicons in FLR3-1 cells. HCV replication (white bars) and cell viability (black bars) were determined after 72 hr of treatment; $^{**}P < 0.01$, compared with the medium control. **C**: Effect of BO-653 on the levels of HCV NS3 protein and β-actin (Western blotting). **D**: Immunofluorescent staining of HCV NS3 protein (green) in FLR3-1 cells in the absence or presence (111 μM) of BO-653; nuclei were stained with 4′,6-diamidino-2-phenylindole (blue). **E**: Effect of BO-653 on the HCV RNA titer and cell viability of HuH-7 cells infected with HCV genotype 1a (RMT-tri) after 72 hr of treatment; $^{*}P < 0.05$, compared with the medium control. Statistical analyses were performed by using ANOVA with post-hoc Dunnett's (n = 3 replicates).

compounds had any cytotoxicity at the concentrations tested (Fig. 3B).

Anti-HCV Activity of BO-653 and PEG-IFN in Chimeric Mice Infected With HCV

As demonstrated above using an in vitro assay with FLR3-1 cells, lipophilic antioxidants, including BO-653, exhibited strong anti-HCV activity. The anti-HCV activity of BO-653 was assessed further in vivo by using the compound to treat humanized chimeric mice infected with HCV. First, to measure the pharmacokinetics of BO-653, two chimeric mice were administered orally BO-653 at 800 or 2,000 mg/kg. Twenty-four hours after administration, the mice had mean BO-653 plasma concentrations of 25.0 and 83.1 μM, respectively (Table II). Thus, the BO-653 plasma concentration at the higher dose level exceeded the IC_{50} of BO-653 (36.0 μM) demonstrated previously by the in vitro assay (Fig. 1B), suggesting that oral administration of 2,000 mg/kg BO-653 might be relevant therapeutically for chimeric mice infected with HCV. These mice were infected persistently with HCV genotype 1b by injection with the serum of a HCV-infected patient (see Materials and Methods Section). To determine the anti-HCV activity of BO-653 in early phase of treatment, BO-653 (oral) and/or PEG-IFN (subcutaneous) were then administered over a period of 14 days, according to the schedule shown in Table I, and serum and liver specimens were collected. No adverse effect of the treatment, such as loss of body weight or decreased human albumin secretion, was observed in any of the study groups (Fig. 4A and B). In the mice treated with PEG-IFN, which received a dose 20-fold higher dose than that used in the clinic, the serum HCV RNA titers fell approximately 30-fold and 50-fold in weeks 1 and 2, respectively. Treatment with BO-653 alone at 2,000 mg/kg orally once daily did not reduce the HCV RNA serum titers. However, the combination of BO-653 and PEG-IFN was effective, with the combination demonstrating 200-fold decrease in serum

