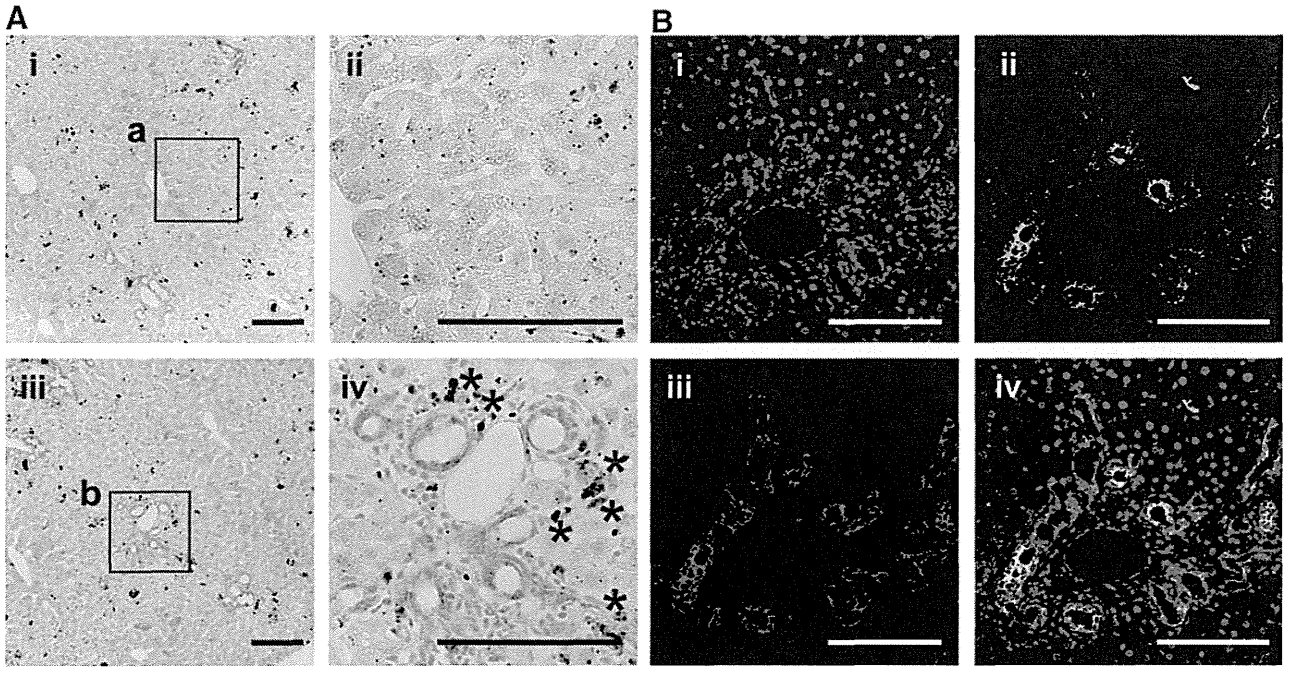

FIG. 4. Duct-like cells express NS-GFP in liver of adult mice fed a 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet. **(A, B)** GFP in hepatocytes of adult NS-GFP Tg mice fed a DDC diet. **(A)** Sections were stained with an anti-GFP antibody. **a** and **b** are lower power views of the areas shown at higher power in **(ii)** and **(iv)**, respectively. *Deposition of iron hemes, visible as *brown* clots. **(B)** Sections were stained with anti-GFP (*green*) and anti-CK19 (*red*) antibodies and DAPI (nuclear staining, *blue*). **(i)** DAPI, **(ii)** GFP, **(iii)** CK19, **(iv)** merged. Scale bars, 100 μm.

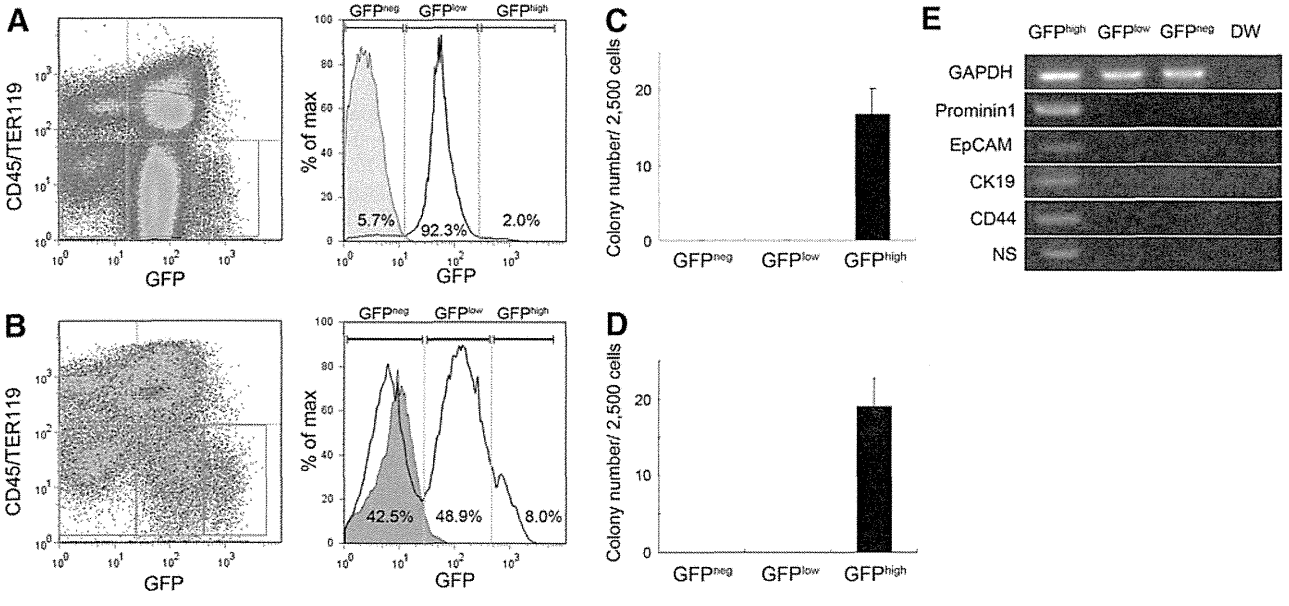

FIG. 5. Increased ratios of NS-GFP^{high} hepatic precursor cells are seen following a DDC diet. **(A, B)** Flow cytometric analyses of GFP expression in nonparenchymal cells of adult NS-GFP Tg mice without **(A)** and with **(B)** a DDC diet. Flow cytometry with CD45/Ter119 and GFP, and a histogram of GFP in CD45$^-$Ter119$^-$ cells are shown in the *left* and *right* panels, respectively. Nonhematopoietic nonparenchymal cells (CD45$^-$Ter119$^-$) were fractionated into 3 distinct subpopulations (GFP^{neg}, GFP^{low}, and GFP^{high} cells). Values in panels are the percentage of the specified subpopulation among CD45$^-$ Ter119$^-$ cells. The data shown are representative of 3 independent experiments. **(C, D)** Hepatic colony formation of subpopulations. Fractionated cells were cultured for 7 days. Data shown are the mean ratio ± SD of colonies derived from mice fed a control **(C)** and DDC **(D)** diet ($n = 3$ each). **(E)** Gene expression in NS-GFP subpopulations. Total RNA was purified from the subpopulations indicated in **(B)**, and mRNA levels of the indicated molecules were evaluated by reverse transcription–polymerase chain reaction. DW, distilled water.

