on TaqMan chemistry using the forward primer R6-130-S17 (nucleotides 130–146), 5′-CGGGAGAGCCATAGTGG-3′; the reverse primer R6-290-R19 (nucleotides 290–272), 5′-AGTACCA-CAAGGCCTTTCG-3′; and the Taq-Man probe R6-148-S21FT (nucleotides 148–168), 5′-FAM-CTGCGGAACCGGTGAGTACAC-TAMRA3′, as described previously (Takeuchi et al., 1999). HCV RNA was extracted from PYC-treated, persistently-infected JFH-1/K4 HCV cells, using the ISOGEN RNA extraction kit (Nippon Gene, Japan).

2.5. In vivo effects of PYC

We produced chimeric mice by transplanting human primary hepatocytes into severe combined immunodeficient mice carrying a urokinase plasminogen activator transgene controlled by the albumin promoter (Mercer et al., 2001; Tateno et al., 2004). All animals received humane care according to National Institute of Health criteria outlined in the Guide for Care and Use of Laboratory Animals. The hepatocytes were infected with HCV-G9 (genotype 1a) (Inoue et al., 2007). HCV 1a RNA levels reached 2.9–18.0 $\times$ 10^6 -copies/mL in mice sera after 1–2 months of infection. PYC (40 mg/kg) was administered intraperitoneally once daily. PEG-IFN (30 μg/kg) was administered subcutaneously at 0, 3, 7, and 10 days either alone or in combination with PYC. Each treated group contained at least 3 chimeric mice. HCV RNA was purified from 2 μL chimeric mouse serum using SepaGene RV-R (Sanko Junyaku Co., Ltd., Tokyo, Japan). HCV RNA levels were quantified using qRT-PCR as reported previously (Takeuchi et al., 1999).

2.6. ROS assay

Formation of ROS in the HuH-7 cell-based HCV-replicon-harbouring cell line (R6FLR-N), and in R6FLR-N cured of HCV by interferon treatment (Blight et al., 2002) was measured using the OxiSelect ROS assay kit (Cell Biolabs, San Diego, CA, USA) according to the manufacturer's instructions. Duplicate samples at 1 $\times$ 10^7 -cells/mL from each culture were then incubated with dichlorodihydrofluorescein DiOxyQ (DCFH-DiOxyQ). Under these conditions, ROS species rapidly oxidise DCFH into the highly fluorescent 2′, 7′-dichlorodihydrofluorescein (DCF). Fluorescence intensity, which is proportional to the total ROS levels in the sample, was measured with a fluorescence spectrophotometer reader at 480-nm excitation and 530-nm emission.

2.7. Statistical analysis

Data are presented as means ± standard error of triplicate experiments. Data were analysed using Kruskal–Wallis test and Mann–Whitney *U* tests. A *p*-value <0.05 was considered statistically significant. To evaluate the synergistic or antagonistic effects of combinations of PYC and IFN-alpha or PYC and telaprevir, we calculated the combination index (CI) using CalcuSyn (Biosoft, Ferguson, MO, USA), where CI < 1 indicates synergism, CI = 1 indicates an additive effect, and CI > 1 indicates antagonism. The weighted average CI was calculated using the formula: CI = [CI_{50} + 2CI_{75} + 3CI_{90} + 4CI_{95}]/10, where CI_{50}, CI_{75}, CI_{90}, and CI_{95} are the CI values at 50%, 75%, 90% and 95% inhibition, respectively (Bassit et al., 2008; Chou and Talalay, 1984).

3. Results

3.1. PYC inhibits HCV replication and protein synthesis in vitro

We assessed the effect of PYC on HCV in R6FLR-N and FLR3-1 cell lines after 72 h (Fig. 1). The data are expressed as relative values using the relative light unit count for the 0 μg/mL treatment sample as 100% (Fig. 1A). The results showed that PYC inhibited luciferase activity in R6FLR-N cells (50% inhibitory concentration [IC_{50}] = 5.78 ± 3.75 μg/mL, 50% effective concentration [EC_{50}] = 4.33 μg/mL (2.2–8.5) in a dose-dependent manner. To rule out the possibility that the antiviral activity was caused by cytotoxic effects, cell proliferation was analysed using the WST-8 assay; no significant differences in cell viability (50% cytotoxic concentration [CC_{50}] > 60 μg/mL PYC; Selectivity index [SI] > 14.1) (Fig. 1B). These results indicate that PYC suppresses HCV (genotype 1b) replication.

Consistent with results showing the inhibitory effects of PYC on HCV replication, we observed that HCV NS3 protein levels decreased significantly in PYC and IFN-alpha-treated HCV replicon cell lines (Fig. 1C). HCV NS3 and NS5B proteins levels were progressively suppressed in HCV replicon cell lines at various PYC concentrations (0, 5, 10, and 20 μg/mL) (Fig. 1D). These results suggest that HCV protein synthesis was inhibited by PYC in a concentration-dependent manner.

3.2. Combinations of IFN-alpha, RBV, and PYC inhibit HCV replication

R6FLR-N cells were treated with IFN-alpha and RBV alone or in combination with several concentrations of PYC and incubated for 48 h (Fig. 2A). HCV replication was suppressed by approximately 20% following treatment with 5 μg/mL RBV, and by approximately 40% following treatment with 1 IU/mL IFN-alpha. Treatment with both RBV and IFN-alpha led to approximately 50% suppression. PYC showed a dose-dependent additive effect when administered in combination with RBV and IFN-alpha (Fig. 2A). Treatment with both PYC (5 μg/mL) and IFN-alpha (1 IU/mL) showed a synergistic effect (CI = 0.253) in suppressing HCV replication without cytotoxicity (Fig. 2A and B).

3.3. Effect of PYC on HCV JFH-1 replication

JFH Luc3-13-N cells were inoculated with IFN-alpha (5 IU/mL) or several concentrations of PYC (5–50 μg/mL) and incubated for 72 h (Fig. 2C). HCV (genotype 2a) replication was suppressed by approximately 50% following treatment with 40 μg/mL PYC (Fig. 2C) without significant cytotoxicity (Fig. 2D).

3.4. Anti-HCV activity of PYC in the HCV JFH-1 infection system

PYC, IFN-alpha, and RBV treatments were also evaluated in JFH-1/K4 HCV (genotype 2a) infected cells (Fig. 2E). HCV RNA levels decreased in the presence of PYC (10 or 20 μg/mL) to levels comparable to treatment with 1 IU/mL IFN-alpha in cell culture supernatant after 72 h. Treatment with a combination of IFN-alpha (1 IU/mL), RBV (5 μg/mL), and PYC (10 or 20 μg/mL) had a greater effect than IFN-alpha or PYC alone (Fig. 2E).

3.5. PYC inhibits HCV replication in telaprevir-resistant replicon cells and inhibits additively with telaprevir

We next examined the efficacy of PYC in DAA-resistant HCV. To select telaprevir-resistant replicons, cells with genotype 1b HCV replicons were treated for 14 passages with 1.8 μM and 2.7 μM telaprevir, concentrations 4–6 times the reported IC_{50} (Katsume et al., 2013). These telaprevir-resistant replicon cells showed some cross-resistance to another protease inhibitor, simeprevir (Supplementary Fig. 2). We investigated whether incubation of the wild-type HCV and telaprevir-resistant replicon with PYC alone or with telaprevir would inhibit HCV replication. The susceptibility of the replicon to PYC was measured after treating the cells with increasing concentrations of PYC and telaprevir for 72 h (Fig. 3). Fig. 3A shows that PYC reduced luciferase activity in

