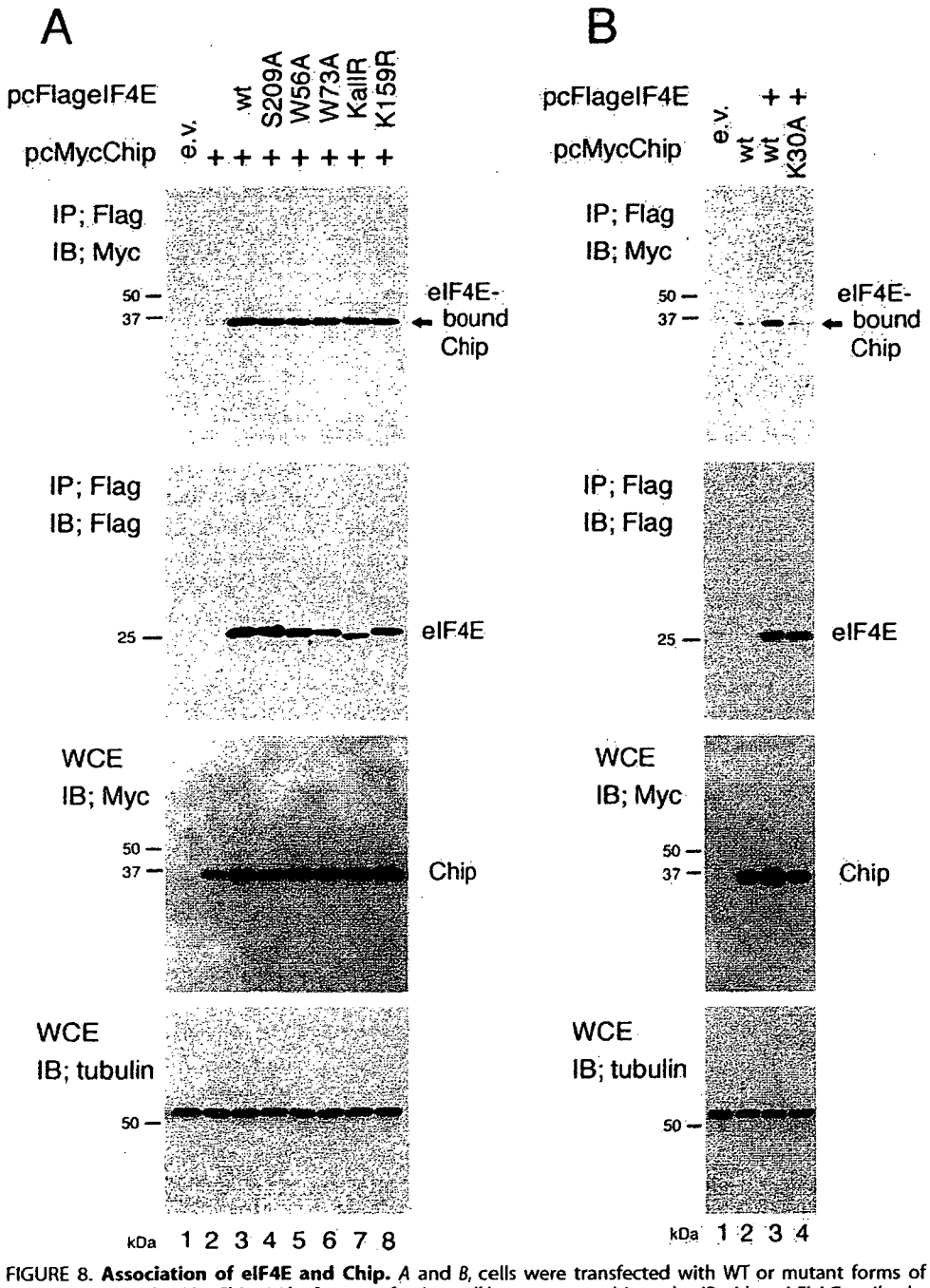

FIGURE 8. **Association of eIF4E and Chip.** *A* and *B*, cells were transfected with WT or mutant forms of pcFLAGeIF4E and pcMycChip. 24 h after transfection, cell lysates were subjected to IP with anti-FLAG antibody, followed by IB with anti-Myc antibody (*top panels*). The membrane was then stripped and reprobed with anti-FLAG antibody (*2nd panels*). The WCE was subjected to IB with anti-Myc (*3rd panels*) or tubulin (*bottom panels*) antibody.

Heat shock and Chip expression could additively or synergistically enhance eIF4E ubiquitination. We co-transfected cells with FLAG-tagged eIF4E, HA-Ub, and/or Myc-Chip, and samples were collected 2 h after heat shock (45 °C 10 min) (Fig. 9*C*). Although heat shock or Chip expression independently induced the ubiquitination of eIF4E (Fig. 9*C*, *top panel*, *lanes 3* and *4*), the combination of heat shock and Chip expression did not increase ubiquitination levels (*lane 5*). It is likely that each condition alone induced maximal eIF4E ubiquitination.

Ubiquitination and Degradation of Endogenous eIF4E—We then wished to see the ubiquitination of endogenous eIF4E (Fig. 10*A*). It was not visible under normal condition, but mono-ubiquitinated endogenous eIF4E was detected (Fig. 10*A*, *middle panel*, *lane 3*) when cells were heat shocked, eIF4E protein was concentrated by m^7GTP-Sepharose precipitation, and highly effective ECL solution was used. The 24-kDa band for endogenous eIF4E whited out (Fig. 10*A*, *middle panel*) because the ECL solution was too strong.

In Fig. 10, *B* and *C*, we carried out pulse-chase labeling of endogenous eIF4E. With heat shock, the levels of eIF4E decreased more rapidly (down to 55% at 15 h) and addition of MG132, an inhibitor of proteasome-dependent degradation pathway, restored the levels. These results indicate that endogenous eIF4E protein is also degraded in the Ub/proteasome-dependent manner.

DISCUSSION

Protein synthesis is a tightly controlled process essential for cell survival, and in this study we described the ubiquitination and proteasome-dependent degradation of the cap-binding protein eIF4E. We summarize our working hypothesis in supplemental Fig. S2.

We identified the most probable site for Ub conjugation as Lys-159. The crystal structure of eIF4E has been solved (23–25), and Lys-159 is in the middle of two β-sheets, S5 and S6. The side chain of this residue protrudes outward (25), suggesting it is readily accessible for Ub conjugation by E3 ligases. Interestingly, there is a relationship between Lys-159 and the phosphorylated residue Ser-209. Phosphorylation of Ser-209 causes a retractable salt bridge to form with Lys-159 to clamp the cap moiety and thereby lead to increased binding of capped mRNA (23, 26). In addition, because the K159A mutant exhibited reduced association with cap analogues (27), Lys-159 is involved in the binding of capped mRNA. However, we saw no reduction in m^7GTP association with the K159R mutant (Fig. 3). Additionally, we did not observe reduced m^7GTP association with ubiquitinated eIF4E. The attachment of a Ub molecule to Lys-159 might stabilize the distance between Ser-209

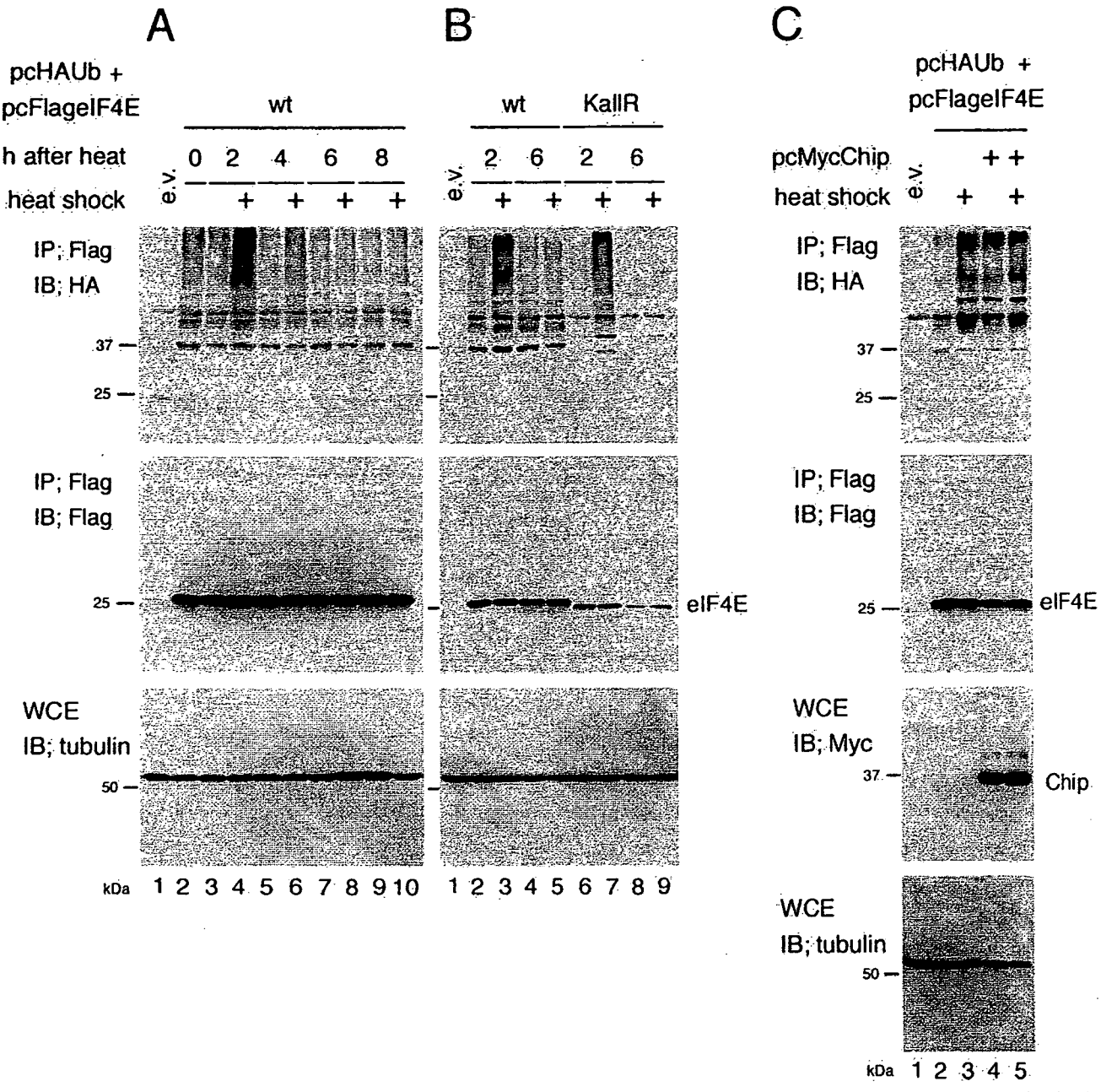

FIGURE 9. **Increased ubiquitination of eIF4E following heat shock.** *A* and *B*, heat shock enhanced eIF4E ubiquitination. Cells were transfected with pcHAUb and WT (*A*, *B*) or the KallR mutant (*B*) of pcFLAGeIF4E. 24 h after transfection, cells were heat shocked at 45 °C for 10 min (+) or not, followed by incubation at 37 °C for the indicated time. The cell lysates were subjected to IP with anti-FLAG antibody, followed by IB with anti-HA antibody (*top panels*). The membrane was then stripped and reprobed with anti-FLAG antibody (*middle*). Levels of tubulin in the WCE were also examined (*bottom*). *C*, heat shock and Chip did not additively increase eIF4E ubiquitination. Cells were transfected with pcHAUb, pcFLAGeIF4E, and/or with pcMycChip. 24 h after transfection cells were heat shocked at 45 °C for 10 min (+) or not, followed by incubation at 37 °C for 2 h. The cell lysates were subjected to IP with anti-FLAG antibody, followed by IB with anti-HA antibody (*top panel*). The membrane was then stripped and reprobed with anti-FLAG antibody (*2nd panel*). Levels of Chip (*3rd panel*) and tubulin (*bottom*) in the WCE were also assessed.

