1 **Materials and Methods**

2 ***Compounds.*** DEBIO-025 is a synthetic compound derived from CsA. Sarcosine

3 (N-methyl-D-glycine) at position 3 and N-methyl-D-leucine at position 4 are substituted for

4 N-methyl-D-alanine and N-ethyl-D-valine respectively (Fig. 1A).[16] DEBIO-025 was obtained

5 from Debiopharm (Lausanne, Switzerland). CsA was purchased from Fulka Chemie (Buchs,

6 Switzerland) and Peg-IFN was purchased from Chugai Pharmaceutical Co. (Tokyo, Japan).

7 ***Anti-HCV Assay in HuH-7 Cells Harboring Subgenomic Replicons.*** We used two HCV

8 subgenomic replicon cell lines, FLR3-1[17] and R6FLR-N[18], which were constructed as shown in

9 Fig. 2A. They were seeded at a density of 5×10^3 per well in 96-well tissue culture plates, in

10 complete Dulbecco's modified Eagle's medium GlutaMax I (DMEM-GlutaMaxI; Invitrogen,

11 Carlsbad, CA) and containing 5% FCS (Invitrogen).[17,18] The genome of the two replicons was

12 genotype 1b. Following incubation for 24 hours at 37°C (5% CO_2), the medium was removed and

13 serial dilutions of DEBIO-025 or CsA in growth medium were added. After 72 hours, luciferase

14 activity was determined using the Bright-Glo luciferase assay kit (Promega Madison, WI). The

15 luciferase signal was measured in triplicate using an LB940 luminometer (Berthold, Freiburg,

16 Germany) and the results were expressed as the average percentage of control. IC_{50} values of

1 DEBIO-025 and CsA were calculated by nonlinear curve fitting following the equation:
2 $Y=100-(Y_{Bottom} \times X/(IC_{50}+X))$, where Y represents percent inhibition and X represents the
3 concentration of the agent. The viability of replicon cells was measured using the WST-8 cell
4 counting kit according to the manufacturer's instructions (Dojindo, Kumamoto, Japan).
5 ***Western Blot Analysis of HCV NS3 and β-Actin.*** HCV replicon cells (1×10^6) were lysed with
6 100 μL of lysis buffer (1% SDS, 0.5% Nonidet P-40, 150 mmol/L NaCl, 0.5 mmol/L EDTA, 1
7 mmol/L dithiothreitol, and 10 mmol/L Tris, pH 7.4). Five μg of total protein was electrophoresed
8 on a 10% SDS-polyacrylamide gel and subsequently transferred to a polyvinylidene difluoride
9 membrane (Immobilon-P; Millipore, Billerica, MA). Nonstructural protein 3 (NS3) of HCV was
10 detected using the rabbit anti-NS3 (R212) polyclonal antibody that was prepared in our laboratory.
11 β-actin was detected using anti-β-actin monoclonal antibody (Sigma, St. Louis, MO).
12 ***Immunosuppressive Activity of DEBIO-025 and CsA by Interleukin-2 Reporter Gene Assay In***
13 ***Vitro.*** We examined the immunosuppressive activities of DEBIO-025 and CsA using an nuclear
14 factor of activated T cells (NF-AT)-dependent interleukin-2 (IL-2) reporter gene assay (Fig.
15 1B).[19] We used Jurkat T-cells stably expressing lac-Z controlled by the IL-2 promoter. The cells
16 were grown in RPMI 1640 medium containing 10% FCS, 2 mmol/L glutamine, 50 μmol/L