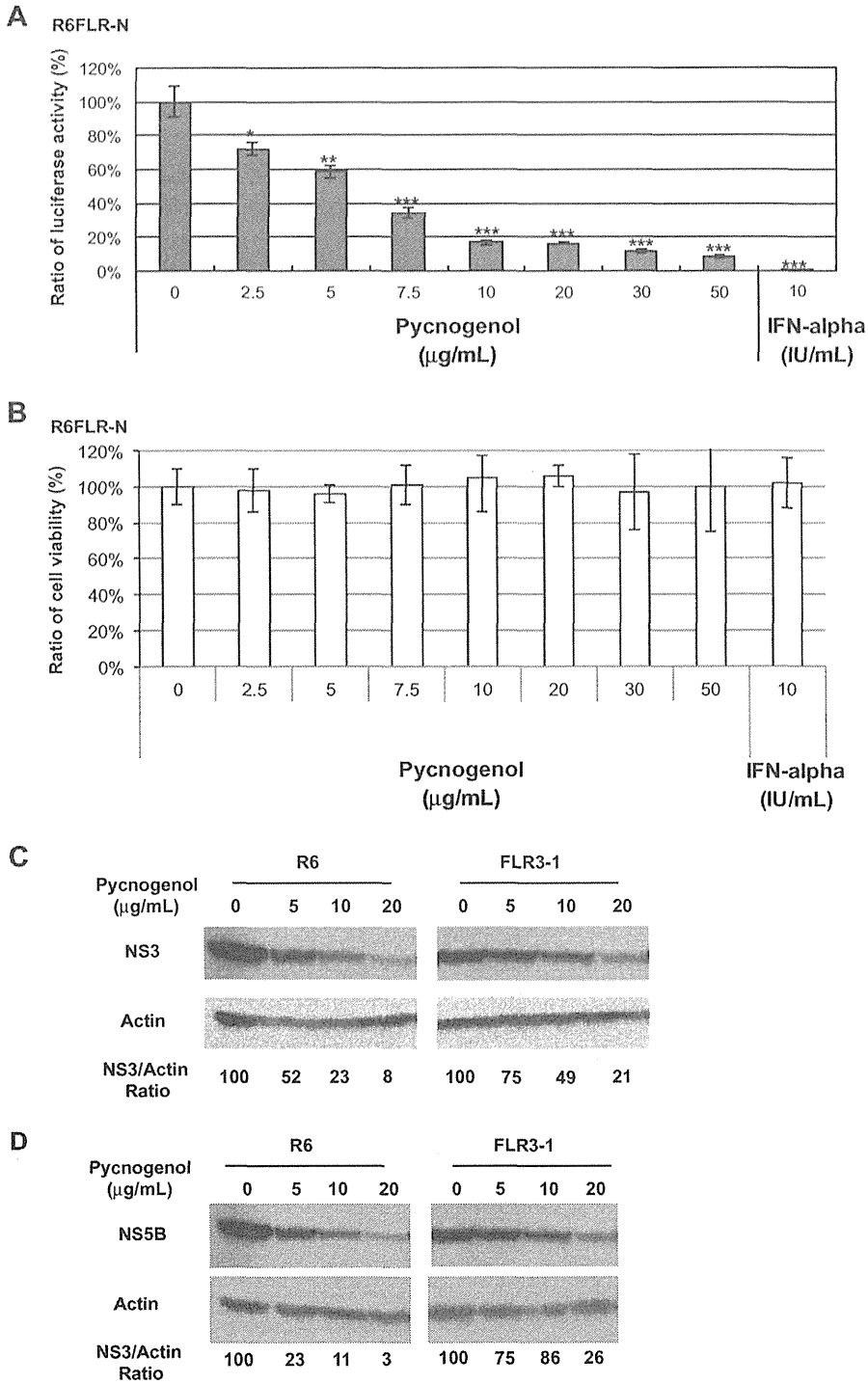

Fig. 1. Effect of PYC on HCV RNA replication in HCV replicon cell lines. (A) Dose-dependent inhibition of HCV by PYC in the R6 replicon cell line. (B) Cytotoxicity in the R6 HCV-replicon cell line treated with PYC. The ratio (%) of viability compared to the no-treatment control value is indicated. (C and D) HCV protein expression level decreased with PYC treatment in the HCV R6 and FLR3-1 replicon cell lines. (C and D) Dose-dependent effects of PYC on HCV protein synthesis (C NS3, D NS5B) in R6 and FLR3-1 replicon cell lines. Error bars = standard deviation. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. The ratio of luciferase was calculated as compared to non-treatment values.

a dose dependant manner in a wild-type HCV replicon and 2 telaprevir-resistant replicon cell lines. In addition, PYC had an additive effect with telaprevir (CI = 1.05) (Fig. 3B). Further, inhibition was greater in telaprevir (1.8 μM) than telaprevir (2.7 μM) and combined PYC (10 μg/mL) and telaprevir (1.8 μM and 2.7 μM) treatment reduced luciferase levels to those reached by PYC alone at 10 μg/mL. Moreover, the resistant mutants remain as sensitive to IFN-alpha as the wild-type replicon (Fig. 3A). After a 72-h incubation with PYC and telaprevir, no significant cytotoxicity, as evaluated in the WST-8 based cell viability assay, was observed in the replicon cells (Fig. 3C).

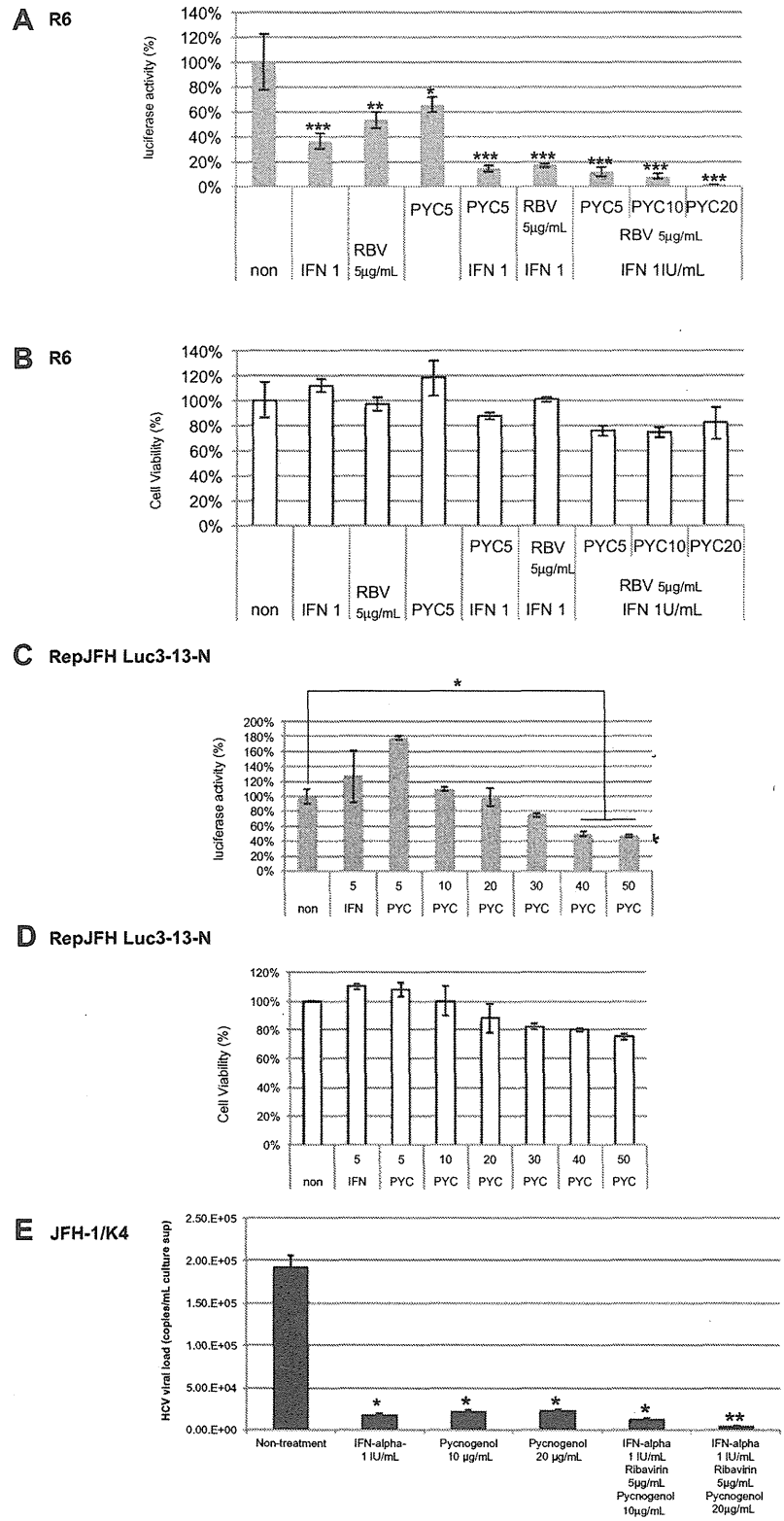

Fig. 2. Effect of PYC and IFN-alpha on HCV replication. (A) Effect of PYC (5 μg/mL), IFN-alpha (1 IU/mL), and RBV (5 μg/mL) on antiviral activity in the R6 HCV replicon cell line. (B) Cytotoxicity in the R6 HCV replicon cell line treated with PYC, IFN-alpha, and RBV. (C) Effect of IFN-alpha (5 IU/mL), and PYC (5–50 μg/mL) on JFH-1 replicon cells (RepJFH Luc3-13-N) (replication; left, viability; right). (D) Cytotoxicity in the RepJFH Luc3-13-N cell treated with PYC. (E) Synergistic effect of PYC, IFN-alpha, and RBV in the culture supernatant of HCV infection system in JFH-1/K4 cell line. Error bars = standard deviation. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