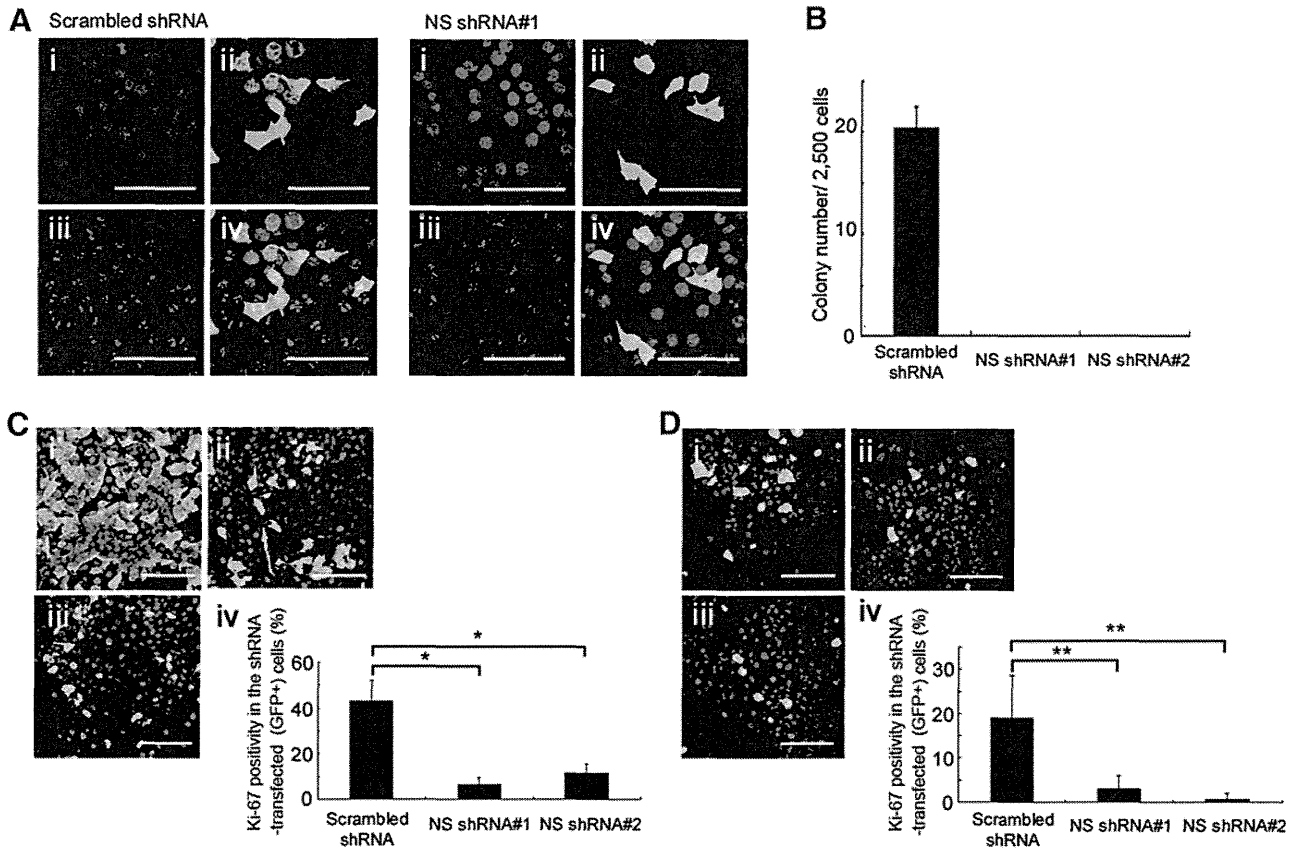

FIG. 6. Inhibition of proliferation of hepatic precursor cells following NS downregulation in vitro. **(A)** NS knockdown in a hepatic cell line. A scrambled shRNA (control, *left panels*) or NS shRNA#1 (*right panels*) was introduced into Hepa1-6 cells (a murine hepatocellular carcinoma cell line) by lentiviral infection, followed by staining with anti-GFP (*green*) and anti-NS (*red*) antibodies and DAPI (*blue*). **(i)** DAPI, **(ii)** GFP, **(iii)** NS, **(iv)** merged. NS protein expression (*red*) was reduced in GFP^+ cells treated with shRNA#1 but not the control shRNA. **(B)** Colony formation of Hepa1-6 cells following NS knockdown. GFP^+ Hepa1-6 cells, indicating those transduced with shRNA, were isolated and cultured for 10 days. Data shown are the mean number ± SD of colonies ($n = 3$). **(C, D)** Proliferation of Hepa1-6 cells **(C)** and Dlk^+ hepatic precursors from fetal liver **(D)** following NS knockdown. Hepa1-6 cells and Dlk^+ fetal liver cells were infected with the shRNA lentivirus, and then cultured for 3 **(C)** or 10 **(D)** days. Cells were stained with an anti-Ki-67 antibody (*red*), anti-GFP antibody (*green*), and DAPI (*blue*). Representative data are shown in **(i)**, **(ii)**, and **(iii)** for the scrambled control, NS shRNA#1, and NS shRNA#2, respectively. Data shown in **(iv)** are the mean ratio ± SD of Ki-67 positivity among cells transfected with the indicated shRNA plasmids (GFP^+ cells) ($n = 3$). $^*P < 0.01$, $^{**}P < 0.05$. Scale bars, 100 μm.

proportion of Ki-67-positive cells among transfected (GFP^+) cells in the hepatic cell line (Fig. 6C) and in the hepatic colonies derived from freshly isolated fetal liver precursor cells (Fig. 6D). Thus, NS downregulation inhibits proliferation of hepatic precursor cells.

NS plays an essential role in hepatocyte proliferation in response to liver injury in vivo

The observation that NS is upregulated in hepatocytes after partial hepatectomy suggested that NS is essential for hepatocyte proliferation. To examine the effect of loss NS function in hepatocytes in vivo, we introduced the shRNA plasmids into liver cells by hydrodynamic injection of plasmid DNA via the tail vein [20]. Partial hepatectomy was performed 3 days later and we found that hepatocytes were successfully transfected with shRNA plasmids by detection of GFP expression. Three days after partial hepatectomy, we found that NS knockdown in the hepatocytes significantly suppressed expression of the Ki-67 antigen (Fig. 7), indicating that NS is essential for hepatocyte proliferation in response to liver injury in vivo.

Discussion

In this study, we examined the expression and function of NS in developing and injured liver, and we evaluated the capacity of hepatic NS-expressing cells to form colonies by using an NS-GFP system. As previously reported [19], DDC treatment induced the emergence of ductal cells that express both cholangiocellular and hepatocytic markers, called "oval cells," in periportal regions. Several studies have identified markers of oval cells, including Ep-CAM and CD133 [19,24,25]. NS-GFP was not specific for oval cells, since most hepatocytes and nonparenchymal cells also expressed GFP. Therefore, NS-GFP expression alone cannot be used to purify hepatic stem/precursor cells. However, since a distinct subpopulation of cells expressing high GFP levels (GFP^{high}) showed higher clonogenic potential, combining this system with evaluation of other stem cell markers could enable

