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Anti-hepatitis C Virus Activity of Tamoxifen Reveals the Functional Association of Estrogen Receptor with Viral RNA Polymerase NS5B*

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Hepatitis C virus (HCV) is a major causative agent of hepatocellular carcinoma. HCV genome replication occurs in the replication complex (RC) around the endoplasmic reticulum membrane. However, the mechanisms regulating the HCV RC remain widely unknown. Here, we used a chemical biology approach to show that estrogen receptor (ESR) is functionally associated with HCV replication. We found that tamoxifen suppressed HCV genome replication. Part of ESRα resided on the endoplasmic reticulum membranes and interacted with HCV RNA polymerase NS5B. RNA interference-mediated knockdown of endogenous ESRα reduced HCV replication. Mechanistic analysis suggested that ESRα promoted NS5B association with the RC and that tamoxifen abrogated NS5B-RC association. Thus, ESRα regulated the presence of NS5B in the RC and stimulated HCV replication. Moreover, the ability of ESRα to regulate NS5B was suggested to serve as a potential novel target for anti-HCV therapeutics.

Estrogen receptor (ESR)[2] belongs to the steroid hormone receptor family of the nuclear receptor superfamily (1). ESR consists of two subtypes, ESRα and ESRβ. As a primary physiological function, ESR is involved in the transcription for downstream genes in response to stimulation by the ligand, estradiol. In the normal state, ESR is mainly located in the cytoplasm and nucleus. Upon binding of the ligand, ESR dimerizes and translocates into the nucleus, where it binds to the ESR-responsive elements (ERE) in the DNA promoter of downstream genes and drives transcription. In addition to this classical genomic action, a portion of ESR is located on the membrane, such as the plasma membrane, and involved in the nongenomic function of triggering signal transduction pathways, such as mitogen-activated protein kinase, phosphatidylinositol 3-kinase, and protein kinase C (2–4). Although the molecular basis of ESR membrane retention is not fully understood, one mechanism involves a membrane protein, caveolin (CAV); ESRα interacted with CAV, and this interaction facilitated ESRα localization to the membrane (5, 6). It was also reported that ESRα localizes to the lipid rafts on the plasma membrane (7). The lipid rafts are microdomains of the membrane that form platforms enriched in cholesterol and glycosphingolipids. However, the characteristics and relevance of membrane-associated ESR have not been fully disclosed. Here, we report the novel role of ESRα in the regulation of viral replication.

Hepatitis C virus (HCV), a causative agent of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma, constitutes a serious health problem worldwide (8). HCV has a positive strand RNA genome that produces at least 10 functional viral proteins: core, envelope 1, envelope 2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B (9, 10). NS5B is an RNA-dependent RNA polymerase, which plays a central role in viral genome replication (11, 12). HCV genome replication can be evaluated using a HCV subgenomic replicon system, which Lohmann *et al.* (13) first established. In this system, cells carry an HCV subgenome RNA encoding NS3 to NS5B. Using this system, it has been proposed that HCV genome replication occurs in the replication complex (RC), which contains the viral genome RNA and HCV NS proteins. The RC forms on the surface of the intracellular membranes, including the endoplasmic reticulum (ER) membrane, and is surrounded by a membrane structure (14–17). It also has been reported that HCV genome replication associates with the lipid rafts on these intracellular membranes, such as the ER membrane (14, 18). These lipid rafts accumulate CAV2, and HCV proteins involved in viral genome replication cofractionate with CAV2 (18). However, it is largely unknown how the RC is formed and under what mechanism the HCV proteins participate in the RC.

A chemical biology approach is a useful method to analyze the molecular mechanism of viral life cycles as well as cellular physiological processes (19). We employed forward chemical genetics in which we analyzed HCV replication activity as a phenotypic indicator of a cell-based assay to screen chemical

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[2] The abbreviations used are: ESR, estrogen receptor; HCV, hepatitis C virus; RC, replication complex; ER, endoplasmic reticulum; TAM, tamoxifen; ERE, ESR-responsive element(s); CAV, caveolin; NS, nonstructural protein; MM, microsomal membrane; siRNA, small interfering RNA; si-ESR, small interfering ESR; GST, glutathione *S*-transferase; aa, amino acid(s); RT, reverse transcription; NS3, NS4A, NS4B, NS5A, and NS5B, nonstructural protein 3, 4A, 4B, 5A, and 5B, respectively.

compounds that inhibited HCV replication. Using this system, we previously identified an immunosuppressant, cyclosporin A, as an anti-HCV compound (20). We also reported that cyclophilin B regulated the RNA binding activity of NS5B (21). In the current study, this chemical screening approach linked ESRα to HCV replication. We showed that tamoxifen (TAM) suppressed HCV genome replication. Using TAM as a bioprobe, we found that ESRα interacted with NS5B and regulated the participation of NS5B in the RC.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Huh-7 and cured MH-14 cells (21) were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, minimal essential medium nonessential amino acid (Invitrogen), and kanamycin (Meiji). MH-14 cells, carrying HCV subgenomic replicon (16), and LucNeo#2 cells, carrying luciferase-containing subgenomic replicon (22), were cultured in the same medium supplemented with 300 μg/ml G418 (Invitrogen). Hus-E7/DN24 cells, a human hepatocyte cell line established by immortalization with HPV E6E7 and hTERT from human primary hepatocytes and introduction with a dominant negative form of interferon regulatory factor-7 (23), were cultured with Dulbecco's modified Eagle's medium with 20 mM Hepes (Invitrogen), 15 g/ml L-proline, 0.25 g/ml insulin (Sigma), 50 nM dexamethasone (Sigma), 44 mM $NaHCO_3$, 10 mM nicotinamide, 5 ng/ml epidermal growth factor, 0.1 mM Asc-2P, 100 IU/ml penicillin G (Invitrogen), 100 μg/ml streptomycin (Invitrogen), 5% fetal bovine serum, 1% Dulbecco's modified Eagle's medium, and 2 UG/ml Fungizone (Invitrogen) (24). Plasmid transfection was performed with FuGENE 6 transfection reagent (Roche Applied Science), as described previously (25). RNA transfection was achieved using DMrie-C transfection reagent (Invitrogen), as described previously (21). siRNA was transfected by using siLentFect (Bio-Rad) (21).

Plasmid Construction—pCMV-FL-ESRα, encoding the whole open reading frame of ESRα fused with a FLAG tag, was generated by inserting the PCR product using 5′-GTTGAATTCATGACCATGACCCTCCAC-3′ and 5′-GTTGATCTCGAGTCAGACTGTGGCAGGGAAAC-3′ as primer set and human lymphocyte cDNA library (Clontech) as a template into the EcoRI-XhoI site of pCMV-FLAG vector (21). pCAG-HA-NS5B, encoding the NS5B protein fused with a hemagglutinin tag, was made by subcloning the PCR product with 5′-GTTGCGGCCGCTATGTCAATGTCCTACTCA-3′ and 5′-GTTCTCGAGTCACCGGTTGGGGAGCAGGTA-3′ as primers and pMH14 as a template into NotI-XhoI digestion of PCAG-HA vector (21). Expression plasmids for HCV NS3, NS4B, NS5A, and NS5B (pcDNA-NS3, pcDNA-NS4B, pcDNA-NS5A, and pcDNA-NS5B, respectively) were described in Ref. 21. pGEX-ESRα A/B, C, D, and E/F, expressing the fusion protein of the domain A/B, C, D, and E/F of ESRα with GST, were prepared by the insertion of the PCR product with pCMV-FL-ESRα as a template and appropriate primers into the EcoRI-XhoI site of pGEX-6P1 vector (Clontech). The expression plasmids for the point mutants of ESRα, ESRα(L540Q), ESRα(255M), and ESRα(258M), of which Leu at aa 540, IRK at aa 255–257, and DRR at aa 258–260 were replaced by Gln, TGT, and ANT, respectively, was generated by oligonucleotide-directed mutagenesis. pCMV-FL-CAV2, encoding FLAG-tagged CAV2, was prepared by inserting the PCR product amplified with 5′-GTTGTCGACTATGGGGCTGGAGAC-3′ and 5′-GTTAAGCTTTCAATCCTGGCTC-3′ as primers and human liver cDNA library (Clontech) as a template into the SalI-HindIII site of pCMV-FLAG vector (21). The mammalian expression vector for the C domain of ESRα was generated by replacing the EcoRI-XhoI digestion of pCMV-FLAG vector (21) by that of pGEX-ESRα C. pLMH14 was described previously (26). pGL3-EREX3-TATA-Luc, pcDNA3-ERα, pcDNA3-hERβ were kindly provided by Dr. Kato (Institute of Molecular and Cellular Biosciences, University of Tokyo). JFH1 expression plasmid was provided by Dr. Wakita (National Institute of Infectious Diseases).