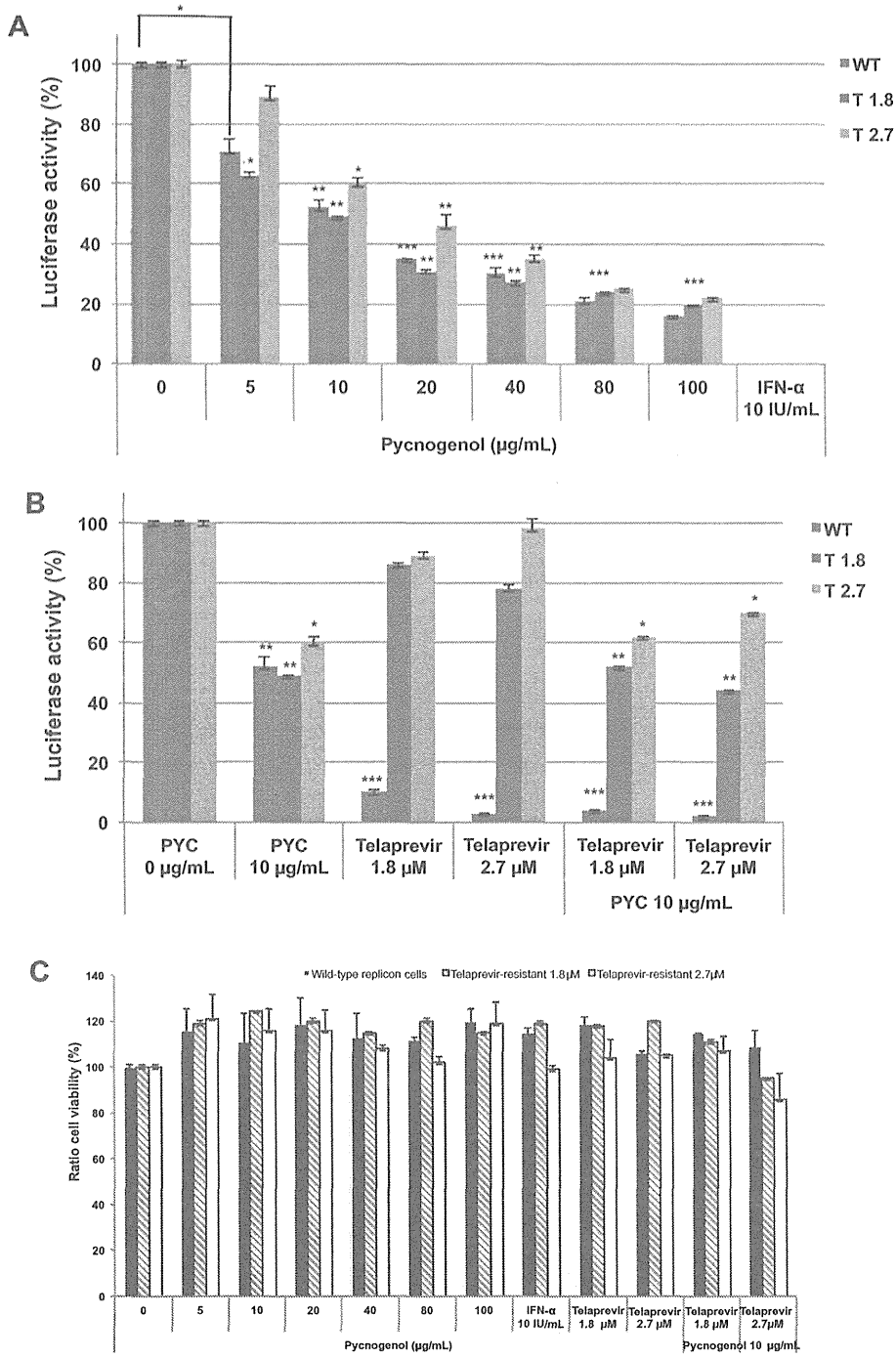

Fig. 3. Effect of PYC on HCV replication in telaprevir-resistant replicon cells. (A) Dose-dependent inhibition of HCV by PYC in wild type (WT) and telaprevir-resistant replicon cell lines (V1.8, V2.7). (B) Effect of PYC and telaprevir in the R6 replicon cell line. (C) PYC cytotoxicity in the R6 HCV replicon cell line treated with PYC, IFN-alpha, and telaprevir. Ratio of viability (%) to cells without PYC is indicated. Error bars = standard deviation. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

3.6. PYC suppresses HCV replication more effectively than procyanidin or taxifolin

Because procyanidin and taxifolin are the main constituents of PYC (Lee et al., 2010), we examined their ability to suppress HCV replication (Supplementary Fig. 3). Procyanidin could not inhibit HCV replication in R6FLR-N cells at concentrations between 15 and 60 μg/mL (Supplementary Fig. 3A). Cytotoxicity was not observed even at this high dose (data not shown). In JFH-1/K4 HCV-infected cell lines, procyanidin suppressed supernatant HCV RNA levels after 72 h and worked synergistically with IFN-alpha (Supplementary Fig. 3B). Moreover, we also examined taxifolin efficacy, but did not observe any effect on HCV replication (Supplementary Fig. 3C) or HCV infection in JFH-1/K4 cells (data not shown).

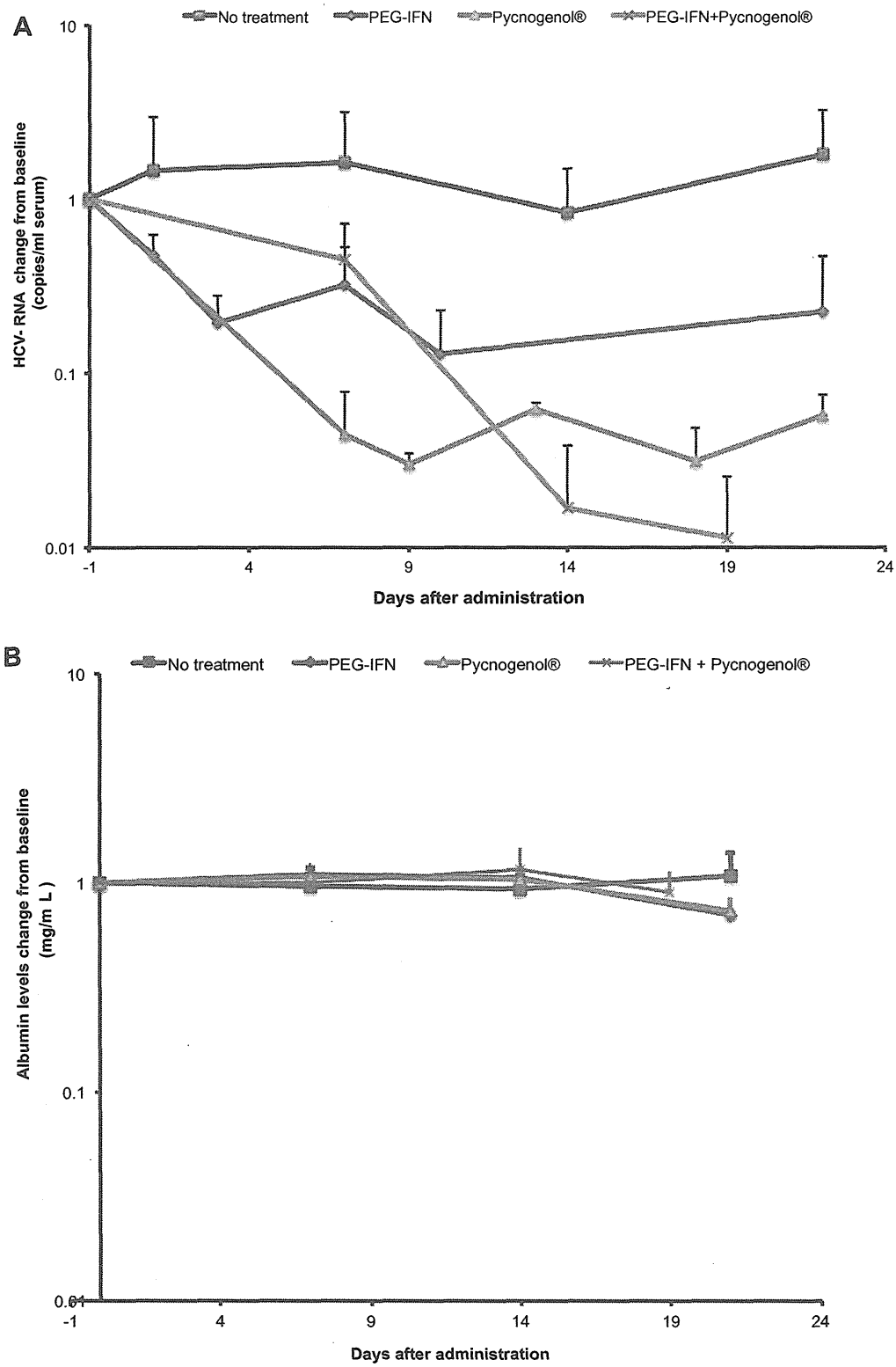

Fig. 4. *In vivo* effects of PYC on HCV replication. (A) Median change from baseline serum relative HCV RNA levels (HCV RNA amount at the beginning; average 4.8×10^7 copies/mL) in chimeric mice treated with PYC (40 mg/kg/day, intraperitoneally) or PEG-IFN (30 μg/kg, subcutaneously) alone, in combination with PEG-IFN, and untreated controls (in each group, $n = 3$). (B) Serum human albumin levels in chimeric mice during treatment.

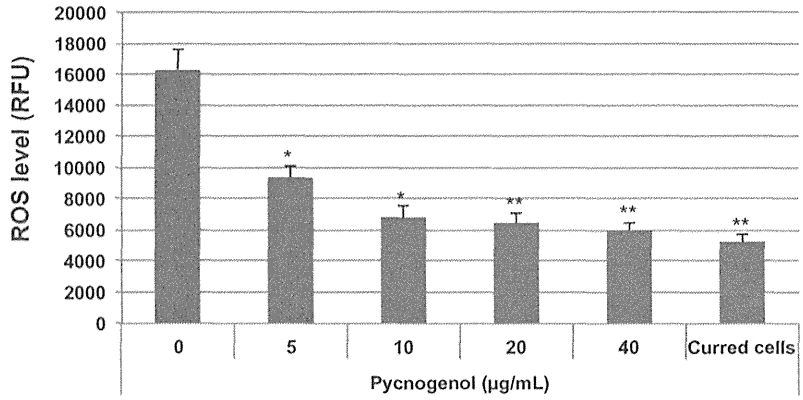

Fig. 5. Effect of PYC on ROS production in HCV replicon cells. HCV replicon and cured cell lines were incubated for 72 h with various concentrations of PYC and without PYC treatment. Data are shown as mean ± standard error of mean. ROS levels in HCV-negative cured cells are also shown. $^{*}P < 0.05$; $^{**}P < 0.01$.

3.7. PYC inhibits HCV replication in chimeric mice

To evaluate the *in vivo* effects of PYC on HCV, we used chimeric mice with a humanized liver infected with HCV G9 (genotype 1a). In the untreated control group ($n = 3$ mice), no decrease in HCV genome RNA levels was observed. In the group treated with PYC (40 mg/kg/day) ($n = 3$ mice), serum HCV RNA levels decreased rapidly, and within 9 days the effect was greater than with PEG-IFN treatment (30 μg/kg) ($n = 3$ mice) (Fig. 4A). Treatment with both PYC (40 μg/kg) and PEG-IFN (30 μg/kg) significantly reduced HCV RNA levels after 14 days compared to either PEG-IFN or PYC monotherapy (Kruskal–Wallis test, $p = 0.0008$). To exclude the possibility that this decline was caused by indirect effects on human hepatocyte viability, we measured human albumin levels in chimeric mice and found no significant variation during treatment ($p = 0.728$) (Fig. 4B), indicating that PYC has an antiviral effect and acts synergistically with PEG-IFN in chimeric mice with humanized livers infected with HCV.

