

EXPERIMENTAL PROCEDURES

Infection of Human Hepatocyte-Chimeric Mice with HBV and In Vivo Treatment with ϵ RNA

To generate human hepatocyte-chimeric mice, $uPA^{+/-}/SCID$ mice, were transplanted with commercially available cryopreserved human hepatocytes (a 2-year-old Hispanic female; BD Bioscience) as described previously (Tateno et al., 2004). Chimeric mice were intravenously infected with HBV-C (10^6 copies per mouse) derived from patient with chronic hepatitis (Sugiyama et al., 2006). Total RNAs were isolated from liver tissues at 0, 4, or 5 weeks after infection and subjected to quantitative RT-PCR (qRT-PCR). In preparation for ϵ RNA treatment, HBV infection at 3 weeks after infection was confirmed by measuring the number of viral genome copies in the sera of HBV-infected chimeric mice by qPCR analysis, and at 4-week postinfection, ϵ RNA expression vector or empty vector loaded in a liposomal carrier, a multifunctional envelope-type nanodevice (MEND), was administered intravenously at a dose of 0.5 mg/kg of body weight ($n = 3$ per group) every two days for 14 days. Serum samples were subjected to qPCR for the quantification of DNA copy numbers of HBV as described previously (Nakagawa et al., 2013). All animal protocols described in this study were performed in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Animal Welfare Committee of Phoenix Bio Co., Ltd.

HBV Infection in Human Hepatocytes and Quantification of Encapsidated HBV DNA

HBV infection in PHH or HepG2-hNTCP-C4 cells was performed at 10 or 100 genome equivalents per cell, respectively, in the presence of 4% PEG8000 at 37°C for 24 hr as previously described (Sugiyama et al., 2006; Watashi et al., 2013; Iwamoto et al., 2014). Lamivudine (50 μ M; Sigma) was added in Huh-7 culture media during HBV production. HBV DNA was purified from intracellular core particles as described previously (Turelli et al., 2004; Fujiwara et al., 2005). Briefly, cells were suspended in lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 1% NP-40. Nuclei were pelleted by centrifugation at 4°C and 15,000 rpm for 5 min. The supernatant was adjusted to 6 mM MgOAc and treated with DNase I (200 μ g/ml) and of RNase A (100 μ g/ml) for 3 hr at 37°C. The reaction was stopped by addition of 10 mM EDTA and the mixture was incubated for 15 min at 65°C. After treatment with proteinase K (200 μ g/ml), 1% SDS, and 100 mM NaCl for 2 hr at 37°C, viral nucleic acids were isolated by phenol:chloroform:isoamyl alcohol (25:24:1) extraction and ethanol precipitation with 20 μ g glycogen. Copy numbers of HBV DNA were measured by qPCR with the indicated primers (Table S3).

qRT-PCR Analysis

Total RNAs were isolated from culture cells or frozen liver tissue using Isogen (Nippon Gene) and were treated with DNase I (Invitrogen). cDNA was prepared from total RNAs using ReverTra Ace (TOYOBO). qRT-PCR was performed using SYBR Premix Ex Taq (TAKARA) and analyzed on a StepOnePlus real-time PCR system (Applied Biosystems). Detailed information about the primers used here is shown in Table S3. Data were normalized to the expression of *GAPDH* or HBV RNAs for each sample.

RIP Assay

After 2 hr incubation with the antibody as indicated for immunoprecipitation, cell lysates were mixed with Protein-G Dynabeads (Invitrogen) and further incubated for 1 hr with gentle shaking. After washing three times, the precipitated RNAs were analyzed by qRT-PCR with appropriate primers to detect the target RNA. The amount of immunoprecipitated RNAs is represented as the percentile of the amount of input RNA (percentage input). The detail was described in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, four tables, and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.immuni.2014.12.016>.

AUTHOR CONTRIBUTIONS

S.S., K.L., T.K., and T.H. carried out most of the experiments and analyzed data. Y.T. provided materials of HBV and designed the protocol for infection. Y.I. conducted HBV infection in chimeric mice and preparation of human hepatocytes, and S.M., T. Watanabe, S.I., S.T., and Y.T. performed the related analysis and contributed to the interpretation of the results. C.M.R. offered critical advice on the whole manuscript and provided a pair of Huh-7 and Huh-7.5 cell lines. K.W. and T. Wakita provided HepG2-hNTCP-C4 cells. T.K. carried out FRET analysis. Y. Sakurai, Y. Sato, H.A., and H.H. syntheses of plasmid-loaded MEND, and M.K. contributed to establishment of the protocols for in vivo treatment. A.T. supervised the project, designed experiments and wrote the manuscript with critical input from the coauthors, and all authors contributed to discussing the results.

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Inhibitory effects of Pycnogenol[®] on hepatitis C virus replication



Sayeh Ezzikouri^{a,b,c,1}, Tomohiro Nishimura^{d,1}, Michinori Kohara^e, Soumaya Benjelloun^a, Yoichiro Kino^d, Kazuaki Inoue^f, Akira Matsumori^g, Kyoko Tsukiyama-Kohara^{b,c,*}

^a Virology Unit, Viral Hepatitis Laboratory, Pasteur Institute of Morocco, Casablanca, Morocco

^b Transboundary Animal Diseases Centre, Joint Faculty of Veterinary Medicine, Kagoshima University, Kagoshima, Japan

^c Laboratory of Animal Hygiene, Joint Faculty of Veterinary Medicine, Kagoshima University, Kagoshima, Japan

^d Chemo-Sero Research Institute, Kikuchi Research Center, Kumamoto, Japan

^e Department of Microbiology and Cell Biology, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan

^f Division of Gastroenterology, Showa University, Fujigaoka Hospital, Kanagawa, Japan

^g Tokyo Medical University, Tokyo, Japan

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ABSTRACT

Chronic hepatitis C virus (HCV) infection increases the risk of liver cirrhosis and hepatocellular carcinoma. In the last decade, the current standard HCV treatment, pegylated interferon and ribavirin, have limited efficacy and significant side effects. Novel direct acting antivirals show promise, but escape mutants are expected, along with potential side effects. Pycnogenol[®], a French maritime pine extract, has been reported to have antioxidant and antiviral effects. Here, we evaluated the effect of Pycnogenol[®] on HCV replication.