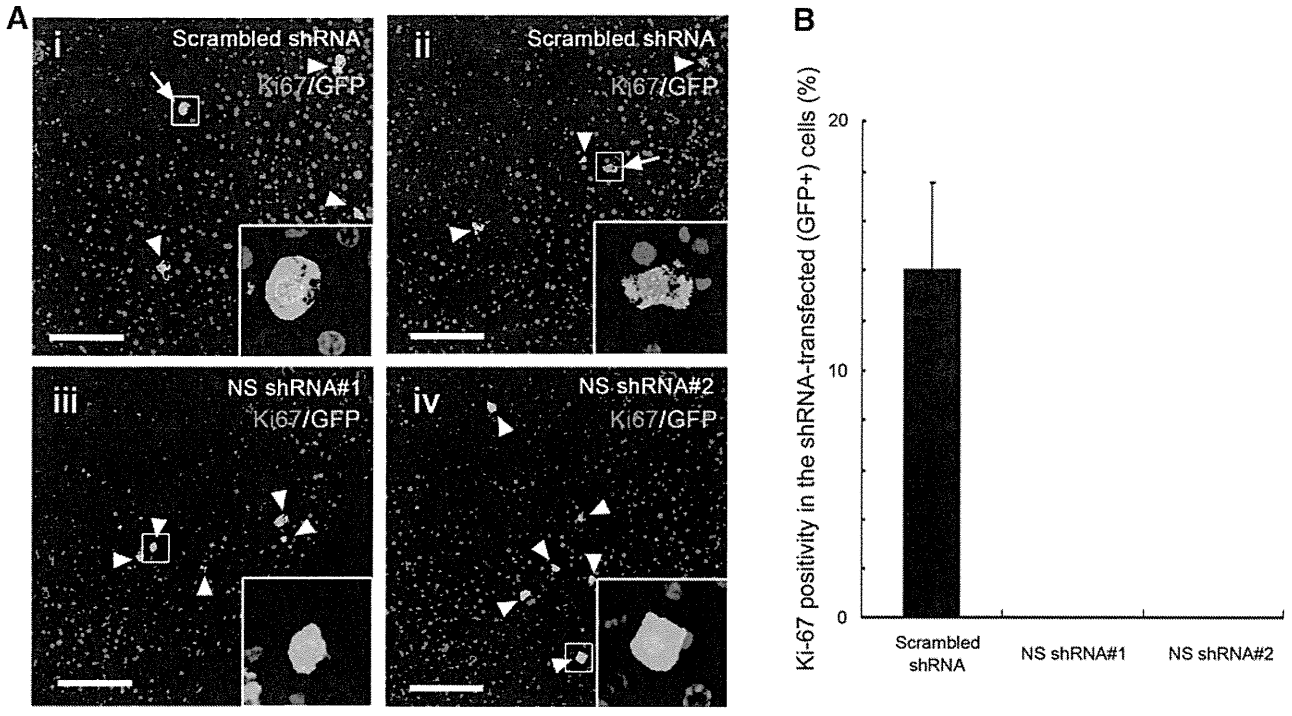

FIG. 7. Inhibition of hepatocyte proliferation following NS downregulation in vivo. NS shRNA plasmids were introduced into liver cells by hydrodynamic injection of the plasmid DNA via the tail vein, followed by partial hepatectomy 3 days after injection. Liver tissue specimens were prepared 3 days later, and sections were stained with an anti-GFP antibody (*green*), anti-Ki-67 antibody (*red*), and DAPI (*blue*). Ki-67 expression was evaluated in more than 60 GFP^+ cells for each sample. **(A)** Representative data for the scrambled control **(i, ii)**, NS shRNA#1 **(iii)**, and NS shRNA#2 **(iv)**. *Arrows*; $GFP^+Ki\text{-}67^+$ cells, *arrowheads*; $GFP^+Ki\text{-}67^-$cells. *Insets* are magnified views of the indicated areas. **(B)** Mean percentage ± SD of Ki-67 positivity in hepatocytes transfected with the indicated shRNA plasmids (GFP^+ cells) ($n=3$). Scale bars, 100 μm.

efficient enrichment of a stem/precursor cell population. In addition, NS may be particularly important for the development of liver, since the expression level of NS-GFP in developing liver cells appeared to be higher than that in adult non-parenchymal cells. NS may therefore play a critical role in expansion of the hepatic stem/precursor cells during liver development.

A previous study demonstrated that NS is required for rRNA processing [9,26], suggesting that NS expression regulates protein synthesis. Enhanced protein synthesis requires activation of ribosomal biogenesis. NS belongs to the class of nucleolar GTPases that includes yeast Nug1, which exports pre-60S ribosomal subunits out of the nucleolus [27]. In *Caenorhabditis elegans*, *nst-1* mutants exhibit reduced rRNA levels, suggesting a critical role of NS in ribosome biogenesis [9]. NS knockdown apparently delays processing of 32S pre-rRNA into 28S rRNA and is accompanied by a substantial decrease in protein synthesis and in the levels of rRNAs and some mRNAs [26]. Because protein synthesis is required for cell growth and proliferation, NS expression in both hepatic precursor cells and hepatocytes may be important for tissue regeneration. On the other hand, protein synthesis appears to be enhanced in resting hepatocytes for reasons unrelated to regeneration. Mature hepatocytes exhibit high levels of protein synthesis to maintain serum protein levels, and protein translation actively occurs even in non-dividing hepatocytes. Thus, NS expression may be controlled by several different signals.

One possible regulator of NS is Myc, which is upregulated by partial hepatectomy [28]. When Myc is overexpressed in mouse hepatocytes in vivo using recombinant adenovirus, hepatocytes enlarge in the absence of significant cell proliferation, an event associated with upregulation of large- and small-subunit ribosomal and nucleolar genes [29]. In addition, a recent study identified the NS gene as a direct transcriptional target of the Myc oncoprotein [22]. Therefore, NS may function to increase cell mass in response to Myc activation following partial hepatectomy. It has also been reported that, constitutive activation of Myc generates hepatocellular carcinoma, whereas Myc inactivation promotes differentiation of tumor cells into hepatocytes and biliary cells, which form bile duct structures [30], suggesting that Myc maintains cells in an undifferentiated status. NS may have a similar function in the case of hepatic malignancy. In addition, Myc may control post-translational regulation of NS protein. A recent study revealed that NS is a target of reactive oxygen species (ROS) [31]. In transformed hematopoietic cells, Myc activation leads to high ROS levels, resulting in impaired NS protein degradation. Therefore, Myc activation may stabilize NS protein in regenerating liver. Because GFP would not be stabilized in the same way as the NS protein, these findings suggest that NS protein levels may not be precisely correlated with NS-GFP levels. Nonetheless, both NS protein levels and NS-GFP expression are consistently upregulated by partial hepatectomy. These findings suggest that overall NS expression is likely regulated by both transcriptional and protein stability.

In conclusion, we have demonstrated that NS is essential for proliferation of both hepatic precursor cells and

hepatocytes. Understanding the mechanisms regulating NS expression and function may contribute to development of methodologies useful for enhancing liver regeneration in pathological states.

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Author Disclosure Statement

No competing financial interests exist.

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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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Conflict of interest: Dr. Sudoh is an employee of Chugai Pharmaceutical Co., Ltd. The other authors declare no potential conflicts of interest.

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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).

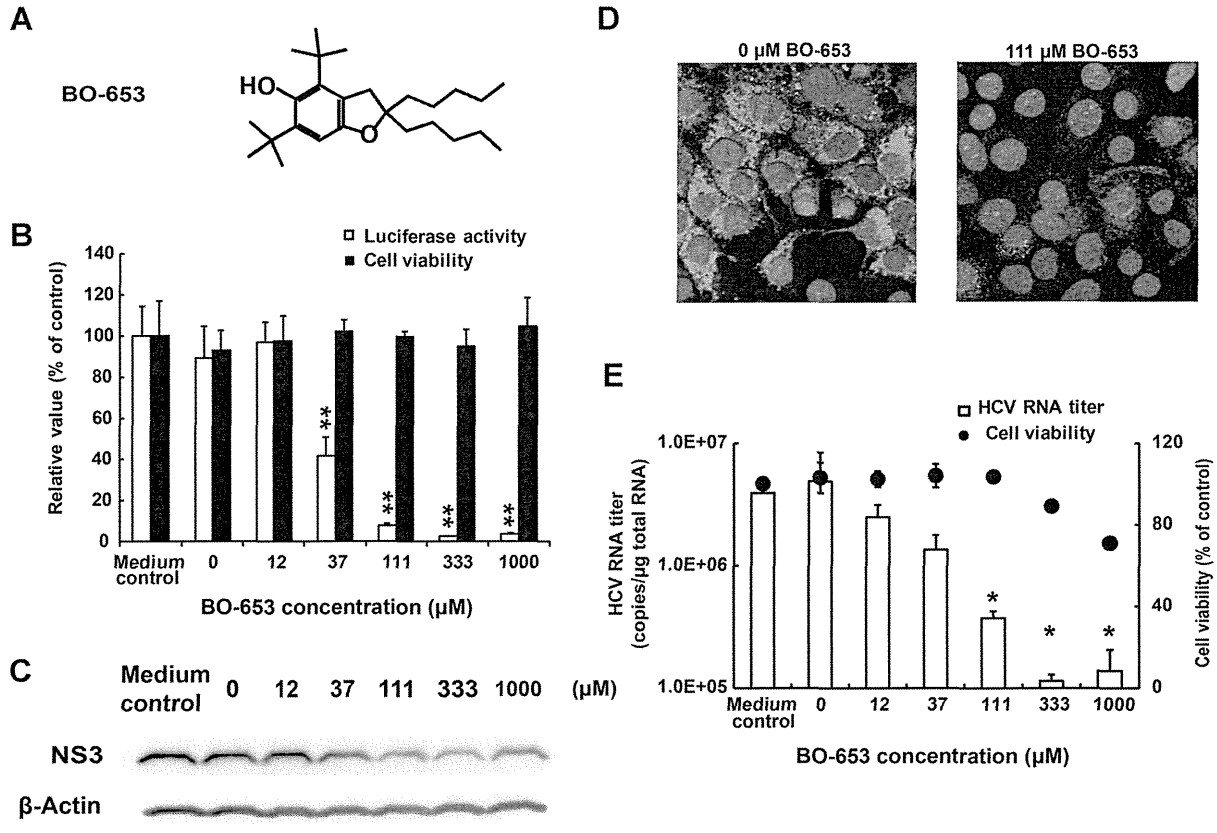

Fig. 1. In vitro anti-HCV activity of BO-653. **A**: The chemical structure of BO-653. **B**: The inhibitory effect of BO-653 on the replication of HCV subgenomic replicons in FLR3-1 cells. HCV replication (white bars) and cell viability (black bars) were determined after 72 hr of treatment; $^{**}P < 0.01$, 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