3.8. PYC suppresses intracellular ROS production in R6FLR-N cells

A ROS assay was used to assess the ability of PYC to act as a free radical scavenger. Fluorescence intensity was measured for each sample. Total ROS production was significantly decreased by PYC in the HCV replicon cell line in a dose-dependent manner (Fig. 5). Treatment with PYC at 40 μg/mL reduced ROS to levels comparable to cells cured of the HCV replicon by IFN treatment (Blight et al., 2002), suggesting that PYC may scavenge ROS in HCV replicon cell lines.

4. Discussion

Oxidative stress has been identified as a key mechanism of HCV-induced pathogenesis (de Mochel et al., 2010; Ke and Chen, 2012; Quarato et al., 2013; Tardif et al., 2005). Moreover, several studies have reported a correlation between oxidative stress and IFN treatment response, and have observed that oxidative stress was reduced to normal levels after viral eradication (Levent et al., 2006; Serejo et al., 2003). These data provide a firm theoretical basis for investigation of antioxidants as therapeutics. PYC is a mixture of various chemical groups and exhibits radical-scavenging antioxidant, anti-inflammatory, and antiviral activities (Maimoona et al., 2011). In addition, PYC protects biomolecules such as proteins against oxidative damage (Voss et al., 2006). To our knowledge, this is the first report to demonstrate a direct antiviral effect of PYC against HCV. Our results show that PYC inhibits HCV replication in HCV replicon cell lines and JFH-1 without cytotoxicity. Moreover, this result is in line with a recent report, based on data obtained from 5723 subjects that showed side effect incidence rates of 2.4% and 0.19% in patients and healthy subjects, respectively (American Botanical Council, 2010). The study also found PYC to be nontoxic at doses of 20–100 mg/day for extended periods (months) and 100–300 mg for shorter periods (American Botanical Council, 2010).

Treatments of replicon and JFH-1 cell lines using combinations of PYC with RBV, IFN, and telaprevir showed that co-administration of these compounds increased HCV antiviral activity. In addition, we found that PYC suppressed HCV replication in telaprevir-resistant replicon cells and may improve the response to protease inhibitors. In this report, we found that procyanidins, oligomeric compounds formed from catechin and epicatechin, but not taxifolin, inhibited HCV replication at doses between 15 and 60 μg/mL and had a synergistic effect with IFN treatment without cytotoxicity. Moreover, procyanidin B1 extracted from *Cinnamomum cassia* cortex suppresses hepatitis C virus replication (Li et al., 2010). Other studies have also shown that epicatechin, catechin-derived compounds, and caffeic acid phenethyl ester inhibit HCV replication and attenuate the inflammation induced by the virus (Khachatoorian et al., 2012; Lin et al., 2013; Shen et al., 2013). However, PYC showed greater antiviral effects than procyanidin and taxifolin. PYC efficacy was much stronger than procyanidin or taxifolin; therefore, a combination of components or unknown factor(s) in PYC may contribute to inhibition of viral replication.

Constitutive activation of NF-kappa B and STAT-3 by HCV is implicated in acute and chronic liver disease (Gong et al., 2001; Waris et al., 2003, 2005). Consistent with these data, a previous study showed that PYC inhibits NF-kappa B and activator protein-1, and abolishes the degradation of I-kappa B alpha (Cho et al., 2000). Moreover, a recent study showed that PYC also inhibits its expression and secretion of tumour necrosis factor-alpha and interleukin 6, reducing calcium uptake and suppressing NF-kappa B activation (Choi and Yan, 2009). We observed PYC free radical scavenging activity against ROS in HCV replicon cell lines. These data support our finding that PYC exerts its antioxidant effects directly by scavenging of ROS and indirectly by enhancing cellular antioxidant enzymes (Packer et al., 1999).

5. Conclusion

Our study shows that the natural product PYC inhibits HCV replication both *in vitro* and *in vivo*. Our results indicate that *in vitro* combinations of PYC/IFN-alpha/RBV and PYC/telaprevir lead to a

much stronger antiviral response than with either agent alone and that PYC suppresses replication in telaprevir-resistant replicon cells. Future clinical trials are necessary to assess which patients, for example, naïves, non-responders, or those with severe liver disease, could benefit from co-administration of PYC with PEG-IFN-alpha, RBV, or DAAs. Addition of PYC may be a viable strategy to improve the efficacy of HCV therapies using the recently licensed antiviral molecules.

Conflict of interest

The authors declare that they have nothing to disclose regarding funding or conflicts of interest relating to this manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2014.10.017.

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—Review—

Tupaia Belangeri as an Experimental Animal Model for Viral Infection

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Abstract: Tupaias, or tree shrews, are small mammals that are similar in appearance to squirrels. The morphological and behavioral characteristics of the group have been extensively characterized, and despite previously being classified as primates, recent studies have placed the group in its own family, the Tupaiidae. Genomic analysis has revealed that the genus *Tupaia* is closer to humans than it is to rodents. In addition, tupaias are susceptible to hepatitis B virus and hepatitis C virus. The only other experimental animal that has been demonstrated to be sensitive to both of these viruses is the chimpanzee, but restrictions on animal testing have meant that experiments using chimpanzees have become almost impossible. Consequently, the development of the tupaia for use as an animal infection model could become a powerful tool for hepatitis virus research and in preclinical studies on drug development.

Key words: genome, HBV, HCV, *Tupaia*, virus

Taxonomic Classification

Tupaia belangeri belongs to the family Tupaiidae, which consists of four genera and 19 extant species (Table 1) [13, 19]. The members of *Tupaia*, which are colloquially referred to as tree shrews, were first recorded in a sketch by William Ellis on a voyage with Captain Cook in 1780 [7]. With a body weight ranging between 45–350 g (Table 1), members of the genus *Tupaia* are similar in appearance to squirrels (Fig. 1). The natural habitat of *Tupaia* spp. consists of the tropical rainforest in South East Asia where they feed on fruits, insects and small vertebrates [7].

Similarities between *Tupaia* spp. and primates were first reported in the 1920s; for example, Le Gros Clark proposed that tree shrews and primates were closely related based on brain anatomy [20]. However, recent molecular studies have separated tupaias from the primates and placed them in the order Scandentia and within the grandorder Euarchonta, which also contains the Primates and Dermoptera [17].

Handling of Tupaia

Tupaia is active during daytime, and animal rooms are illuminated from 7:00 am to 9:00 pm with a relative humidity of 50–60%, and temperature at 26°C. Their foods are CMS-1M (CREA, Japan) 20 g, apple, banana and boiled egg, everyday. They usually slip into the boxes as soon as somebody enters the room, then we can catch them by net. We can bleed approximately 0.5 ml from the tail or leg vein once in 2 weeks. Tupaias can be

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Table 1. Composition of family Tupaiidae [1]

Taxa	Morphological characteristics	Reproductive characteristics	Weaning and longevity	Distribution
Family: Tupaiidae				
Genus: *Tupaia*				
*Species: *belangeri*	BW: 50–270 g	GP: 41–55 d	W: ca. 30 d	
Subspecies:	HBL: 12–21 cm	L: 1–5	L: 9–12 yr	
belangeri chinensis	NN: 1–3 pairs	NBW: 6–10 g		
**Species:* *chrysogaster, dorsalis, glis, gracilis, javanica, longipes, minor, moellendorffi, montana, nicobarica, palawanensis, picta, splendidula, tana*				Tropical forests in Southeast Asia
Genus: *Anathana ellioti*	BW: 180 g	UK	UK	
	HBL: 19 cm			
	NN: 3 pairs			
Genus: *Dendrogale melanura, murina*	BW: 60 g	GP: 41–55 d	W: ca. 30 d	
	HBL: 13 cm	L: 1–5	L: 9–12 yr	
	NN: 1 pair	NBW: 6–10 g		
Genus: *Urogale everetti*	BW: 220–359 g	GP: 30 d	W: ca. 30 d	
	HBL: 20 cm	L: 1–4	L: 6 yr	
	NN: 2 pairs	NBW: 10 g		

*BW: body weight; HBL: head-body length; NN: number of nipples; GP: gestation period; L: litter size; NBW: Newborn body weight; W: weaning; L: life span; UK: unknown.

Fig. 1. Adult female tupaia (*Tupaia belangeri*) maintained at the Department of Animal Hygiene, Kagoshima University.

breeding after 6–9 months age and easily to give average 4 babies after approximately 45 days of pregnancy. Tupaia usually possesses few health problems, but sometimes shows diarrhea by *Escherichia coli*, *Klebsiella pneumonia* or protozoa, which can be checked by quarantine. The inbred tupaia has not been established yet.

Genetic Characteristics of Tupaia Spp

Evolutionary characterization of 7S RNA-derived short interspersed elements (SINEs) revealed that 7S RNA is a component of the cytoplasmic signal recognition particle [33] in primates [5], tupaia [25] and rodents [18], i.e. all of the members of the placental mammalian order Supraprimates and the superorder Euarchontoglires. The fossil *Alu* monomer was previously considered to be the oldest common ancestor of all 7S RNA-derived SINEs [27], and was thought to be restricted to primates [17]. *Tupaia* possesses specific, chimeric, Tu-type II SINEs, which may share a common ancestor with rodent B1 SINEs [27]. Phylogenetic analysis of 7S L RNA-derived SINEs has shown that tupaias can be grouped with primates and Dermoptera in the Euarchonta, while the Rodentia and Lagomorpha can be grouped with the Glires [17].