Luciferase Assay—A luciferase assay monitoring HCV replication activity was performed as described previously (22, 26). In Fig. 1, *A* and *F*, we used LucNeo#2 cells, stably carrying luciferase-containing subgenomic replicon (22). In Figs. 2 (*D* and *E*), 4*C*, and 6*A*, we transiently transduced luciferase-containing replicon LMH14 RNA together with each expression plasmid into cured MH-14 cells (26). A luciferase assay detecting the transcriptional activity driven from the ERE was performed as described previously (25).

Real Time RT-PCR Analysis—Real time RT-PCR analysis was performed as previously described (20).

Immunoblot Analysis—Immunoblot analysis was performed as previously described (25). The antibodies used in this study are anti-NS5A (kindly provided by Dr. Takamizawa (Osaka University)), anti-NS5B (anti-NS5B#14; a generous gift from Dr. Kohara (Tokyo Metropolitan Institute of Medical Science)), anti-NS5B (NS5B#6; a kind gift from Dr. Fukuya (Osaka University)), anti-tubulin (Oncogene), anti-FLAG (Sigma), anti-IκBα (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-calnexin (StressGen), and anti-caveolin-2 antibodies (BD Biosciences Pharmingen).

Indirect Immunofluorescence Analysis—Indirect immunofluorescence analysis was performed as described previously (25). The antibodies used were anti-NS5A and anti-protein-disulfide isomerase antibodies (StressGen).

siRNA—siRNA duplexes (5′-GUGUGCAAUGACUAUGCUUCA-3′ for si-ESRα and 5′-CGCAUCGGGAUAUCACUAUGG-3′ for si-ESRβ) were synthesized (Proligo). A randomized siRNA, si-control, was purchased from Dharmacon (nonspecific control duplex IX).

Enzyme-linked Immunosorbent Assay—HCV core was quantified in the culture medium of the cells transfected with JFH1 RNA (29) with an enzyme-linked immunosorbent assay according to the manufacturer's protocol (HCV antigen enzyme-linked immunosorbent assay test; Ortho-Clinical Diagnostics).

RT-PCR Analysis—RT-PCR analysis was performed as described (20) by using the following primer sets: 5′-CCTACTACCTGGAGAACG-3′ and 5′-GCTGGACACATATAGTCG-3′ for the detection of ESRα and 5′-AGCCATGACATTCTATAGC-3′ and 5′-CCACTTCGTAACACTTCC-3′ for ESRβ.

GST Pull-down Assay—The GST pull-down assay was conducted as described previously (25).

Immunoprecipitation Analysis—Immunoprecipitation analysis was performed as described previously (25). The antibodies

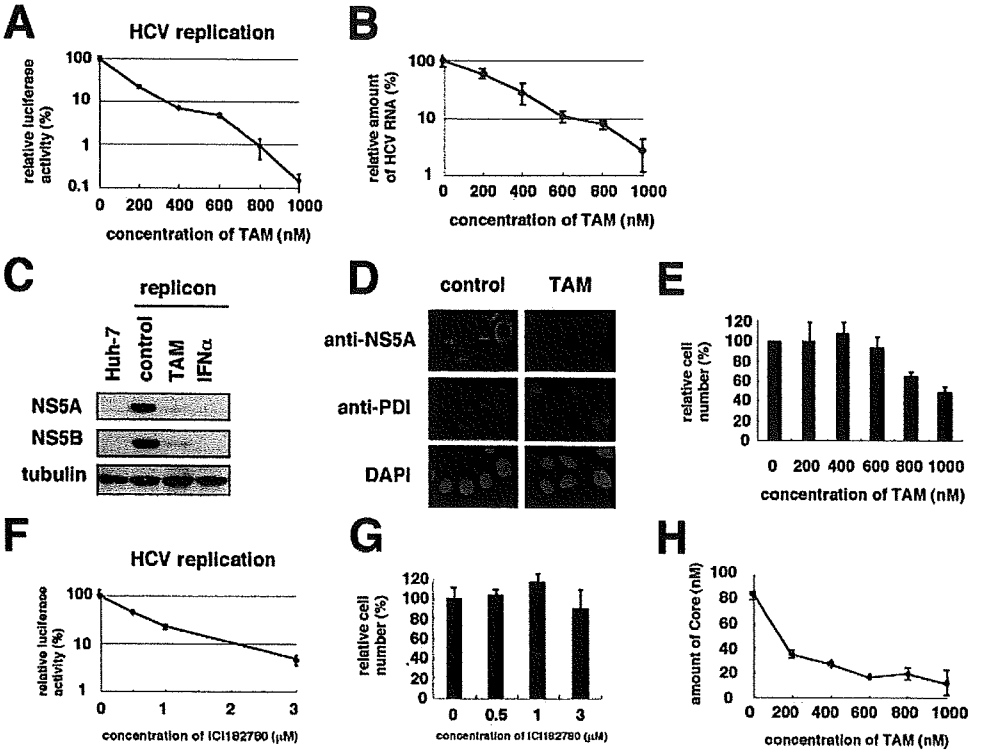

FIGURE 1. **TAM suppressed the replication of the HCV genome.** *A*, luciferase activities were measured using the LucNeo#2 cells, which carried a luciferase-containing replicon RNA, upon treatment with TAM at the indicated doses for 7 days. Relative luciferase activities are plotted against the concentrations of TAM. The data show the means of three independent experiments. The *error bars* are indicated. *B*, HCV RNA was quantified by real time RT-PCR analysis using the lysates from MH-14 cells, harboring the HCV subgenomic replicon, treated with the indicated doses of TAM for 7 days. Relative amounts of HCV RNA are shown. *C*, HCV NS5A and NS5B proteins as well as tubulin as an internal control were detected by immunoblot analysis in the lysates from MH-14 cells (replicon) treated without (control) or with 500 nM TAM or 100 IU/ml interferon-α as a positive control for 7 days and Huh-7 cells. *D*, HCV NS5A and protein-disulfide isomerase (*PDI*) as an internal control were detected by indirect immunofluorescence analysis in the cells treated without (control) or with 500 nM TAM for 7 days. 4′,6-Diamidino-2-phenylindole (*DAPI*) shows a nuclear staining. *E*, cell number was counted after 5 days upon treatment with various concentrations of TAM. Relative cell numbers are shown. *F*, luciferase activities with LucNeo#2 cells treated with various concentrations of ICI182780 were measured as described in *A*. *G*, cell number was counted under treatment with ICI182780 at the indicated concentrations. *H*, core in the culture medium of JFH1 RNA-transfected cells upon treatment with TAM was quantified as described under "Experimental Procedures."

used in this study were mouse normal IgG as a negative control (Zymed Laboratories), anti-NS5B (anti-NS5B#10; a generous gift from Dr. Kohara at the Tokyo Metropolitan Institute of Medical Science), anti-FLAG, and anti-caveolin-2 antibodies.

Fractionation of Cell Extracts—MH-14 cells transfected with the expression plasmid for FLAG-tagged ESRα were fractionated essentially as described previously (25).

HCV Replication Complex Assay—Isolation of HCV RC was done as described previously (16, 21).

In Vitro HCV Infection Experiment—*In vitro* HCV infection was conducted essentially as described (23). Briefly, HCV-infected serum ($\sim 2 \times 10^5$ copies) was inoculated into HuS-E7/DN24 cells (5×10^4 cells) for 24 h. After washes, cells were cultured in the medium supplemented with 10 μM PD98059 to stimulate HCV translation (27) (*scheme* in Fig. 6*B*). To observe HCV amplification, HCV RNA in the cells was quantified, since HCV RNA was hardly detected significantly in the culture medium (23).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide Assay—The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed to examine the cell viability using Cell Proliferation kit II, XTT (Roche Applied Science) according to the manufacturer's protocol.