Wild-type and protease inhibitor (VX-950; telaprevir)-resistant HCV replicon cells were treated with Pycnogenol[®], Pycnogenol[®] and interferon-alpha, and ribavirin and telaprevir. Pycnogenol[®] effects on replication were also evaluated in HCV-infected chimeric mice.

Pycnogenol[®] treatment showed antiviral effects without cytotoxicity at doses up to 50 µg/mL. Pycnogenol[®] in combination with interferon-alpha or ribavirin showed synergistic effects. Moreover, Pycnogenol[®] inhibited HCV replication in telaprevir-resistant replicon cells; telaprevir and Pycnogenol[®] acted additively to reduce HCV RNA levels in wild-type HCV replicon cells without significantly increasing cytotoxicity. Pycnogenol[®] antiviral activity was higher than its components procyanidin and taxifolin. Further, treatment of infected chimeric mice with Pycnogenol[®] suppressed HCV replication and showed a synergistic effect with interferon-alpha. In addition, Pycnogenol[®] treatment resulted in dose-dependent reduction of reactive oxygen species in HCV replicon cell lines.

Pycnogenol[®] is a natural product that may be used to improve the efficacy of the current standard antiviral agents and even to eliminate resistant HCV mutants.

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Abbreviations: CC₅₀, 50% cytotoxic concentration; CI, combination index; DAA, direct acting antivirals; EC₅₀, 50% effective concentration; HCV, hepatitis C virus; IC₅₀, 50% inhibitory concentration; IRES, internal ribosome entry site; NS, non-structural protein; NF-kappa B, nuclear factor-kappa B; PC, procyanidin; PEG-IFN-alpha-2a, pegylated interferon-alpha-2a; PEG-IFN-alpha-2b, pegylated interferon-alpha-2b; RBV, ribavirin; PYC, Pycnogenol[®]; qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction; ROS, reactive oxygen species; SI, selectivity index.

* Corresponding author at: Transboundary Animal Diseases Centre & Department of Animal Hygiene, Joint Faculty of Veterinary Medicine, Kagoshima University, 1-21-24 Korimoto, Kagoshima-city 890-0065, Japan. Tel./fax: +81 99 285 3589.

E-mail addresses: sayeh.ezzikouri@pasteur.ma (S. Ezzikouri), kkohara@vet.kagoshima-u.ac.jp (K. Tsukiyama-Kohara).

¹ These authors contributed equally to the study.

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1. Introduction

Approximately 130–170 million people are chronically infected with HCV, leading to 54,000 deaths and 955,000 disability-adjusted life-years associated with acute HCV infection (Mohd Hanafiah et al., 2013). Chronic hepatitis C can lead to a large spectrum of diseases, including steatosis, fibrosis, cirrhosis, and hepatocellular carcinoma (Perz and Alter, 2006). To date, no protective vaccine is available for HCV infection; over the last decade, therapy has consisted of a 24–48-week course of peginterferon-alpha-2a (PEG-IFN-alpha-2a) or peginterferon-alpha-2b (PEG-IFN-alpha-2b) in combination with the guanosine analogue, ribavirin (RBV). The therapy leads to sustained virologic response (SVR) in 42–52%, 65–85%, and 76–82% of individuals infected with HCV genotype 1; 4, 5, or 6; and 2 or 3, respectively (Antaki et al., 2010; Hoofnagle and Seeff, 2006). The recently approved non-structural

protein (NS) 3/4A protease inhibitors (PIs) boceprevir (approved by the FDA on May 13, 2011) and telaprevir (approved by the FDA on May 23, 2011), used in combination with PEG-IFN-alpha and RBV for HCV genotype 1 infections, have increased cure rates to approximately 70% (Bacon et al., 2011; Jacobson et al., 2011; Poordad et al., 2011). However, these triple-therapy regimens may result in unfavourable side effects and emergence of drug-resistant HCV (Bacon et al., 2011; Jacobson et al., 2011; Poordad et al., 2011), which may reduce virus susceptibility and applicability of current HCV triple therapies (Ozeki et al., 2011). Recently, two more effective compounds have been approved for HCV treatment: the protease inhibitor simeprevir (approved by the FDA in November, 2013) and the nucleotide polymerase inhibitor sofosbuvir (approved by the FDA on December 6, 2013). Among patients infected with HCV, less than 10% are treated and cured, and the major challenge in controlling HCV infections is the identification of those already infected, most of whom are situated in the poorest regions of the world (Thomas, 2013), and to find the most effective, tolerable and affordable direct acting antivirals (DAA) combination that can cure people in the shortest period (Poveda et al., 2014). In the NEUTRINO phase III trial of treatment-naïve patients, 12 weeks of triple combination therapy with sofosbuvir (400 mg) once daily resulted in SVR rates of 89% in patients with HCV genotype 1 (92% for subtype 1a and 82% for subtype 1b), and 96% in patients with genotype 4 (Lawitz et al., 2013). Moreover, in the FISSION trial of HCV-2/3 treatment-naïve patients receiving sofosbuvir/RBV for 12 weeks, 95% of patients with genotype 2 and 56% of patients with genotype 3 achieved an SVR (Lawitz et al., 2013). In addition, most DAA agents are characterised by a low genetic barrier to the development of resistance, except sofosbuvir, which showed a very high resistance barrier. This is the reason most current DAA-based therapies under evaluation must be co-administered with either PEG-IFN-alpha and ribavirin or different compounds belonging to different DAA classes (Poveda et al., 2014).

Pycnogenol® (PYC; trademark of Horphag Research, Geneva, Switzerland) is a French maritime pine extract produced from the outer bark of *Pinus pinaster* ssp. *atlantica*, and is generally considered safe for human use (American Botanical Council, 2010). The main PYC constituents are procyanidins (68.4%), taxifolin (21.87%), ferulic acid (3.70%), catechin (2.53%), and caffeic acid (3.51%) (Lee et al., 2010). PYC has been reported to have antioxidative and anti-inflammatory effects, and to reduce cardiovascular risk factors associated with type 2 diabetes (Maimoona et al., 2011; Zibadi et al., 2008). A recent report suggests that PYC can inhibit encephalomyocarditis virus replication in the mouse heart by suppressing expression of proinflammatory cytokines, and genes related to cardiac remodelling and mast cells (Matsumori et al., 2007). PYC has also been reported to inhibit binding of human immunodeficiency virus type-1 to host cells, and to cause other significant changes, including increased expression of manganese superoxide dismutase (Feng et al., 2008).