(S7-S8 loop) and Lys-159 (S5-S6 loop), similar to phosphorylation (23). Alternatively, the Ub molecule may form a bridge between Ser-209 (S7-S8 loop) and Lys-159 (S5-S6 loop) and hold the triphosphate moiety tightly. Additionally, ubiquitination of the S209A mutant of eIF4E was comparable with WT, suggesting that phosphorylation does not affect ubiquitination (Fig. 1*C*).

The W73A mutant of eIF4E, which only weakly binds eIF4G/4E-BP, showed enhanced ubiquitination and proteasome-dependent degradation (Fig. 1). What is more, expression of 4E-BP clearly reduced the levels of eIF4E ubiquitination and degradation (Fig. 6). Because the only known role for 4E-BP has been as inhibitor of cap-mediated translation initiation, we are suggesting a novel role for 4E-BP as a protector of eIF4E. 4E-BP can inhibit cap-mediated protein synthesis by binding eIF4E, but at the same time may preserve a population of inactive eIF4E-mRNA complexes by preventing eIF4E degradation. Several reports have linked 4E-BP to cellular stress (28–30). 4E-BP binds eIF4E under conditions of stress or reduced growth stimuli. When cells are removed from stress and growth conditions become favorable,

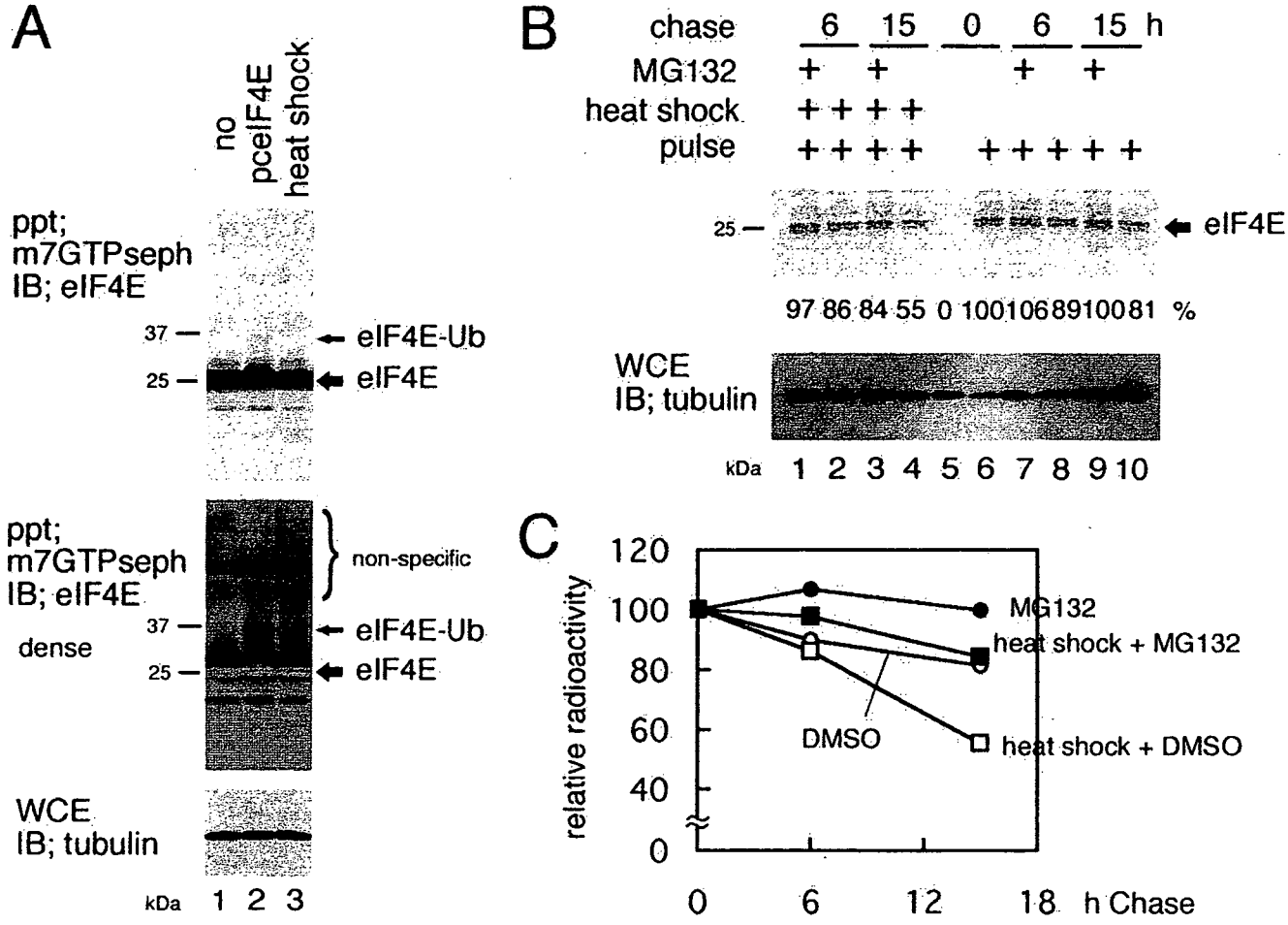

FIGURE 10. **Ubiquitination and degradation of endogenous eIF4E.** *A*, ubiquitination of endogenous eIF4E. Cells were heat shocked (*lane 3*) or mock treated (*lane 1*). As a positive control, cells were transfected with pceIF4E (*lane 2*) for 24 h. Lysates were precipitated with m^7GTP-Sepharose and subjected to SDS-PAGE and IB with anti-eIF4E antibody (*top* and *2nd panels*). As a control, a portion of the WCE was directly subjected to SDS-PAGE and IB was performed with anti-tubulin antibody (*bottom panel*). To assess ubiquitination levels of endogenous eIF4E, the membrane in the *top panel* was overexposed in the *2nd panel*, using highly efficient ECL solution (see "Experimental Procedures"). *B* and *C*, proteasome-dependent degradation of endogenous eIF4E. Cells were pulse labeled with [^{35}S]Met (36) for 3 h, washed extensively, and then incubated with cold chase medium with or without MG132 for the indicated hours. Cells for *lanes 1–4* were heat shocked immediately after the pulse label. The lysates were purified with m^7GTP-Sepharose and subjected to SDS-PAGE, and the radioactivity was visualized using BAS2000 system (*B, upper panel*). As a control, a portion of the WCE was directly subjected to IB with anti-tubulin antibody (*lower panel*). *C*, radioactivities in *panel B* (*upper*) were determined and shown as a line chart.

pre-existing eIF4E-mRNA complexes can be used for immediate protein synthesis after 4E-BP release. Thus, 4E-BP buffers stress by suppressing protein synthesis and preparing cells for a swift recovery.

It is quite interesting to find that the levels of endogenous eIF4E ubiquitination were very low (*e.g.* Fig. 10*A*), whereas degradation of the protein was relatively clear (*e.g.* Fig. 10, *B* and *C*). Ubiquitinated fraction was <1% in Fig. 10*A*, whereas the eIF4E decreased by 45% at 15 h after heat shock in Fig. 10, *B* and *C*. This is similar to the ubiquitination/degradation of the W73A mutant in that most of the W73A mutant protein stayed non-ubiquitinated (Fig. 1*C*, *middle panel*, *lane 6*), whereas >90% of eIF4E degraded at 48 h (Fig. 1*E*, *upper panel*, *lane 4*). We thus speculate that the efficiency of Ub conjugation is the bottleneck, and once ubiquitinated, it is proteolyzed rapidly.

The mechanism of eIF4E ubiquitination/degradation is similar to that of the endoplasmic reticulum membrane-tethered dolichol-phosphate-mannose (DPM) synthase (31). DPM1 is tethered by DPM3 to the membrane. When tethering is abolished and DPM1 becomes free, DPM1 is rapidly ubiquitinated by Chip and degraded by the proteasome. Telomeric repeat binding factor 1 and E2F transcription factors are also protected from Ub targeting and degradation by binding their partners, and these proteins are degraded after binding partner release (32, 33). Because these proteins function as complexes, it is likely important to regulate the levels of the free proteins. Thus, this may be a common mechanism of protein expression regulation in cells.

When examining the bottom panel of Fig. 6*B*, levels of 4E-BP, especially 4E-BP2 and 3, were increased by the addition of MG132. This suggests that 4E-BP is degraded in a Ub/proteasome-dependent manner. As 4E-BP is an important regulator of protein synthesis, the regulation of this process is of great interest.

During the preparation of this report, Othumpangat *et al.* (11) reported that eIF4E is proteolyzed after ubiquitination when cells are exposed to cadmium chloride. We confirmed this finding (data not shown), and because cadmium presumably acts as a cell stressor, this finding supports our hypothesis that Ub modification of eIF4E is enhanced following cell stress.

Cells respond to stress or apoptotic stimuli through regulating protein synthesis levels (21, 22). Nevertheless, the molecular mechanisms regulating cell reactions to such stimuli remain elusive. Chip, an E3 ligase, and stresses, such as heat shock and cadmium, enhanced the Ub conjugation of eIF4E. Because Chip is involved in the quality control of proteins in cells (34, 35), the ubiquitination/degradation of eIF4E may be, at least in part, controlling protein synthesis in response to stress.

eIF4E plays an important role in translation initiation, and it is important to understand the processes regulating eIF4E protein expression levels, including its degradation. Because eIF4E functions in association with many factors, future studies should examine the role of eIF4E ubiquitination within the context of the entire translation initiation complex.