RESULTS

Tamoxifen Suppressed HCV Genome Replication—We screened for agents that suppressed HCV genome replication using a HCV subgenomic replicon system (13, 16). Among the compounds tested, we observed that TAM inhibited HCV genome replication. HCV replication activity, monitored by luciferase activity (22), and the amount of HCV RNA were decreased with TAM treatment in a dose-dependent manner (Fig. 1, *A* and *B*). The expression of HCV proteins, NS5A and NS5B, detected by immunoblot (Fig. 1*C*) and indirect immuofluorescence analyses (Fig. 1*D*), also drastically decreased by treatment with TAM. A high concentration of TAM decreased cell proliferation (Fig. 1*E*). However, TAM suppressed HCV replication without any cytotoxicity in another cell line, HuS-E7/DN24 cells (Fig. 6, *C* and *D*). In addition, a pure anti-estrogen compound ICI182780, which had little cytotoxic effect, reduced HCV RNA (Fig. 1, *F* and *G*). Moreover, TAM inhibited the production of core in the culture medium of HCV JFH1-transfected cells, in a recently developed system of the production of infectious HCV particles (Fig. 1*H*) (28–30). The above data indicate that TAM suppresses HCV genome replication.

ESR Was Involved in HCV Genome Replication—Next, we investigated which cellular protein TAM targets to suppress HCV replication. It has been reported that TAM targets 1) ESR (31), 2) P-glycoprotein (32, 33), 3) calmodulin (34), 4) protein kinase C (35, 36), etc. Although other compounds targeting P-glycoprotein, calmodulin, and protein kinase C did not affect HCV replication in our screening (data not shown), ESR was suggested to play a role in HCV replication as shown below.

RNAi-mediated specific knockdown of endogenous ESRα and ESRβ (Fig. 2*A*) reduced HCV RNA in replicon-containing cells to ~20–40% and 60–70%, respectively (Fig. 2*B*). Transient transfection with ESRα and ESRβ expression plasmids, which activated ERE-driven transcription 4–5-fold (Fig. 2*C*), showed that ectopically expressed ESRα augmented HCV replication activity in a dose-dependent manner, whereas ESRβ did not (Fig. 2*D*). ESRα-induced augmentation of the replication was reversed upon TAM treatment (Fig. 2*D*). These results suggested a significant role of ESR, especially ESRα, in HCV

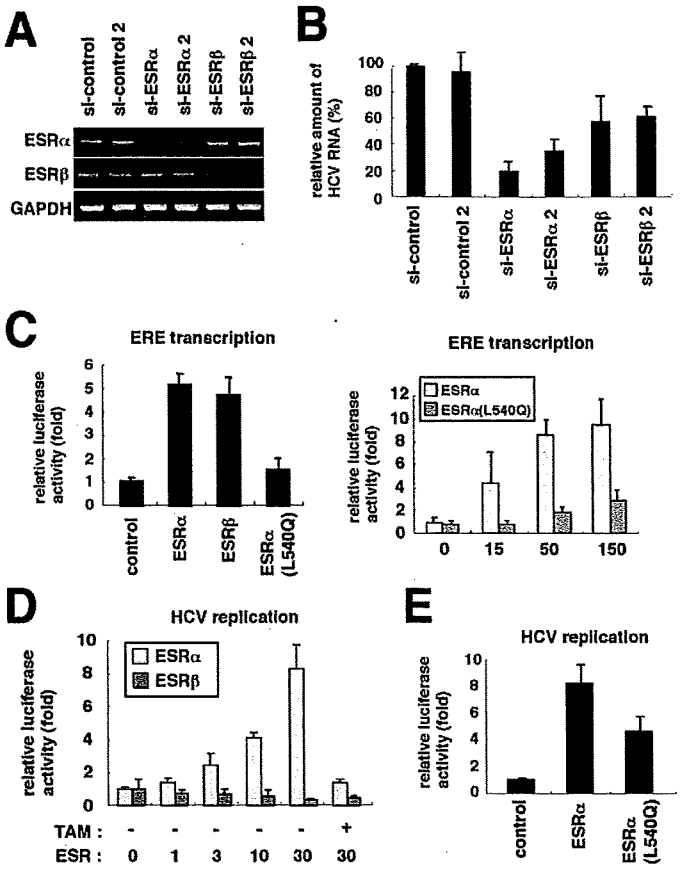

FIGURE 2. **ESR was involved in HCV genome replication.** *A*, specific knockdown of endogenous ESRα and ESRβ. RT-PCR analysis was performed to detect the expression of ESRα, ESRβ, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as an internal control in the cells transfected with siRNA recognizing ESRα (si-ESRα, si-ESRα2), ESRβ (si-ESRβ, si-ESRβ2), or randomized siRNA (si-control, si-control2). *B*, HCV RNA was quantified as shown in Fig. 1*B*, using the cells transfected with si-control, si-control2, si-ESRα, si-ESRα2, si-ESRβ, and si-ESRβ2 for 5 days. *C*, the ERE-mediated transcriptional activities were measured by a luciferase assay using the lysates from the cells transfected with pGL3-EREX3-TATA-Luc reporter plasmid together with pcDNA3-ERα (ESRα), pcDNA3-hERβ (ESRβ), pcDNA-ESRα(L540Q), or the empty vector (control) (*left*) or varying amounts (ng) of pcDNA3-ERα (ESRα) or pcDNA-ESRα(L540Q) (*right*) and treated with 100 nM estradiol for 36 h. *D* and *E*, HCV replication activities were examined by quantifying the luciferase activities using cured MH-14 cells transfected with the indicated doses (ng) of ESRα or ESRβ (*D*) or 30 ng of ESRα, ERα(L540Q), or the empty vector (control) (*E*) together with 0.125 μg of LMH14 RNA without or with 1 μM TAM for 4 days.

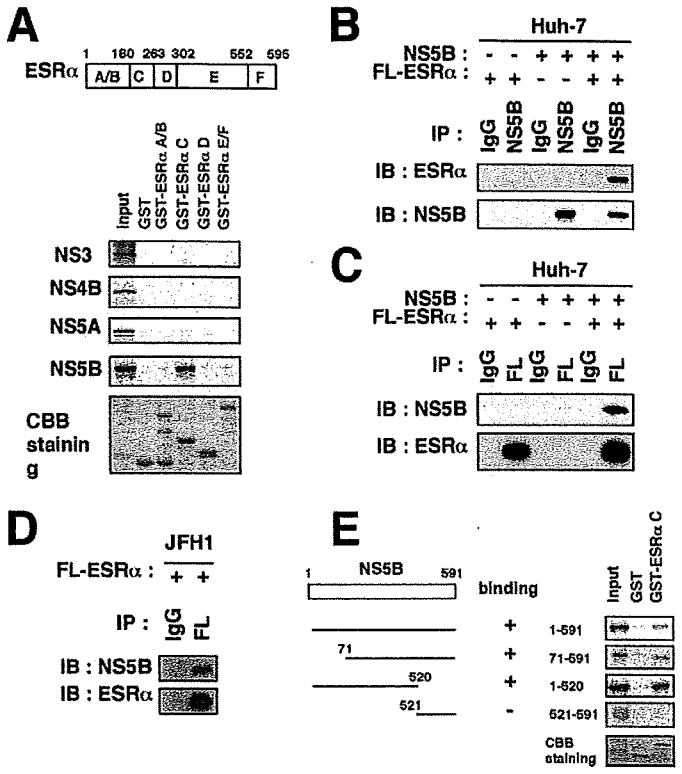

FIGURE 3. **ESRα interacted with HCV NS5B.** *A, top*, schematic representation of the primary structure of ESRα. ESRα consists of domains A–F. The amino acid numbers are also shown. *Bottom*, GST pull-down assays were performed using the recombinant proteins of the A/B, C, D, and E/F domain of ESRα fused with GST and *in vitro* translated HCV NS3, NS4B, NS5A, and NS5B protein. *Input*, the one-fifth amount of protein used for the pull-down assay. The Coomassie Brilliant Blue staining pattern of the precipitated fraction is also shown in the *bottom panel*. *B–D*, the lysates from the cells ectopically expressing NS5B (*B* and *C*) or the whole open reading frame of the HCV JFH1 strain (*D*) and/or FLAG-tagged ESRα were immunoprecipitated (*IP*) with anti-NS5B (*B*; NS5B), anti-FLAG antibody (*C* and *D*; FL), or mouse normal IgG as a negative control followed by the detection of ESRα and NS5B by immunoblot analysis (*IB*). *E*, deletion mutants of NS5B were subjected to a GST pull-down assay with GST-fused C domain of ESRα as described in *A*. The *left panel* shows a schematic representation of the full-length and truncated mutants of NS5B. The *numbers* indicate the amino acid numbers in NS5B.

genome replication. ESRα(L540Q), carrying a leucine to glutamine point mutation at aa 540 within the L*XX*LL motif (aa 536–540) of ESRα (37), had much lower transactivation activity driven from ERE (Fig. 2*C*). However, ESRα(L540Q) stimulated HCV replication activity ~5-fold, although the stimulation was less than that by wild-type ESRα (Fig. 2*E*). Thus, ESRα having lower transactivating capacity could still facilitate HCV replication.