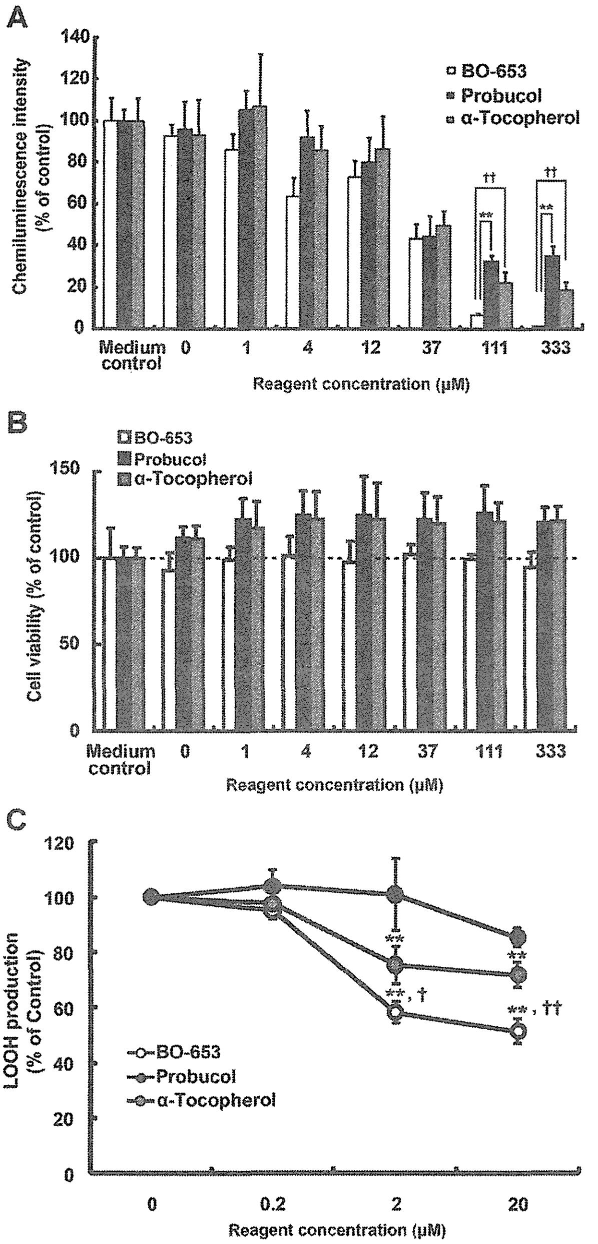

Fig. 2. Comparison of the in vitro anti-HCV activities of lipophilic antioxidants. A: The anti-HCV activity and (B) cytotoxicity of BO-653, α-tocopherol, and probucol were measured in FLR3-1 cells after 72 hr exposure to the compounds; $^{**}P < 0.01$, compared with probucol at the same concentration, $^{††}P < 0.01$, compared with α-tocopherol at the same concentration. C: Antioxidant activity of BO-653, α-tocopherol, and probucol in the peroxidation of methyl linoleate with AMVN (FOX method); $^{**}P < 0.01$, compared with probucol at the same concentration, and $^{†}P < 0.05$ and $^{††}P < 0.01$, compared with α-tocopherol at the same concentration. Statistical analyses were performed using ANOVA with post-hoc Tukey's.

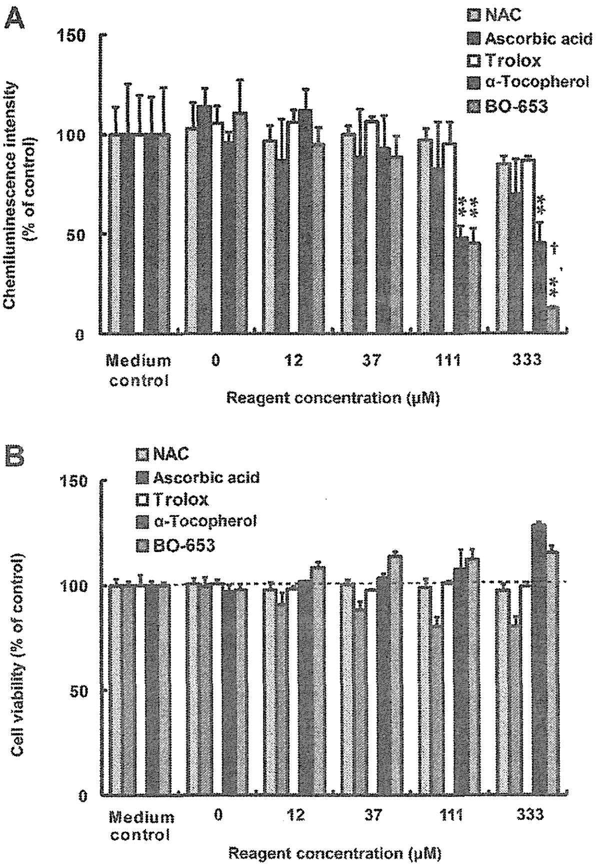

Fig. 3. Comparison of the anti-HCV activity of lipophilic and hydrophilic antioxidants. A: The anti-HCV activity and (B) cytotoxicity of BO-653 and α-tocopherol (lipophilic antioxidants) and *N*-acetyl cysteine (NAC), ascorbic acid, and trolox (hydrophilic antioxidants) in FLR3-1 cells after 72 hr of exposure to the compounds. $^{**}P < 0.01$, compared with the hydrophilic antioxidants at the same concentration, and $^{†}P < 0.05$, compared with α-tocopherol at the same concentration. Statistical analyses were performed using ANOVA with post-hoc Tukey's.

HCV titer at 2 weeks; the effect was statistically significant compared to treatment with PEG-IFN alone (Fig. 4C). In the liver samples, a decrease in the titer of HCV RNA to 7–34% of the value in untreated mice was also observed in the mice given the combination therapy (Fig. 4D).

DISCUSSION

Several lines of evidence indicate that chronic HCV infection is associated with persistently elevated levels of ROS, resulting in oxidative stress and thus contributing to the development of hepatic dam-

TABLE II. Concentration of BO-653 in Plasma

Dose	Mouse ID	Concentration of BO-653 in plasma (μM)	Mean concentration (μM)
800 mg/kg	1	30.2	25.0
	2	19.8	
2,000 mg/kg	1	100.9	83.1
	2	65.2	