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Evaluation of a Cyclophilin Inhibitor in Hepatitis C Virus–Infected Chimeric Mice *In Vivo*

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Cyclosporin A (CsA) inhibits replication of the HCV subgenomic replicon, and this effect is believed to not be mediated by its immunosuppressive action. We found that DEBIO-025, a novel non-immunosuppressive cyclophilin inhibitor derived from CsA, inhibited HCV replication *in vitro* more potently than CsA. We also examined the inhibitory effect of DEBIO-025 on naive HCV genotypes 1a or 1b *in vivo* using chimeric mice with human hepatocytes. These mice were treated for 14 days with DEBIO-025, pegylated-interferon α−2a (Peg-IFN), a combination of either drugs, or CsA in combination with Peg-IFN. In mice treated with Peg-IFN, serum HCV RNA levels decreased approximately 10-fold whereas DEBIO-025 treatment alone did not induce any significant change. In mice treated with both DEBIO-025 and Peg-IFN, HCV RNA levels decreased more than 100-fold. All mice treated with Peg-IFN combined with CsA died within 4 days. The combination treatment of DEBIO-025 and Peg-IFN reduced HCV RNA levels and core protein expression in liver, indicating that the HCV RNA levels reduction in serum was attributable to intrahepatic inhibition of HCV replication. *Conclusion:* We demonstrated that DEBIO-025 was better tolerated than CsA, and that its anti-HCV effect appeared to be synergistic in combination with Peg-IFN *in vivo*. (HEPATOLOGY 2007;45:921-928.)

Hepatitis C virus is a small enveloped RNA virus that belongs to the *Flaviviridae* family.[1] A hallmark of HCV infection is its high propensity to establish a persistent infection that evades the host immune response, leading to chronic liver disease, chronic hepatitis, cirrhosis, and hepatocellular carcinoma.[2,3] Although approximately 170 million individuals are infected with HCV worldwide, drugs that are specifically active against hepatitis C are not yet available.

Currently, the main therapy for chronic hepatitis C is a combination of pegylated interferon alpha (Peg-IFN) and ribavirin. In the intention-to-treat analysis, this combination therapy led to a sustained virological response in approximately 55%[4,5] of patients infected with any HCV genotype and in 42%[4] to 46%[5] of patients with genotype 1. The results of clinical trials were based on selected patients. The proportion of elderly patients was low, and patients with HBV or HIV coinfection, renal disease, post-transplantation status, or hematological disorders were excluded.[4-8] Because approximately 50% of patients show a poor response to combined treatment with Peg-IFN and ribavirin, effective therapies are urgently needed.

We previously reported that combination therapy of interferon (IFN) α-2b and cyclosporin A (CsA) for 24 weeks produced a sustained virological response in 42% of patients with both HCV genotype 1b and high viral levels.[9] High blood levels of CsA correlate with virological response during treatment for HCV, but occasionally can cause adverse events related to immunosuppression.[10] CsA also suppresses HCV replication *in vitro*, by inhibiting the interaction between HCV nonstructural protein 5B and cyclophilin.[11]

Abbreviations: CsA, cyclosporin A; Peg-IFN, pegylated-interferon α-2a.

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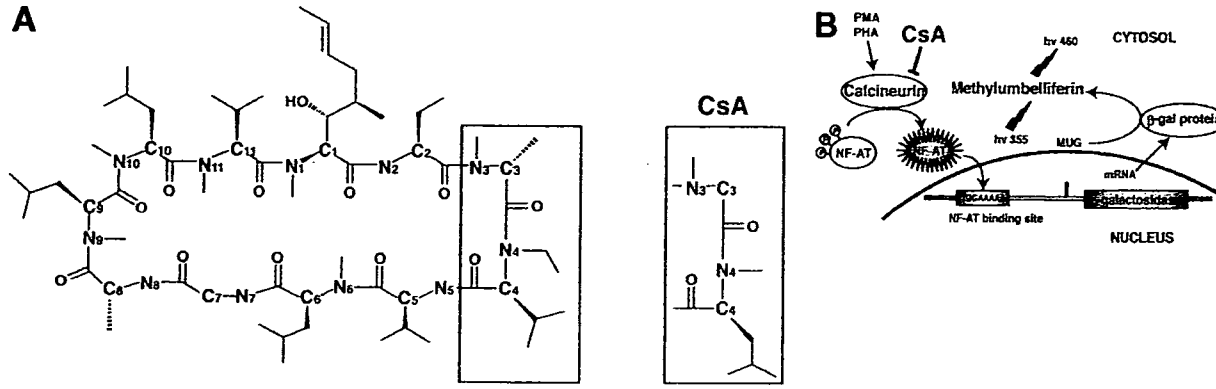

Fig. 1. (A) Structure of DEBIO-25, which was derived from CsA by substitution of amino acids at positions 3 and 4. (B) Scheme for IL-2 reporter gene assay. Nuclear factor of activated T cells (NF-AT), phorbol-12-myristate-13-acetate (PMA), phytohemagglutinin (PHA), 4-methyl umbelliferyl-β-D-galactoside (MUG).

CsA is an immunosuppressive agent widely used to improve graft survival after organ transplantation.[12] It was isolated as a metabolite from *Beauveria nivea* and consists of a cyclic polypeptide of 11 amino acids.[13] DEBIO-025 is a synthetic compound showing a more potent cyclophilin inhibitory activity as compared with CsA[14] and differing from CsA by the substitution of 2 amino acids (Fig. 1A; see Materials and Methods).[15] DEBIO-025 lacks immunosuppressive effects, although it still has remarkable inhibitory effects on HCV replication *in vitro*.[16]

We report the *in vivo* effectiveness and tolerability of DEBIO-025 administered in combination with Peg-IFN in chimeric mice with human hepatocytes that were infected with HCV genotypes 1a or 1b.

Materials and Methods

Compounds. DEBIO-025 is a synthetic compound derived from CsA. Sarcosine (*N*-methyl-D-glycine) at position 3 and *N*-methyl-D-leucine at position 4 are substituted for *N*-methyl-D-alanine and *N*-ethyl-D-valine, respectively (Fig. 1A).[16] DEBIO-025 was obtained from Debiopharm (Lausanne, Switzerland). CsA was purchased from Fluka Chemie (Buchs, Switzerland), and Peg-IFN was purchased from Chugai Pharmaceutical Co. (Tokyo, Japan).

Anti-HCV Assay in HuH-7 Cells Harboring Subgenomic Replicons. We used 2 HCV subgenomic replicon cell lines, FLR3-1[17] and R6FLR-N,[18] which were constructed as shown in Fig. 2A. They were seeded at a density of 5×10^3 per well in 96-well tissue culture plates, in complete Dulbecco's modified Eagle's medium GlutaMax I (DMEM-GlutaMaxI; Invitrogen, Carlsbad, CA) and containing 5% fetal bovine serum (Invitrogen).[17,18] The genome of the 2 replicons was genotype 1b. After incubation for 24 hours at 37°C (5% CO_2), the medium was removed, and serial dilutions of DEBIO-025 or CsA in growth medium were added. After 72 hours, luciferase activity was determined using the Bright-Glo luciferase assay kit (Promega Madison, WI). The luciferase signal was measured in triplicate using an LB940 luminometer (Berthold, Freiburg, Germany), and the results were expressed as the average percentage of control. IC_{50} values of DEBIO-025 and CsA were calculated by nonlinear curve fitting following the equation: $Y = 100 - (Y_{Bottom} \times X/(IC_{50} + X))$, where Y represents percentage inhibition and X represents the concentration of the agent. The viability of replicon cells was measured using the WST-8 cell counting kit according to the manufacturer's instructions (Dojindo, Kumamoto, Japan).

Western Blot Analysis of HCV NS3 and β-Actin. HCV replicon cells (1×10^6) were lysed with 100 μl of lysis buffer (1% SDS, 0.5% Nonidet P-40, 150 mmol/l NaCl, 0.5 mmol/l EDTA, 1 mmol/l dithiothreitol, and 10 mmol/l Tris, pH 7.4). Five micrograms total protein was electrophoresed on a 10% SDS-polyacrylamide gel and subsequently transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Billerica, MA). Nonstructural protein 3 (NS3) of HCV was detected using the rabbit anti-NS3 (R212) polyclonal antibody that was prepared in our laboratory. Beta actin was detected using anti–β-actin monoclonal antibody (Sigma, St. Louis, MO).

***Immunosuppressive Activity of DEBIO-025 and CsA by Interleukin-2 Reporter Gene Assay* In Vitro.** We examined the immunosuppressive activities of DEBIO-025 and CsA using a nuclear factor of activated T cells–dependent IL-2 reporter gene assay (Fig. 1B).[19] We used Jurkat T-cells stably expressing lac-Z controlled by the IL-2 promoter. The cells were grown in RPMI-1640 medium containing 10% fetal bovine serum, 2

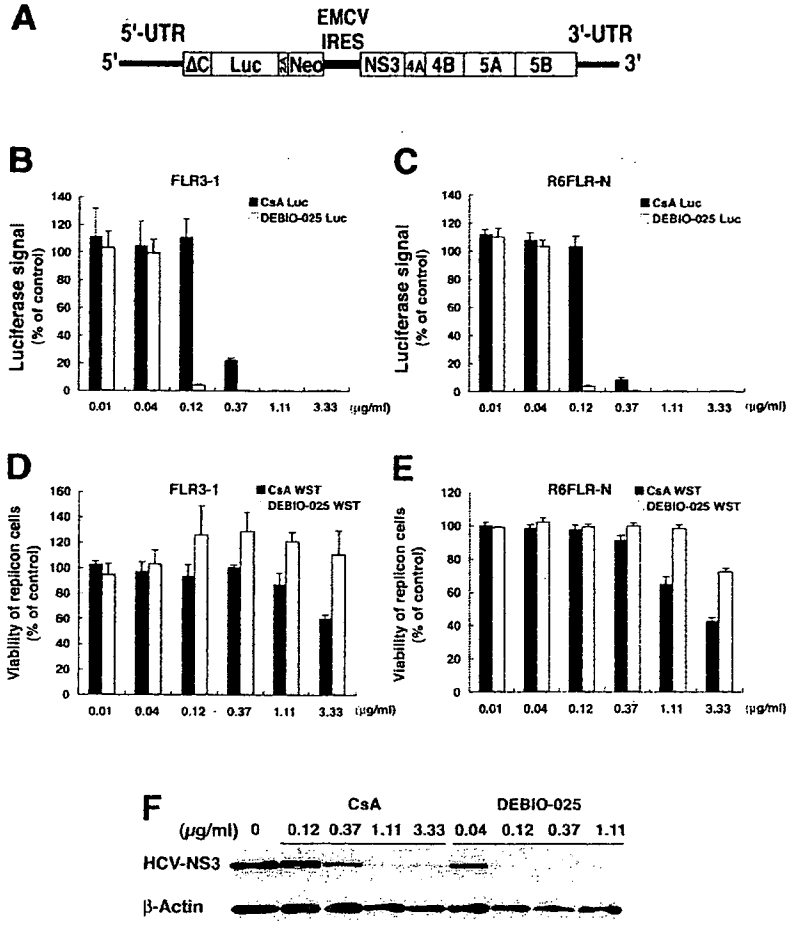

Fig. 2. (A) Structure of HCV replicon genome. FLR3-1 and R6FLR-N were of similar construction. Encephalomyocarditis virus (EMCV), internal ribosomal entry site (IRES). untranslated region (UTR). (B, C) Effect of DEBIO-025 or cyclosporin A (CsA) on HCV replication, as monitored in triplicate by luciferase signal in the 2 HCV replicon systems. Data are expressed as percentages of the untreated control. Error bars indicate SD. (D,E) Effect of DEBIO-025 or CsA on viability of replicon-containing cells, as measured in triplicate by WST-8. Data are expressed as percentages of the untreated control. Error bars indicate SD. (F) Effect of DEBIO-025 or CsA on HCV NS3 protein or β-actin expression, shown by western blotting.