ESRα Interacted with HCV NS5B—Thus, the chemical biology approach revealed the involvement of ESR in HCV genome replication. Then we investigated the molecular mechanism of ESR-induced HCV replication. A binding assay between ESRα and HCV proteins expressed in the HCV subgenomic replicon showed that the C domain of ESRα coprecipitated with NS5B but not NS3, NS4B, and NS5A (Fig. 3*A*). Other ESRα domains, A/B, D, and E/F, did not bind to any HCV proteins. A coimmunoprecipitation assay also indicated the presence of ESRα in the immunoprecipitate by anti-NS5B antibody (Fig. 3, *B* and *D*), and *vice versa* (Fig. 3*C*). Thus, ESRα specifically interacted with NS5B. Deletion analysis indicated that the region of 71–591 and 1–520 but not 521–591 of NS5B coprecipitated with the recombinant C domain of ESRα (Fig. 3*E*). This binding profile is different from that between cyclophilin B and NS5B, which we previously reported (21).

The ESRα-NS5B Interaction Was Important for the Regulation of HCV Genome Replication—To examine whether the interaction between ESRα and NS5B was essential for the ESRα-mediated regulation of HCV replication or not, we searched for a point mutant of ESRα that could not bind to NS5B by alanine-scanning mutation analysis. ESRα mutants, ESRα(255M) and ESRα(258M), in which IRK at aa 255–257 and DRR at aa 258–260 was replaced by TGT and AQT, respectively, had little affinity with NS5B (Fig. 4*A*) but still possessed the ERE-mediated transactivation capacity (Fig. 4*B*). However, both ESRα(255M) and ESRα(258M) caused only weak activations of HCV replication, compared with wild type ESRα (Fig. 4*C*). The data suggest that the interaction of ESRα with NS5B is

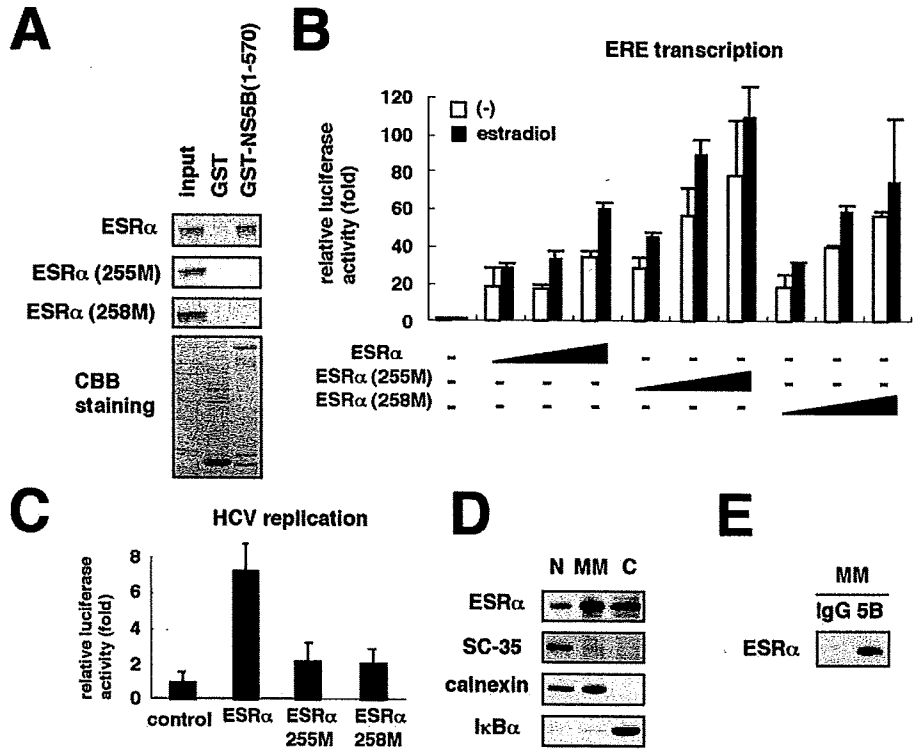

FIGURE 4. **The interaction of NS5B mediated the regulation of HCV genome replication by ESRα.** *A*, GST pull-down assays were performed as described in Fig. 3*A* using the wild type ESRα or point mutant of ESRα, ESRα(255M), and ESRα(258M). *B*, the mutation within ESRα(255M) and ESRα(258M) did not reduce the activation capacity of ERE-mediated transcription. Huh-7 cells were transfected with the expression plasmids for ESRα, ESRα(255M), or ESRα(258M) at doses of 10, 30, and 100 ng each together with pGL3-EREX3-TATA-Luc reporter plasmid and treated without (*white bar*) or with 100 nM estradiol (*black bar*) to quantify the luciferase activity. *C*, HCV replication activities were examined by quantifying the luciferase activities as described in the legend to Fig. 2*D* in the cells upon transfection with the expression plasmids for wild type ESRα, ESRα(255M), or ESRα(258M). *D*, the cells were fractionated into the nucleus (*N*), MM, and cytoplasm (*C*). Each fraction was detected for FLAG-tagged ESRα, SC-35, calnexin, and IκBα, respectively, by immunoblot analysis. Calnexin, an ER marker protein, was detected in the nucleus as well as MM, probably because of the existence of the nuclear membrane in the nuclear fraction. *E*, the MM fraction obtained in *D* was subjected to a coimmunoprecipitation assay using anti-NS5B or IgG followed by immunoblot analysis for the detection for ESRα.

critical for ESRα-mediated regulation of HCV genome replication.

Thus, ESRα interaction with NS5B regulates HCV replication. NS5B is mainly located on the cytoplasmic surface of the ER membrane (21, 38). On the other hand, ESRα as a nuclear hormone receptor is normally distributed in the cytoplasm and translocates into the nucleus upon ligand stimulation. In addition, a part of ESRα localizes on the membrane fraction. In our experiment, NS5B was mainly located around the ER, colocalized with the ER marker, protein-disulfide isomerase (data not shown) (21). Ectopically expressed ESRα showed diffuse distribution in the cells (data not shown). We fractionated cell homogenates and observed that a part of the ESRα resided in the microsomal membrane (MM) fraction (Fig. 4*D*). Moreover, ESRα in the MM fraction was coprecipitated with NS5B (Fig. 4*E*). It suggests the possibility that the interaction between NS5B and ESRα, at least in part of them, occurs on the ER membrane.