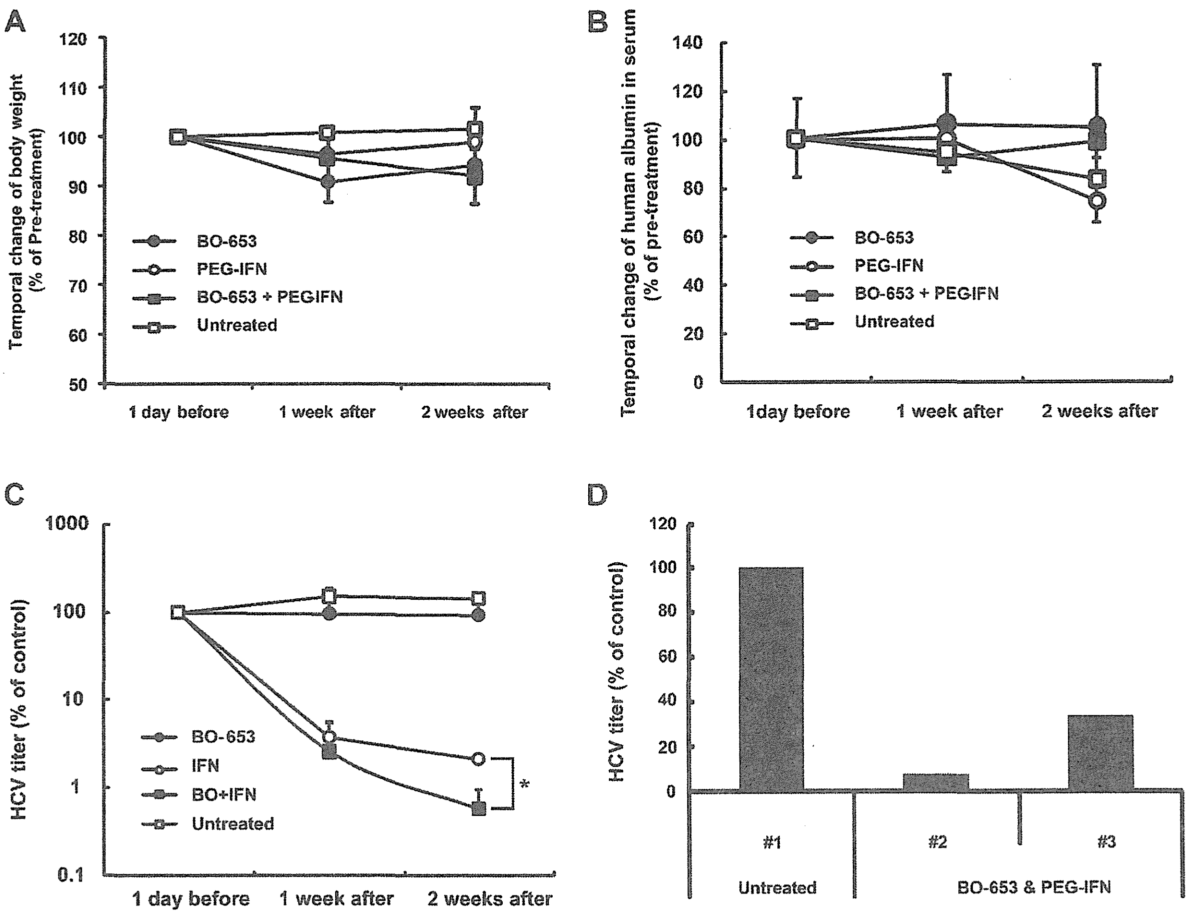

Fig. 4. Anti-HCV effect of BO-653 in chimeric mice infected with HCV. Temporal changes in the (A) body weight, (B) human albumin concentration in the serum, and (C) HCV RNA titer in the serum of chimeric mice infected with HCV after the indicated treatments. All groups included 3–5 mice, except for the untreated group (n = 2). The mice received once-daily oral gavage with 2,000 mg/kg BO-653 and/or twice-weekly subcutaneous injection with 30 μg/kg PEG-IFNα-2a (see Table I). The HCV RNA titers were normalized to the pre-treatment (Day-1) titer in the respective mouse. $^{*}P < 0.05$, PEG-IFN and BO-653 combination treatment (n = 5) was compared with PEG-IFN monotherapy (n = 3) of the same duration. Statistical analyses were performed using Student's *t*-test. **D**: The HCV RNA titer in the liver of chimeric mice treated with or without PEG-IFN and BO-653 combination treatment (untreated group, n = 1; combination treatment group, n = 2).

age [Shimoda et al., 1994; Bureau et al., 2001; Gong et al., 2001; Waris et al., 2005; Levent et al., 2006]. On the other hand, the influence of the intracellular redox state on HCV replication is controversial. A previous study demonstrated inhibition of HCV subgenomic replicon replication by lipid peroxidation and restoration of the replication by treatment with vitamin E [Huang et al., 2007]. A similar result was reported by Choi et al. [2004], who showed inhibition of HCV replication by exogenous hydrogen peroxide treatment. These results indicate that elevated levels of ROS, higher than those induced by natural HCV infection, can lead to inhibition of HCV replication. Yano et al. [2007] reported that several antioxidants, including vitamin E and β-carotene, enhance the replication of HCV genome-length replicons at relatively low concentrations (<10 μM). In contrast, the antioxidant pyrrolidine dithiocarbamate can suppress HCV replication via the inhibition of STAT-3 activation [Waris et al., 2005]. Notably, these studies have been performed primarily in in vitro systems, using either subgenomic replicon-containing cells, full-genome replicon-containing cells, or cells persistently infected with HCV. Therefore, clarification of the effect of antioxidants on HCV replication will require further work, including the use of in vivo models.

BO-653 (2,3-dihydro-5-hydroxy-2,2-dipentyl-4,6-di-*tert*-butylbenzofuran), a lipophilic (hydrophobic) antioxidant, was investigated clinically for potential treatment of atherosclerosis and prevention of post-angioplasty restenosis [Cynshi et al., 1998; Meng, 2003]. Starting in November 2001, a phase II trial tested BO-653 for treatment of atherosclerosis and the prevention of post-angioplasty restenosis, but subsequent trials of the compound were suspended due to lack of efficacy. The present investigation of BO-653 as a potential anti-HCV drug reflects subsequent identification of the compound in random screening for antiviral activity.