1 2-mercaptoethanol, and 100 U/mL hygromycin B. Jurkat T-cells were stimulated with
2 phorbol-12-myristate-13-acetate (2.4 μmol/L) and phytohemagglutinin (75 μg/mL) in the
3 presence or absence of DEBIO-025 or CsA (10^{-9} to 2×10^{-5} mol/L). After incubation at 37°C for
4 20 hours, cells were harvested by lysis buffer (50 mmol/L Na_2HPO_4, pH 9.0, 10 mmol/L KCl, 1
5 mmol/L $MgSO_4$, and 1% Triton X-100), and then β-galactosidase activity in the lysate was
6 measured using 4-methylumbelliferyl-β-D-galactoside (0.5 mmol/L; Sigma).
7 ***HCV Infection Into Chimeric Mice.*** We purchased chimeric mice from PhenixBio (Hiroshima,
8 Japan). The chimeric mice were generated by transplanting human primary hepatocytes into
9 severe combined immunodeficient (SCID) mice carrying the urokinase plasminogen activator
10 transgene controlled by an albumin promoter (uPA/SCID).[20] The chimeric mice used in this study
11 were improved from the original ones, as described by Tateno et al.,[21] and had a high substitution
12 rate of human hepatocytes. Six weeks after hepatocyte transplantation, we intravenously injected
13 each mouse with patient serum containing 10^6 copies of HCV genotype 1a (HCG9) or 1b
14 (HCR6).[22] HCV inoculations, drug administration, blood collection, and sacrifice were performed
15 under ether anesthesia. Blood samples were taken from the orbital vein and sera were
16 immediately isolated. The protocols for animal experiments were approved by the local ethics

1 committee. The animals received humane care according to guidelines of the National Institutes

2 of Health. Patients gave written informed consent before sampling.

3 ***Measurement of Human Serum Albumin.*** Human serum albumin in the blood of chimeric mice

4 was measured with a commercially available kit according to the manufacturer's instructions

5 (Alb-II kit; Eiken Chemical, Tokyo, Japan).

6 ***Schedule for Administration of Agents Into Chimeric Mice Infected With HCV Genotype 1b or***

7 ***1a.*** Treatment was started 12 weeks after HCV inoculation and continued during 14 days (Fig. 4A

8 and Fig. 5A). There were 3 animals in each treatment group. Peg-IFN and DEBIO-025 in mice

9 with HCV genotype 1a or 1b were administered as follows: either Peg-IFN (30 μg/kg) was

10 injected subcutaneously twice weekly alone or DEBIO-025 (100 mg/kg) was given orally every

11 day alone or a combination of both drugs was given. CsA (100 mg/kg) was given orally every day

12 combined with Peg-IFN (30 μg/kg) subcutaneously twice weekly only to chimeric mice

13 inoculated with genotype 1a.

14 ***Measurement of HCV Core Protein in Liver.*** Liver tissues were homogenized in lysis buffer (10

15 mmol/L Tris pH 7.5, 1% SDS, 0.5% NP-40, and 150 mmol/L NaCl) and centrifuged for 60

16 seconds at 15,000 rpm. HCV core protein was quantified using a commercially available kit

1 (Ortho Clinical Diagnostics, Tokyo, Japan).[23]

2 ***Quantification of HCV RNA by Real-time RT-PCR.*** HCV RNA in serum or liver tissue was

3 extracted using the acid guanidinium-phenol-chloroform method. Quantification of HCV RNA

4 was performed using real-time RT-PCR based on Taq-Man chemistry, as described previously.[24]

5 ***Immunohistochemistry.*** Liver tissues obtained from mice were embedded in OCT compound

6 (Ted Pella, Redding, CA). The frozen tissues were cut into thin sections (6 μm) and placed on

7 glass slides. The sections were fixed in 10% buffered formalin and then treated with 0.1% Triton

8 X-100. To detect HCV protein, the slides were incubated with rabbit anti-core protein IgG and

9 then donkey anti-rabbit IgG polyclonal antibody (Fab fragment, labeled with HRP; Dako,

10 Glostrup, Denmark). The HRP label was amplified with FITC-conjugated tyramide according to

11 the manufacturer's instructions (Molecular Probes, Eugene, OR). To detect human hepatocytes,

12 liver sections were probed by anti-human hepatocyte monoclonal antibody (Dako), followed by

13 anti-mouse IgG-Alexa 546 (Molecular Probes). Nuclei were stained by DAPI (Molecular Probes).