ESRα Promoted the Participation of NS5B in the HCV Replication Complex—It was reported that HCV proteins involved in the replication machinery was associated with the lipid raft on the ER and cofractionated with CAV2. A coimmunoprecipitation assay showed that NS5B associated with CAV2 (Fig. 5*A*). In the experiment investigating the role of ESRα in NS5B-CAV2 association, the coprecipitation of NS5B with CAV2 was decreased upon the knocking down of ESRα (Fig. 5*B*). Treatment with TAM abrogated the association of NS5B with CAV2 (Fig. 5*C*), although the total amount of NS5B in the cells is similar in the presence and absence of TAM for 24 h in this experiment (data not shown). Thus, ESRα was suggested to promote the association between NS5B and CAV2. Since a part of CAV2 resided on the lipid raft on the ER (18), ESRα-mediated binding between NS5B and CAV2 was possible to affect the localization of NS5B to the HCV RC. To see the consequential relevance of ESRα on NS5B function, we analyzed the HCV RC by treatment with digitonin/protease as described previously (16). HCV proteins involved in the RC and surrounded by the membrane structure are resistant to the treatment with digitonin followed by protease, whereas those unrelated to the replication outside the RC are digested by the treatment. By using this technique measuring the sensitivity to protease, HCV RC can be distinguished from the ER that is not related to the replication, although the RC and the nucleus cannot be separated. The experimental condition for fractionation was confirmed with the detection with IκBα and calnexin; a cytosolic protein IκBα was washed out following the treatment with digotinin (Fig. 5*D*, *lanes 1* and *2*), and ER protein calnexin, which did not accumulate in the RC, was digested by treatment with digotinin/protease (Fig. 5*D*, *lanes 2–4*). An ER lipid raft component, CAV2, was still detected under the digitonin/protease treatment (the RC-containing fraction) (Fig. 5*D*, *lanes 3* and *4*). Under this condition, a part of NS5B was detected in the digitonin/protease-resistant fraction, as described previously (16) (Fig. 5*D*, *lanes 3* and *4*). However, NS5B in this fraction was decreased upon treatment with TAM (Fig. 5*D*, *lanes 3*, *4*, *7*, and *8*). On the other hand, the amount of NS5A was not significantly changed by TAM treatment. Knocking down of ESRα also disrupted the association of NS5B with the RC-containing fraction (Fig. 5*E*). From the above results, it was suggested that ESRα promoted the participation of NS5B in the RC (also see "Discussion").

ESRα Could Serve as a Molecular Target of Anti-HCV Agents—Finally, we assessed the possibility that the association of ESRα with NS5B could serve as a target of anti-HCV agents. By introducing a decoy peptide against ESRα-NS5B interaction, consisting of the C domain of ESRα into replicon-bearing cells,

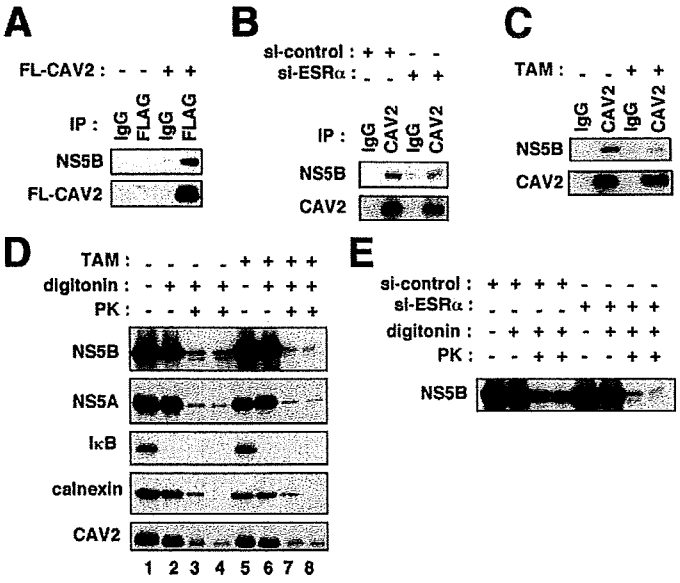

FIGURE 5. **ESRα promoted the participation of NS5B in HCV RC.** *A–C*, a coimmunoprecipitation assay (*IP*) was performed with anti-FLAG (*A*), anti-CAV2 (*B* and *C*) antibody, or mouse normal IgG from the lysates of the cells transfected without or with FLAG-tagged CAV2 (*A*), transfected with si-control or si-ESRα (*B*), or treated without or with 1 μM TAM (*C*). NS5B (*top*) and CAV2 (*bottom*) were detected by immunoblot analysis. *D*, detection of the amount of NS5B in the digitonin/protease-resistant fraction. MH-14 cells were treated without (*lanes 1–4*) or with 1 μM TAM (*lanes 5–8*) for 24 h. Cells were then treated without (*lanes 1* and *5*) or with digitonin (*lanes 2–4* and *6–8*), followed by digestion with proteinase K (0 μg/ml for *lanes 2* and *6*, 0.3 μg/ml for *lanes 3* and *7*, and 1 μg/ml for *lanes 4* and *8*). NS5B, NS5A, IκBα, calnexin, and CAV2 were detected by immunoblot analysis. *E*, HCV RC was isolated as described in *D* using the cells transfected with si-control or si-ESRα, and NS5B was detected. A similar result was obtained by using si-ESRα2.

HCV replication activity was reduced in a dose-dependent manner (Fig. 6*A*). To further observe the significance of ESRα in a physiological condition, we performed an *in vitro* infection experiment using serum from an HCV-infected patient as a nascent virus inoculum and nonneoplastic human hepatocytes as highly infection-permissive cells (Fig. 6*B*). Treatment with 1 μM TAM did not show a cytotoxic effect on these cells in any time course examined (Fig. 6*C*). However, treatment with TAM as well as cyclosporin A as a positive control inhibited the multiplication of viral genome RNA in the cells along with the time course (Fig. 6*D*). Thus, ESRα could serve as a potent molecular target of anti-HCV agents.

DISCUSSION

In general, viruses take advantage of host cell factors for their replication. So far, some factors have been shown to relevantly regulate HCV replication, including hVAP33 (39, 40), FBL2 (41), and cyclophilin B (21). Among these, FBL2 and cyclophilin B were identified by a chemical biological approach; FBL2 from the observation of an anti-HCV activity of lovastatin and an inhibitor of geranylgeranyl transferase (41–43); cyclophilin B from the inhibitory effect of cyclosporin A on HCV replication (20, 21). In this study, we found a suppressive capacity of TAM to HCV genome replication. Through further examination using TAM, we revealed ESRα as a host cell factor regulating HCV replication and suggested its regulation mechanism.

Currently, it is proposed that HCV RC that replicates the HCV genome is formed on the intracellular membrane, including the ER membrane (14–17). It was also reported that HCV genome replication was associated with the lipid raft on the intracellular membrane (18). Most HCV proteins are not related to the RC, whereas only a minor portion of HCV proteins take part in the RC to drive the viral replication (16). It has remained widely unknown, however, how HCV proteins are regulated to participate in the RC. It was reported that hVAP-33 binds to NS5A and NS5B, and this protein is related to the amount of NS5B in the lipid raft (40). hVAP-33 was speculated to recruit NS5B to the lipid raft, although its molecular mechanism has not been analyzed. This study suggested the interaction between ESRα and NS5B in the ER fraction, although we did not show the existence of ESRα in the RC, since the RC and the nucleus cannot be separated in the digitonin/protease treatment experiment. ESRα promoted the interaction of NS5B with CAV2. Previous papers reported that ESRα bound to CAV1 and CAV2 (6). From these observations, ESRα is