mmol/l glutamine, 50 μM 2-mercaptoethanol, and 100 U/ml hygromycin B. Jurkat T-cells were stimulated with phorbol-12-myristate-13-acetate (2.4 μM) and phytohemagglutinin (75 μg/ml) in the presence or absence of DEBIO-025 or CsA (10^{-9} to 2×10^{-5} mol/l). After incubation at 37°C for 20 hours, cells were harvested by lysis buffer (50 mmol/l Na_2HPO_4, pH 9.0, 10 mmol/l KCl, 1 mmol/l $MgSO_4$, and 1% Triton X-100), and then β-galactosidase activity in the lysate was measured using 4-methyl umbelliferyl-β-D-galactoside (0.5 mmol/l; Sigma).

HCV Infection into Chimeric Mice. We purchased chimeric mice from PhenixBio (Hiroshima, Japan). The chimeric mice were generated by transplanting human primary hepatocytes into severe combined immunodeficient (SCID) mice carrying the urokinase plasminogen activator transgene controlled by an albumin promoter.[20] The chimeric mice used in this study were improved from the original ones, as described by Tateno et al.,[21] and had a high substitution rate of human hepatocytes. Six weeks after hepatocyte transplantation, we intravenously injected each mouse with patient serum containing 10^6 copies of HCV genotype 1a (HCG9) or 1b (HCR6).[22] HCV inoculations, drug administration, blood collection, and killing were performed under ether anesthesia. Blood samples were taken from the orbital vein and sera were immediately isolated. The protocols for animal experiments were approved by the local ethics committee. The animals received humane care according to NIH guidelines. Patients gave written informed consent before sampling.

Measurement of Human Serum Albumin. Human serum albumin in the blood of chimeric mice was measured with a commercially available kit according to the manufacturer's instructions (Alb-II kit; Eiken Chemical, Tokyo, Japan).

Schedule for Administration of Agents into Chimeric Mice Infected with HCV Genotype 1b or 1a. Treatment was started 12 weeks after HCV inoculation and continued during 14 days (Fig. 3A and Fig. 4A). Each treatment group comprised 3 animals. Peg-IFN and DEBIO-025 in mice with HCV genotype 1a or 1b were administered as follows: either Peg-IFN (30 μg/kg) was injected subcutaneously twice weekly alone or DEBIO-

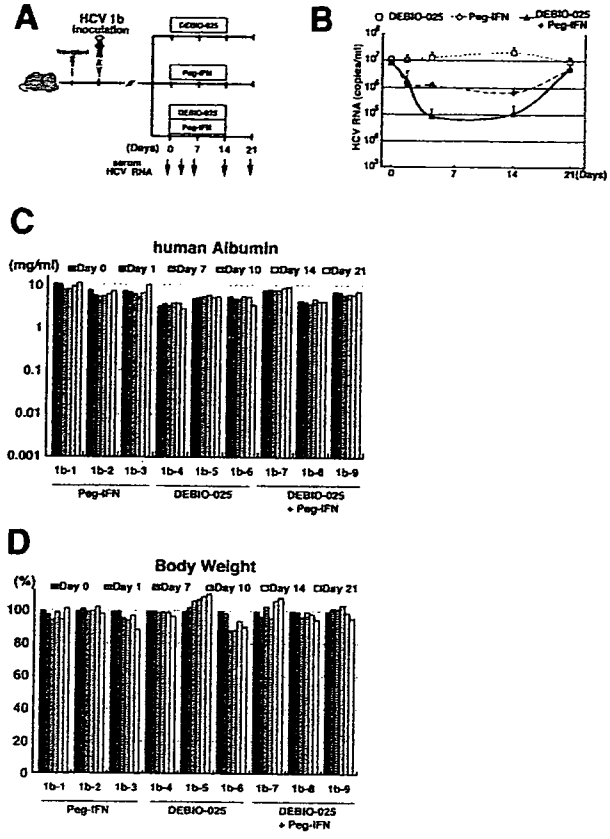

Fig. 3. (A) Schedule of experiments using chimeric mice infected with HCV genotype 1b. The mice were treated for 14 days with DEBIO-025 100 mg/kg/day orally, Pegylated-interferon $\alpha-2a$ (Peg-IFN) 30 μg/kg subcutaneously twice weekly, or DEBIO-025 100 mg/kg/day orally combined with Peg-IFN 30 μg/kg subcutaneously twice weekly. (B) Time course of serum HCV RNA levels in mice treated with DEBIO-025 (open squares), Peg-IFN (gray diamonds), or DEBIO-025 with Peg-IFN (closed triangles). Error bars indicate SD. (C) Human albumin concentrations in the sera of individual mice during the experimental period. (D) Body weight of individual mice during the experimental period.

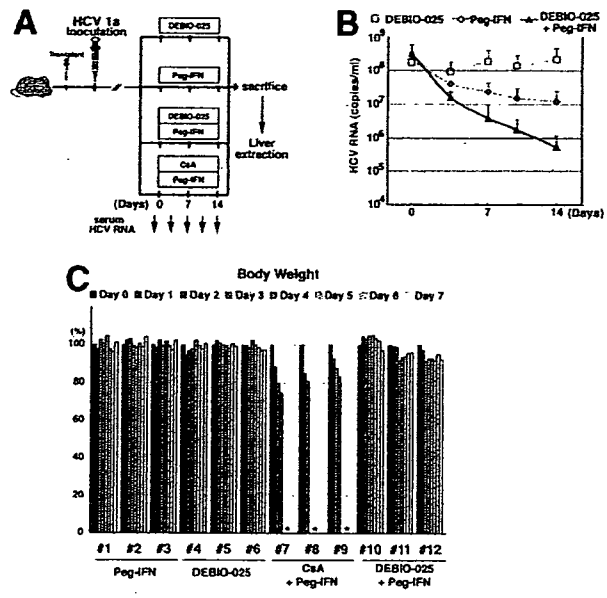

Fig. 4. (A) Schedule of experiments using chimeric mice infected with HCV genotype 1a. The mice were treated for 14 days with DEBIO-025 100 mg/kg/day orally, Peg-IFN 30 μg/kg subcutaneously twice weekly, DEBIO-025 100 mg/kg/day orally combined with Peg-IFN 30 μg/kg subcutaneously twice weekly, or CsA 100 mg/kg/day orally combined with Peg-IFN 30 μg/kg subcutaneously twice weekly. (B) Time course of serum HCV RNA levels in mice treated with DEBIO-025 (open squares), Peg-IFN (gray diamonds), or DEBIO-025 with Peg-IFN (closed triangles). Error bars indicate SD. (C) Body weight of individual mice during the first 7 days of the experimental period. All mice treated with CsA combined with Peg-IFN died within 4 days.

025 (100 mg/kg) was given orally every day alone, or a combination of both drugs was given. CsA (100 mg/kg) was given orally every day combined with Peg-IFN (30 μg/kg) subcutaneously twice weekly only to chimeric mice inoculated with genotype 1a.

Measurement of HCV Core Protein in Liver. Liver tissues were homogenized in lysis buffer (10 mM Tris pH 7.5, 1% SDS, 0.5% NP-40, and 150 mM NaCl) and centrifuged for 60 seconds at 16,000 g. HCV core protein was quantified using a commercially available kit (Ortho Clinical Diagnostics, Tokyo, Japan).[23]

Quantification of HCV RNA by Real-Time Reverse Transcription PCR. HCV RNA in serum or liver tissue was extracted using the acid guanidinium-phenol-chloroform method. Quantification of HCV RNA was performed using real-time reverse transcription PCR based on TaqMan chemistry, as described.[24]

Immunohistochemistry. Liver tissues obtained from mice were embedded in OCT compound (Ted Pella, Redding, CA). The frozen tissues were cut into thin sections (6 μm) and placed on glass slides. The sections were fixed in 10% buffered formalin and then treated with 0.1% Triton X-100. To detect HCV protein, the slides were incubated with rabbit anti-core protein IgG and then donkey anti-rabbit IgG polyclonal antibody [Fab fragment, labeled with horseradish peroxidase; Dako, Glostrup, Denmark]. The horseradish peroxidase label was amplified with FITC-conjugated tyramide according to the manufacturer's instructions (Molecular Probes, Eugene, OR). To detect human hepatocytes, liver sections were probed by anti-human hepatocyte monoclonal antibody (Dako), followed by anti-mouse IgG-Alexa 546 (Molecular Probes). Nuclei were stained by DAPI (Molecular Probes). Normal rabbit IgG was used as a control.

Results

Antiviral Activity of DEBIO-025 in HCV Subgenomic Replicon Cells. The anti-HCV effects of DEBIO-025 and CsA were initially confirmed using

HCV replicon cells. Both inhibited the replication of HCV replicon RNA in a concentration-dependent manner. The IC_{50} values of DEBIO-025 and CsA against replicon cell line of FLR3-1 were 0.06 μg/ml and 0.31 μg/ml respectively (Fig. 2B). The IC_{50} values of DEBIO-025 and CsA against replicon cell line of R6FLR-N were 0.07 μg/ml and 0.27 μg/ml, respectively (Fig. 2C). The inhibitory effect of DEBIO-025 was approximately 5-fold greater than that of CsA. When cell viabilities were monitored using WST-8, DEBIO-025 differed from CsA by showing a reduction of cell viability only in R6FLR-N cells (CsA reduced cell viability in both types of replicon cells; Fig. 2D-E). In R6FLR-N cells, DEBIO-025 at 3.33 μg/ml reduced cell viability by an average of 27.8%, whereas CsA at the same concentration reduced cell viability by an average of 57.2% (Fig. 2E). Western blotting of FLR3-1 cells showed that expression levels of NS3 protein, but not β-actin, were decreased by treatment with DEBIO-025 or CsA (Fig. 2F).