Whole-genome analysis by several groups ([8], Tsukiyama-Kohara *et al.*, *in preparation*) revealed a genetic relationship between tupaias and humans. Similarly, phylogenetic analysis based on whole genome sequences showed that humans are closer to tupaias than they are to mice (Fig. 2). Further, several of the same highly conserved and variable genes have been identified

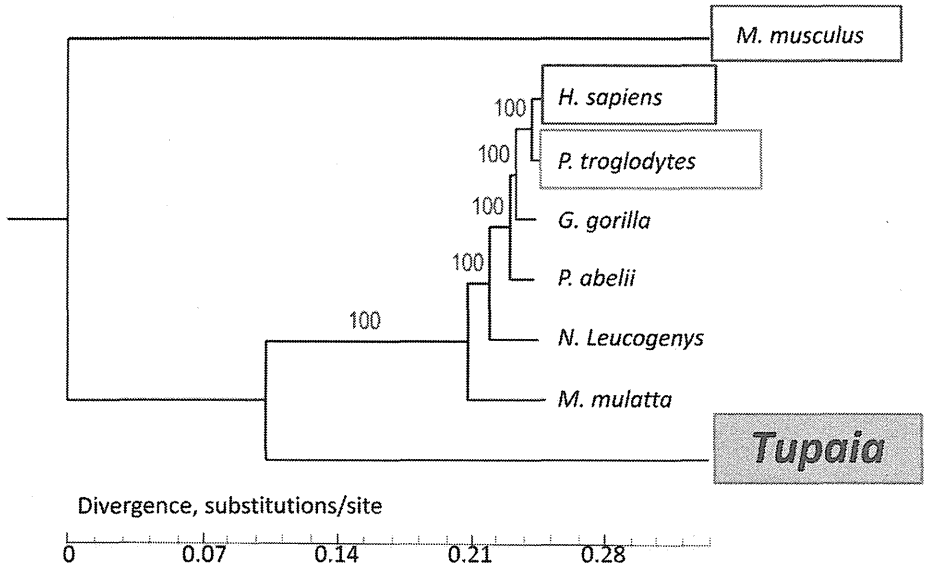

Fig. 2. Dendrogram showing relationships between primates, tree shrews and rodents. Phylogenetic tree constructed using orthologous genes at 4-fold degenerate sites by the maximum likelihood method. Branch lengths represent the neutral divergence rate and blue characters indicate bootstrap values.

in both tupaia and humans. For example, relatively high homology has been observed between human and *Tupaia* hepatitis C virus (HCV) viral receptor CD81 (Fig. 3A), scavenger receptor class B member I (SR-BI), the tight junction proteins claudin I and occludin I [16], as well as the hepatitis B virus (HBV) receptor, sodium-taurocholate cotransporting polypeptide (NTCP) (Fig. 3B) [38], particularly in the receptor and virus envelope surface glycoprotein regions that interact with the transmembrane proteins. It is possible that these highly conserved molecules could be a missing link during the evolution of tupaia, and detailed analysis of this hypothesis is currently underway.

Tupaia as an Experimental Animal Model

The high degree of genetic homology between several neuromodulator receptor proteins in tree shrews and primates has meant that *Tupaia* has been extensively utilized in preclinical research, particularly in the areas of toxicology and virology [10]. Although adult male tupaias exhibit strong territoriality in their natural habitat, the coexistence of two males in visual and olfactory contact in the laboratory leads to the establishment of a stable dominant-subordinate relationship, with subordinates showing distinct stress-induced alterations to behavior, physiology and central nervous activity [9]. These alterations exhibited by the subordinate male tupaias are similar to those observed in depressed human patients, and could be applicable to preclinical research of antidepressant drugs [11]. Various aspects of human behavior, infant development, communication and social structure could also potentially be studied in tupaia [22, 23].

Tupaia as Viral Hepatitis Model

Tupaia have also been employed in studies of viral infection, especially on hepatitis B and C viruses (HBV and HCV) [12]. For these viruses, the only existing natural-infection animal model is the chimpanzee. However, because chimpanzees are long-lived (>50 years), very expensive, and subject to stringent animal welfare regulations, several groups have attempted to develop *Tupaia* for use as an animal infection model. Pathogenesis of HCV was characterized using various transgenic mouse animal models and they can develop chronic hepatitis, liver cirrhosis and hepatocellular carcinoma [30], however natural infection is difficult to be established in these mice. HCV can successfully establish infection in the humanized chimeric mice liver [15, 24], but they do not have immune response, therefore, patho-

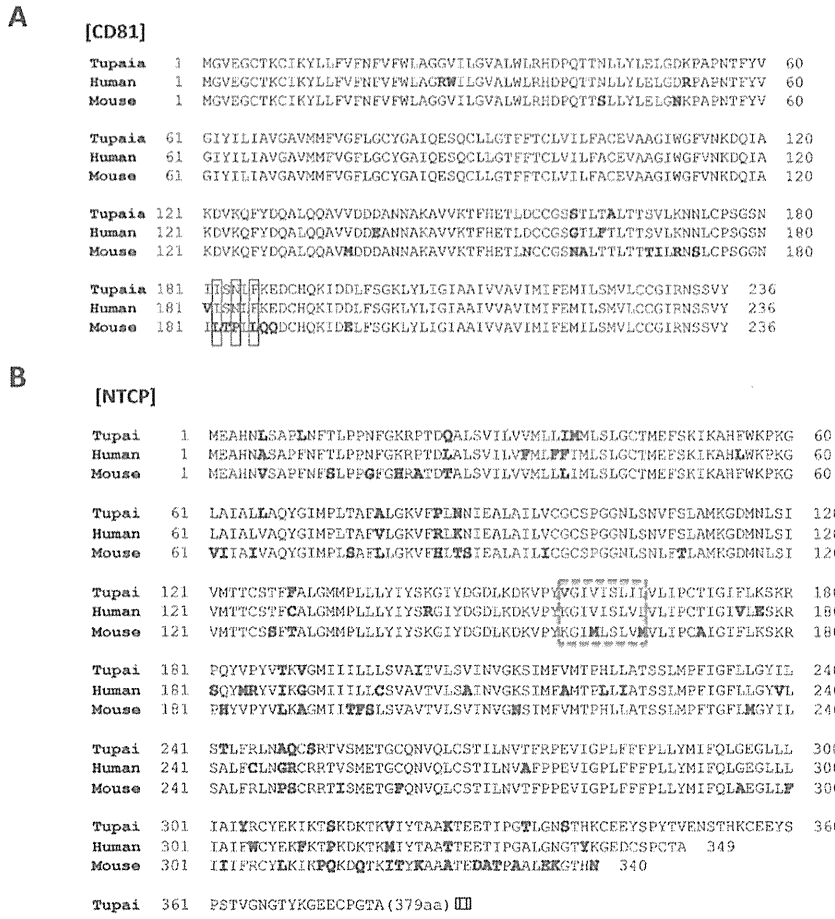

Fig. 3. Alignment of amino acid sequences of viral receptors. (A) Alignment of CD81 amino acid sequences from tupaia, human and mouse. Different amino acids were indicated with red colour. Significant amino acids for binding to HCV E2 protein were surround by square (Ile182, Asn184 and Phe186) [14, 6]. (B) Alignment of NTCP amino acid sequences from tupaia, human and mouse. Different amino acids were indicated with red colour. HBV pre-S1 binding region [37] was surrounded by break line box.

genicity of HCV could not be characterized.

We previously conducted infection experiments using HCV in *Tupaia* and characterized the pathogenesis in this animal [2]. Chronic HCV infection, which manifests as liver cirrhosis and hepatocellular carcinoma, is easily established [1]. Currently, approximately 170 million people around the world may be infected with HCV [35]. The current standard therapy for chronic hepatitis C is a combination of pegylated interferon (IFN) alpha-2a and nucleoside analog ribavirin. Recently, IFN-free combinations of direct-acting antiviral agents have been tested for clinical use and can achieve significant antiviral activity [29]. However, no vaccines against HCV infection have been developed to date, mainly because of the lack of suitable animal experimental systems.

We injected tupaias with serum from a chronic hepatitis C patient (HCR6; 3.7×10^4 50% chimpanzee infectious dose/ml) or reconstituted virus (RCV; genotype 1b). Inoculation with patient serum caused marked fluctuations in the serum alanine aminotransferase (ALT) concentrations − from 2–5 fold in both tupaias − suggesting acute hepatitis (Figs. 4 and 5). Quantitation of viral RNA by reverse transcription PCR revealed HCV viremia in *Tupaia* (Tup. 5 and 6, Fig. 5A). Inoculation with RCV showed sustained viremia for up to 10 weeks (Tup. 4 and 8; Fig. 5B). Histological examination re-

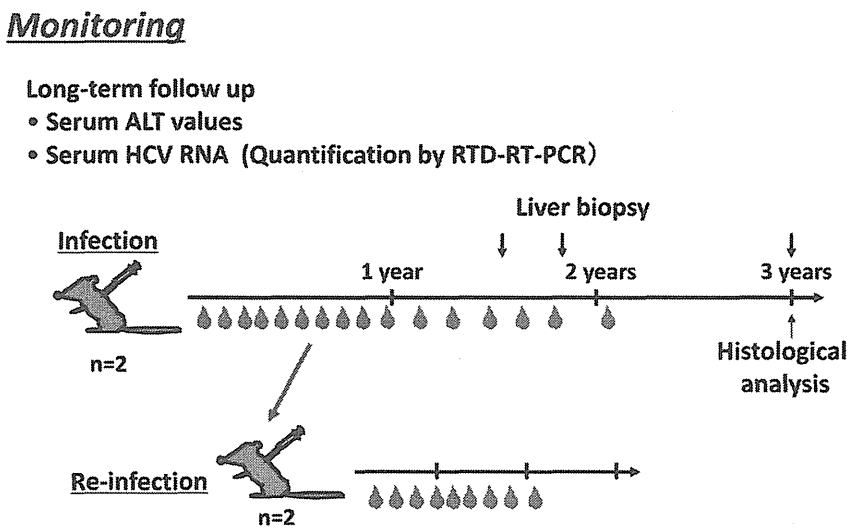

Fig. 4. Experimental design of HCV infection and re-infection of tupaia.