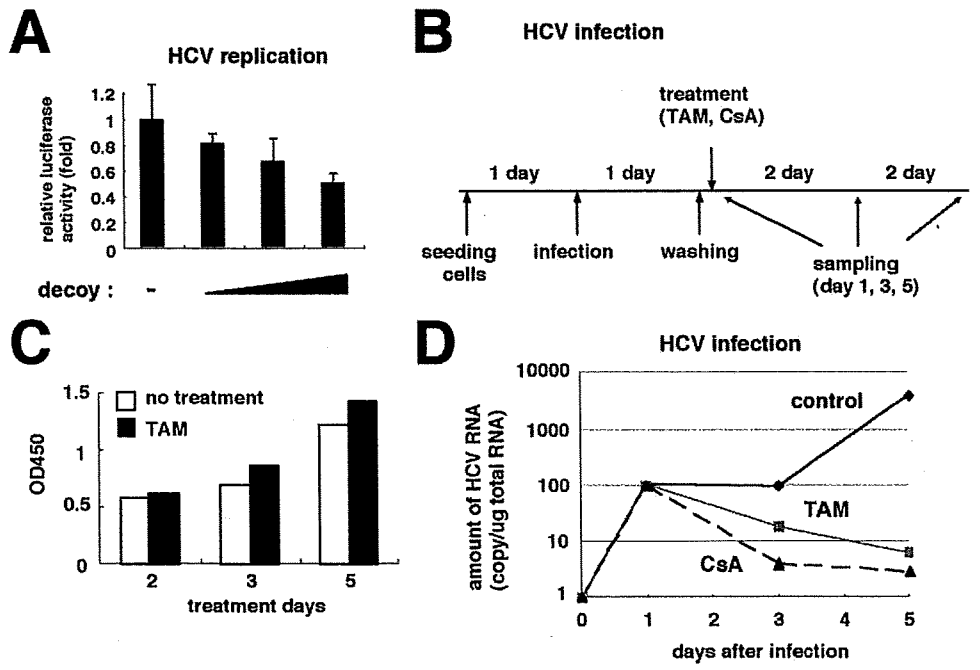

FIGURE 6. **ESRα could serve as a molecular target for anti-HCV agents.** *A*, HCV replication activity was measured by quantifying the luciferase activity as described in the legend to Fig. 2*D* in the cells overexpressing a decoy peptide consisting of the C domain of ESRα. *B*, experimental scheme of *in vitro* HCV infection experiment. After seeding the HuS-E7/DN24 cells, HCV-positive serum was inoculated for 24 h. After extensive washes, the cells were cultured with the medium supplemented without (control) or with 1 μM TAM or 3 μg/ml cyclosporin A. HCV genome RNA was quantified along with the time course (days 1, 3, and 5 postinoculation) by real time RT-PCR analysis. *C*, the treatment with 1 μM TAM did not show any cytotoxic effect on HuS-E7/DN24 cells. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays were performed as described under "Experimental Procedures" to examine the viability of the cells at days 2, 3, and 5 postinoculation. *D*, HCV genome RNA was quantified as described in *B* and plotted against the time course.

likely to function as a bridging factor that connects NS5B to CAV2, although we cannot fully neglect the possibility that ESRα augments NS5B-CAV2 binding via another function, such as transcriptional activity. Because CAV2 resided on the lipid raft of the intracellular membrane (18), this action of ESRα may recruit NS5B to the lipid raft and the HCV RC. In fact, ESRα promoted the participation of NS5B in the HCV RC. Thus, ESRα is suggested to escort NS5B to the HCV RC, although it is also possible that ESRα augments the number of the RC itself. However, ESRα at least augments the amount of NS5B involved in HCV replication machinery to stimulate the replication. It was reported that the membrane-associated ESRα served as a platform where signalsomes, including receptor tyrosine kinase, nonreceptor tyrosine kinase Src, and G proteins, assembled and activated downstream signaling pathways (44–46). HCV may also take advantage of such platform characteristics of ESRα to form the RC for their efficient replication. Although the mechanisms of the nuclear receptor function of ESRα have been extensively elucidated, the functions of membrane-associated ESRα have not been widely characterized so far. This study suggested a novel physiological relevance of membrane-associated ESRα as a regulator of the viral replication.

Until now, there are no clinical studies that report a direct interaction of TAM treatment with HCV replication in patients infected with HCV. Given our results, examinations on the effect of TAM or other anti-estrogen drugs may be one of the useful approaches to develop a new anti-HCV strategy. On the other hand, we disclosed the mechanism of ESR-mediated regulation of HCV genome replication. Screening for compounds that inhibit this mechanism expectedly led to novel types of anti-HCV agents. Further analyses on ESR are needed to develop anti-HCV therapeutics as well as reveal the regulation mechanism of HCV replication.

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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).

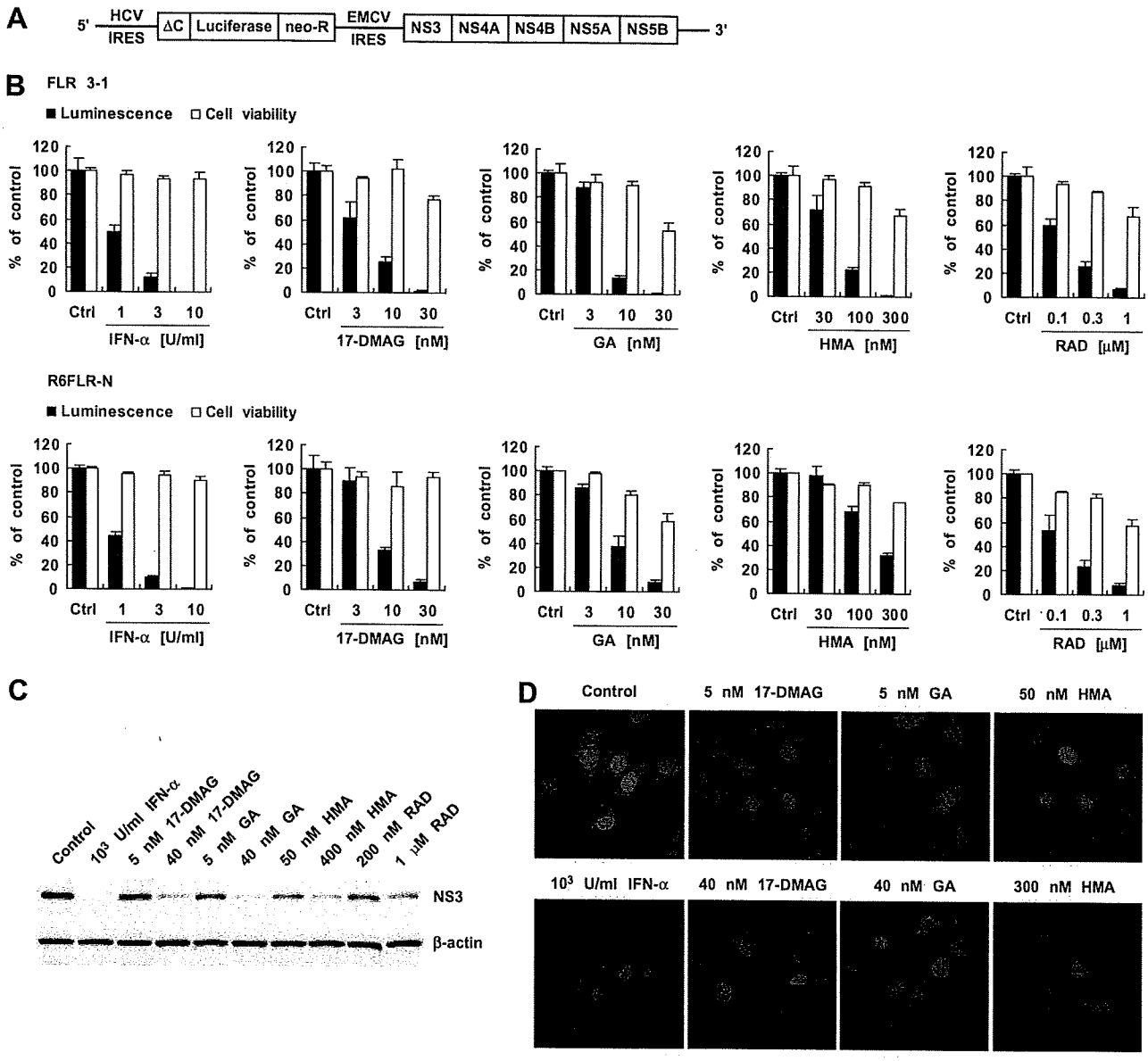

Fig. 1. Effect of Hsp90 inhibitors on HCV replication and cell viability in HCV replicon cells. (A) Schematic representation of the HCV subgenomic replicons used in this study. ΔC, first 45 nucleotides of HCV core protein gene; neo-R, neomycin phosphotransferase gene. (B) Inhibition of replication of 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).