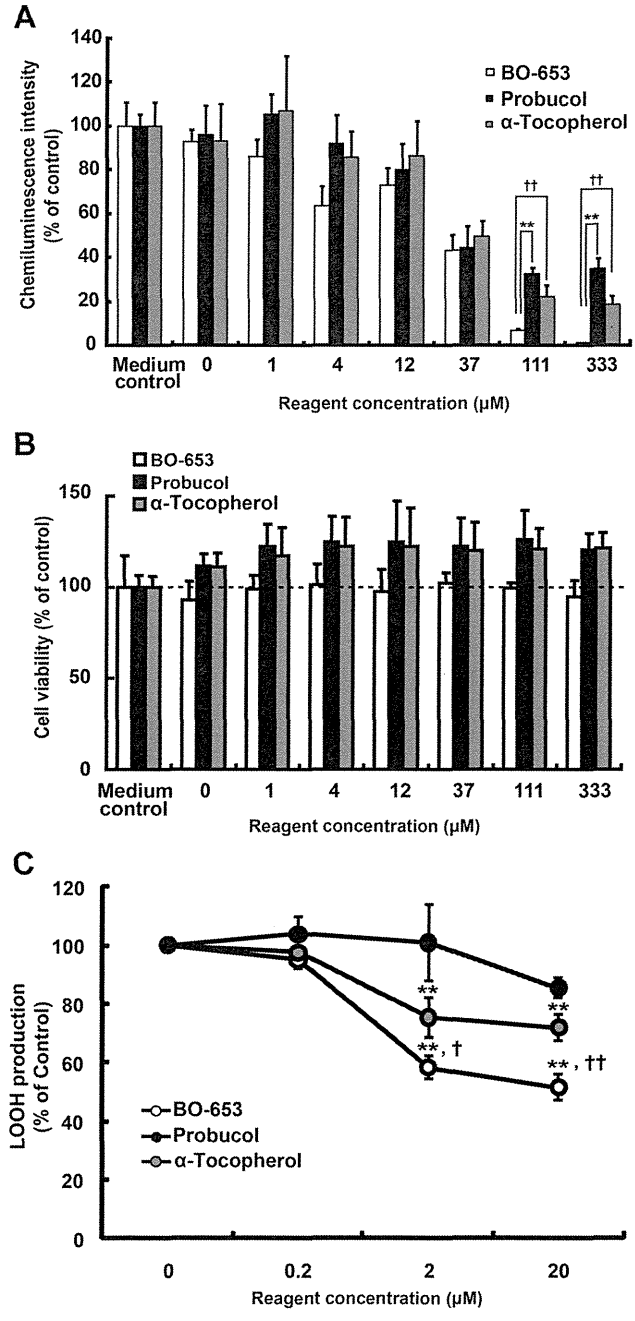

Fig. 2. Comparison of the in vitro anti-HCV activities of lipophilic antioxidants. **A**: The anti-HCV activity and (**B**) cytotoxicity of BO-653, α-tocopherol, and probucol were measured in FLR3-1 cells after 72 hr exposure to the compounds; $^{**}P < 0.01$, compared with probucol at the same concentration, $^{\dagger\dagger}P < 0.01$, 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 $^{\dagger}P < 0.05$ and $^{\dagger\dagger}P < 0.01$, 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

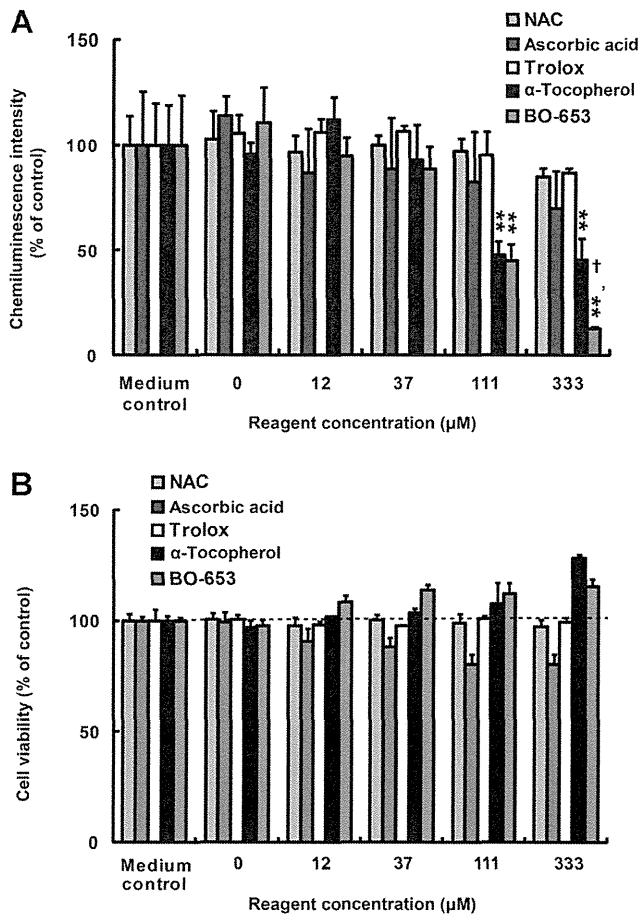

Fig. 3. Comparison of the anti-HCV activity of lipophilic and hydrophilic antioxidants. **A**: The anti-HCV activity and (**B**) cytotoxicity of BO-653 and α-tocopherol (lipophilic antioxidants) and *N*-acetyl cysteine (NAC), ascorbic acid, and trolox (hydrophilic antioxidants) in FLR3-1 cells after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