HCV gene expression elevates reactive oxygen species (ROS) levels via calcium signalling. In addition, HCV Core, NS3, and NS5A proteins have all been shown to induce oxidative stress (Choi et al., 2004). The reported link between HCV and oxidative stress makes this pathway a promising anti-HCV therapeutic strategy. To date, however, the effect of PYC on HCV infection has not been investigated. This study evaluated the inhibitory effects of Pycnogenol® on HCV replication *in vitro* and *in vivo*.

2. Materials and methods

2.1. Cell culture and reagents

Genotype 1b HCV subgenomic replicon cell lines, R6FLR-N (R6, genotype 1b, strain N) (Watanabe et al., 2006), FLR3-1 (genotype

1b, Con-1) (Sakamoto et al., 2005) and Rep JFH Luc3-13 genotype 2a (Takano et al., 2011), strain JFH-1 (Wakita et al., 2005) (Supplementary Fig. 1) were cultured at 37 °C (5% CO₂) in Dulbecco's modified Eagle medium-GlutaMAX-I (DMEM-GlutaMAX-I; Invitrogen, Carlsbad, CA, USA) containing 10% foetal bovine serum and 0.5 mg/mL G418 (Invitrogen, Carlsbad, CA, USA) (Sakamoto et al., 2005; Watanabe et al., 2006). The JFH-1/K4 cell line, which comprises HuH-7 cells persistently infected with the HCV JFH-1 strain, was maintained in DMEM with 10% FCS (Takano et al., 2011).

PYC was supplied by Horphag Research Co., Pegylated IFN-alpha-2a was obtained from Chugai Pharmaceutical Co., Japan.

2.2. HCV replicon cell reporter assay

Cells were seeded into 96-well plates (5×10^3 /well). After incubation for 24 h at 37 °C (5% CO₂), the medium was removed and replaced with growth medium containing serial dilutions of PYC, IFN-alpha, RBV, telaprevir or simeprevir (Janssen Pharma Co., Tokyo, Japan). After 72 h, luciferase activity was measured using the Bright-Glo luciferase assay kit (Promega, Madison, WI). Measurements were made in triplicate using an AccuFLEX Lumi 400 luminometer (Aloka, Tokyo, Japan), and the results expressed as the average percentage of the control.

2.3. Generation of telaprevir-resistant replicon cell lines and analysis

Telaprevir-resistant R6FLR-N subgenomic replicon cell lines were established as described previously (Katsume et al., 2013). Briefly, wild-type R6FLR-N replicon cells were seeded in 10-cm dishes in the presence of 0.5 mg/mL G418 and treated with telaprevir. The cells were incubated for 51 days with no-compound control or telaprevir (1.8 μM and 2.7 μM serially diluted in media). Fresh media and telaprevir were added every 3 days. Most cells incubated with 2.7 μM telaprevir died; however, after 3 weeks small colonies started to appear and were expanded for 4 weeks. Deep sequencing was performed as described previously (Katsume et al., 2013) and revealed a mutation profile in NS3 (V36A, T54V and A156T) and NS5A (Q181H, P223S and S417P) which confer resistance to telaprevir. Resistant replicon cells were seeded at 5×10^3 /well. After incubation for 24 h at 37 °C (5% CO₂), culture medium was removed and replaced with growth medium containing serial dilutions of PYC or telaprevir alone or in combination. After 72 h, luciferase activity was determined using the Bright-Glo luciferase assay kit (Promega, Madison, WI, USA). Measurements were made in duplicate using a GloMax-Multi detection system (Promega, Madison, WI, USA). Cytotoxicity was measured using WST-8 cell counting kit (Dojindo, Kumamoto, Japan). Western blot analysis was performed, as described previously (Nishimura et al., 2009). Briefly, HCV replicon cells (2×10^5) were grown in a 60-mm cell culture dish. After 24 h, cells were treated with PYC for 72 h. Cells were collected and lysed with radioimmunoprecipitation buffer (1% sodium dodecyl sulphate, 0.5% Nonidet P-40, 150 mmol NaCl, 0.5 mmol ethylenediaminetetraacetic acid, 1 mmol dithiothreitol, and 10 mmol Tris, pH 7.4). Total protein (30 μg) was electrophoresed on a 12% sodium dodecyl sulphate-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Billerica, MA, USA). HCV NS3 and NS5B proteins were detected using rabbit NS3 (R212) polyclonal antibody or anti-NS5B (5B14) monoclonal antibody. Beta-actin was detected using an actin monoclonal antibody (Sigma, St. Louis, MO, USA).

2.4. Quantitative real-time polymerase chain reaction

Quantification of HCV RNA was performed using real-time reverse transcription polymerase chain reaction (qRT-PCR) based

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 $2.9\text{--}18.0 \times 10^6$ 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-harboring 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 1×10^7 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 \pm 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 $[IC_{50}] = 5.78 \pm 3.75$ 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, simprevir (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