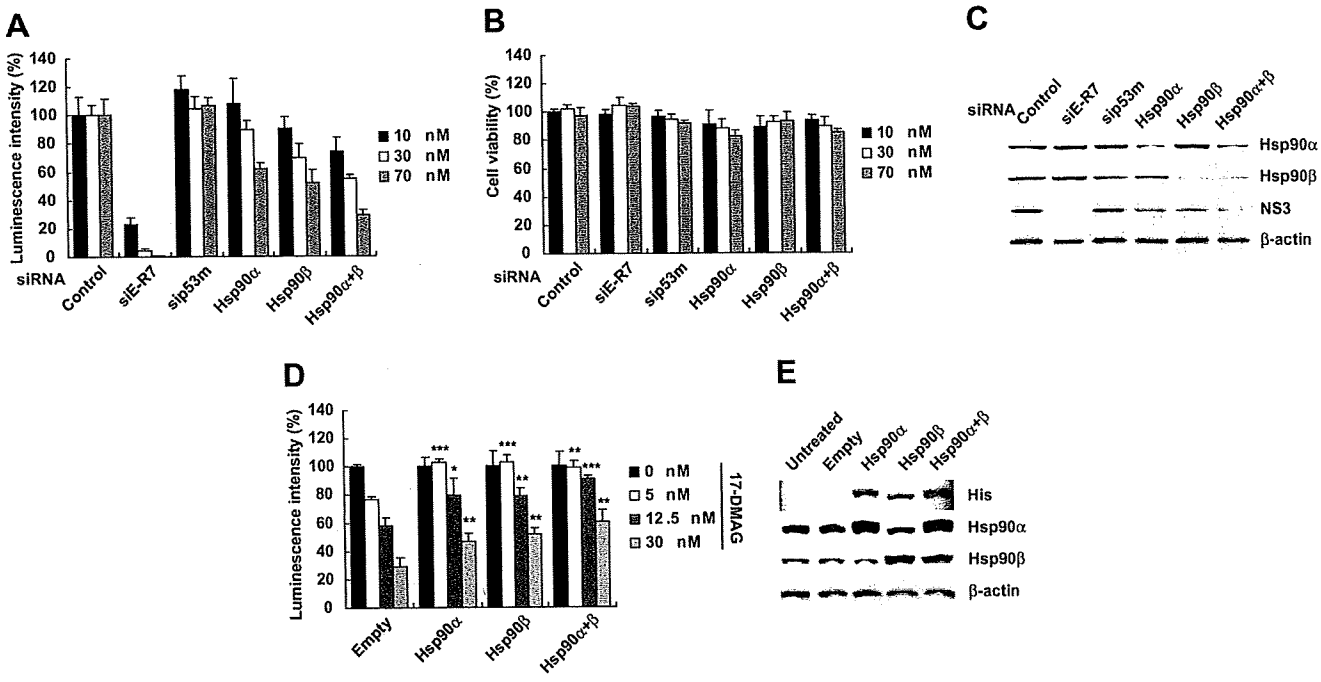

Fig. 2. Effect of knockdown or overexpression of Hsp90 on replication of the HCV replicon. (A,B) FLR 3-1 replicon cells were transfected with HCV-directed siRNA (siE-R7), negative-control siRNA (sip53m) or Hsp90-directed siRNA. Seventy-two hours after siRNA transfection, HCV replicon levels were determined 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

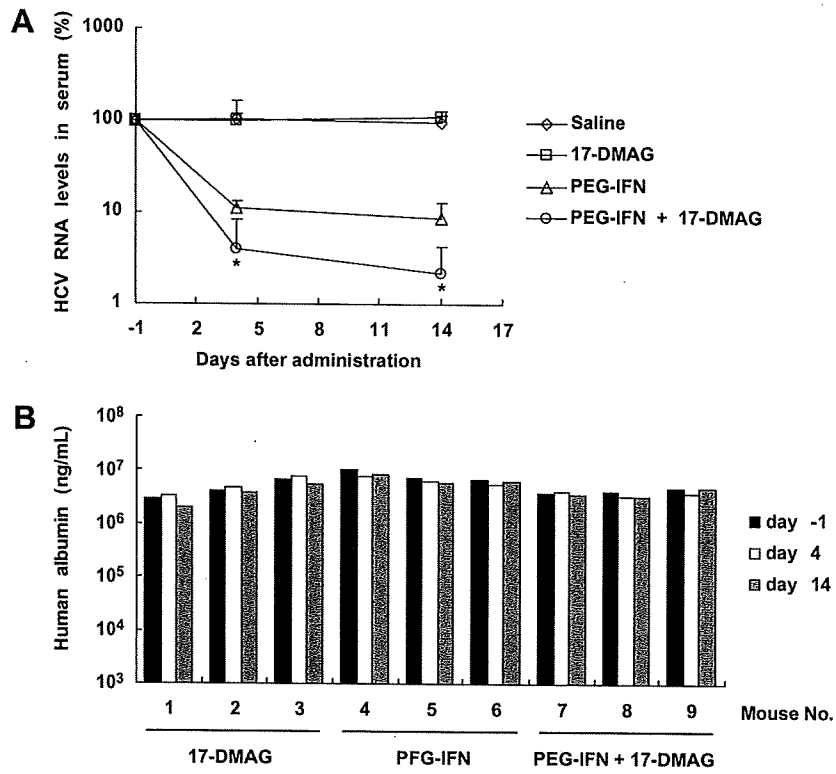

Fig. 3. Anti-HCV effect of 17-DMAG in HCV-infected chimeric mice. (A) HCV genome RNA levels in the serum of chimeric mice were determined by real-time PCR. (B) Human albumin concentrations in the sera of chimeric mice. The numbers 1–9 represent individual mice. Numbers 1, 2, and 3 were in the group treated 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.

Acknowledgments

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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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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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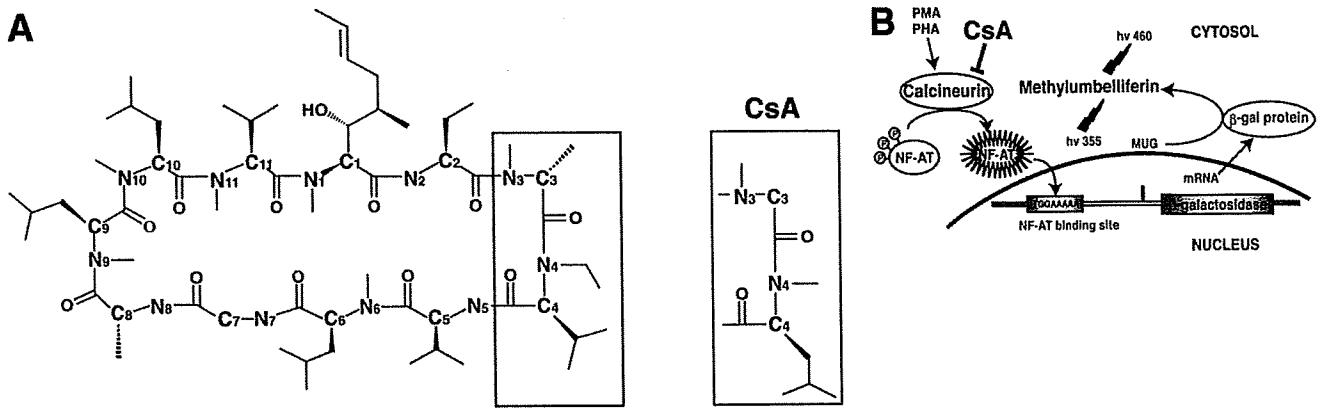

Fig. 1. (A) Structure of DEBIO-25, which was derived from CsA by substitution of amino acids at positions 3 and 4. (B) Scheme for IL-2 reporter gene assay. Nuclear factor of activated T cells (NF-AT), phorbol-12-myristate-13-acetate (PMA), phytohemagglutinin (PHA), 4-methyl umbelliferyl-β-D-galactoside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