Immunosuppressive Activity of DEBIO-025. To examine the immunosuppressive activity of DEBIO-025, we used an nuclear factor of activated T cells–dependent IL-2 reporter gene assay. DEBIO-025 showed only a slight inhibitory effect on this system, with an activity that was 7,000-fold lower than that of CsA (data not shown). This indicates that the substitution of 2 amino acids in CsA to produce DEBIO-025 resulted in a greatly reduced immunosuppressive activity.

Human Albumin Levels in Mouse Serum After Transplantation of Human Hepatocytes. The concentration of human albumin in the serum of the chimeric mice was measured to provide an index of the substitution rate of mouse to human hepatocytes after transplantation.[21] The concentration measured 20 days after transplantation of human hepatocytes was 3.5 to 6.0 mg/ml, indicating that human hepatocytes had settled into the chimeric mice. At 6 weeks after transplantation, we inoculated the mice with patient serum containing HCV genotypes 1a or 1b. We repeatedly measured the concentrations of human albumin after inoculation and found that they reached a plateau at approximately 6.5 mg/ml. Although the mice were infected with HCV, significant reductions of the human albumin concentrations were not observed (Fig. 5A-B).

Persistent Infection of HCV in Chimeric Mice. To determine whether the chimeric mice were persistently infected with HCV, we measured HCV RNA levels in serum weekly after the inoculation. HCV RNA disappeared at the first week and was then detected from 2 weeks after the inoculation. Four weeks after infection, HCV RNA levels reached 10^8 to 10^9 copies/ml in the genotype 1a group (Fig. 5C) and 10^6 to 10^7 copies/ml in the genotype 1b group (Fig. 5D). These results showed that our patient sera containing HCV had infected the chimeric mice. Furthermore, the increase of HCV levels in the serum was time dependent, indicating that HCV replicated and accumulated in the human hepatocytes of the chimeric mice.

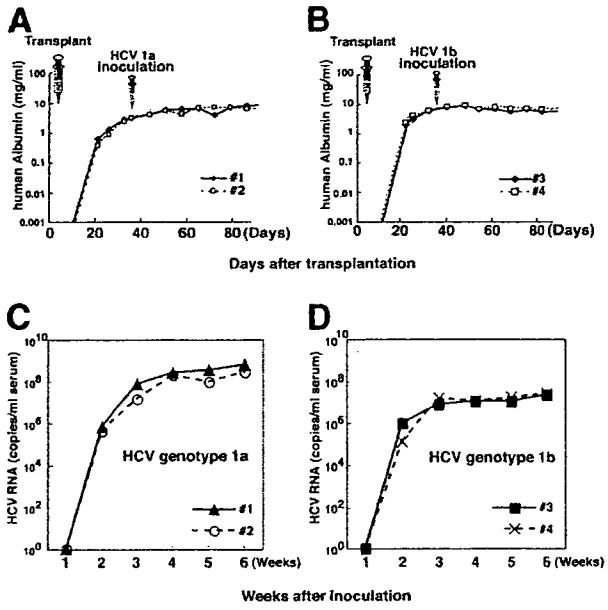

Fig. 5. Time course studies in 4 mice inoculated with human serum samples positive for HCV genotypes 1a or 1b. (A,B) Human albumin concentrations in mouse serum after transplantation of hepatocytes. (C,D) HCV RNA levels in mouse serum after inoculation.

Effect on HCV RNA Levels of DEBIO-025 and/or Peg-IFN in Mice Infected with HCV Genotype 1b. DEBIO-025 alone did not inhibit HCV replication, but Peg-IFN alone reduced serum HCV RNA levels approximately 10-fold from day 3 to day 14 (Fig. 3B). A 100-fold reduction was observed with the combined treatment (Fig. 3B). These results indicated an effect of DEBIO-025 that appeared to be synergistic with Peg-IFN against genotype 1b. The concentration of human serum albumin and the body weight of the mice did not change significantly during this period (Fig. 3C, D). After cessation of treatment, HCV RNA levels returned to 10^7 copies/ml.

Comparison of DEBIO-025 and CsA Effect in Chimeric Mice Infected with HCV Genotype 1a. The serum HCV RNA levels with the administration of DEBIO-025 or Peg-IFN alone seemed to be similar at day 7 and at day 14 as compared with those seen in mice infected with genotype 1b (Fig. 4B). The combined administration of DEBIO-025 with Peg-IFN resulted in a 600-fold reduction of HCV RNA levels at day 14 (Fig. 4B). The combined administration of CsA and Peg-IFN resulted in the death of all treated mice within 4 days. The

body weight of all CsA-treated mice was reduced by more than 20% during this period (Fig. 4C). The concentration of human serum albumin in the mice treated with CsA did not change significantly (data not shown). This toxicity was not observed with DEBIO-025 and Peg-IFN.

Quantification of Hepatic HCV RNA and Core Protein Levels and Immunohistochemistry at the End of Treatment in Chimeric Mice Infected with Genotype 1a. At the end of treatment, hepatic HCV RNA was quantified by real-time reverse transcription PCR, and core protein levels were quantified by enzyme-linked immunosorbent assay (Fig. 6A,B). DEBIO-025 monotherapy (1a-3 mouse) reduced HCV RNA by 3-fold compared with the nontreated mouse (1a-4 mouse). Peg-IFN reduced both HCV RNA and core protein levels by approximately 10-fold (1a-2 mouse). Combined treatment with DEBIO-025 and Peg-IFN resulted in an approximately 100-fold reduction in HCV RNA and HCV core protein levels (1a-1 mouse). Moreover, immunohistochemistry was performed. In 1a-4 mouse, HCV core protein was detected in human hepatocytes. In 1a-1 mouse, HCV core protein was not detected by immunohistochemistry; however, reduced HCV core protein was quantified by enzyme-linked immunosorbent assay, which is more sensitive than immunohistochemistry (Fig. 6C, D).

Discussion

Development of new anti-HCV drugs has been significantly impeded by the lack of a suitable cell culture model for the propagation of HCV in laboratories. This obstacle has been partially overcome by the development of the replicon system, which can be used for evaluating the *in vitro* anti-HCV effect of compounds. However, because adaptive mutation into the replicon genome and host permissiveness enable particularly efficient replication in cultured hepatoma cell lines,[25] evaluation of HCV drugs using replicon systems alone is considered insufficient. The only animal species readily infected with HCV has been the chimpanzee, which is labor-intensive and expen-

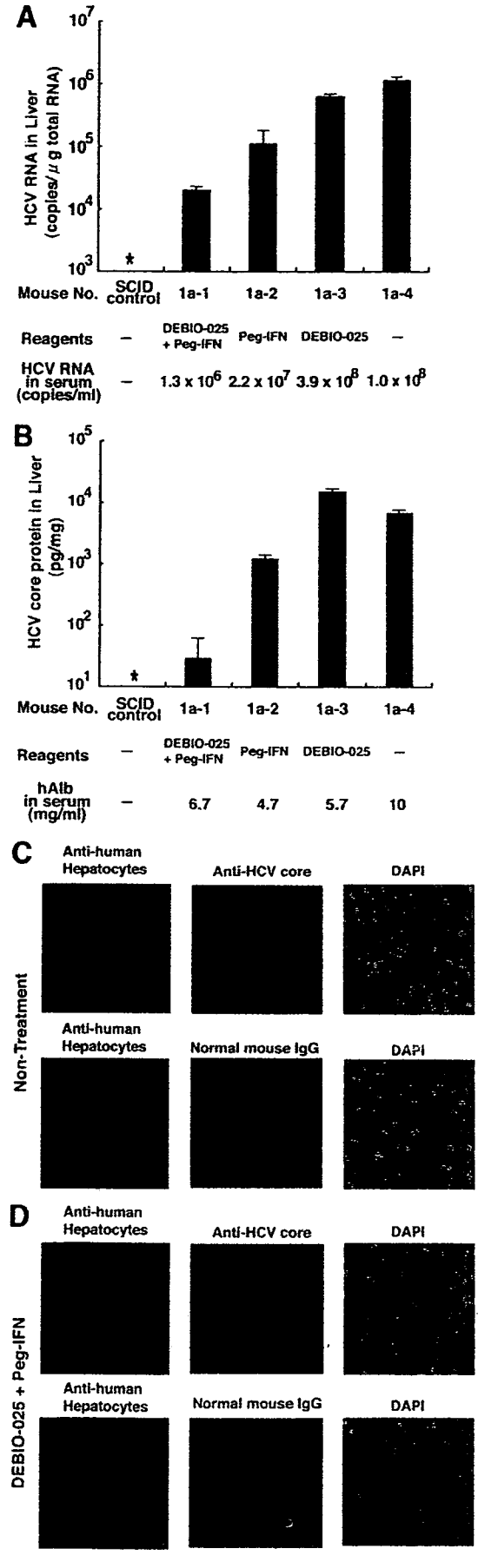

Fig. 6. Analysis of liver tissue from chimeric mice infected with HCV genotype 1a. (A) HCV RNA, and (B) HCV core protein, measured in triplicate in the livers of mice undergoing different treatment protocols. Severe combined immunodeficient (SCID) control: noninfected SCID mouse; 1a-1, mouse treated with DEBIO-025 combined with Peg-IFN; 1a-2, mouse treated with Peg-IFN; 1a-3, mouse treated with DEBIO-025; 1a-4, nontreated mouse infected with HCV. (C,D) Immunofluorescent labeling of human hepatocytes and HCV core protein, and fluorescent staining of nuclei. HCV core protein was labeled in human hepatocytes of nontreated chimeric mouse (C), but was not apparent in chimeric mouse treated with DEBIO-025 combined with Peg-IFN (D).

sive to use, and is associated with ethical problems. The chimeric mouse with human hepatocytes has recently been developed as a practical small animal model that can be infected with HCV.[20] This model is promising for the evaluation of new anti-HCV drugs because the mice are easy to handle, grow rapidly, and are well characterized genetically and immunologically. In this study, we used chimeric mice to bridge the gap between the replicon system and naive HCV replication in human liver, and to examine the anti-HCV effect of DEBIO-025, a novel cyclophilin inhibitor and non-immunosuppressive cyclosporin.