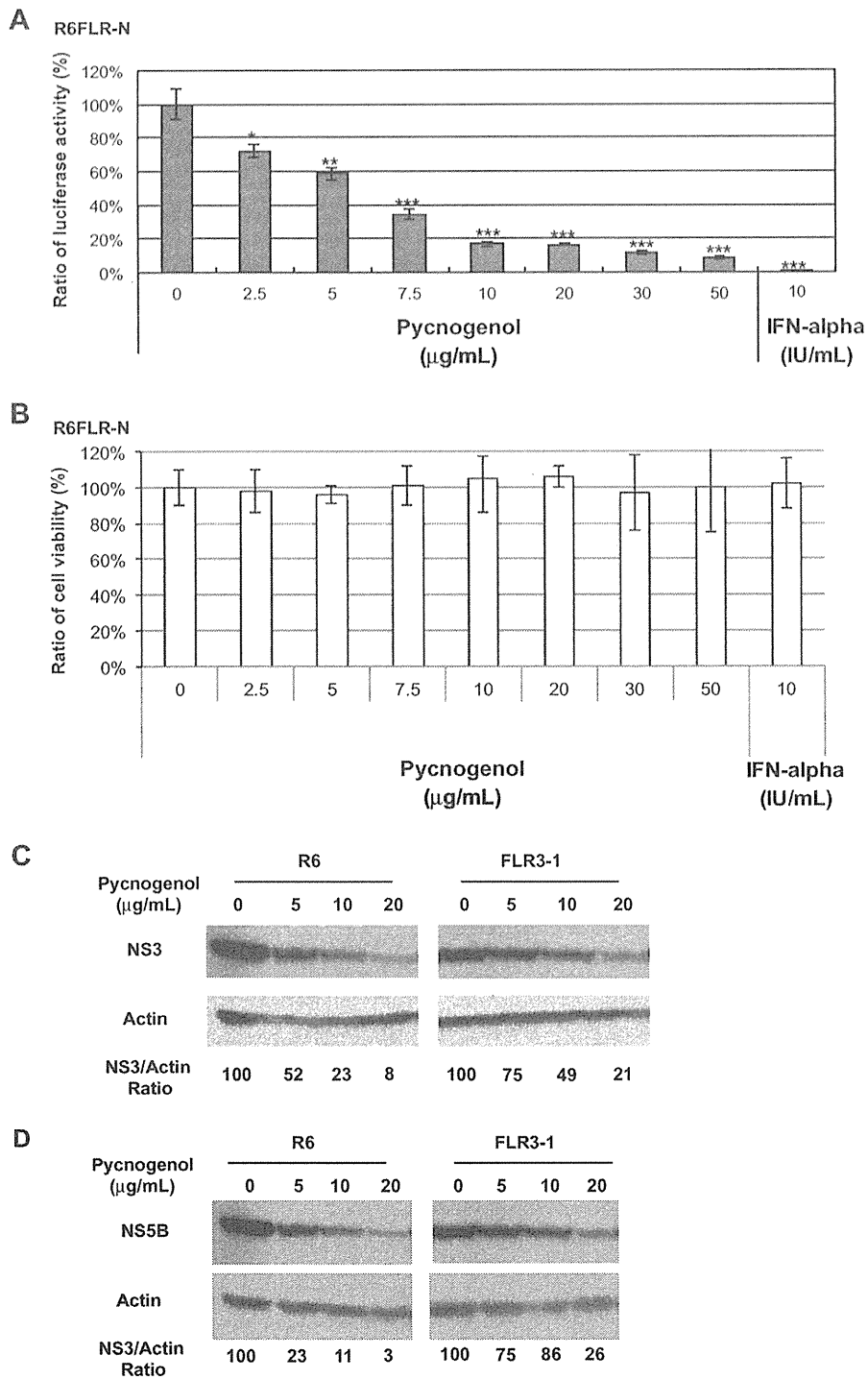


Fig. 1. Effect of PYC on HCV RNA replication in HCV replicon cell lines. (A) Dose-dependent inhibition of HCV by PYC in the R6 replicon cell line. (B) Cytotoxicity in the R6 HCV-replicon cell line treated with PYC. The ratio (%) of viability compared to the no-treatment control value is indicated. (C 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).