14 Normal rabbit IgG was used as a control.

1 **Results**

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

15 ***Immunosuppressive Activity of DEBIO-025.*** To examine the immunosuppressive activity of
16 DEBIO-025, we used an NF-AT-dependent IL-2 reporter gene assay. DEBIO-025 showed only a

1 slight inhibitory effect on this system, with an activity that was 7000-fold lower than that of CsA
2 (data not shown). This indicates that the substitution of 2 amino acids in CsA to produce
3 DEBIO-025 resulted in a greatly reduced immunosuppressive activity.
4 ***Human albumin Levels in Mouse Serum After Transplantation of Human Hepatocytes.*** The
5 concentration of human albumin in the serum of the chimeric mice was measured to provide an
6 index of the substitution rate of mouse to human hepatocytes following transplantation.[21] The
7 concentration measured 20 days after transplantation of human hepatocytes was 3.5 to 6.0 mg/mL,
8 indicating that human hepatocytes had settled into the chimeric mice. At 6 weeks after
9 transplantation, we inoculated the mice with patient serum containing HCV genotypes 1a or 1b.
10 We repeatedly measured the concentrations of human albumin after inoculation, and found that
11 they reached a plateau at about 6.5 mg/mL. Although the mice were infected with HCV,
12 significant reductions of the human albumin concentrations were not observed (Fig. 3A-B).
13 ***Persistent Infection of HCV in Chimeric Mice.*** To determine whether the chimeric mice were
14 persistently infected with HCV, we measured HCV RNA levels in serum weekly after the
15 inoculation. HCV RNA disappeared at the first week and was then detected from 2 weeks after
16 the inoculation. Four weeks after infection, HCV RNA levels reached 10^8 to 10^9 copies/mL in the

1 genotype 1a group (Fig. 3C) and 10^6 to 10^7 copies/mL in the genotype 1b group (Fig. 3D). These
2 results showed that our patient sera containing HCV had infected the chimeric mice. Furthermore,
3 the increase of HCV levels in the serum was time-dependent, indicating that HCV replicated and
4 accumulated in the human hepatocytes of the chimeric mice.

5 ***Effect on HCV RNA levels of DEBIO-025 and/or Peg-IFN in mice infected with HCV genotype***
6 ***1b.*** DEBIO-025 alone did not inhibit HCV replication, but Peg-IFN alone reduced serum HCV
7 RNA levels approximately 10-fold from day 3 to day 14 (Fig. 4B). A 100-fold reduction was
8 observed with the combined treatment (Fig. 4B). These results indicated an effect of DEBIO-025
9 that appeared to be synergistic with Peg-IFN against genotype 1b. The concentration of human
10 serum albumin and the body weight of the mice did not change significantly during this period
11 (Fig. 4C-D). After cessation of treatment, HCV RNA levels returned to10^7 copies/mL.

12 ***Comparison of DEBIO-025 and CsA effect in Chimeric Mice infected with HCV genotype 1a.***
13 The serum HCV RNA levels with the administration of DEBIO-025 or Peg-IFN alone seemed to
14 be similar at day 7 and at day 14 as compared to those seen in mice infected with genotype 1b (Fig.
15 5B). The combined administration of DEBIO-025 with Peg-IFN resulted in a 600-fold reduction
16 of HCV RNA levels at day 14 (Fig. 5B). Of importance the combined administration of CsA and

1 Peg-IFN resulted in the death of all treated mice within 4 days. The body weight of all
2 CsA-treated mice was reduced by more than 20% during this period (Fig. 5C). The concentration
3 of human serum albumin in the mice treated with CsA did not change significantly (data not
4 shown). This toxicity was not observed with DEBIO-025 and Peg-IFN.

5 ***Quantification of Hepatic HCV RNA and Core Protein Levels and Immunohistochemistry at***
6 ***the End of Treatment in Chimeric Mice Infected With Genotype 1a.*** At the end of treatment,
7 hepatic HCV RNA was quantified by real-time RT-PCR and core protein levels were quantified
8 by ELISA (Fig. 6A-B). DEBIO-025 monotherapy (1a-3 mouse) reduced HCV RNA by 3-fold
9 compared with the nontreated mouse (1a-4 mouse). Peg-IFN reduced both HCV RNA and core
10 protein levels by approximately 10-fold (1a-2 mouse). Combined treatment with DEBIO-025 and
11 Peg-IFN resulted in an approximately 100-fold reduction in HCV RNA and HCV core protein
12 levels (1a-1 mouse). Moreover, immunohistochemistry was performed. In 1a-4 mouse, HCV core
13 protein was detected in human hepatocytes. In 1a-1 mouse, HCV core protein was not detected by
14 immunohistchemistry, however, reduced HCV core protein was quantified by ELISA which is
15 more sensitive than immunohistchemistry (Fig. 6C-D).