We found that HCV from our patient sera were able to infect the chimeric mice and persistently replicate over several weeks. HCG9 (1a) and HCR6 (1b) reached 10^8 to 10^9 copies/ml and 10^6 to 10^7 copies/ml, respectively, resulting in HCV RNA levels in serum that were higher than those previously reported.[20] This was probably because of a high substitution rate of human hepatocytes in the chimeric mice. When Mercer et al.[20] initially developed chimeric mice infected with HCV, they reported that human albumin concentrations in sera of the mice reached 2 mg/ml and that the substitution rate of liver from mouse to human was approximately 50%. In our study, the human albumin concentration in the chimeric mice reached 6.5 mg/ml, which would be consistent with a higher substitution rate of 80% to 90%.[21] In addition, our findings also indicate that the plateau point of HCV RNA in serum depends on the type of inoculum, because the HCV RNA levels were different for HCG9 and HCR6. Taken together, the results suggest that our chimeric mice propagated large amounts of HCV in their livers.

Although DEBIO-025 strongly inhibited replication of the HCV replicon, it did not affect the replication of naive HCV *in vivo* when given as monotherapy. These results probably indicate differences between the replication of naive HCV *in vivo* and the replicon system. The sensitivity of HCV strains to CsA and non-immunosuppressive cyclosporins was variable, depending on their cyclophilin requirement for their replication.[26] Cyclophilin polymorphism and its role in HCV replication will be the focus of future study.

The HCV RNA levels are known to decline biphasically in most patients treated with IFN.[27] During the first phase, there is a rapid drop in viremia that reflects the direct inhibition of HCV replication. During the second phase, there is a slower decline in serum HCV RNA levels, which appears to reflect the elimination of infected cells by host immune responses. In chimeric mice, the second-phase decline is not obvious, because they lack T cells and B cells (being SCID). Thus, it appears that DEBIO-025 accelerates the decline in HCV RNA levels induced by Peg-IFN during the first phase. There is no evidence that DEBIO-025 enhances the interferon pathway. Also, recent *in vitro* findings show that cyclosporins do not modify the IFN-α signal transduction pathway as assessed by 2′, 5′-oligoadenylate synthetase (2′, 5′-OAS) levels.[28] It therefore seems likely that the apparent synergistic effect of DEBIO-025 seen in our *in vivo* model is not solely related to the antiviral effect mediated by IFN. The DEBIO-025 inhibition of cyclophilin may produce a proper anti-HCV effect by interacting with the RNA-dependent RNA polymerase.[11]

CsA was originally used as an immunosuppressive agent, and we previously demonstrated in clinical trials that CsA has an anti-HCV effect.[9] However, CsA is not devoid of adverse effects, such as hypertension, neurotoxicity, and nephrotoxicity, limiting its therapeutic usefulness against HCV.[29] The immunosuppressive action of CsA occurs by inhibition of calcineurin. Our findings showing that DEBIO-025 exhibits a 7,000-fold lower immunosuppressive activity than CsA suggest that it has less affinity to calcineurin and may lead to fewer adverse effects in patients.

In conclusion, our results indicate that naïve HCV replication *in vivo* is inhibited by the combined administration of the cyclophilin inhibitor DEBIO-025 and Peg-IFN. These findings support further evaluation of DEBIO-025 as a promising drug for the treatment of chronic hepatitis C.

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Hsp90 inhibitors suppress HCV replication in replicon cells and humanized liver mice

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Abstract

Persistent infection with hepatitis C virus (HCV) is a major cause of liver diseases such as chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. Here we report that inhibition of heat shock protein 90 (Hsp90) is highly effective in suppressing HCV genome replication. In HCV replicon cells, HCV replication was reduced by Hsp90 inhibitors and by knockdown of endogenous Hsp90 expression mediated by small-interfering RNA (siRNA). The suppression of HCV replication by an Hsp90 inhibitor was prevented by transfection with Hsp90 expression vector. We also tested the anti-HCV effect of Hsp90 inhibition in HCV-infected chimeric mice with humanized liver. Combined administration of an Hsp90 inhibitor and polyethylene glycol-conjugated interferon (PEG-IFN) was more effective in reducing HCV genome RNA levels in serum than was PEG-IFN monotherapy. These results suggest that inhibition of Hsp90 could provide a new therapeutic approach to HCV infection.

Keywords: HCV; Hsp90; Replication; Replicon; Chimeric mouse with humanized liver

Infection with hepatitis C virus (HCV), the major causative agent of non-A, non-B hepatitis [1–3], can lead to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [4]. An estimated 170 million people worldwide are currently infected with HCV [5]. Combination therapy comprising polyethylene glycol-conjugated interferon (PEG-IFN) and ribavirin represents the current standard treatment for chronic HCV infection, although it has demonstrated limited success and causes some serious side effects [6–8]. The development of safer and more effective drugs for the treatment of HCV infection is therefore an urgent necessity.

HCV, a member of the *Flaviviridae* family, has a single-stranded RNA genome of positive polarity. The genome encodes a large precursor polyprotein which is cleaved by host and viral proteases to generate at least 10 functional viral proteins: core, envelope1, envelope2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B [9,10].

Lohmann et al. [11] have established an HCV replicon system in which HCV subgenomic RNA autonomously replicates in HuH-7, a human hepatoma cell line (HCV replicon cells). This HCV replicon system allows one to investigate HCV genome replication in cell culture.

In this study we performed random screening with natural-product libraries using HCV subgenomic replicon cells and found that inhibitors of heat shock protein 90 (Hsp90)

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inhibited HCV replication. We found that Hsp90 was an important host-derived factor that regulates HCV replication in an HCV replicon system. Using HCV-infected chimeric mice with humanized liver, which have recently been developed as a practical small animal model of HCV infection [12–14], we also demonstrated inhibition of native HCV replication in human liver cells by Hsp90 inhibitors.

Materials and methods

HCV replicon cells. Two types of human hepatoma HuH-7 cells carrying an HCV subgenomic replicon, FLR 3-1 (genotype 1b, Con-1) [15] and R6FLR-N (genotype 1b, strain N) [16], were maintained in Dulbecco's modified Eagle's medium supplemented with GlutaMAX™ I (Invitrogen, Carlsbad, CA, USA) and 0.5 mg/ml of G418 at 37 °C in 5% CO_2. The replicons were constructed as shown in Fig. 1A.

Infection of chimeric mice with HCV. Chimeric mice harboring a functional human liver cell xenograft were purchased from PhenixBio (Hiroshima, Japan). The chimeric mice were generated by transplanting human primary hepatocytes into severe combined immunodeficient (SCID) mice carrying the urokinase plasminogen activator transgene controlled by an albumin promoter. The chimeric mice used in this study had been improved over the original mice described by Tateno et al. [14], and they had a higher human hepatocyte substitution rate. Six weeks after hepatocyte transplantation, we intravenously injected each mouse with an HCV-infected patient's serum containing 10^6 copies of HCV genotype 1b (HCR6; Accession No. AY045702). The protocols for animal experiments were approved by the Ethics Committee of The Tokyo Metropolitan Institute of Medical Science. The animals received humane care according to the guidelines of the National Institutes of Health. The HCV-infected patient who provided the serum gave written informed consent before blood sampling.

Administration of 17-DMAG and/or PEG-IFN. Starting on day 0, HCV-infected chimeric mice with humanized liver received intravenous injections of 4 mg/kg of 17-DMAG and/or subcutaneous injections of 30 μg/kg PEG-IFNα-2a (Chugai, Tokyo, Japan) according to the schedule shown in Table 1.

Quantification of HCV RNA by real-time PCR. Total RNA was purified from 1 μl of serum from chimeric mice with humanized liver by the acid guanidinium–phenol–chloroform method, and HCV RNA was quantified by real-time PCR as described by Takeuchi et al. [17].

Results

Effect of Hsp90 inhibitors on HCV replication in HCV replicon cells

We have developed two modified HCV replicon cell lines, FLR 3-1 (genotype 1b, Con-1) [15] and R6FLR-N (genotype 1b, strain N) [16]. The HCV replicons are composed of the HCV 5′-untranslated region (UTR) containing an internal ribosomal entry site (IRES), the first 45 nucleotides of the core protein gene, fusion genes for luciferase and neomycin phosphotransferase, the encephalomyocarditis virus (EMCV) IRES, HCV NS3 through NS5B, and the HCV 3′-UTR (Fig. 1A). The construct allows quantification of replication levels by measuring luciferase activity. Taking advantage of this feature, we performed random screening of potential anti-HCV compounds using FLR 3-1 cells. Geldanamycin and radicicol, both of which are well-known Hsp90 inhibitors, were included among the hits. To assess the effects of Hsp90 inhibition on the intracellular replication of HCV in more detail, we treated the two different types of HCV replicon cells (FLR 3-1 and R6FLR-N) with each of four different Hsp90 inhibitors, 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG), geldanamycin, herbimycin A, and radicicol. All of the Hsp90 inhibitors suppressed replication of the HCV replicon in both cell types in a dose-dependent manner. The half-maximal inhibitory concentration (IC_{50}) for HCV replication in FLR 3-1 cells was 4.4 nM for 17-DMAG, 5.5 nM for geldanamycin, 50 nM for herbimycin A, and 133 nM for radicicol (Fig. 1B, upper panel). In R6FLR-N cells, the IC_{50} was 8.1 nM for 17-DMAG, 7.8 nM for geldanamycin, 180 nM for herbimycin A, and 125 nM for radicicol (Fig. 1B, lower panel). We also assessed the levels of HCV NS3 protein, which plays a key role in HCV replication, by Western blotting and immunostaining. Hsp90 inhibitors brought about a dose-dependent decrease in the levels of this protein (Fig. 1C and D).

Effect of Hsp90 knockdown by siRNA on HCV replication in HCV replicon cells

To examine whether suppression of the replication of the HCV replicon by Hsp90 inhibitors resulted from functional inhibition of Hsp90, we used small-interfering RNA (siRNA) to perform knockdown of Hsp90. In mammalian cells there are two Hsp90 isoforms, Hsp90α and Hsp90β, which are encoded by separate genes, so we transfected siRNA targeting Hsp90α, Hsp90β, or both Hsp90α and Hsp90β into FLR 3-1 cells. As a positive control, we used siE-R7 [16], an siRNA that is sequence specific for HCV IRES. As a negative control we used sip53m [16], an siRNA that is sequence specific for p53 mRNA except for two nucleotides. Replication of the HCV replicon was suppressed by siRNA against either Hsp90α or Hsp90β in a dose-dependent manner with no effect on cell viability (Fig. 2A and B). Double knockdown of both Hsp90α and Hsp90β suppressed replication of the HCV replicon more effectively than knockdown of either Hsp90α or Hsp90β alone. HCV replication was reduced to 50–60% of control levels when cells were treated with 70 nM siRNA targeting either Hsp90α or Hsp90β, but it fell to 30% of control when cells were treated with 35 nM siRNA against Hsp90α combined with 35 nM siRNA against Hsp90β (Fig. 2A). To confirm the functionality of the siRNAs, cells were treated with 100 nM siRNA and the expression of Hsp90 protein was assessed by Western blotting. siRNA against Hsp90α or Hsp90β specifically inhibited the expression of the corresponding Hsp90 protein (Fig. 2C). It was also observed that the expression of NS3 protein was downregulated in cells transfected with siRNA targeting either Hsp90α or Hsp90β (Fig. 2C). Moreover, when cells were cotransfected with siRNA against Hsp90α and siRNA against Hsp90β (each at a concentration of 50 nM), the expression of NS3 protein was more effectively downregulated than when cells were transfected with 100 nM of siRNA targeting either Hsp90α or Hsp90β alone (Fig. 2C).