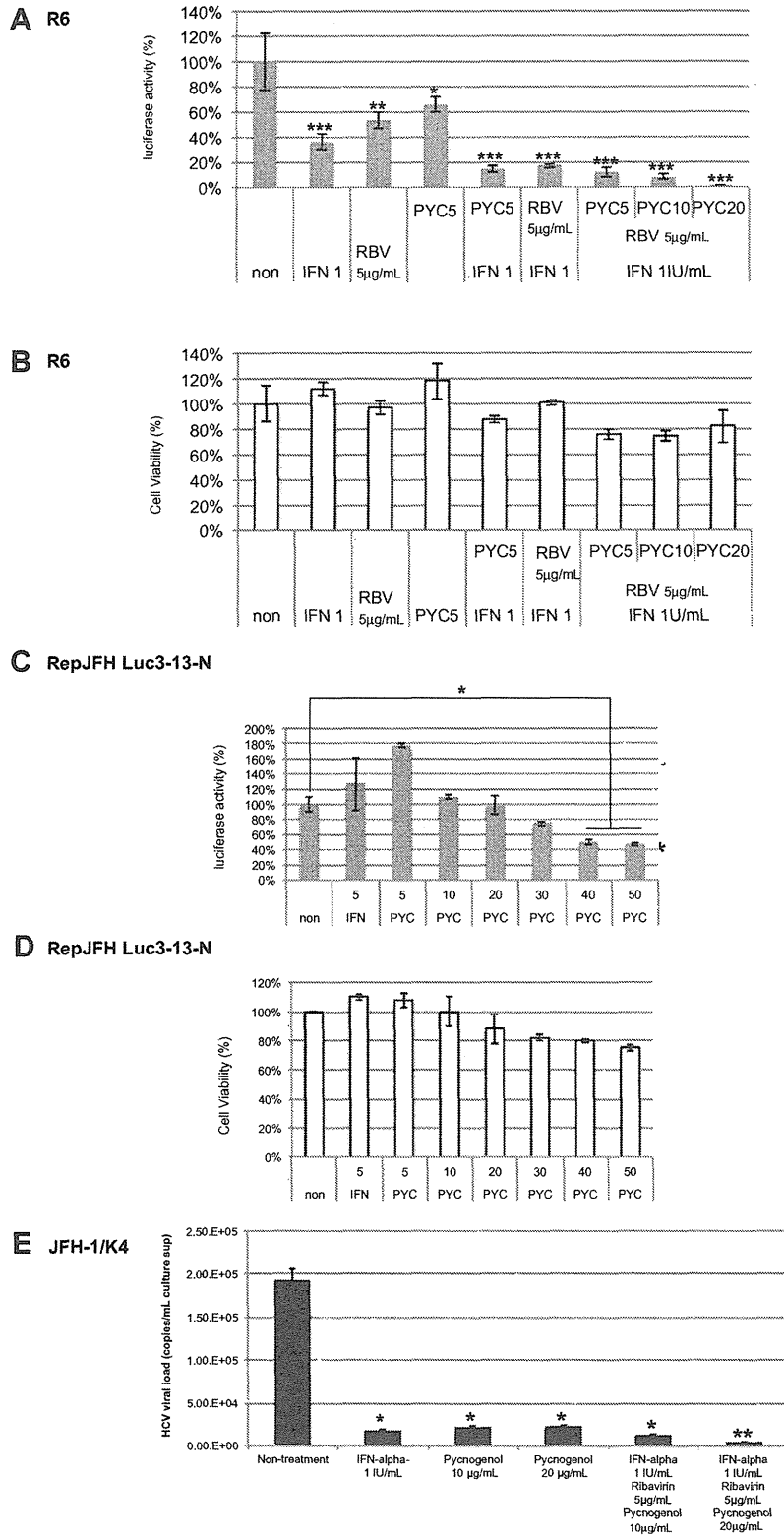


Fig. 2. Effect of PYC and IFN-alpha on HCV replication. (A) Effect of PYC (5 µg/mL), IFN-alpha (1 IU/mL), and RBV (5 µg/mL) on antiviral activity in the R6 HCV replicon cell line. (B) Cytotoxicity in the R6 HCV replicon cell line treated with PYC, IFN-alpha, and RBV. (C) Effect of IFN-alpha (5 IU/mL), 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.

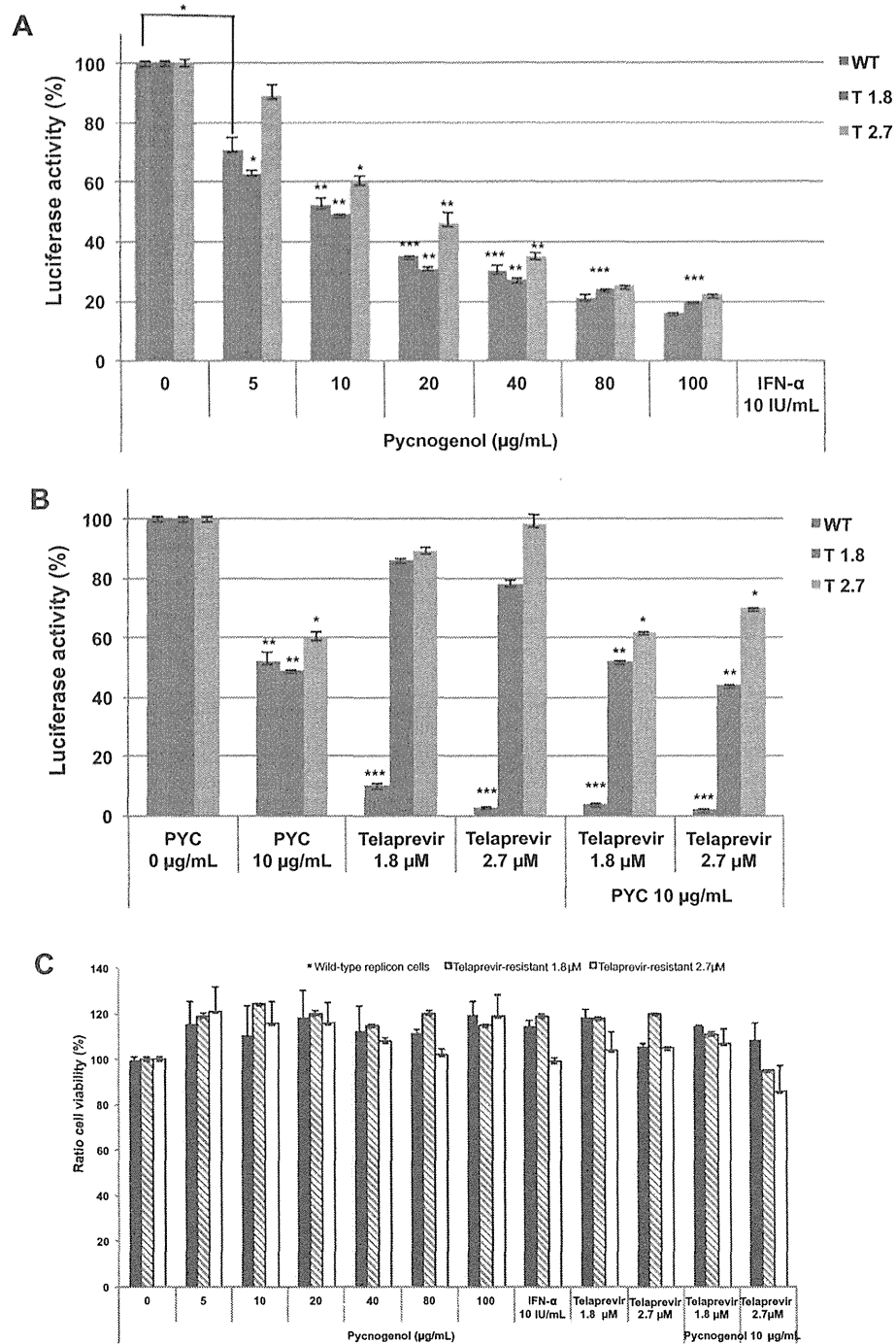


Fig. 3. Effect of PYC on HCV replication in telaprevir-resistant replicon cells. (A) Dose-dependent inhibition of HCV by PYC in wild type (WT) and telaprevir-resistant replicon cell lines (V1.8, V2.7). (B) Effect of PYC and telaprevir in the R6 replicon cell line. (C) PYC cytotoxicity in the R6 HCV replicon 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).