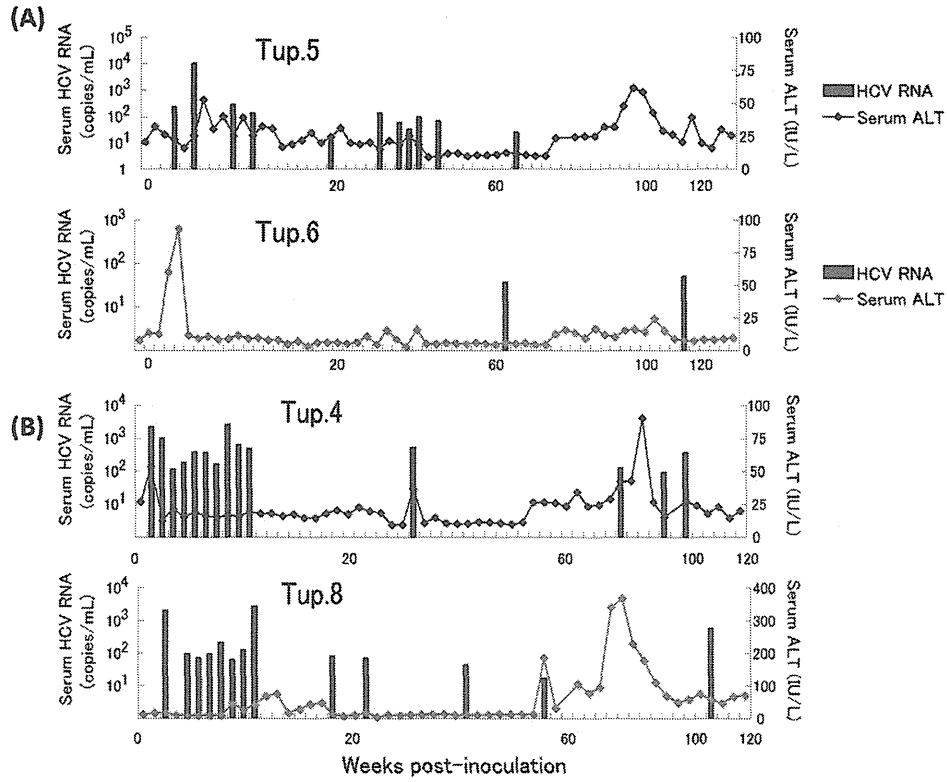

Fig. 5. Course of HCV infection in tupaia. (A) Tupaias No. 5 and 6 were inoculated with patient serum HCR6. Serum ALT (IU/ml) and viral loads, measured as amount of HCV RNA (copies/ml), were measured for over 120 weeks. Set point for serum ALT in untreated tupaias was 22.3 IL/ml (n=23). Negative control animals showed no significant ALT fluctuations for more than 2 years (n=3). No HCV RNA was detected in the negative controls after more than 2 years (n=3). (B) Tupaias No. 4 and 8 were inoculated with RCV as for the HCR6 inoculated animals.

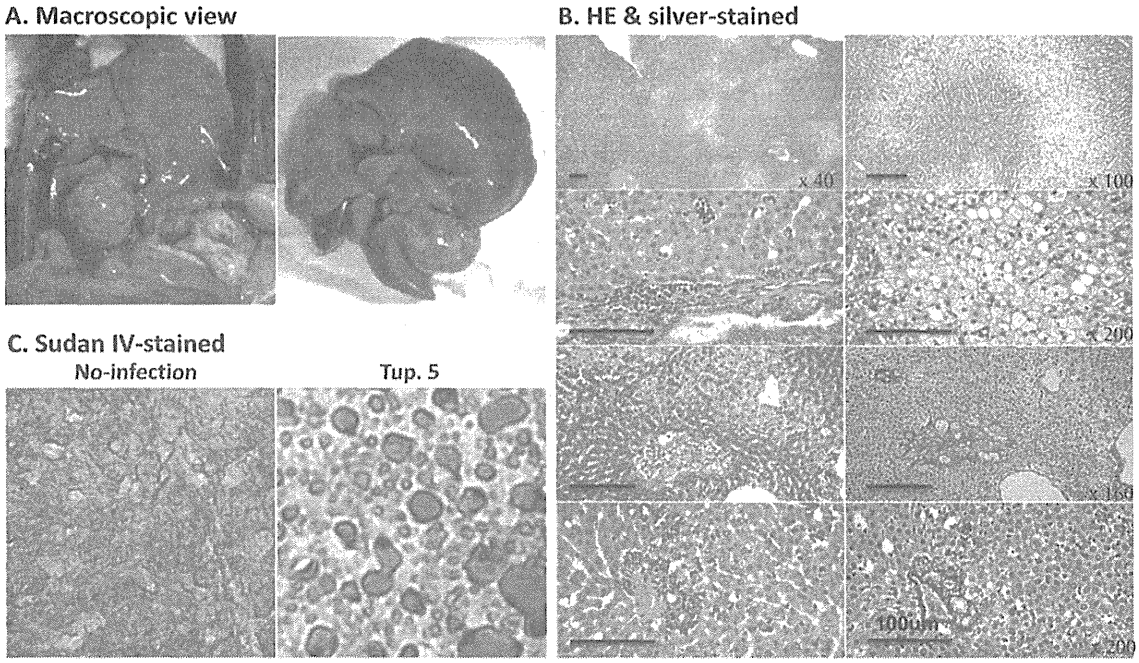

Fig. 6. (A) Macroscopic view of liver inoculated with patient serum HCR6 after 2 years (Tup.5, serum ALT value was 25 IU/l at autopsy). (B) HE staining (×40, ×100, ×200; scale bars indicate **) and silver staining (×160, ×200) of liver tissue (Tup 5) were indicated. Lymphocytic infiltration, steatosis and fibrogenesis were observed. (C) Sudan IV staining of the liver tissue of Tup5 (right) and non-infection (left).

vealed that HCV caused chronic hepatitis, fibrosis and cirrhosis (Fig. 6), with progressive lipid degeneration observed in tupaias over the course of infection. Macroscopic observations also indicated that liver cirrhosis worsened and large surface nodules were observed (Fig. 6). Transmission of viral RNA-positive serum to naïve animals reproduced acute hepatitis and viremia, indicating that HCV infection could reproduce the pathogenesis typically associated with acute and chronic hepatitis in tupaia. However, sustained seroconversion was not observed in tupaia and production of HCV and antibody only occurred at specific time points. To increase the susceptibility of tupaia to HCV infection and to develop a sensitive HCV infection model, these differences between HCV infection in tupaias and humans should be examined in future. HCV infection studies in tupaias have been examined using x-rays [41] and metabolic analysis [31], and the efficacy of natural products for treating HCV-infected tupaia has also been evaluated [39].

Several groups have successfully infected tupaias with HBV, as follows. In culture medium, infection by HBV has been shown to produce HBs antigen (Ag) and HBeAg. HBV infection in newborn and adult tupaias induced the production of HBsAg, HBsAb, HBcAb and HBeAb; all of the adults were successfully infected [34]. Experimental infection of tupaias with HBV was successful in approximately 55% of the animals inoculated [38]. HBV infection and aflatoxin B1 exhibited a synergistic effect in hepatocarcinogenesis [21]. To establish chronic infection by HBV, newborn tree shrews were infected with HBV [36]. Six of 46 newborn babies were found to be susceptible to HBV infection at 48 weeks post inoculation. Histological analysis of liver tissues from infected tupaias revealed chronic hepatitis symptoms, such as hydropic, fatty and eosinophilic degeneration of hepatocytes, lymphocytic infiltration, and hyperplasia of small bile ducts in the portal area [28]. One tupaia infected with HBV for more than 6 years showed multiple necrotic areas [28]. These findings show that although the efficacy of infection needs to be improved in future, tupaias are potentially well suited for use as a model for HBV infection.

Tupaias have also been reported to be infected by

specific viruses, such as tupaia herpes virus, which induces tumorigenicity [4], and potentially with non-pathogenic tupaia paramyxovirus [32]. Tupaias have also been infected with TTV [26], tupaia adenovirus [3], and influenza virus [40].

Conclusion

Tupaia shares considerable genetic homology with both humans and primates, and is considered to be well suited for use as a model for studies on viral infection and preclinical drug development. At present, difficulties associated with maintaining and handling tupaia are major factors limiting the widespread adoption of this animal for use in infection studies. However, optimizing these issues will facilitate the use of tupaias as an experimental animal. In addition, development of genetic methods for modifying the tupaia genome would also increase the potential value of tupaia as a model animal, as this would facilitate detailed studies of virus pathogenesis and drug evaluation.