1 **Discussion**

2 ________Development of new anti-HCV drugs has been significantly impeded by the lack of a
3 suitable cell culture model for the propagation of HCV in laboratories. This obstacle has been
4 partially overcome by the development of the replicon system, which can be used for evaluating
5 the *in vitro* anti-HCV effect of compounds. However, because adaptive mutation into the replicon
6 genome and host permissiveness enable particularly efficient replication in cultured hepatoma
7 cell lines,[25] evaluation of HCV drugs using replicon systems alone is considered insufficient. The
8 only animal species readily infected with HCV has been the chimpanzee, which is labor-intensive
9 and expensive to use, and is associated with ethical problems. The chimeric mouse with human
10 hepatocytes has recently been developed as a practical small animal model that can be infected
11 with HCV.[20] This model is promising for the evaluation of new anti-HCV drugs because the mice
12 are easy to handle, grow rapidly and are well characterized genetically and immunologically. In
13 this study, we used chimeric mice to bridge the gap between the replicon system and naive HCV
14 replication in human liver, and to examine the anti-HCV effect of DEBIO-025, a novel
15 cyclophilin inhibitor and non-immunosuppressive cyclosporin.

16 ________We found that HCV from our patient sera were able to infect the chimeric mice and

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

12 Although DEBIO-025 strongly inhibited replication of the HCV replicon, it did not
13 affect the replication of naive HCV *in vivo* when given as monotherapy. These results probably
14 indicate differences between the replication of naive HCV *in vivo* and the replicon system. Of
15 interest, it was recently shown that the sensitivity of HCV strains to CsA and
16 non-immunosuppressive cyclosporins was variable, depending on their cyclophilin requirement

1 for their replication.[26] Cyclophilin polymorphism and its role in HCV replication will be the focus
2 of future study.
3 The HCV RNA levels are known to decline biphasically in most patients treated with
4 IFN.[27] During the first phase, there is a rapid drop in viremia that reflects the direct inhibition of
5 HCV replication. During the second phase, there is a slower decline in serum HCV RNA levels,
6 which appears to reflect the elimination of infected cells by host immune responses. In chimeric
7 mice, the second-phase decline is not obvious, because they lack T cells and B cells (being SCID).
8 Thus it appears that DEBIO-025 accelerates the decline in HCV RNA levels induced by Peg-IFN
9 during the first phase. There is no evidence that DEBIO-025 enhances the interferon pathway.
10 Also, recent *in vitro* findings show that cyclosporins do not modify the IFN α signal transduction
11 pathway as assessed by 2', 5'-oligoadenylate synthetase (2', 5'-OAS) levels.[28] It therefore seems
12 likely that the apparent synergistic effect of DEBIO-025 seen in our *in vivo* model is not solely
13 related to the antiviral effect mediated by IFN. It can be speculated that the DEBIO-025 inhibition
14 of cyclophilin produces a proper anti-HCV effect by interacting with the RNA-dependant RNA
15 polymerase.[11]
16 CsA was originally used as an immunosuppressive agent, and we previously

1 demonstrated in clinical trials that CsA has an anti-HCV effect.[9] However, CsA is not devoid of
2 adverse effects, such as hypertension, neurotoxicity and nephrotoxicity, limiting its therapeutic
3 usefulness against HCV.[29] The immunosuppressive action of CsA occurs via inhibition of
4 calcineurin. Our findings showing that DEBIO-025 exhibits 7000-fold lower immunosuppressive
5 activity than CsA suggests that it has less affinity to calcineurin and may lead to fewer adverse
6 effects in patients.
7 ________In conclusion, our results indicate that naive HCV replication *in vivo* is inhibited by the
8 combined administration of the cyclophilin inhibitor DEBIO-025 and Peg-IFN. This findings
9 support further evaluation of DEBIO-025 as a promising drug for the treatment of chronic
10 hepatitis C.
11
12

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3 maintenance of the chimeric mice.