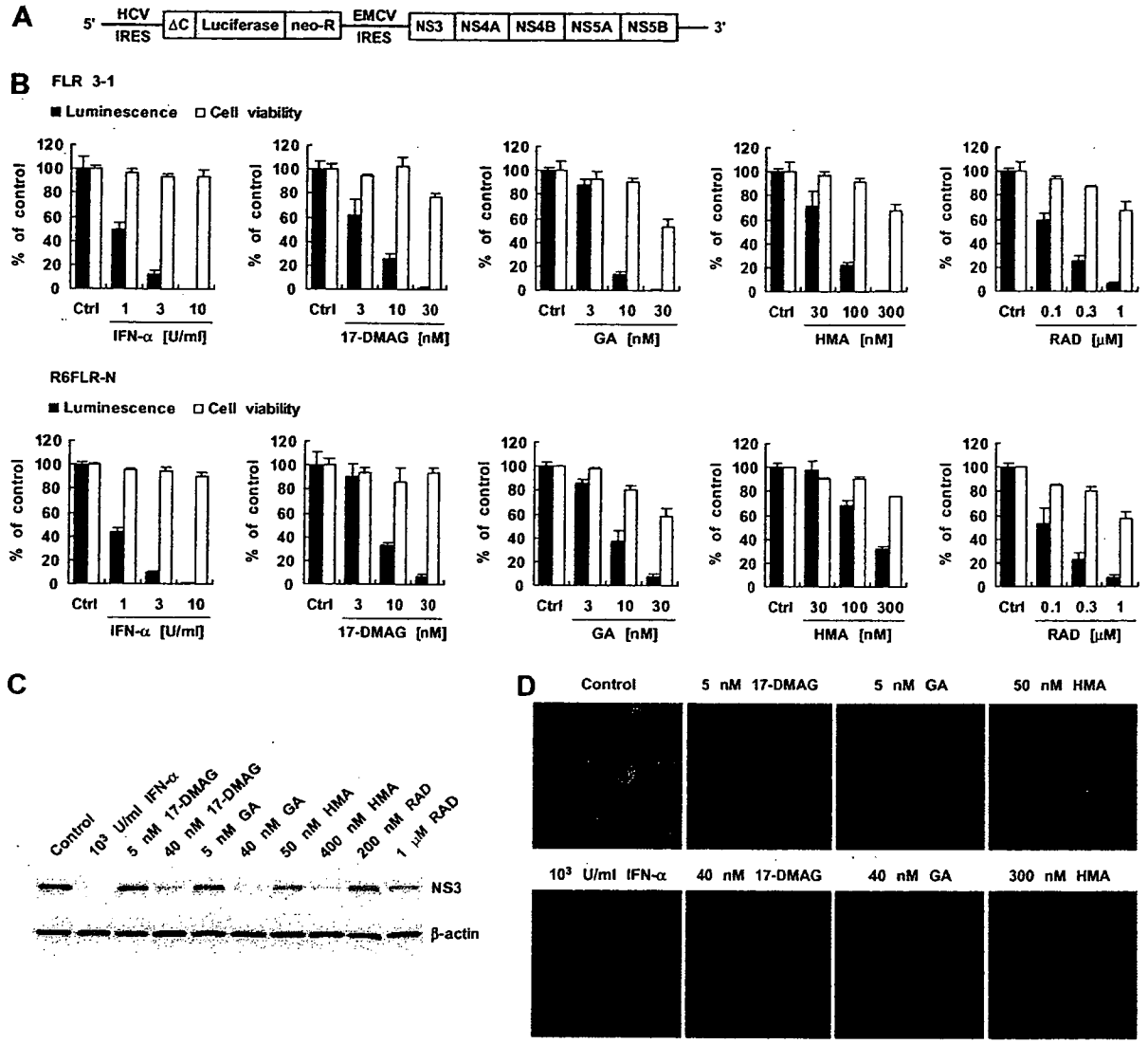

Fig. 1. Effect of Hsp90 inhibitors on HCV replication and cell viability in HCV replicon cells. (A) Schematic representation of the HCV subgenomic replicons used in this study. ΔC, first 45 nucleotides of HCV core protein gene; neo-R, neomycin phosphotransferase gene. (B) Inhibition of replication of the HCV replicon by the Hsp90 inhibitors 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG), geldanamycin (GA), herbimycin A (HMA), and radicicol (RAD) in FLR 3-1 replicon cells (upper panels) and R6FLR-N replicon cells (lower panels). Interferon-α (IFN-α) was used as a positive control. Seventy-two hours after treatment, HCV replication was determined by luciferase assay and cell viability by WST-8 assay. The data represent the mean of four values and the bars indicate the standard deviation. (C) FLR 3-1 cells were treated with IFN-α or Hsp90 inhibitors for 72 h. Saline was added to control cells. The levels of NS3 protein or β-actin were assessed by Western blotting. (D) FLR 3-1 cells were immunostained with anti-NS3 antibody (green) and diamidino-2-phenylindole (red). Cells were treated with saline (control) or with 10^3 U/ml IFN-α, 5 nM 17-DMAG, 40 nM 17-DMAG, 5 nM GA, 40 nM GA, 50 nM HMA or 300 nM HMA as indicated.

Table 1
Schedule of blood sampling 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
17-DMAG		D	D	D	D	D	D	D	D	D	D	D	D	D	D	
PEG-IFN		I			I				I			I				
17-DMAG + PEG-IFN		D/I	D	D	D/I	D	D	D	D/I	D	D	D/I	D	D	D	

B indicates sampling of blood; D, intravenous injection of 17-DMAG (4 mg/kg); I, subcutaneous injection of PEG-IFN (30 μg/kg).

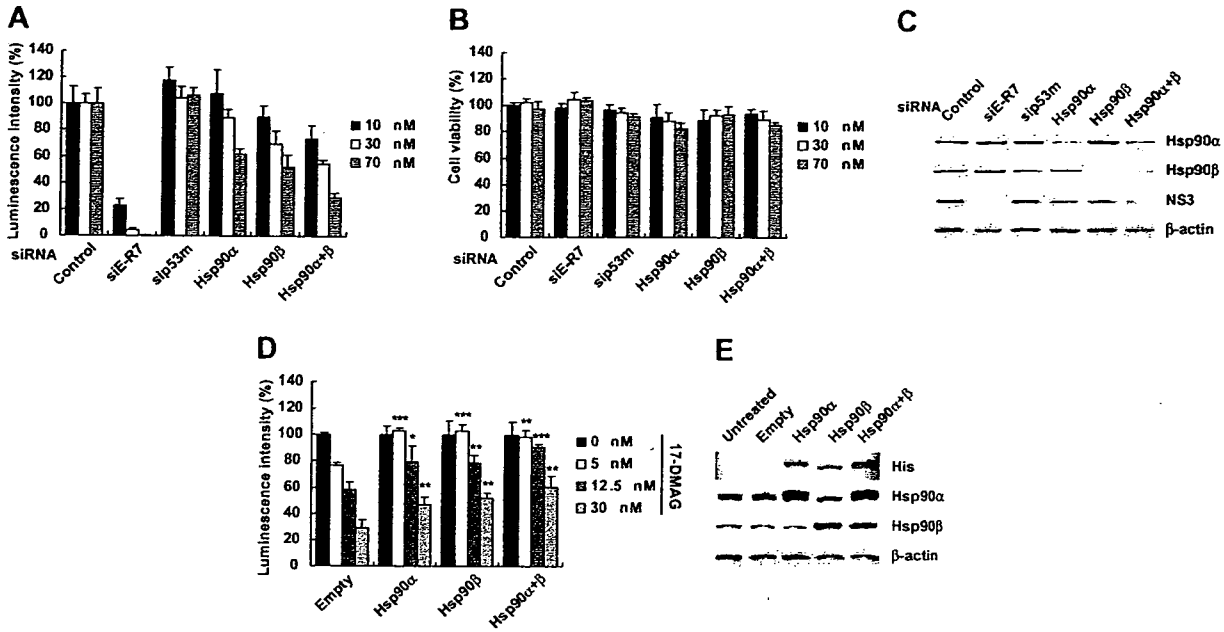

Fig. 2. Effect of knockdown or overexpression of Hsp90 on replication of the HCV replicon. (A,B) FLR 3-1 replicon cells were transfected with HCV-directed siRNA (siE-R7), negative-control siRNA (sip53m) or Hsp90-directed siRNA. Seventy-two hours after siRNA transfection, HCV replicon levels were determined by luciferase assay (A) and cell viability was determined by WST-8 assay (B). Saline was added to control cells. The data represent the mean of four values and the bars indicate the standard deviation. (C) Seventy-two hours after siRNA transfection, NS3, Hsp90α, and Hsp90β protein were quantified by Western blotting. (D) FLR 3-1 replicon cells were transfected with an expression vector encoding His-tagged Hsp90α, Hsp90β, or both Hsp90α and Hsp90β, or control empty vector and treated with 17-DMAG for 6 h. Forty-eight hours after treatment with 17-DMAG, HCV replicon levels were determined by luciferase assay. The data represent mean of four values and the bars indicate the standard deviation. The asterisks indicate significant differences between the Hsp90 expression vector and control vector groups at the corresponding concentration ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; Student's t-test). (E) Seventy-two hours after transfection with expression vector encoding His-tagged Hsp90, His-tag, Hsp90α, and Hsp90β protein were quantified by Western blotting.

Effect of complementation with Hsp90

We tested whether the reduction in HCV replicon replication caused by Hsp90 inhibitors could be prevented by transfection with an expression vector encoding Hsp90. FLR 3-1 cells were transfected with an expression vector encoding histidine (His)-tagged Hsp90α, Hsp90β, or both Hsp90α and Hsp90β, or an control empty vector (Fig. 2E), and pulse-stimulated with 17-DMAG for 6 h. As expected, replication of the HCV replicon in cells transfected with control vector was suppressed by 17-DMAG in a dose-dependent manner. In contrast, replication of the HCV replicon in cells transfected with an expression vector encoding Hsp90α, Hsp90β, or both Hsp90α and Hsp90β was significantly higher than in cells transfected with control empty vector, when cells were treated with 17-DMAG (Fig. 2D).