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Resistance to cyclosporin A derives from mutations in hepatitis C virus nonstructural proteins

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ABSTRACT

Cyclosporine A (CsA) is an immunosuppressive drug that targets cyclophilins, cellular cofactors that regulate the immune system. Replication of hepatitis C virus (HCV) is suppressed by CsA, but the molecular basis of this suppression is still not fully understood. To investigate this suppression, we cultured HCV replicon cells (Con1, HCV genotype 1b, FLR-N cell) in the presence of CsA and obtained nine CsA-resistant FLR-N cell lines. We determined full-length HCV sequences for all nine clones, and chose two (clones #6 and #7) of the nine clones that have high replication activity in the presence of CsA for further analysis. Both clones showed two consensus mutations, one in NS3 (T1280V) and the other in NS5A (D2292E). Characterization of various mutants indicated that the D2292E mutation conferred resistance to high concentrations of CsA (up to 2 μM). In addition, the missense mutation T1280V contributed to the recovery of colony formation activity. The effects of these mutations are also evident in two established HCV replicon cell lines—HCV-RMT ([1], genotype 1a) and JFH1 (genotype 2a). Moreover, three other missense mutations in NS5A—D2303H, S2362G, and E2414K—enhanced the resistance to CsA conferred by D2292E; these double or all quadruple mutants could resist approximately 8- to 25-fold higher concentrations of CsA than could wild-type Con1. These four mutations, either as single or combinations, also made Con1 strain resistant to two other cyclophilin inhibitors, N-methyl-4-isoleucine-cyclosporin (NIM811) or Debio-025. Interestingly, the changes in IC_{50} values that resulted from each of these mutations were the lowest in the Debio-025-treated cells, indicating its highest resistant activity against the adaptive mutation.

1. Introduction

The genome of the hepatitis C virus (HCV) is a single-stranded RNA with positive polarity and is classified in the *Flaviviridae* family. HCV frequently establishes chronic infections that lead to liver cirrhosis and hepatocellular carcinoma (HCC) [2]. An estimated 130–200 million people worldwide are now infected with HCV [3]. HCVs have been classified into six major genotypic groups (genotypes 1–6); genotype 1 is the most prevalent over most of the world. Treatments with alpha interferon (IFNα), together with the nucleoside analog ribavirin (RBV), greatly increased the percentage of HCV chronically infected patients able to reach a sustained anti-viral response (SVR). Covalent attachment of polyethylene glycol (PEGylated) IFN-α-plus-RBV therapy has a success rate of ~80% in patients with genotype 2 or 3 infections, but only ~50% in patients with genotype 1 infections [4,5]. The recently approved protease inhibitors boceprevir and telaprevir each improved the efficacy of IFN-α-plus-RBV therapy [6]. These direct-acting agents (boceprevir, simeprevir, sofosbuvir, faldaprevir and telaprevir, etc.) each have the advantage of being highly specific, but each may select for specific resistant mutations, limiting their long-time efficacy. Therefore, antiviral inhibitors targeting host factors crucial for viral replication should be developed to overcome these problems.

Abbreviations: HCV, hepatitis C virus; CsA, cyclosporine A; HCC, hepatocellular carcinoma; IFNα, alpha interferon; Cyp, cyclophilins; SVR, sustained anti-viral response.

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Reportedly, several HCV proteins interact with cyclophilins (Cyp) and modulate HCV replication [7–9]. To date, three Cyp inhibitors—Debio-025, NIM811, and SCY-635—have been deemed safe and effective for patients with HCV in phase I and II studies [10–12]. Development of Debio-025 has advanced the farthest through phase II studies, and Debio-025 has approved and showed a great deal of promise for decreasing HCV viremia in infected patients. However, emergence of drug-resistant HCV mutants could limit the therapeutic potential of CsA and Cyp inhibitors.

The HCV genome is a positive-sense, single-stranded RNA (about 9.6 kb) that encodes at least 10 viral proteins; these are categorized as structural core proteins (E1, E2) or nonstructural (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) [13,14]. The nonstructural proteins are involved in HCV RNA replication [14]. NS5A protein comprises three domains linked by two low-complexity sequences (LCS) that are either serine or proline rich; domain I is a highly structured zinc binding domain whose three-dimensional structure shows two dimeric conformations [15,16]. Domains II and III have been shown to be unstructured in their native states, but nuclear magnetic resonance and circular dichroism have shown that elements of secondary structure run throughout each of these domains [17–19]. NS5A is anchored to membranes by an N-terminal amphipathic helix and is an essential component of the viral genome replication complex; it also interacts with other non-structural proteins [20] or cellular factors. NS5A domain II is a substrate for the peptidyl-prolyl cis/trans isomerase activity of Cys A and B [21], and NS5A domain III is reportedly a substrate of CypA [22].

In this study, we used CsA to select for and isolate drug-resistant HCV mutants; we then performed virus genome sequencing to investigate the molecular mechanisms of this drug resistance.

2. Materials and methods

2.1. Cells, electroporation and ethics statement

HuH-7 cells were cultured in DMEM-GlutaMax-I (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, penicillin, and streptomycin (Invitrogen). Replicon cells were maintained in the same medium supplemented with 300 μg/ml G418 (Invitrogen). Cells were passaged three times a week, and at each passage, each culture was split into four subcultures. Electroporation of replicon RNA and G418 selection were performed as previously described [23]. All experimental protocol was approved by the regional research institute.

2.2. Establishment of cyclosporin A resistant replicon clones

FLR3-1 cells derived from Con1 (AJ238799)-based, luciferase-harboring HCV sub-genomic replicon cell were treated with both 2 μM of cyclosporin A and 0.5 mg/ml of G418 for 24 days. Surviving cells were further treated with 3 μM CsA for 2 days, 4 μM for 4 another days, and finally 6 μM for the last 10 days. Using limiting dilution cloning, we established nine clonal cell lines. Using real-time RT-PCR (ABI 7700 system, Applied Biosystems, Foster City, CA, USA) as described previously [24], we systematically measured HCV RNA copy number in each of these nine clonal lines.

2.3. Determination of consensus sequence of resistant clones

LongRange Reverse transcriptase (QIAGEN, Valencia, CA, USA) and an oligonucleotide primer (antisense sequence 9549–9569 of HCV-Con1) were used to reverse transcribe purified RNA (1 μg). The resulting cDNA, Phusion DNA polymerase (Finnzymes, Vantaa, Finland), and primers recognizing each non-coding region were used for PCR amplification of the entire non structural protein coding region of the sub-genomic replicon. The TA cloning kit (Invitrogen) was used to introduce each fragment into a separate plasmid; we picked up eight clones from each resistant cell line and their nucleotide sequences were determined.

2.4. Construction and RNA transcription

The pFK I389neo/NS3-3′/5.1 and pFK I389luc/NS3-3′/5.1 plasmids (ReBlikon, Baden-Württemberg, Germany) were used to generate HCV constructs with regions of the sub-genomic replicon with mutations (Fig. 2A). The QuikChangeII kit (Stratagene, La Jolla, CA, USA) was used to introduce specific mutations into the HCV sequences. To generate RNA, plasmids were digested with *XbaI* and used as a template for RNA transcription; RiboMax (Promega, Madison, WI, USA) was used for each transcription reaction.

2.5. Drug treatment

For the drug resistance assays, established CsA-resistant replicon clones were seeded onto 24-well tissue culture plates (10,000 cells/well) and cultivated overnight. Then cells were treated with various concentrations of CsA (0–8 μM) for 4 days. Surviving cells were stained with crystal violet.

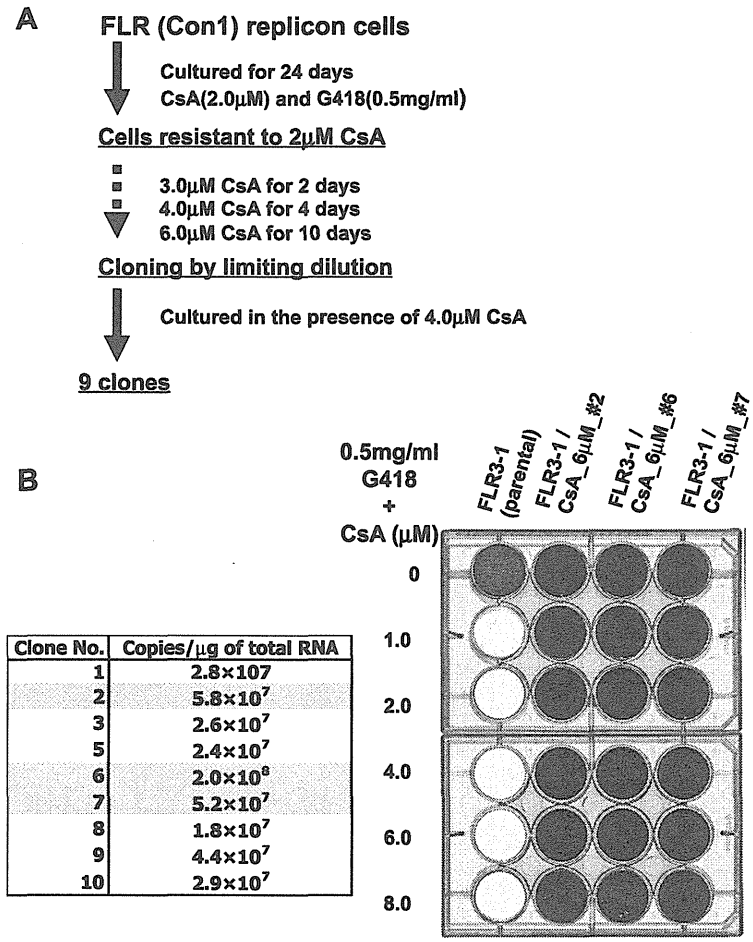
For HCV replication inhibition assays, replicon cells were seeded in 96-well tissue culture plates (5000 cells/well) and cultivated overnight. Serial dilutions of CsA (Fluka Chemie, Buchs, Switzerland) or NIM811 (Novartis) and Debio025 (Debiopharma) were then added to sets of wells. After incubation for 72 h, ABI prizm 6100 (Applied Biosystems) was used to extract total RNA from cells, and HCV-RNA was measured as described above. Each assay was carried out in triplicate.