In the present study, the anti-HCV activity of BO-653 was assessed in an in vitro system, and analyzed further in an in vivo system that used chimeric mice harboring human hepatocytes infected with HCV. In addition, the anti-HCV activity of BO-653 was compared with that of several other antioxidants, including hydrophilic and lipophilic compounds. Among these antioxidants, BO-653 had the strongest anti-HCV activity against HCV subgenomic replicons, followed by α-tocopherol. A previous study showed that the replication of a HCV replicon was increased in the presence of lower concentrations of vitamin E [Yano et al., 2007]. However, the results of the present study suggest that vitamin E exhibits anti-HCV activity when present in cell culture at concentrations exceeding 37 μM. Also, in chimeric mice infected persistently with HCV, the combination of PEG-IFN and BO-653 was more effective than PEG-IFN alone, although BO-653 alone did not have any anti-HCV activity in this mouse model. Monotherapy with DEBIO-025, a non-immunosuppressive cyclosporine A derivative and novel anti-HCV drug candidate, can decrease the viral load in patients with chronic HCV infection but not in chimeric mice infected with HCV [Inoue et al., 2007; Flisiak et al., 2009]; therefore, BO-653 monotherapy should be evaluated further in patients infected with HCV. Di Bona et al. [2006] reported impaired IFN-α signaling following oxidative stress, suggesting that oxidative stress causes resistance to the antiviral activity of IFN-α in patients infected with HCV. Taken together, these findings imply that lipophilic antioxidants such as BO-653 support the effects of PEG-IFN via antioxidant activity.

Several reports indicate that antioxidants, especially hydrophilic compounds, have antiviral activity [Docherty et al., 1999; Docherty et al., 2006; Ho et al., 2009; Geiler et al., 2010; Tian et al., 2010]. *N*-acetyl cysteine can inhibit the replication of H5N1 influenza A virus in a cell culture system [Geiler et al., 2010], and the antiviral effect of epigallocatechin gallate on enterovirus 71 may be associated with the modulation of the cellular redox state [Ho et al., 2009]. In contrast, the inhibitory effects of the lipophilic antioxidants on HCV RNA replication were stronger than those of the hydrophilic antioxidants. These lipophilic antioxidants inhibited the replication of HCV without cytotoxicity at concentrations higher than 37 μM. The role of the redox state of the lipid membrane on HCV RNA replication is of great interest, given reports of the association of RNA replication with lipid rafts [Shi et al., 2003; Sakamoto et al., 2005]. Further studies will be needed to elucidate the precise mechanism(s) underlying the suppression of HCV replication by BO-653.

In conclusion, the present study demonstrated that lipophilic antioxidants have stronger anti-HCV activity than hydrophilic antioxidants and that BO-653 has the strongest anti-HCV activity of the antioxidants tested. The combination of PEG-IFN and BO-653 was more effective than PEG-IFN alone in chimeric mice infected persistently with HCV. Further development of this compound would require additional considerations such as the route of administration and pharmacokinetics. Taken together, these findings provide insights into the influence of the intracellular redox state on the life-cycle of HCV.

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ORIGINAL ARTICLE

Hepatitis C virus kinetics by administration of pegylated interferon-α in human and chimeric mice carrying human hepatocytes with variants of the *IL28B* gene

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ABSTRACT

Objective Recent studies have demonstrated that genetic polymorphisms near the *IL28B* gene are associated with the clinical outcome of pegylated interferon α (peg-IFN-α) plus ribavirin therapy for patients with chronic hepatitis C virus (HCV). However, it is unclear whether genetic variations near the *IL28B* gene influence hepatic interferon (IFN)-stimulated gene (ISG) induction or cellular immune responses, lead to the viral reduction during IFN treatment.

Design Changes in HCV-RNA levels before therapy, at day 1 and weeks 1, 2, 4, 8 and 12 after administering peg-IFN-α plus ribavirin were measured in 54 patients infected with HCV genotype 1. Furthermore, we prepared four lines of chimeric mice having four different lots of human hepatocytes containing various single nucleotide polymorphisms (SNP) around the *IL28B* gene. HCV infecting chimeric mice were subcutaneously administered with peg-IFN-α for 2 weeks.

Results There were significant differences in the reduction of HCV-RNA levels after peg-IFN-α plus ribavirin therapy based on the *IL28B* SNP rs8099917 between TT (favourable) and TG/GG (unfavourable) genotypes in patients; the first-phase viral decline slope per day and second-phase slope per week in TT genotype were significantly higher than in TG/GG genotype. On peg-IFN-α administration to chimeric mice, however, no significant difference in the median reduction of HCV-RNA levels and the induction of antiviral ISG was observed between favourable and unfavourable human hepatocyte genotypes.

Conclusions As chimeric mice have the characteristic of immunodeficiency, the response to peg-IFN-α associated with the variation in *IL28B* alleles in chronic HCV patients would be composed of the intact immune system.