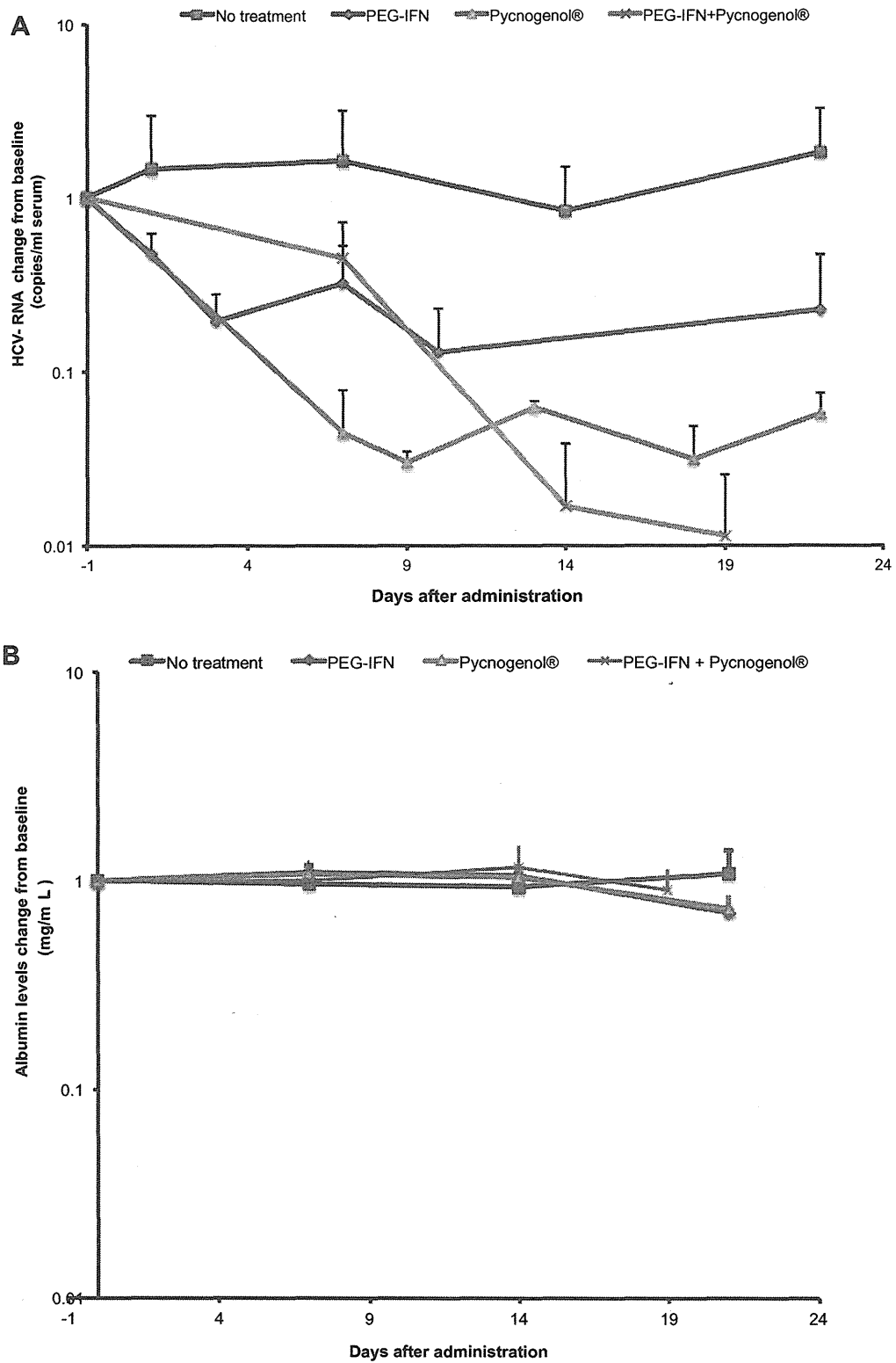


Fig. 4. *In vivo* effects of PYC on HCV replication. (A) Median change from baseline serum relative HCV RNA levels (HCV RNA amount at the beginning; average 4.8×10^7 copies/mL) in chimeric mice treated with PYC (40 mg/kg/day, intraperitoneally) or PEG-IFN (30 µg/kg, subcutaneously) alone, in combination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

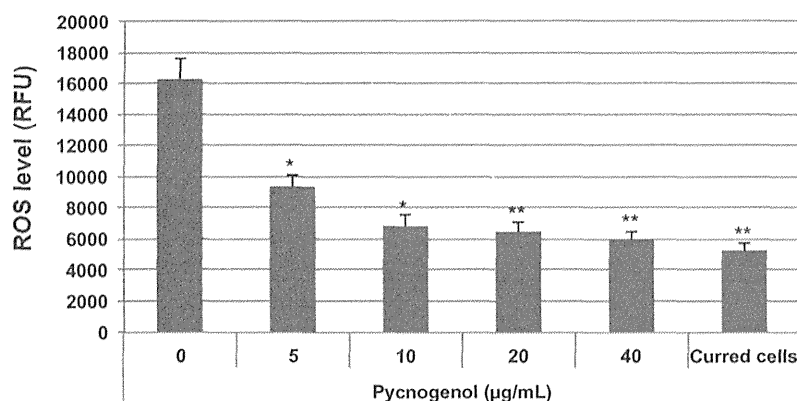


Fig. 5. Effect of PYC on ROS production in HCV replicon cells. HCV replicon and cured cell lines were incubated for 72 h with various concentrations of PYC and without PYC treatment. Data are shown as mean \pm standard error of mean. ROS levels in HCV-negative cured cells are also shown. * $P < 0.05$;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

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 (Khachatourian 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 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- α , 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

Kyoko TSUKIYAMA-KOHARA^{1,2)}, and Michinori KOHARA³⁾

¹⁾Transboundary Animal Diseases Center, Joint Faculty of Veterinary Medicine, Kagoshima University, 1-2-24 Korimoto, Kagoshima 890-0065, Japan

²⁾Laboratory of Animal Hygiene, Joint Faculty of Veterinary Medicine, Kagoshima University, 1-2-24 Korimoto, Kagoshima 890-0065, Japan

³⁾Department of Microbiology and Cell Biology, Tokyo Metropolitan Institute of Medical Science, Tokyo 113-8613, Japan

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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Address Corresponding: K. Tsukiyama-Kohara, and M. Kohara, Department of Microbiology and Cell Biology, Tokyo Metropolitan Institute of Medical Science, Tokyo 113-8613, Japan

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Table 1. Composition of family Tupaiidae [1]