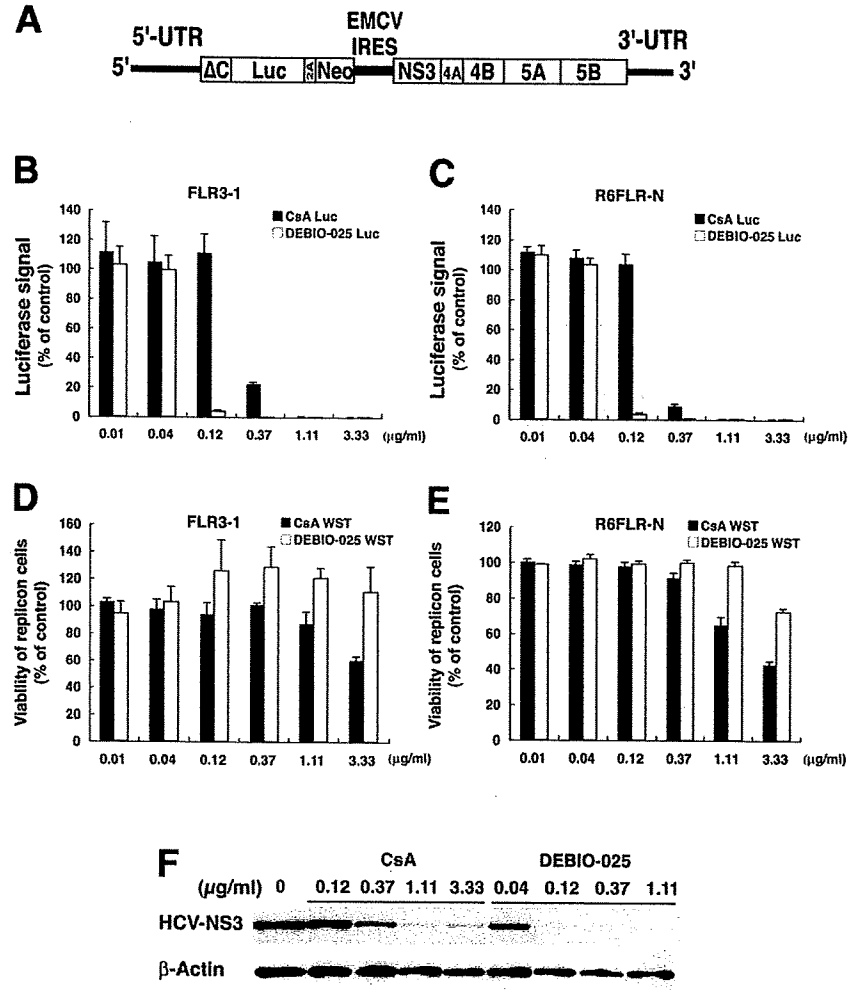

Fig. 2. (A) Structure of HCV replicon genome. FLR3-1 and R6FLR-N were of similar construction. Encephalomyocarditis virus (EMCV), internal ribosomal entry site (IRES), untranslated region (UTR). (B, C) Effect of DEBIO-025 or cyclosporin A (CsA) on HCV replication, as monitored in triplicate by luciferase 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-

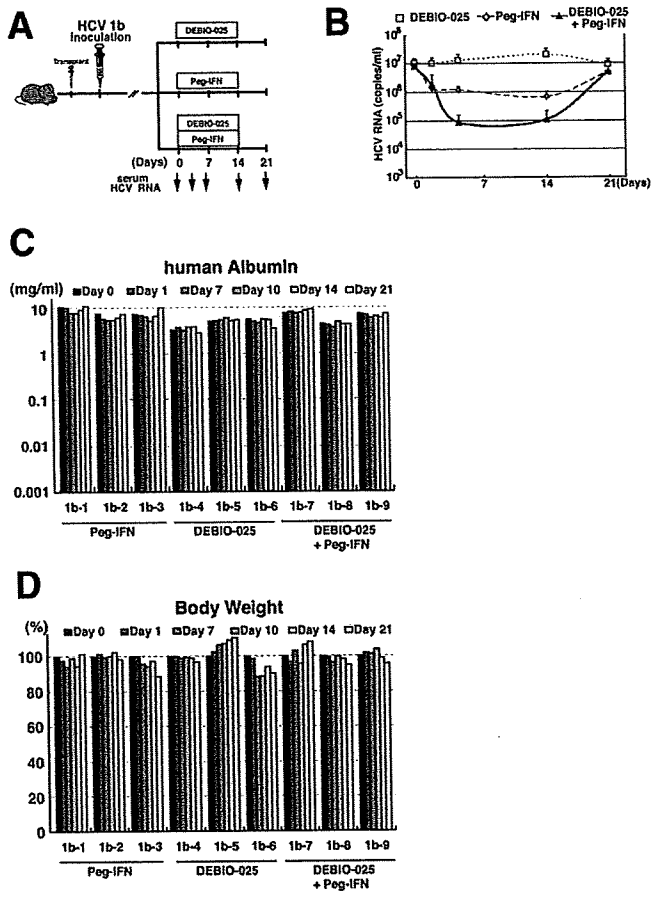

Fig. 3. (A) Schedule of experiments using chimeric mice infected with HCV genotype 1b. The mice were treated for 14 days with DEBIO-025 100 mg/kg/day orally, Pegylated-interferon $\alpha-2a$ (Peg-IFN) 30 μg/kg subcutaneously twice weekly, or DEBIO-025 100 mg/kg/day orally combined with Peg-IFN 30 μg/kg 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.

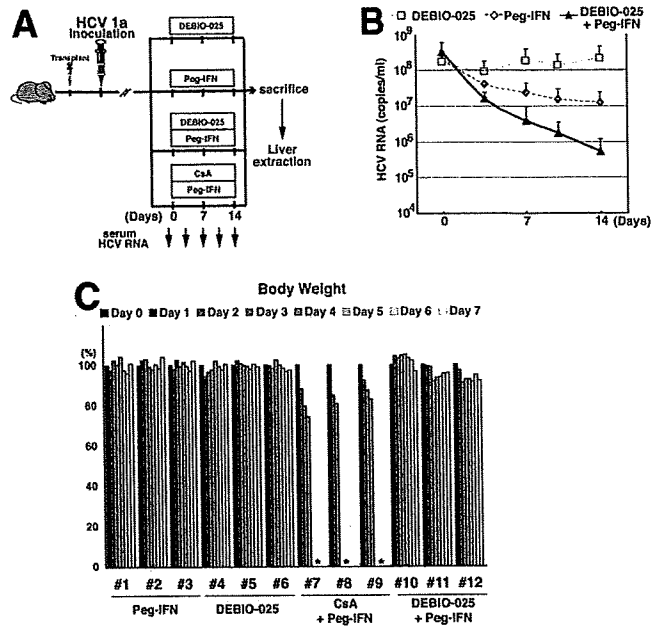

Fig. 4. (A) Schedule of experiments using chimeric mice infected with HCV genotype 1a. The mice were treated for 14 days with DEBIO-025 100 mg/kg/day orally, Peg-IFN 30 μg/kg subcutaneously twice weekly, DEBIO-025 100 mg/kg/day orally combined with Peg-IFN 30 μg/kg subcutaneously twice weekly, or CsA 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.

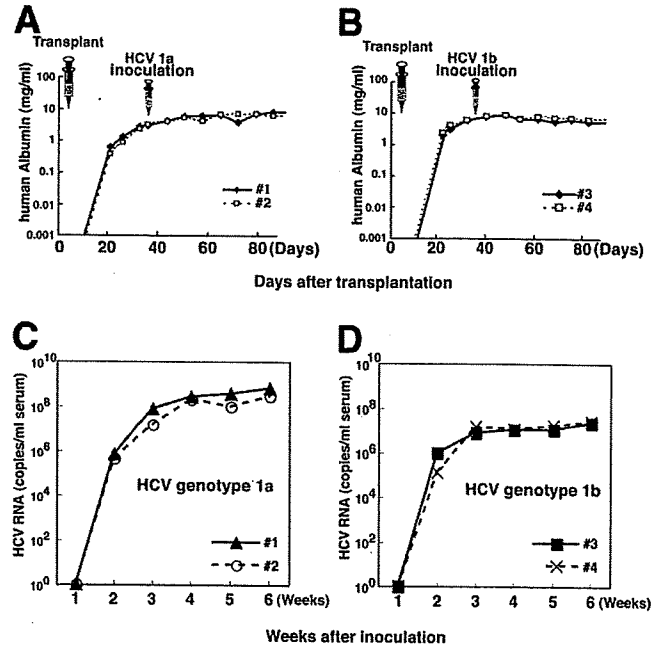

Fig. 5. Time course studies in 4 mice inoculated with human serum samples positive for HCV genotypes 1a or 1b. (A,B) Human albumin concentrations in mouse serum after transplantation of hepatocytes. (C,D) HCV RNA levels in mouse serum after inoculation.

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