Significance of this study

What is already known on this subject?

- Genetic polymorphisms near the *IL28B* gene are associated with a chronic HCV treatment response.
- HCV-infected patients with the *IL28B* homozygous favourable allele had a more rapid decline in HCV kinetics in the first and second phases by peg-IFN-α-based therapy.
- During the acute phase of HCV infection, a strong immune response among patients with the *IL28B* favourable genotype could induce more frequent spontaneous clearance of HCV.

What are the new findings?

- In chronically HCV genotype 1b-infected chimeric mice that have the characteristic of immunodeficiency, no significant difference in the reduction in serum HCV-RNA levels and the induction of antiviral hepatic ISG by the administration of peg-IFN-α was observed between favourable and unfavourable human hepatocyte *IL28B* genotypes.
- By comparison of serum HCV kinetics between human and chimeric mice, the viral decline in both the first and second phases by peg-IFN-α treatment was affected by the variation in *IL28B* genotypes only in chronic hepatitis C patients.

How might it impact on clinical practice in the foreseeable future?

- The immune response according to *IL28B* genetic variants could contribute to the first and second phases of HCV-RNA decline and might be critical for HCV clearance by peg-IFN-α-based therapy.

INTRODUCTION

Hepatitis C is a global health problem that affects a significant portion of the world's population. The WHO estimated that, in 1999, 170 million hepatitis C virus (HCV)-infected patients were present worldwide, with 3–4 million new cases appearing per year.[1]

The standard therapy for hepatitis C still consists of pegylated interferon-α (peg-IFN-α), administered once weekly, plus daily oral ribavirin for 24–48 weeks in countries where protease inhibitors are not available.[2] This combination therapy is quite successful in patients with HCV genotype 2 or 3 infection, leading to a sustained virological response (SVR) in approximately 80–90% of patients treated; however, in patients infected with HCV genotype 1 or 4, only approximately half of all treated individuals achieved a SVR.[3 4]

Table 1 Characteristics of 54 patients infected HCV genotype 1

	IL28B SNP rs8099917		
	TT (n=34)	TG (n=19) + GG (n=1)	p Value
Age (years)	55.6±10.1	54.7±11.3	0.746
Gender (male %)	70	50	0.199
Body mass index (kg/m^2)	24.6±3.1	24.7±3.3	0.870
Viral load at therapy (log IU/ml)	6.0±0.7	5.8±0.8	0.357
SVR rate (%)	50	11	0.012
Serum ALT level (IU/l)	100.3±80.8	79.3±45.0	0.226
Platelet count (×10^4/μl)	17.1±9.0	16.5±5.8	0.771
Fibrosis (F3+4 %)	42	40	0.877

HCV, hepatitis C virus; SNP, single nucleotide polymorphism; SVR, sustained virological response.

Host factors were shown to be associated with the outcome of the therapy, including age, sex, race, liver fibrosis and obesity.[5] Genome-wide association studies have demonstrated that genetic variations in the region near the interleukin-28B (*IL28B*) gene, which encodes interferon (IFN)-λ3, are associated with a chronic HCV treatment response.[6–10] Furthermore, it was demonstrated that genetic variations in the *IL28B* gene region are also associated with spontaneous HCV clearance.[11–12]

Interestingly, a recent report showed the effect of genetic polymorphisms near the *IL28B* gene on the dynamics of HCV during peg-IFN-α plus ribavirin therapy in Caucasian, African American and Hispanic individuals;[13] HCV-infected patients with the *IL28B* homozygous favourable allele had a more rapid decline of HCV in the first phase, which is associated with the inhibition of viral replication as well as the second phase associated with immuno-destruction of viral-infected hepatocytes.[14] However, it is unknown how a direct effect by the *IL28B* genetic variation, such as the induction of IFN-stimulated genes (ISG) or cellular immune responses, would influence the viral kinetics during IFN treatment. Over recent periods, engineered severe combined immunodeficient (SCID) mice transgenic for urokinase-type plasminogen activator (uPA) received human hepatocyte transplants (hereafter referred to as chimeric mice)[15–17] and are suitable for experiments with hepatitis viruses in vivo.[18 19] We have also reported that these chimeric mice carrying human hepatocytes are a robust animal model to evaluate the efficacy of IFN and other anti-HCV agents.[20 21]

The purpose of this study was to reveal the association between genetic variations in the *IL28B* gene region and viral decline during peg-IFN-α treatment in patients with HCV, and to clarify the association between different *IL28B* alleles of human hepatocytes in chimeric mice and the response to peg-IFN-α without immune response. These studies will elucidate whether the immune response by the *IL28B* genetic variation affects the viral kinetics during peg-IFN-α treatment.

MATERIALS AND METHODS

Patients

Fifty-four Japanese patients with chronic HCV genotype 1 infection at Nagasaki Medical Center and Nagoya City University were enrolled in this study (table 1). Patients received peg-IFN-α2a (180 μg) or 2b (1.5 μg/kg) subcutaneously every week and were administered a weight-adjusted dose of ribavirin (600 mg for <60 kg, 800 mg for 60–80 kg, and 1000 mg for >80 kg daily), which is the recommended dosage in Japan. Patients with other hepatitis virus infection or HIV coinfection were not included in the study. The study protocol conformed to the ethics guidelines of the 1975 Declaration of Helsinki as reflected by earlier approval by the institutions' human research committees.