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	

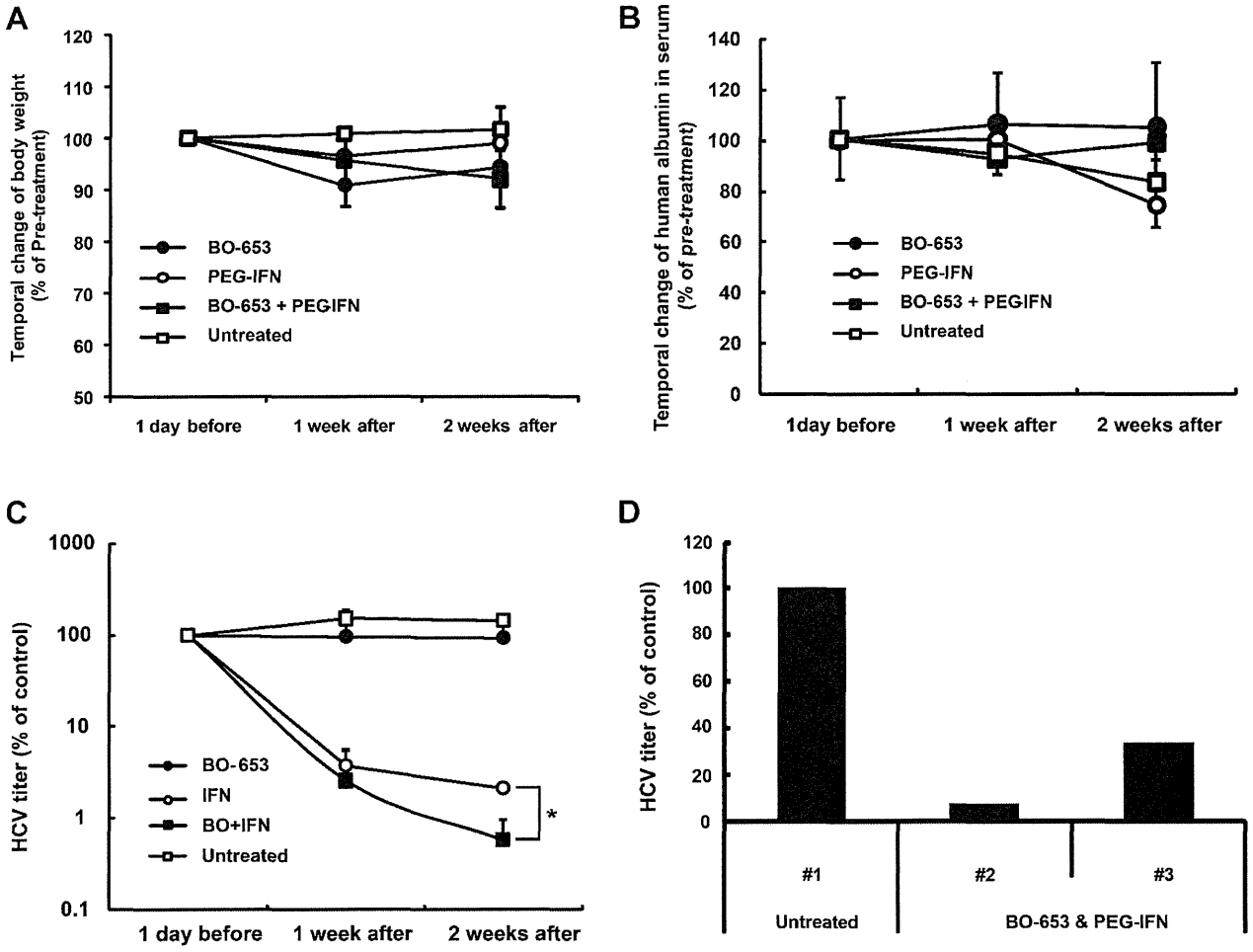

Fig. 4. Anti-HCV effect of BO-653 in chimeric mice infected with HCV. Temporal changes in the (**A**) body weight, (**B**) human albumin concentration in the serum, and (**C**) HCV RNA titer in the serum of chimeric mice infected with HCV after the indicated treatments. All groups included 3–5 mice, 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.

ACKNOWLEDGMENTS

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Hepatitis C Virus Promotes Expression of the 3β-Hydroxysterol Δ24-Reductase Through Sp1

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Hepatitis C virus (HCV) establishes chronic infection, which often causes hepatocellular carcinoma. Overexpression of 3β-hydroxysterol Δ24-reductase (DHCR24) by HCV has been shown to impair the p53-mediated cellular response, resulting in tumorigenesis. In the present study, the molecular mechanism by which HCV promotes the expression of *DHCR24* was investigated. A significant increase in *DHCR24* mRNA transcription was observed in a cell line expressing complete HCV genome, whereas no significant difference in the expression of *DHCR24* was seen in cell lines expressing individual viral proteins. The 5′-flanking genomic region of *DHCR24* was characterized to explore the genomic region and host factor(s) involved in the transcriptional regulation of *DHCR24*. As a result, the HCV response element (−167/−140) was identified, which contains AP-2α, MZF-1, and Sp1 binding motifs. The binding affinity of the host factor to this response element was increased in nuclear extracts from cells infected with HCV and corresponded with augmented affinity of Sp1. Both mithramycin A (Sp1 inhibitor) and small interfering RNA targeting Sp1 prevented the binding of host factors to the response element. Silencing of Sp1 also downregulated the increased expression of *DHCR24*. The binding affinity of Sp1 to the response element was augmented by oxidative stress, whereas upregulation of *DHCR24* in cells expressing HCV was blocked significantly by a reactive oxygen species scavenger. Elevated phosphorylation of Sp1 in response to oxidative stress was mediated by the ATM kinase. Thus, activation of Sp1 by oxidative stress is involved in the promotion of expression of *DHCR24* by HCV. ***J. Med. Virol. 84:733–746, 2012.*** © 2012 Wiley Periodicals, Inc.

KEY WORDS: HCV; DHCR24; Sp1; oxidative stress

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INTRODUCTION

Hepatitis C virus (HCV) causes chronic hepatitis and hepatocellular carcinoma [Koike, 2007]. The estimated worldwide prevalence of HCV infection is 2.2–3.0% (130–170 million people) [Lavanchy, 2009], and chronic HCV infection is a major global public health concern. The most effective current treatment for HCV infection comprises combination therapy with PEGylated interferon-α and ribavirin [Bruchfeld et al., 2001; Lu et al., 2008]. However, this therapy has limited clinical efficacy, as sustained virological responses develop in only about half of patients infected with HCV genotype 1 [Kohara et al., 1995; Nakamura et al., 2002]. Efforts to develop therapies to treat HCV are also hindered by the high level of viral variation and the capacity of HCV to cause chronic infection. Therefore, there is an urgent need to develop effective treatments against chronic HCV infection.

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A cell line that expresses complete HCV genome (RzM6-LC) was established to investigate the effects of persistent expression of HCV on cell growth [Tsukiyama-Kohara et al., 2004]. A monoclonal antibody (2-152a mAb) against the RzM6-LC cell line was also developed to produce clones that recognize both cell surface and intracellular molecules. As a result, 3β-hydroxysterol Δ24-reductase (DHCR24) was identified as the target of 2-152a mAb [Nishimura et al., 2009].

DHCR24 is an oxidoreductase with a broad expression pattern and shares homology with a family of flavin-adenine dinucleotide-dependent reductases [Waterham et al., 2001]. In mammals, DHCR24 functions as an enzyme to catalyze the conversion of desmosterol to cholesterol in the post-squalene cholesterol biosynthetic pathway, and it is essential for normal tissue development and maintenance [Waterham et al., 2001; Crameri et al., 2006]. DHCR24 regulates cholesterol synthesis and promotes recruitment of domain components into detergent-resistant membrane fractions [Crameri et al., 2006]. An absence of DHCR24 leads to desmosterolosis—a rare disorder of cholesterol biosynthesis [Waterham et al., 2001]. Expression of DHCR24 is downregulated in areas of the brain affected by Alzheimer's disease [Greeve et al., 2000], suggesting that DHCR24 has alternative functions. Indeed, DHCR24 is also known as seladin-1 (the selective Alzheimer's disease indicator 1), reflecting the association between DHCR24/seladin-1 and the selective vulnerability of the neurons in the affected areas of the brain. High levels of DHCR24/seladin-1 exert protective effects, conferring resistance against oxidative stress and preventing apoptotic cell death [Greeve et al., 2000; Benvenuti et al., 2005; Di Stasi et al., 2005; Luciani et al., 2005; Lu et al., 2008]. Endogenous DHCR24/seladin-1 levels are upregulated in response to acute oxidative stress [Wu et al., 2004; Benvenuti et al., 2006; Kuehnle et al., 2008], whereas the expression declines to low levels upon chronic exposure [Benvenuti et al., 2006; Kuehnle et al., 2008]. Therefore, DHCR24/seladin-1 may be involved in integrating cellular responses to oxidative stress. DHCR24 also functions as a hydrogen peroxide scavenger [Lu et al., 2008]. Based on these findings, DHCR24 may play a crucial role in maintaining cellular physiology by regulating both cholesterol synthesis and cellular defense against oxidative stress.

HCV infection impairs apoptosis induced by oxidative stress and inhibits p53 function via overexpression of DHCR24 [Nishimura et al., 2009]. Augmented expression of DHCR24 also facilitates efficient replication of HCV [Takano et al., 2011b]. Since DHCR24 may play a significant role in viral replication and in the tumorigenicity of the hepatocellular carcinoma related to HCV, the molecular mechanism of overexpression of DHCR24 in response to HCV was examined in the present study.