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

*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 tupaia 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 tupaia and humans. Similarly, phylogenetic analysis based on whole genome sequences showed that humans are closer to tupaia than they are to mice (Fig. 2). Further, several of the same highly conserved and variable genes have been identified

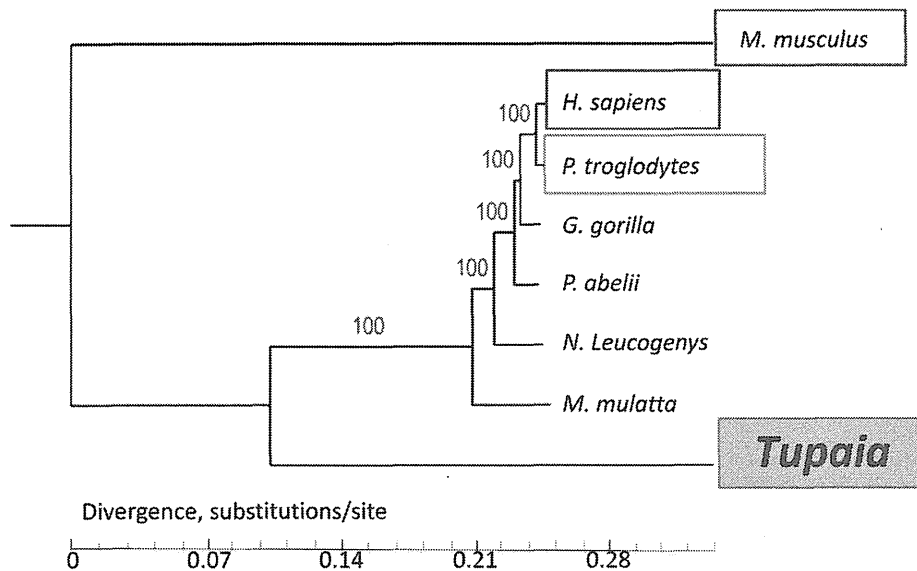


Fig. 2. Dendrogram showing relationships between primates, tree shrews and rodents. Phylogenetic tree constructed using orthologous genes at 4-fold degenerate sites by the maximum likelihood method. Branch lengths represent the neutral divergence rate and blue characters indicate bootstrap values.

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 tupaia 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 be-

havior, physiology and central nervous activity [9]. These alterations exhibited by the subordinate male tupaia 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-

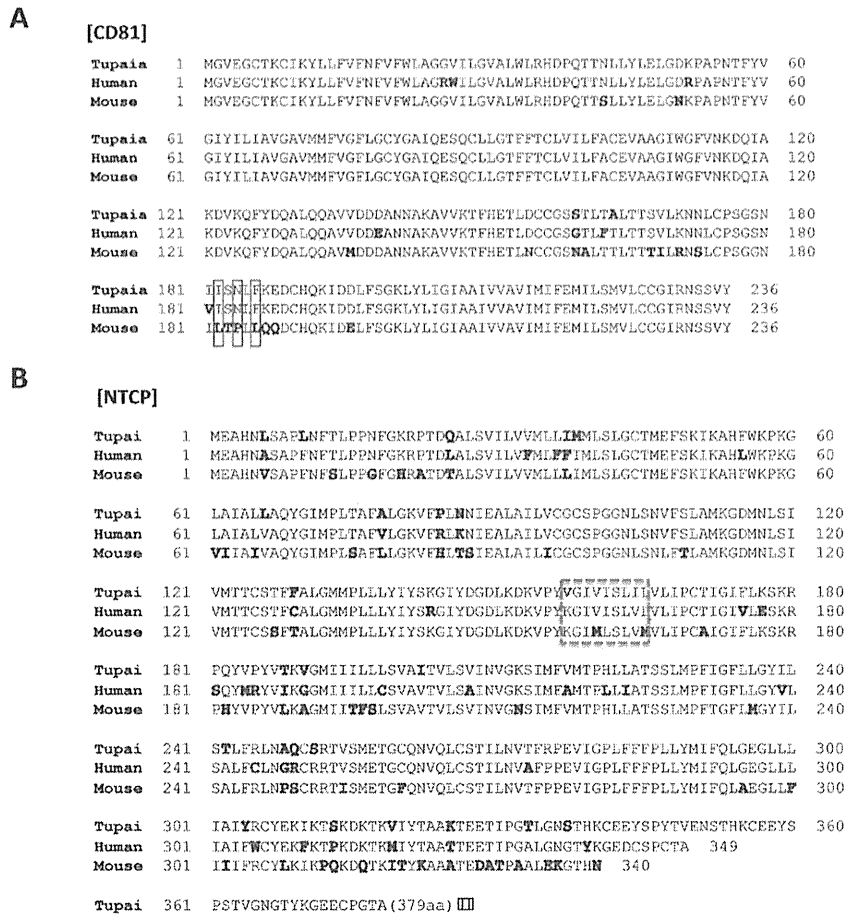


Fig. 3. Alignment of amino acid sequences of viral receptors. (A) Alignment of CD81 amino acid sequences from tupaia, human and mouse. Different amino acids were indicated with red colour. Significant amino acids for binding to HCV E2 protein were surrounded by square (Ile182, Asn184 and Phe186) [14, 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 tupaia 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 tupaia – 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-

Monitoring

Long-term follow up

- Serum ALT values
- Serum HCV RNA (Quantification by RTD-RT-PCR)

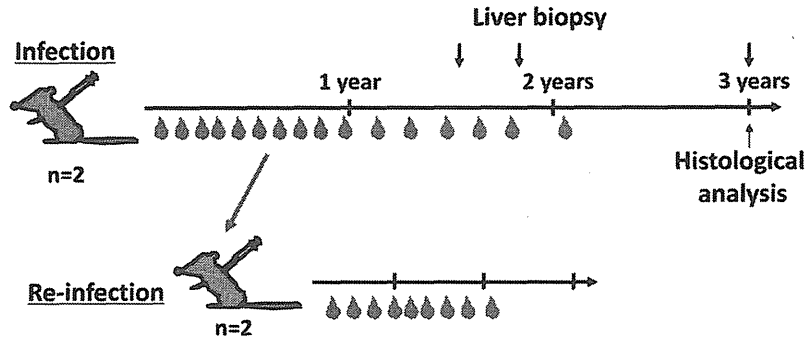


Fig. 4. Experimental design of HCV infection and re-infection of tupaia.