Laboratory tests

Blood samples were obtained before therapy, as well as on day 1 and at weeks 1, 2, 4, 8 and 12 after the start of therapy and were analysed for the HCV-RNA level by the commercial Abbott Real-Time HCV test with a lower limit of detection of 12 IU/ml (Abbott Molecular Inc., Des Plains, Illinois, USA). Genetic polymorphism in the *IL28B* gene (rs8099917), a single nucleotide polymorphism (SNP) recently identified to be associated with treatment response,[6–8] was tested by the TaqMan SNP genotyping assay (Applied Biosystems, Foster City, California, USA).

HCV infection of chimeric mice with the liver repopulated for human hepatocytes

SCID mice carrying the uPA transgene controlled by an albumin promoter were injected with 5.0–7.5×10^5 viable hepatocytes through a small left-flank incision into the inferior splenic pole, thereafter chimeric mice were generated. The chimeric mice were purchased from PhoenixBio Co, Ltd (Hiroshima, Japan).[17] Human hepatocytes with the *IL28B* homozygous favourable allele, heterozygous allele or homozygous unfavourable allele were imported from BD Biosciences (San Jose, California, USA) (table 2). Murine serum levels of human albumin and the body weight were not significantly different among four chimeric mice groups, providing a reliable comparison for anti-HCV agents.[22] Three different serum samples were obtained from three chronic HCV patients (genotype 1b).[21 22] Each mouse was intravenously infected with serum sample containing 10^5 copies of HCV genotype 1b. Administration of peg-IFN-α2a (Pegasys; Chugai Pharmaceutical Co., Ltd., Tokyo, Japan) at the dose formulation (30 μg/kg) was consecutively applied to each mouse on days 0, 3, 7 and 10 (table 3).

HCV-RNA quantification

HCV-RNA in mice sera (days 0, 1, 3, 7 and 14) was quantified by an in-house real-time detection PCR assay with a lower quantitative limit of detection of 10 copies/assay, as previously reported.[21]

Quantification of IFN-stimulated gene-expression levels

For analysis of endogenous ISG levels, total RNA was isolated from the liver using the RNeasy RNA extraction kit (Qiagen, Valencia, California, USA) and complementary DNA synthesis

Table 2 Four lines of uPA/SCID mice from four different lots of human hepatocytes (donor) containing various SNP around the *IL28B* gene

uPA/SCID mice	Donor	Race	Age	Gender	rs8103142	rs12979860	rs8099917
PXB mice	A	African American	5 Years	Male	CC	TT	TG
	B	Caucasian	10 Years	Female	CC	TT	TG
	C	Hispanic	2 Years	Female	TT	CC	TT
	D	Caucasian	2 Years	Male	TT	CC	TT

PXB mice; urokinase-type plasminogen activator/severe combined immunodeficiency (uPA/SCID) mice repopulated with approximately 80% human hepatocytes.
SCID, severe combined immunodeficient; SNP, single nucleotide polymorphism.

Table 3 Dosage and time schedule of pegIFN-α2a* treatment for HCV genotype 1b infected chimeric mice

				Dose			
Donor hepatocytes†	**No of chimeric mice**	**Inoculum**	**Test compound**	**Level (μg/kg)**	**Concentrtion (μg/ml)**	**Volume (ml/kg)**	**Frequency**
A	3	Serum A	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
B	4	Serum A	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
C	3	Serum A	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
D	3	Serum A	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
A	2	Serum B	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
C	2	Serum B	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
A	2	Serum C	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10
C	2	Serum C	Peg-IFN-α2a	30	3	10	Day 0, 3, 7, 10

*Pegasys; Chugai Pharmaceutical Co., Ltd., Tokyo, Japan.
†The *IL28B* genetic variation of the donor hepatocytes was indicated in table 2.
HCV, hepatitis C virus; peg-IFN-α, pegylated interferon α.

was performed using 2.0 μg of total RNA (High Capacity RNA-to-cDNA kit; Applied Biosystems). Fluorescence real-time PCR analysis was performed using an ABI 7500 instrument (Applied Biosystems) and TaqMan Fast Advanced gene expression assay (Applied Biosystems). TaqMan Gene Expression Assay primer and probe sets (Applied Biosystems) are shown in the supplementary information (available online only). Relative amounts of messenger RNA, determined using a FAM-Labeled TaqMan probe, were normalised to the endogenous RNA levels of the housekeeping reference gene, glyceraldehyde-3-phosphate dehydrogenase. The delta Ct method ($2^{-(\text{delta Ct})}$) was used for quantitation of relative mRNA levels and fold induction.[23 24]

Statistical analyses

Statistical differences were evaluated by Fisher's exact test or the χ^2 test with the Yates correction. Mice serum HCV-RNA and intrahepatic ISG expression levels were compared using the Mann–Whitney U test. Differences were considered significant if p values were less than 0.05.