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31

1 **Figure Legends**

2 Fig. 1. (A) Structure of DEBIO-25, which was derived from CsA by substitution of amino acids at

3 positions 3 and 4.

4 (B) Scheme for IL-2 reporter gene assay. nuclear factor of activated T cells (NF-AT),

5 phorbol-12-myristate-13-acetate (PMA), phytohemagglutinin (PHA),

6 4-methylumbelliferyl-β-D-galactoside (MUG)

7

8 Fig. 2. (A) Structure of HCV replicon genome. FLR3-1 and R6FLR-N were of similar

9 construction. Encephalomyocarditis virus (EMCV), internal ribosomal entry site (IRES).

10 untranslated region (UTR) (B, C) Effect of DEBIO-025 or cyclosporin A (CsA) on HCV

11 replication, as monitored in triplicate by luciferase signal in the two HCV replicon systems. Data

12 are expressed as percentages of the untreated control. Error bars indicate SD. (D, E) Effect of

13 DEBIO-025 or CsA on viability of replicon-containing cells, as measured in triplicate by WST-8.

14 Data are expressed as percentages of the untreated control. Error bars indicate SD. (F) Effect of

15 DEBIO-025 or CsA on HCV NS3 protein or β-actin expression, shown by Western blotting.

16

1 Fig. 3. Time course studies in 4 mice inoculated with human serum samples positive for hepatitis
2 C virus genotypes 1a or 1b. (A, B) Human albumin concentrations in mouse serum after
3 transplantation of hepatocytes. (C, D) HCV RNA levels in mouse serum after inoculation.
4
5 Fig. 4. (A) Schedule of experiments using chimeric mice infected with HCV genotype 1b. The
6 mice were treated for 14 days with DEBIO-025 100 mg/kg/day orally, Pegylated-interferon α–2a
7 (Peg-IFN) 30 μg/kg subcutaneously twice weekly, or DEBIO-025 100 mg/kg/day orally
8 combined with Peg-IFN 30 μg/kg subcutaneously twice weekly. (B) Time course of serum HCV
9 RNA levels in mice treated with DEBIO-025 (open squares), Peg-IFN (gray diamonds) or
10 DEBIO-025 with Peg-IFN (closed triangles). Error bars indicate SD. (C) Human albumin
11 concentrations in the sera of individual mice during the experimental period. (D) Body weight of
12 individual mice during the experimental period.
13
14 Fig. 5. (A) Schedule of experiments using chimeric mice infected with HCV genotype 1a. The
15 mice were treated for 14 days with DEBIO-025 100 mg/kg/day orally, Peg-IFN 30 μg/kg
16 subcutaneously twice weekly, DEBIO-025 100 mg/kg/day orally combined with Peg-IFN 30

1 μg/kg subcutaneously twice weekly, or CsA 100 mg/kg/day orally combined with Peg-IFN 30
2 μg/kg subcutaneously twice weekly. (B) Time course of serum HCV RNA levels in mice treated
3 with DEBIO-025 (open squares), Peg-IFN (gray diamonds) or DEBIO-025 with Peg-IFN (closed
4 triangles). Error bars indicate SD. (C) Body weight of individual mice during the first 7 days of
5 the experimental period. All of the mice treated with CsA combined with Peg-IFN died within 4
6 days.
7
8 Fig. 6. Analysis of liver tissue from chimeric mice infected with HCV genotype 1a. (A) HCV
9 RNA, and (B) HCV core protein, measured in triplicate in the livers of mice undergoing
10 different treatment protocols.
11 severe combined immunodeficient (SCID) control: non-infected SCID mouse; 1a-1: mouse
12 treated with DEBIO-025 combined with Peg-IFN; 1a-2: mouse treated with Peg-IFN; 1a-3:
13 mouse treated with DEBIO-025; 1a-4: non-treated mouse infected with HCV.
14 (C, D) Immunofluorescent labeling of human hepatocytes and HCV core protein, and
15 fluorescent staining of nuclei. HCV core protein was labeled in human hepatocytes of
16 non-treated chimeric mouse (C), but was not apparent in chimeric mouse treated with