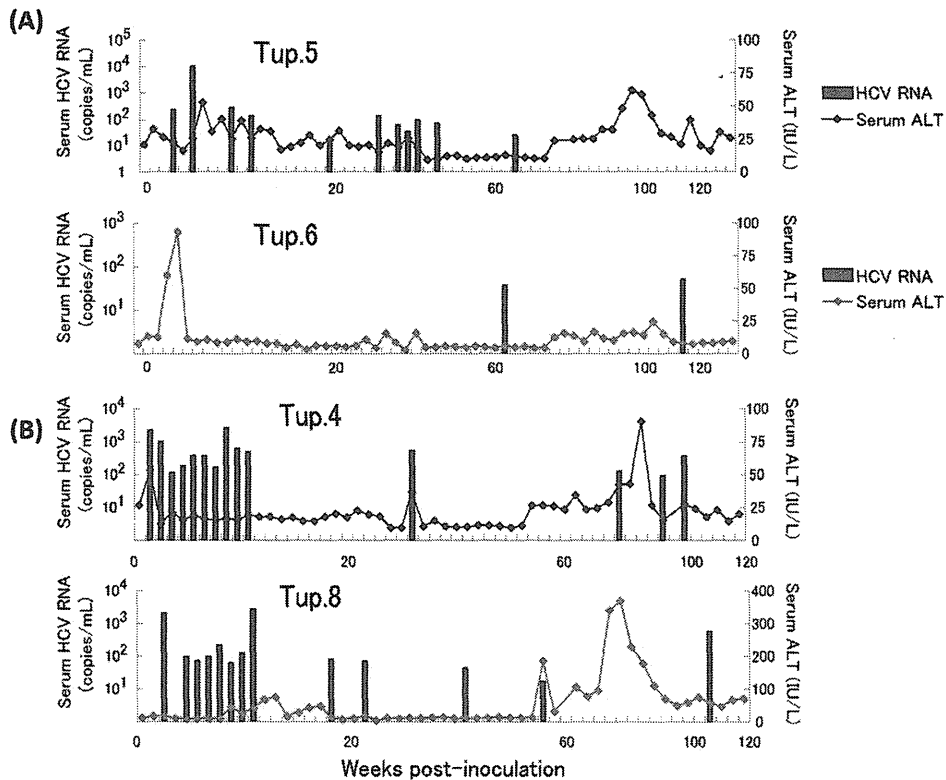


Fig. 5. Course of HCV infection in tupaia. (A) Tupaias No. 5 and 6 were inoculated with patient serum HCR6. Serum ALT (IU/ml) and viral loads, measured as amount of HCV RNA (copies/ml), were measured for over 120 weeks. Set point for serum ALT in untreated tupaia was 22.3 IU/ml (n=23). Negative control 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.

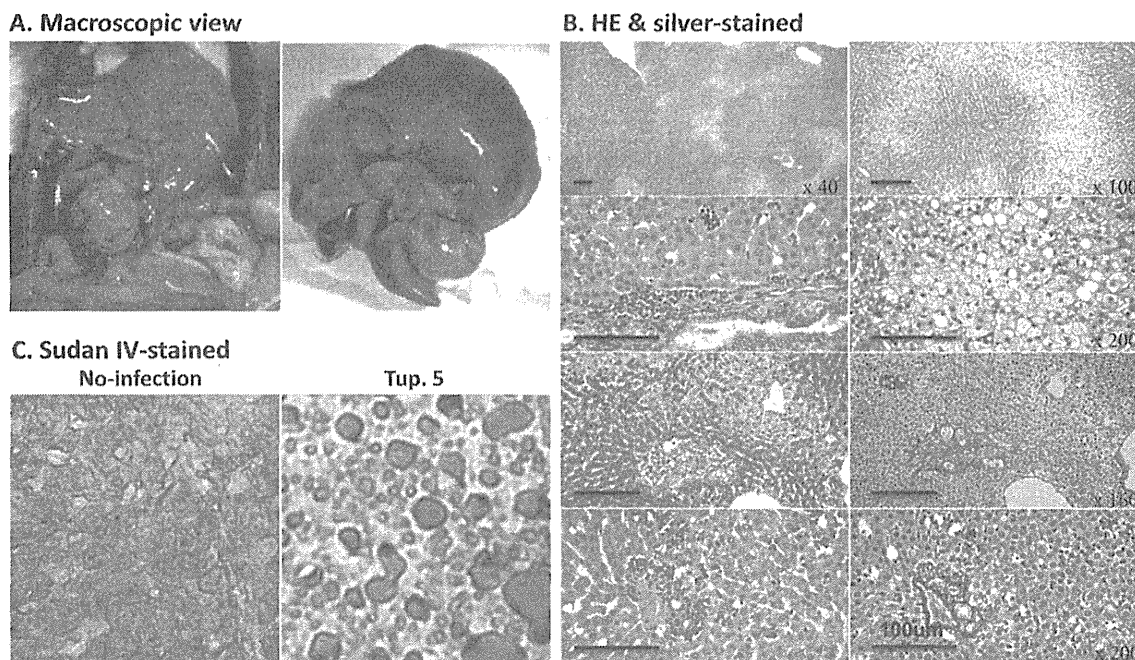


Fig. 6. (A) Macroscopic view of liver inoculated with patient serum HCR6 after 2 years (Tup.5, serum ALT value was 25 IU/l at autopsy). (B) HE staining ($\times 40$, $\times 100$, $\times 200$; scale bars indicate **) and silver staining ($\times 160$, $\times 200$) of liver tissue (Tup 5) were indicated. 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 tupaia 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 tupaia and humans should be examined in future. HCV infection studies in tupaia 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 tupaia 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 tupaia induced the production of HBsAg, HBsAb, HBcAb and HBeAb; all of the adults were successfully infected [34]. Experimental infection of tupaia 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 tupaia 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, tupaia are potentially well suited for use as a model for HBV infection.

Tupaia 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]. Tupaia has 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 tupaia 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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