RESULTS

Characteristics of the study patients

Genotypes (rs8099917) TT, TG and GG were detected in 34, 19 and one patient infected with HCV genotype 1, respectively. SVR rates were significantly higher in HCV patients with genotype TT than in those with genotype TG/GG (50% vs 11%, p=0.012). The initial HCV serum load was comparable between genotypes TT and TG/GG (6.0±0.7 vs 5.8±0.8 log IU/ml). There were no significant differences in sex (male%, 70% vs 50%), age (55.6±10.1 vs 54.7±11.3 years), serum alanine aminotransferase level (100.3±80.8 vs 79.3±45.0 IU/L), platelet count (17.1±9.0 vs 16.5±5.8×10^4/μl) and fibrosis stages (F3/4%, 42% vs 40%) between HCV patients with the favourable (rs8099917 TT) and unfavourable (rs8099917 TG/GG) *IL28B* genotypes (table 1).

Changes in serum HCV-RNA levels in patients treated by peg-IFN-α plus ribavirin

Figure 1 shows the initial change in the serum HCV-RNA level for 14 days after peg-IFN-α plus ribavirin therapy in patients infected with HCV genotype 1 based on the genetic polymorphism near the *IL28B* gene. The immediate antiviral response (viral drop 24 h after the first IFN injection) was significantly higher in HCV patients with genotype TT than genotype TG/GG (−1.08 vs −0.39 log IU/ml, p<0.001). Figure 2 also shows the subsequent change in the serum HCV-RNA reduction after peg-IFN-α plus ribavirin therapy in patients infected with HCV genotype 1. Similarly, during peg-IFN-α plus ribavirin therapy, a statistically significant difference in the median reduction in serum HCV-RNA levels was noted according to the genotype (TT vs TG/GG). The median reduction in the serum HCV-RNA levels (log IU/ml) at 1, 2, 4, 8 and 12 weeks between genotypes TT and TG/GG was as follows: −1.58 vs −0.62, p<0.001; −2.35 vs −0.91, p<0.001;

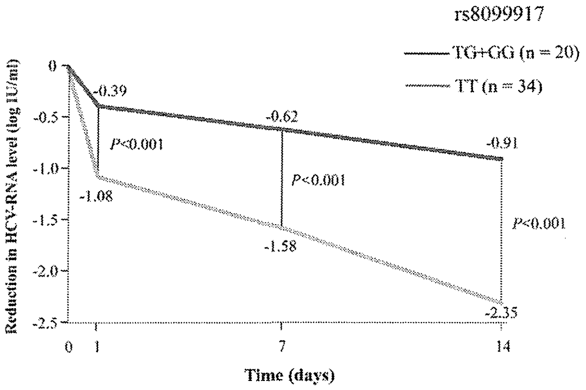

Figure 1 Rapid reduction of median hepatitis C virus (HCV)-RNA levels (log IU/ml) at 1, 7 and 14 days between *IL28B* single nucleotide polymorphisms rs8099917 genotype TT (n=34) and TG/GG (n=20) in HCV genotype 1-infected patients treated with peg-IFN-α plus ribavirin.

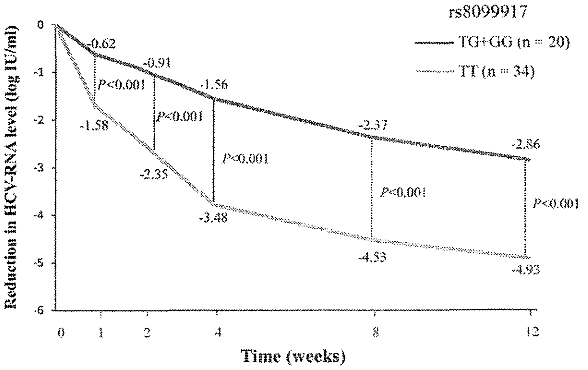

Figure 2 Weekly reduction of median hepatitis C virus (HCV)-RNA levels (log IU/ml) at 1, 2, 4, 8 and 12 weeks between *IL28B* single nucleotide polymorphisms rs8099917 genotype TT (n=34) and TG/GG (n=20) in HCV genotype 1-infected patients treated with pegylated interferon α plus ribavirin.

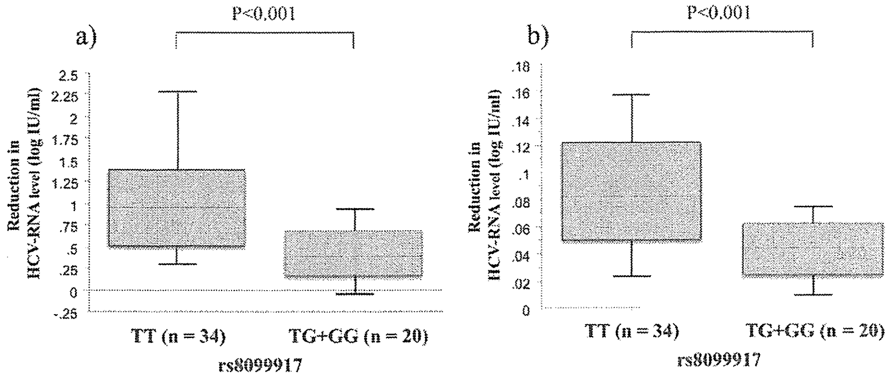

Figure 3 (A) The first-phase viral decline slope per day (Ph1/day) and (B) second-phase viral decline slope per week (Ph2/week) in hepatitis C virus (HCV) genotype 1-infected patients treated with pegylated interferon α plus ribavirin. The lines across the boxes indicate the median values. The hash marks above and below the boxes indicate the 90th and 10th percentiles for each group, respectively.