Anti-HCV effects of 17-DMAG and PEG-IFN in HCV-infected chimeric mice with humanized liver

The anti-HCV effect of Hsp90 inhibitors was investigated in chimeric mice with humanized liver injected with an HCV-infected patient's serum. Four weeks after infection, HCV RNA levels had reached 2.3×10^6–1.4×10^7 copies/ml. We then administered 17-DMAG intravenously and/or PEG-IFN subcutaneously over a period of 14 days according to the schedule shown in Table 1. Blood samples were collected before, during, and after this period (Table 1). In the group treated with 30 μg/kg of PEG-IFN, a dose 10-fold larger than that used in clinical treatment, HCV genome RNA levels had decreased in the serum 8.8-fold by day 4 and 11-fold by day 14 (Fig. 3A). Combined treatment with 4 mg/kg of 17-DMAG and 30 μg/kg of PEG-IFN reduced HCV genome RNA levels significantly more than did PEG-IFN monotherapy. HCV genome RNA levels in the coadministration group had decreased 25-fold by day 4 and 45-fold by day 14 (Fig. 3A). In the group treated with 4 mg/kg of 17-DMAG, no reduction in HCV genome RNA levels was observed at this dose. We monitored the concentration of human albumin over the same period and observed no suppression of this protein during or after administration of 17-DMAG (Fig. 3B).

Discussion

In an effort to develop safer and more effective drug treatments, new antiviral agents, including inhibitors of

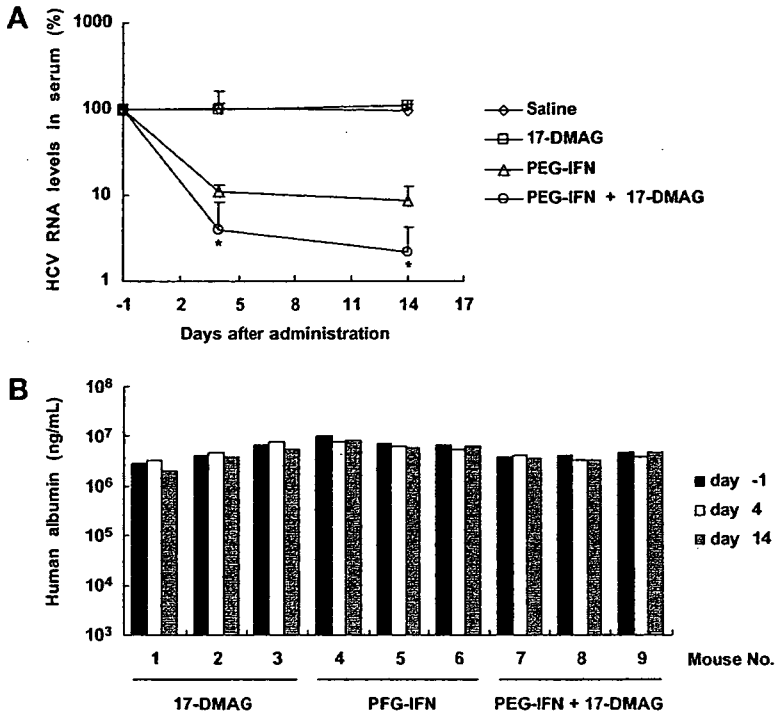

Fig. 3. Anti-HCV effect of 17-DMAG in HCV-infected chimeric mice. (A) HCV genome RNA levels in the serum of chimeric mice were determined by real-time PCR. (B) Human albumin concentrations in the sera of chimeric mice. The numbers 1–9 represent individual mice. Numbers 1, 2, and 3 were in the group treated with 4 mg/kg 17-DMAG, numbers 4, 5, and 6 were in the group treated with 30 μg/kg polyethylene glycol-conjugated interferon (PEG-IFN), and numbers 7, 8, and 9 were in the group treated with 4 mg/kg 17-DMAG and 30 μg/kg PEG-IFN. Asterisks indicate significant differences at the $P < 0.1$ level between the coadministration and PEG-IFN monotherapy groups on the same date.

the HCV NS3/4A serine protease and the HCV NS5B RNA-dependent RNA polymerase, are currently under clinical investigation [18]. However, it is likely that viral resistance will develop against antiviral agents that directly target viral enzymes, because of the error-prone nature of the virus reverse transcriptase and RNA-dependent RNA polymerase [19,20]. Therefore, we believe that anti-HCV therapies that target host-derived factors may be preferable to those that target a viral protein.

Recently, several host-derived factors that regulate replication of the HCV replicon or genome have been identified. Inhibitors of serine palmitoyltransferase have been found to suppress HCV replication in cultured cells and chimeric mice infected with HCV [15,21]. They act by preventing *de novo* synthesis of sphingolipids, major components of the lipid raft where HCV NS proteins associate. In other studies the immunosuppressant cyclosporin A has been shown to inhibit replication of the HCV genome [22,23], and cyclophilin B has been identified as the host-derived target of cyclosporin A [24,25].

In the present study, we tested the effects of Hsp90 inhibitors on HCV replication in cells carrying an HCV subgenomic replicon. All of the Hsp90 inhibitors we tested had potent anti-HCV activity. Using NS2/3 translated in a rabbit reticulocyte lysate and expressed in Jurkat cells, Waxman et al. [26] demonstrated a role for Hsp90 in promoting the cleavage of HCV NS2/3 protein . Because the replicon cells used in our study genetically lacked NS2, our results suggest that Hsp90 may directly or indirectly interact with any of the proteins NS3 through NS5B to regulate replication of the HCV replicon. Recently, Okamoto et al. [27] have reported that FKBP8, an Hsp90 partner protein, directly interacts with NS5A and regulates HCV RNA replication.

We also performed siRNA knockdown of Hsp90 and found suppression of HCV replication, consistent with the results of our experiment with Hsp90 inhibitors. Moreover, the HCV replication suppressed by Hsp90 inhibitors was rescued by the expression of Hsp90. These results indicate that Hsp90 is critical for efficient replication of the HCV replicon and that both Hsp90 isoforms (Hsp90α and Hsp90β) participate in replication.

Although the HCV replicon system is useful for screening compounds with potential anti-HCV activity, the inhibitory effects on replication are not always consistent with those found for HCV genome RNA in HCV-infected human liver. To bridge the gap between the replicon system and native HCV replication in the human liver, we tested the anti-HCV effect of Hsp90 inhibitors in HCV-infected chimeric mice with humanized liver. Of the Hsp90 inhibitors we tested, we chose to administer 17-DMAG to the mice because it is highly specific and therefore less toxic, and also because it

is currently under clinical investigation for the treatment of cancer [28,29]. We found that 17-DMAG exerted an apparently synergistic anti-HCV effect when used in combination with PEG-IFN to treat HCV-infected chimeric mice with humanized liver. Furthermore, 17-DMAG did not alter the levels of human albumin, indicating that it did not cause appreciable damage to the human hepatocytes. These results suggest that 17-DMAG may be a promising agent for the treatment of HCV infection.

In conclusion, our results demonstrate that Hsp90 supports HCV RNA replication both in an HCV replicon system and in a humanized liver mouse model infected with HCV. In addition, our results suggest that inhibition of Hsp90 may provide a feasible therapeutic strategy for the treatment of HCV infection. The precise molecular mechanism by which Hsp90 participates in the replication of HCV RNA remains to be elucidated and is under investigation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.12.117.

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ORIGINAL ARTICLE

Intracellular-diced dsRNA has enhanced efficacy for silencing HCV RNA and overcomes variation in the viral genotype

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RNA interference (RNAi) can be used to inhibit viral replication in mammalian cells and therefore could be a powerful new antiviral therapy. Small interfering RNA (siRNA) may be effective for RNAi, but there are some technical problems that must be solved in each case, for example, predicting the effective siRNA target site and targeting heterogeneous sequences in a virus population. We show here that diced siRNA generated from long double-stranded RNA (dsRNA) is highly effective for inducing RNAi in HuH-7 cells harboring hepatitis C virus (HCV) replicons and can overcome variations in the HCV genotype. However, in mammalian cells, long dsRNA induced an interferon response and caused cell death. Here we describe an improvement of this method, U6 promoter-driven expression of long hairpin-RNA with multiple point mutations in the sense strand. This can efficiently silence HCV RNA replication and HCV protein expression without triggering the interferon response or cell death normally caused by dsRNA. In conclusion, intracellular-diced dsRNA efficiently induces RNAi, and, despite the high rate of mutation in HCV, it should be a feasible therapeutic strategy for silencing HCV RNA.

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Keywords: *RNAi; long dsRNA; dicer; heterogeneous; interferon; U6 vector; HCV*

Introduction

An estimated 170 million people worldwide are persistently infected with hepatitis C virus (HCV).[1] Although the initial infection is frequently asymptomatic, there are several subsequent clinical manifestations, including fibrosis of the liver, cirrhosis and hepatocellular carcinoma. Although combination therapy with interferon (IFN)-α and ribavirin has markedly improved the clinical outcome, less than half of the patients with chronic hepatitis C can be expected to respond favorably to currently available agents.[2] Therefore, developing a new therapy for chronic HCV is a major public health objective.

The genome of HCV, a member of Flaviviridae family, is encoded in an approximately 9.6-kb single-stranded RNA with positive polarity that includes a 5′-untranslated region (UTR)[3] and a 3′-UTR containing a 3′X terminal sequence.[4] Hepatitis C virus displays a high rate of mutation and is classified into distinct genotypes (1–6) and subtypes, whose distribution varies both geographically and between risk groups.[5] Furthermore, several distinct but closely related HCV sequences coexist within each infected individual. These are referred to as quasi-species and reflect the high replication rate of the virus and the lack of a proofreading activity of the RNA-dependent RNA polymerase.[6,7]

Gene targeting with functional nucleic acids is commonly used to determine gene function and has potential as a treatment for viral diseases. Although antisense RNA and ribozyme technologies are successful in some situations, they have been difficult to apply universally and are less effective *in vivo*.[8] A possible alternative, sequence-specific post-transcriptional gene silencing by double-stranded RNA (dsRNA), also known as RNA interference (RNAi), has been found in plants, *Caenorhabditis elegans* and mammalian cells.[9,10] As RNAi with small interfering RNA (siRNA) can inhibit the replication of several viruses, including human immunodeficiency virus type 1 (HIV-1)[11] and poliovirus,[12] it may be a powerful new antiviral therapy. Recently, it has been demonstrated that replication of HCV RNA is also receptive to RNAi machinery,[13–18] but it has been difficult to design highly effective siRNAs against HCV because of the exquisite sequence specificity of the siRNAs coupled with the variation in HCV genotypes and the enormous diversity of HCV sequences between and within infected individuals.

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