MATERIALS AND METHODS

Cell Lines

The HepG2 hepatoblastoma cell line, the HepG2-derived RzM6 cell line, which is capable of conditional control of expression of HCV genome (genotype 1b) based on the Cre/*loxP* system (RzM6-0d, no switching; RzM6-LC, switching of full genome HCV induced by tamoxifen), and HepG2-derived CN5 cell line, in which all HCV proteins were expressed conditionally by cre adenovirus (CN5-Cre) [Tsukiyama-Kohara et al., 2004] were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Using a stable expression system based on lentiviral vectors, HepG2/Lenti cell lines (core, E1, E2, NS2, NS3/4A, NS4B, NS5A, and NS5B) were established [Takano et al., 2011a]. An additional cell line—HepG2-emp—was infected with an empty lentiviral vector. Cells from the human hepatoma HuH-7 cell line were maintained in DMEM supplemented with 10% FBS and 0.4% glucose. The cell lines harboring HCV replicon, namely, R6FLR-N (genotype 1b) and FLR3-1 (genotype 1b), which are derived from HuH-7 [Takano et al., 2011b], were maintained under selective pressure with G418 (500 μg/ml for R6FLR-N and FLR3-1) in DMEM GlutaMAX (Invitrogen, Carlsbad, CA) containing 10% FBS. Cured/HuH-7 K4 cells—cured of HCV by interferon-α treatment—were maintained in DMEM GlutaMAX containing 10% FBS without G418. The JFH/K4 cell line, which shows persistent infection with the HCV JFH-1 strain, was maintained in DMEM containing 10% FBS. The human fetal hepatic cell line WRL68 was obtained from the American Type Culture Collection and maintained in DMEM supplemented with 10% FBS, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids. The human hepatoma cell line PLC/PRF/5 was obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University, and cultured in Eagle's minimum essential medium supplemented with 10% FBS.

Construction of DHCR24 Promoter Reporter Plasmids

Genomic DNA was extracted from HepG2 cells, and the 5′-flanking sequence of the predicted transcription start site of *DHCR24* (~5 kb) was isolated. The genomic region was inserted upstream of the firefly luciferase gene in pGL3-Basic (Promega, Madison, WI). Deletion constructs of the *DHCR24* promoter region were constructed using restriction enzymes and PCR (sense primer for −4956, 5′-GATCCTCGAGCACTCCTGCTCACCACTGAT-3′; sense primer for −2982, 5′-GATCCTCGAGGAGGCTCACATTGTAGAAAG-3′; antisense primer, 5′-GTAGTAGATATCGAAGATAAGCGAGAGCGG-3′) and cloned into pGL3-Basic at the *Xho*I and *Nco*I sites.

Dual Luciferase Reporter Assay

HepG2 cells (1×10^4 cells/well in a 96-well plate) were transfected with each of the 3 *DHCR24* promoter reporter plasmids and their deletion constructs (0.25 μg/well) using cationic lipid (Lipofectamine LTX, Invitrogen). Samples were analyzed with the Dual-Glo Luciferase Assay System (Promega) at 48 h post-transfection, and luminescence was measured using a TriStar LB941 microplate reader (Berthold, Bad Wildbad, Germany). To account for differences in transfection efficiency, the luminescence produced by firefly luciferase (FL) was normalized to that produced by Renilla luciferase (RL), which was expressed by co-transfection with phRL-TK (Promega; 0.025 μg/well).

Electrophoresis Mobility Shift Assay

Nuclear extracts were prepared from 5×10^6 to 1×10^7 cells as described previously [Dignam et al., 1983]. Electrophoresis mobility shift assays (EMSAs) were performed by a nonradioactive method using the DIG Gel Shift Kit (Roche, Indianapolis, IN). Briefly, binding reactions were performed by mixing the following components: 1 μg of poly[d(I-C)], 0.1 μg of poly L-lysine, 40 fmol DIG-labeled double-stranded oligonucleotide probe (HCV response element −167/−140 [28-mer], 5′-CCCCCGCCTCGCGCGGCGGCGGGGAGAA-3′; Sp1 consensus sequence [22-mer], 5′-ATTCGATCGGGGCGGGGCGAGC-3′; MZF1.1-4 consensus sequence [21-mer], 5′-GATCTAAAAGTGGGGAGAAAA-3′; AP-2α consensus sequence [26-mer], 5′-GATCGAACTGACCGCCCGCGGCCCGT-3′), and 10 μg of the nuclear extract in binding buffer (10 mM Tris–HCl, pH 7.5; 50 mM NaCl; 5 mM $MgCl_2$; 0.5 mM EDTA). Where indicated, reactions were supplemented with unlabeled/competitive oligonucleotide at a 50-fold molar excess concentration before addition of the probe. Mithramycin A (MMA; Sigma, St. Louis, MO), which blocks the binding of Sp1 to target sequences, was added at different final concentrations (2.5, 5, and 10 μM) and incubated at 4°C for 1 h. For supershift assays, 1 μg of monoclonal anti-Myc Tag antibody (Upstate Biotechnology, Lake Placid, NY) was added 30 min prior to addition of the probe. Binding reactions were carried out at 25°C for 30 min and electrophoresed on 6% acrylamide–0.5× TBE gels, transferred to positively charged nylon membranes, and detected by a chemiluminescence method (Roche) and a LAS1000 scanner (Fujifilm Co., Tokyo, Japan).

Silencing of Sp1, HCV, and DHCR24 by siRNA

SP1 Validated Stealth RNAi™ siRNA (VHS40867, Invitrogen) was designed with the BLOCK-iT RNAi designer to target the human Sp1 mRNA sequence. RzM6-0d and RzM6-LC cells (1.5×10^6 cells in a 100-mm dish) were transfected with Sp1 siRNA (final concentration, 30 nM) using Lipofectamine RNAiMAX (Invitrogen) in Opti-MEM (Invitrogen) and incubated for 48 h at 37°C. The siRNAs specific for DHCR24 and HCV were designed and utilized as described previously [Nishimura et al., 2009].

Kinase Inhibitors

ATM kinase inhibitor KU55933 (Wako Pure Chemical Industry, Osaka, Japan; final concentration, 10 μM), PI3K inhibitor LY294002 (Cell Signaling Technology, Beverly, MA; 50 μM), and MEK1 inhibitor PD98059 (Cell Signaling Technology; 50 μM) were added to cell cultures, which were incubated for 8 h at 37°C.

Western Blotting

Western blotting was performed as described previously [Tsukiyama-Kohara et al., 2004] with the following primary antibodies: rabbit monoclonal anti-DHCR24/Seladin-1 (C59D8; Cell Signaling Technology); rabbit polyclonal anti-Sp1, anti-phospho-Akt (Ser473), and mouse monoclonal anti-phospho-ERK (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); mouse monoclonal anti-HCV core (clone 31-2), E1 (clone 384), E2 (clone 544), NS4A (c14II-2-1), NS5A (32-2), NS5B (14-5), rabbit polyclonal anti-NS2, NS3 (R212), and NS4B (RR10) [Tsukiyama-Kohara et al., 2004]. Phosphorylation of Sp1 was investigated by 5% SDS–PAGE and immunoblotting with a polyclonal antibody against Sp1 phosphorylated at Ser101 (Active Motif, Carlsbad, CA) or Thr453 (Abcam, Cambridge, MA). Detection of γH2AX was performed by 15% SDS–PAGE and immunoblotting with mouse monoclonal anti-phospho-histone H2AX (Ser139) (JBW301; Upstate Biotechnology). Phosphorylated ATM (Ser1981) and ATR (Ser428) were detected by specific antibodies (Cell Signaling Technology). Monoclonal anti-actin (Sigma), anti-histone H1 (Santa Cruz Biotechnology, Inc.), anti-HAUSP (Calbiochem, San Diego, CA), and anti-heat shock protein 90 (Stressgen, Victoria, BC, Canada) primary antibodies were used for normalization of Western blotting. Bound antibody was detected with a horseradish peroxidase-conjugated secondary antibody and visualization using ECL reagents (GE Healthcare, Piscataway, NJ) and an LAS1000 scanner (Fujifilm). Densitometric analysis of protein bands was performed with Image Quant software (Molecular Dynamics, Sunnyvale, CA).

Quantitative PCR and HCV Infection

Total RNA was extracted from cell lines using ISOGEN, and reverse transcription of total RNA (125 ng) was performed with SuperScript III Reverse Transcriptase and Random Primers (Invitrogen). Synthesized cDNA samples were subjected to a TaqMan gene expression assay (Applied Biosystems, Foster City, CA), and the level of expression of *DHCR24* mRNA in each sample was normalized to the level of expression of *GAPDH* mRNA and represented as a ratio of the control (Hep-emp, CN5-Hep, or RzM6-0d). Infection of the human hepatocytes from human liver-uPA/SCID chimeric mice with HCV was performed,

and HCV RNA, *DHCR24* mRNA, and 18S rRNA were measured by quantitative PCR (qPCR), as described previously [Takano et al., 2011a].