−3.48 vs −1.56, p<0.001; −4.53 vs −2.37, p<0.01; −4.93 vs −2.86, p<0.001. Furthermore, the initial first-phase viral decline slope per day (Ph1/day) and subsequent second-phase viral decline slope per week (Ph2/week) in TT genotype were significantly higher than in genotype TG/GG (Ph1/day 0.94 ±0.83 vs 0.38±0.40 log IU/ml, p<0.001; Ph2/week 0.08±0.06 vs 0.04±0.03 log IU/ml, p<0.001) (figure 3).

Changes in serum HCV-RNA levels in chimeric mice treated by peg-IFN-α

In order to clarify the association between *IL28B* alleles of human hepatocytes and the response to peg-IFN-α, we prepared four lines of uPA/SCID mice and four different lots of human hepatocytes containing various rs8099917, rs8103142 and rs12979860 SNPs around the *IL28B* gene (table 2). The chimeric mice were inoculated with serum samples from each HCV-1b patient, and then HCV-RNA levels had increased and reached more than 10^6 copies/ml in all chimeric mice sera at 2 weeks after inoculation. After confirming the peak of HCV-RNA in all chimeric mice, they were subcutaneously administered with four times injections of the bolus dose of peg-IFN-α2a for 2 weeks (table 3). Figure 4 shows the change in the serum HCV-RNA levels for 14 days during IFN injection into chimeric mice transplanted with *IL28B* favourable or unfavourable human hepatocyte genotypes. On peg-IFN-α administration, no significant difference in the median reduction in HCV-RNA levels in the serum A-infected[22] chimeric mice sera was observed between favourable (n=7) and unfavourable

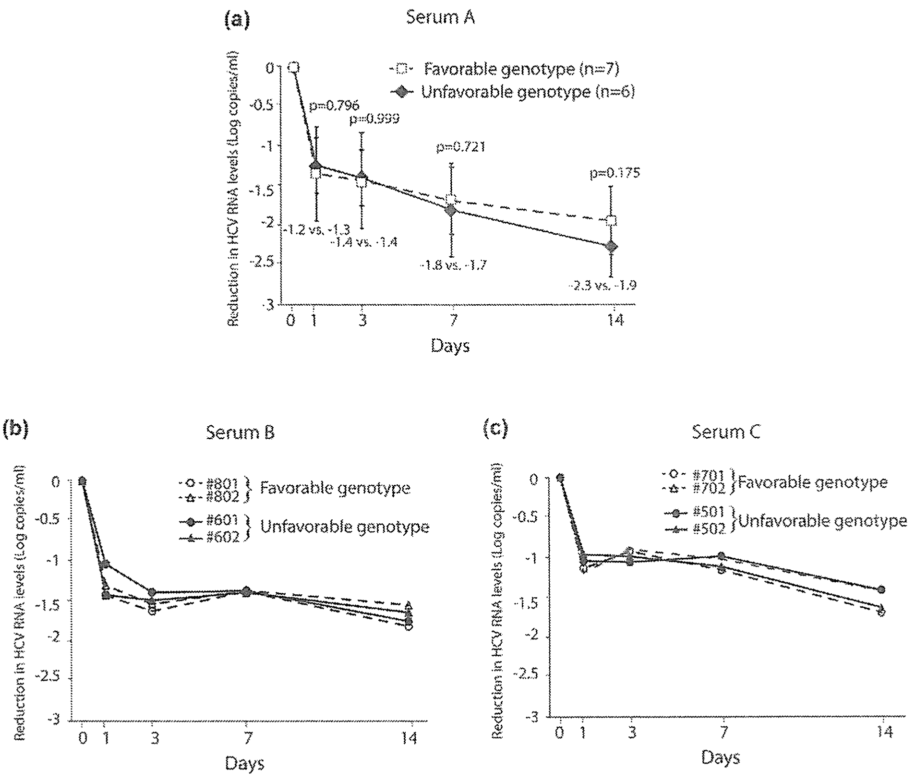

Figure 4 Median reduction of hepatitis C virus (HCV)-RNA levels (log copies/ml) after administering pegylated interferon α to chimeric mice having human hepatocytes containing various single nucleotide polymorphisms around the *IL28B* gene as favourable (rs8099917 TT) and unfavourable (rs8099917 TG) genotypes. Data are represented as mean+SD. Chimeric mice infected with a) serum A (n=7; favourable genotype, n=6; unfavourable genotype), (B) serum B (n=2, each genotype), and (C) serum C (n=2, each genotype). All serum samples were obtained from HCV-1b patients.