For another HCV replication inhibition assay, mutant replicon RNA derived from pFK I389luc/NS3-3′/5.1 plasmid were introduced into HuH7 cells via electroporation, and the transformed/transfected cells were seeded to 96-well tissue culture plates. Drugs were added 24 h after electroporation. Luciferase activities were evaluated 4 h or 72 h after electroporation, which corresponded to 20 h before drug treatment or 48 h after drug treatment, respectively; the Blight-Glo kit (Invitrogen) and Envision (Perkin Elmer, Waltham, MA, USA) were used to take all measurements, and values at 72 h were normalized relative to the values from 4 h.

3. Results

3.1. Establishment of CsA-resistant clones

To establish CsA-resistant clones, we treated HCV FLR-N replicon cells with CsA (Fig. 1A) and obtained nine resistant clonal cell lines. We measured the amount of HCV RNA in each resistant clonal line and chose for further study the three lines that consistently had the largest amount of HCV RNA (Fig. 1B). We then determined the entire HCV sequence from 16 subclones; we isolated two groups of eight subclones (one group each from clones #6 and #7), because we could not establish clone #2; each subclone was isolated by treating a CsA-resistant clone (#6 or #7) with 6 μM CsA (Table 1). Although there were several mutations in the NS3–NS5B protein-coding regions, common mutations were isoleucine (I) to valine (V) at amino acid 1280 (T1280V) and aspartic acid (D) to glutamic acid (E) at amino acid 2292 (D2292E). At 1280, original Con1 has threonine (T) and was mutated into (I) in Con1 replicon cells.



Clone No.	Copies/μg of total RNA
1	2.8×10^{7}
2	5.8×10^{7}
3	2.6×10^{7}
5	2.4×10^{7}
6	2.0×10^{8}
7	5.2×10^{7}
8	1.8×10^{7}
9	4.4×10^{7}
10	2.9×10^{7}

Fig. 1. Basic characteristics of the nine cyclosporin A-resistant clones. (A) Flow chart outlining the selection of cyclosporin A-resistant HCV replicon clones. (B) Real-time PCR was used to determine the copy number of each Cys A-resistant clone. The three clones with the highest HCV genome copy number are highlighted in green (Left). Colony formation assay of mutant #2, 6 and 7 (Right). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Identification of mutations responsible for CsA resistance

To define the mutations responsible for CsA resistance, we constructed various chimeric clones that each contained specific mutation that arose from CsA selection (Fig. 2A). We could thereby evaluate each mutation with regard to its effect on CsA resistance. We found that mutations in two proteins—NS5A and NS4A—significantly enhanced the resistance against CsA treatment (Fig. 2B). We also cultured replicon cells with these mutants in the presence of CsA (up to 2 μM); we found that cells with a D2292E mutation could survive, but cells with wild-type NS5A or T1280V mutation could not (Fig. 3A).

The effect of T1280V mutation on colony formation was further evaluated (Fig. 3B). Introduction of the T1280V mutation in *cis* to the D2292E mutation rescued the colony-formation defect of the D2292E mutant replicon cells; specifically, the T1280V–D2292E double-mutant replicon cells had the same colony-forming ability as the parental replicon cells.

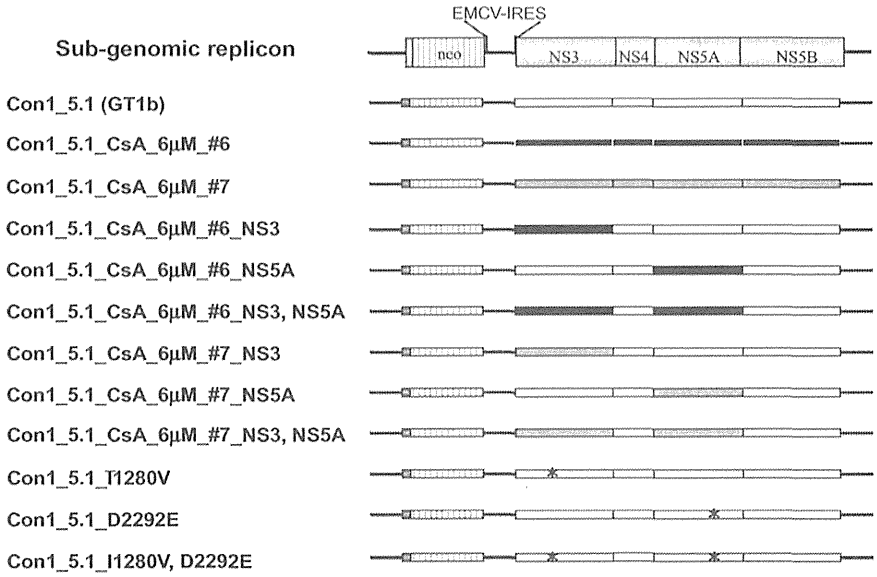
3.3. Evaluation of mutations for CsA resistance in other HCV genotypes

We evaluated whether the mutations that conferred CsA resistance to the HCV Con1 strain (genotype 1b) also conferred CsA resistance to the RMT (genotype 1a; AB520610) and JFH1 (genotype 2a; AB047639) strains (Fig. 4A, B and Table 2) [1]. D2292E conferred CsA resistance to the HCV strains RMT and JFH1, but T1280V did not (Table 2), as observed with HCV Con1 strain (Fig. 2E). The amino acid sequences surrounding mutations other than D2292E showed some differences among three genotypes (1a, 1b, and 2a) (Fig. 4B). D2292E mutants of these three genotypes showed resistance to CsA (Fig. 2E, Table 2) but the fold increase of resistance in genotype 1a and 2a was lower than that of genotype 1b (Tables 2 and 3). Therefore, there might be some residue(s) other than D2292E to influence the resistance to CsA.

3.4. Efficacy of mutations in NS5A for conferring CsA resistance

Although D2292E clearly conferred CsA resistance to HCV, other mutations in NS5A may also have had an effect because constructs with all four of the original NS5A mutations found in clone #6 mutations were more resistant to CsA than were constructs with only the D2292E mutation (Fig. 2B and E). We constructed HCV-luciferase replicons, each with one or more of four mutations (D2292E, D2303H, S2362G, and E2414K). HuH-7 cells were transiently transfected with RNA of each construct; we then treated the transfected cells with CsA (Table 3). Of the four single mutants, all but S2362G conferred some CsA resistance to HCV-luciferase replicons; notably, combinations of mutations had additive effects

A



B

Cyclosporin A	Replacement or Mutation			IC_{50} (μM)	Fold change
	NS3	NS4A	NS5A		
Con1_5.1 (GT1b)	-	-	-	0.18	1.0
	Cs6#6	-	-	0.21	1.2
	-	-	Cs6#6	2.10	11.7
	Cs6#6	-	Cs6#6	2.20	12.2
	Cs6#6	V1681A	Cs6#6	5.40	30.0

C

NIM811	replacement or mutations			IC_{50} (μM)	Fold change
	NS3	NS4A	NS5A		
Con1_5.1 (GT1b)	-	-	-	0.09	1.0
	Cs6#6	-	-	0.10	1.1
	-	-	Cs6#6	0.49	5.4
	Cs6#6	-	Cs6#6	0.50	5.6
	Cs6#6	V1681A	Cs6#6	1.80	20.0

D

Debio025	Replacement or mutation			IC_{50} (μM)	Fold change
	NS3	NS4A	NS5A		
Con1_5.1 (GT1b)	-	-	-	0.05	1.0
	Cs6#6	-	-	0.04	0.8
	-	-	Cs6#6	0.43	7.8
	Cs6#6	-	Cs6#6	0.40	8.0
	Cs6#6	V1681A	Cs6#6	0.75	13.7

E

Cyclosporin A	Replacement or mutation			IC_{50} (μM)	Fold change
	NS3	NS4B	NS5A		
Con1_5.1 (GT1b)	-	-	-	0.54	1.0
	Cs6#7	-	-	0.59	1.1
	-	-	Cs6#7	2.36	4.4
	Cs6#7	-	Cs6#7	2.51	4.6
	Cs6#7	I1797V	Cs6#7	2.44	4.5
	T1280V	-	-	0.57	1.1
	-	-	D2292E	1.71	3.1
	T1280V	-	D2292E	1.76	3.3

Fig. 2. (A) Schematic representations of 12 Con1 replicon-derived constructs. (B–D) Evaluation of Cs6#6 constructs with regard to resistance to CsA or to each of two CsA derivatives (NIM811 and Debio025). Real-time PCR was used to measure HCV sub-genome copy number in cells, and IC_{50}s were then determined from the copy number values. For each construct, the fold change represents the ratio of IC_{50} values from the construct and the parental Con1 replicon (IC50Construct:IC50Parental). (E) Resistance to CsA of three Cs6#7 derivative constructs that represent the T1280V and D2292E mutations as each single mutation or as a double mutation.

and conferred greater CsA resistance than any single mutation. The HCV replicon with all four mutations showed the strongest CsA resistance.

3.5. Evaluation of CsA-resistant mutants for resistance to cyclophilin inhibitors

We further evaluated each of the NS5A mutants for their ability to confer resistance to each of two other cyclophilin inhibitors, N-methyl-4-isoleucine-cyclosporin (NIM811, Table 4) and Debio-025 (Table 5). Of the four single mutants, D2292E conferred the highest resistance, and the combination of all four mutations conferred the overall highest resistance to NIM811 and to Debio-025. When we compared CsA, NIM811, and Debio-025, the mutation-mediated increases in IC_{50} values were lowest with the Debio-025 treatment (Tables 3–5).

4. Discussion

Here, we investigated two of nine HCV sub-genomic replicon cell clones (CsA-resistant HCV mutants) isolated following long-term dual treatment with CsA and G418. Comparing the HCV sequences of these two clones (#6 and #7), only two of many mutant sites were shared between the mutant HCV sequences. Specifically, both clones #6 and #7 had a D2292E missense mutation in NS5A and a T1280V missense mutation in NS3. D2292E is