Statistical Analysis

The Student's *t*-test was used to analyze the statistical significance of the results; P values < 0.05 were considered statistically significant.

RESULTS

DHCR24 Expression Is Upregulated by the Complete HCV Genome But Not by Individual Viral Proteins

Overexpression of *DHCR24* in human hepatocytes from human liver-uPA/SCID chimeric mice has been observed after HCV infection (Fig. 1A). The overexpression of *DHCR24* in cells expressing HCV decreased to a similar extent as that observed in control cells following treatment with HCV siRNA (Fig. 1B). Since these findings suggest that overexpression of *DHCR24* is associated with the expression or infection by HCV, the identity of the viral factor involved in the augmentation of expression of *DHCR24* was examined. The level of expression of *DHCR24* mRNA was measured by quantitative RT-PCR (Fig. 1C) in HepG2-derived cell lines that stably express individual HCV proteins (core, E1, E2, NS2, NS3/4A, NS4B, NS5A, or NS5B; Supplementary Fig. 1). The level of expression of *DHCR24* mRNA was slightly higher in the cells expressing NS4B and NS5A than in control cells; however, there was no significant difference in the expression of *DHCR24* mRNA among these cell

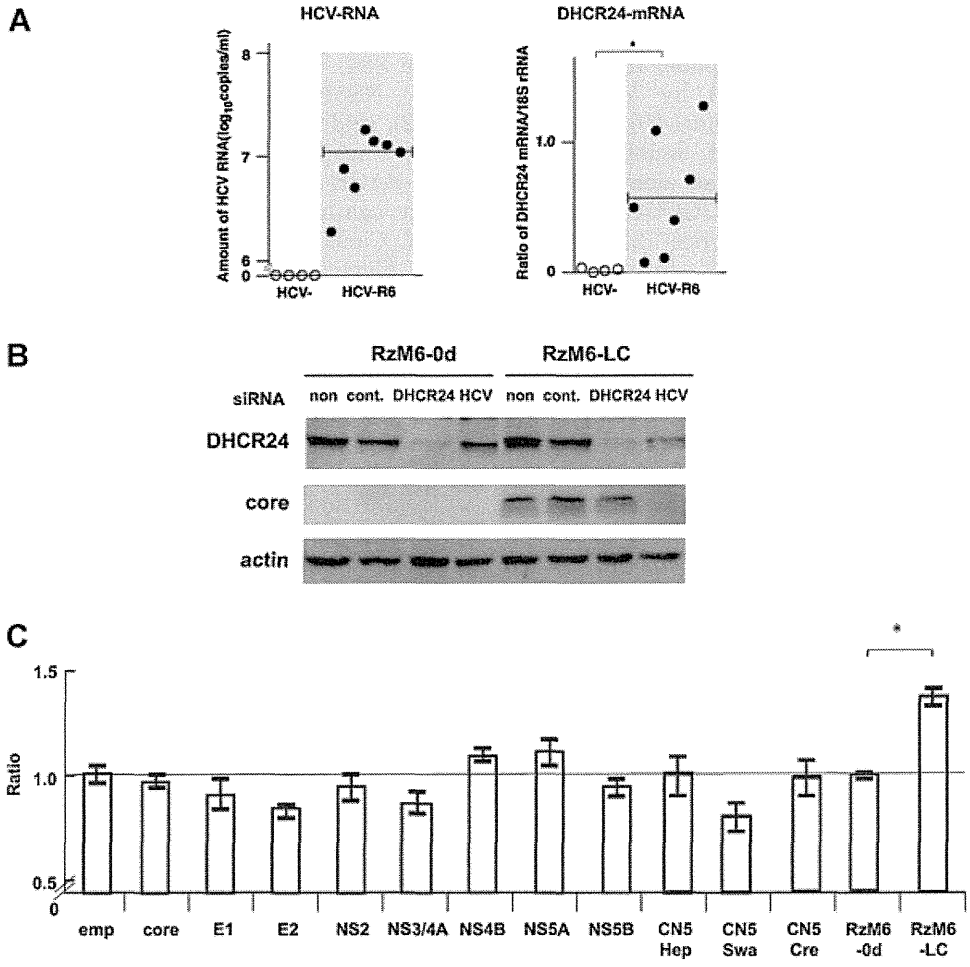

Fig. 1. *DHCR24* expression is induced in the presence of the complete HCV genome. **A**: The amount of HCV RNA in chimeric mice infected with HCV-R6 (genotype 1b) was quantified by qPCR (left panel). The amount of DHCR24 mRNA was measured, and the ratio to the amount of 18S rRNA was calculated in the tissues (right panel). **B**: Western blotting of DHCR24, HCV core, and actin protein in RzM6-0d and LC cells following treatment with the indicated siRNA. **C**: Level of *DHCR24* mRNA expression in cell lines with stable expression of individual HCV proteins, the HCV open reading frame, or the complete HCV genome. Total RNA from HepG2/Lenti cell lines (emp, core, E1, E2, NS2, NS3/4A, NS4B, NS5A, and NS5B), CN5 cell lines (CN5-Hep, CN5-Swa, and CN5-Cre), or RzM6 cell lines (RzM6-0d and RzM6-LC) were prepared, and reverse transcription was performed. Synthesized cDNA was subjected to quantitative PCR. The level of expression of *DHCR24* mRNA for each sample was normalized to that of *GAPDH* mRNA and represented as a ratio of HepG2-emp ($^*P < 0.05$).

lines. No significant upregulation of *DHCR24* mRNA was observed in the CN5-Cre cell line, which expresses all HCV proteins and is negative for viral replication [Tsukiyama-Kohara et al., 2004]. In contrast, significant upregulation of *DHCR24* was observed in a cell line that expresses the complete HCV genome (RzM6-LC) compared with the expression in HCV-negative control cells (RzM6-0d). Thus, expression of viral proteins alone is insufficient to reproduce the augmentation of expression of *DHCR24* induced by HCV.

DHCR24 Promoter Activity Is Potentiated by the Expression of HCV

The 5′-flanking region contains a number of possible transcriptional regulatory elements, including three candidate-binding motifs for the endoplasmic reticulum (ER) stress-responsive transcription factor, XBP1. Cellular ER stress is induced in response to the expression of the HCV gene and infection by HCV [Tardif et al., 2005]. Thus, to explore host factors involved in the transcriptional regulation of *DHCR24*, the 5′-flanking genomic region (~5 kb) of *DHCR24* was isolated. Subsequently, *DHCR24* promoter reporter plasmids that contain the 5′-flanking region of *DHCR24* and the firefly luciferase gene were constructed (Fig. 2A). Relevant regions of the promoter were defined by constructing deletion mutants of the 5′-flanking regions, which were analyzed by a dual luciferase reporter assay in the presence or absence of full-length HCV genome expression—resulting from transfection with pCA-Rz [Tsukiyama-Kohara et al., 2004] or the control pCAGGS vector, respectively (Fig. 2B). Progressive shortening of the 5′-flanking regions did not

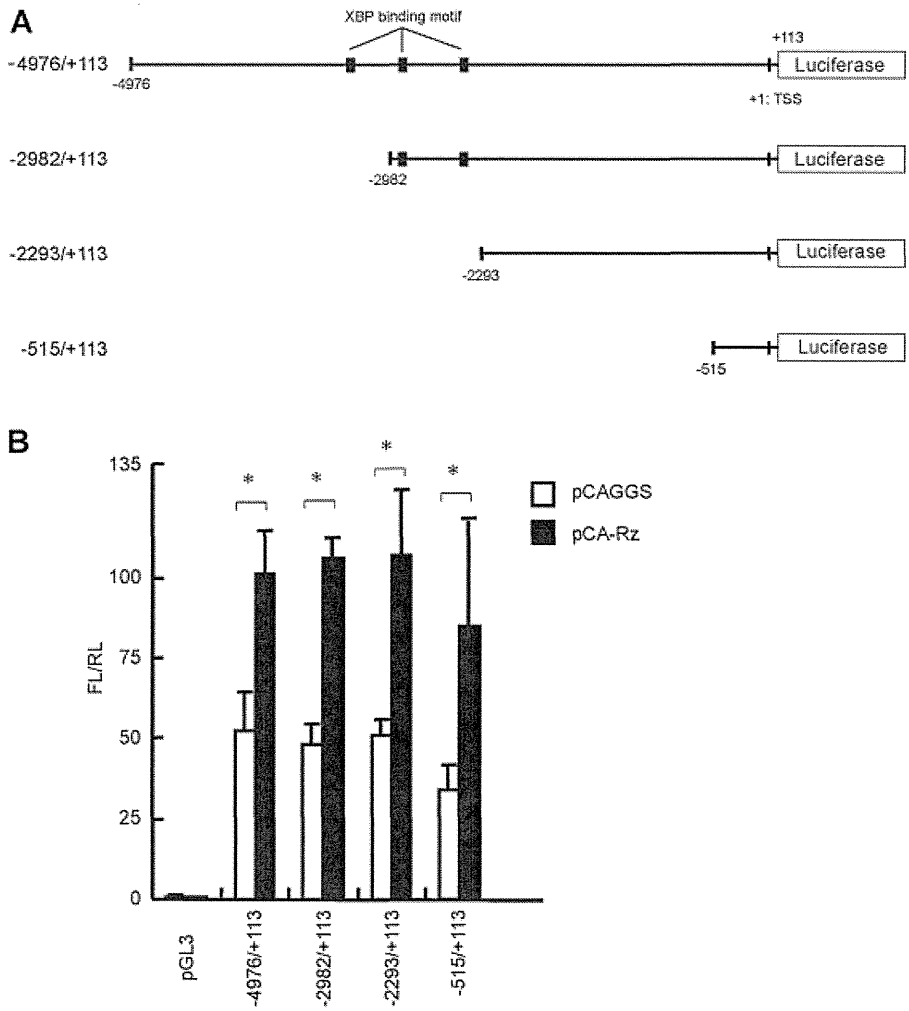

Fig. 2. *DHCR24* promoter activity is augmented by the expression of HCV. **A:** The 5′-flanking region of *DHCR24* was cloned from HepG2 and inserted upstream of the firefly luciferase gene in pGL3 (−4976/+113). A deletion series of the 5′-flanking regions was also constructed (−2982/+113, −2293/+113, and −515/+113). The black boxes indicate potential binding sites for the ER stress-responsive transcription factor, XBP-1. TSS, transcription start site (+1). **B:** HepG2 cells (1×10^4 cells/well in a 96-well plate) were co-transfected with each *DHCR24* promoter reporter plasmid (0.25 μg/well), a Renilla luciferase expression vector (phRL-TK; 0.025 μg/well), and either an expression vector containing the HCV full-length genome (pCA-Rz; 0.5 μg/well) or an empty expression vector (pCAGGS). Luciferase activity at 48 h post-transfection is shown as the ratio of firefly luciferase (FL) to Renilla luciferase (RL). Data are shown as the mean ± SD from 2 representative experiments performed in triplicate ($^*P < 0.05$).

result in significant differences in the basal promoter activity (Fig. 2B). The −515/+113 construct also produced a significant response in the presence of full-length HCV genome expression.

Additional reporter deletion mutants were constructed to define the region in the *DHCR24* promoter that is responsive to HCV expression. To this aim, potential binding motifs for transcription factors were predicted in the minimized *DHCR24* promoter sequence (nucleotides –515/+113; Fig. 3A), and a series of promoter mutants containing sequential 100-bp deletions was constructed. As shown in Fig. 3A, while the promoter activity of −515/+113, −400/+113, −300/+113, and −200/+113 constructs was increased significantly by expression of HCV ($^*P < 0.05$), the promoter activity of the −100/+113 construct was unchanged. Therefore, an HCV-responsive sequence appears to be located in the upstream region (−200 to −100 bp) from the transcriptional start site of *DHCR24*, which includes sequences with similarity to the consensus-binding motifs for AP-2α, Sp1, MZF-1, Pax-4, and NF-Y.

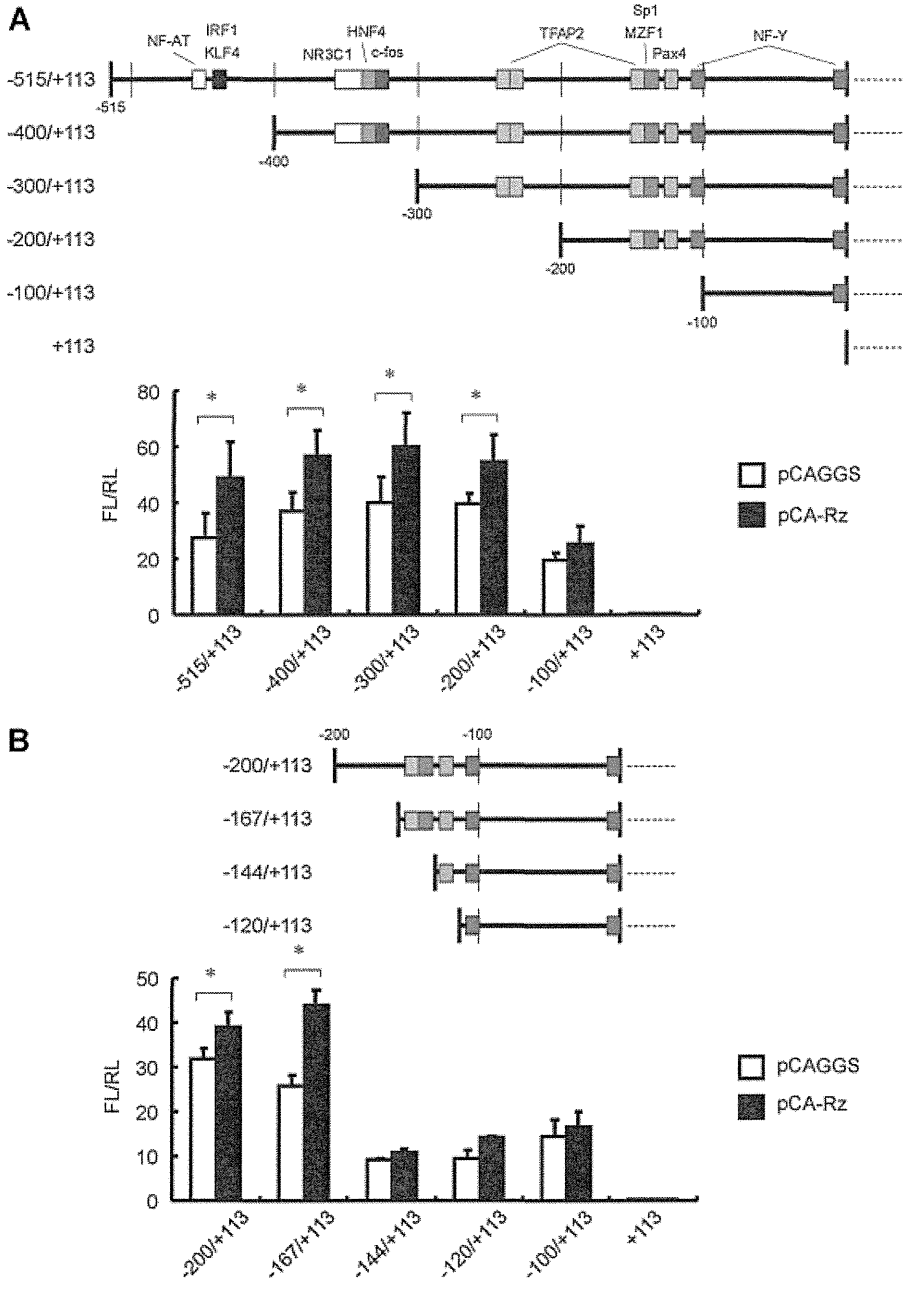

Fig. 3. Responsiveness of the *DHCR24* promoter to the expression of HCV. **A**: A *DHCR24* promoter series with sequential 100-bp deletions was constructed and analyzed as described in the legend to Fig. 2B ($^*p < 0.05$). **B**: An additional deletion series (−167/+113, −144/+113, and −120/+113) was constructed and analyzed as described in (A).