Practical evaluation of a mouse with chimeric human liver model for hepatitis C virus infection using an NS3-4A protease inhibitor

Naohiro Kamiya,1 Eiji Iwao,1 Nobuhiko Hiraga,2,3 Masataka Tsuge,2,3 Michio Imamura,2,3 Shoichi Takahashi,2,3 Shinji Miyoshi,4 Chise Tateno,3,5 Katsutoshi Yoshizato3,5 and Kazuaki Chayama2,3

Correspondence
Kazuaki Chayama
chayama@hiroshima-u.ac.jp

1Pharmacology Department V, Mitsubishi Tanabe Pharma Corporation, Yokohama, Japan
2Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan
3Liver Research Project Center, Hiroshima University, Hiroshima, Japan
4DMPK Department, Mitsubishi Tanabe Pharma Corporation, Kisarazu, Chiba, Japan
5PhoenixBio, Higashihiroshima, Japan

INTRODUCTION

Hepatitis C virus (HCV) is a major cause for concern worldwide. More than 3 % of the world’s population is chronically infected with HCV and 3–4 million people are newly infected each year (Wasley & Alter, 2000). Chronic HCV infection is relatively mild and progresses slowly; however, about 20 % of chronic hepatitis C (CHC) carriers progress to serious end-stage liver disease (Lauer & Walker, 2001; Liang et al., 2000; Poynard et al., 2003). The current standard treatment for HCV infection is administration of pegylated alpha interferon (PEG-IFN) in combination with ribavirin (RBV) for 48 weeks. The overall cure rates with this intervention are 40–50 % for patients with genotype 1 and more than 75 % for patients with genotypes 2 and 3 (Fried et al., 2002; Manns et al., 2001). Several compounds that inhibit specific stages of the virus life cycle have been clinically evaluated (Manns et al., 2007; Pereira & Jacobson, 2009). Telaprevir is a novel peptidomimetic slow- and tight-binding inhibitor of HCV NS3-4A protease, which was discovered using a structure-based drug design approach (Perni et al., 2006). A rapid decline in viral RNA was observed in CHC patients treated with telaprevir (Reesink et al., 2006) and an increased antiviral effect of a combination of telaprevir and PEG-IFN has been reported (Forestier et al., 2007). Recent clinical trials of telaprevir in combination with PEG-IFN and RBV have indicated a promising material advance in therapy for CHC patients (Hézode et al., 2009; McHutchison et al., 2009). First-generation HCV-specific agents have been developed despite the lack of small-animal models for HCV infection. However, early emergence of resistant variants against novel antiviral agents is a concern. Thus, the use of two or more investigation agents is strongly recommended for...
clinical studies in CHC patients (Sherman et al., 2007). To ensure ethical and safe clinical trials, animal models continue to be necessary for the mechanistic evaluation of the ability of specific agents to inhibit the virus life cycle in vivo and to develop better therapeutic strategies, including combination regimens (Boonstra et al., 2009). Several groups have developed a small-animal model for HCV infection using homozygous urokinase-type plasminogen activator (uPA)/severe combined immunodeficiency (SCID) (uPA<sup>+/+</sup>/SCID<sup>+/+</sup>) mice transplanted with human hepatocytes (Mercer et al., 2001). These mice are susceptible to cell culture-grown HCV (HCVcc; Lindenbach et al., 2006) and have been used to evaluate antiviral agents including IFN-α, BILN 2061 (an NS3-4A protease inhibitor) and HCV796 (an NS5B polymerase inhibitor) (Kneteman et al., 2006, 2009; Vanwolleghem et al., 2007). However, the HCV clearance rate in the SCID mouse model and the virion production rate in hepatocytes engrafted in the mouse liver are not fully understood. We also generated a mouse model with an almost humanized liver (Tateno et al., 2004). Using this mouse model, we reported the infection of a genetically engineered hepatitis B virus (Tsuge et al., 2005) and developed a reverse genetics system for HCV genotypes 1a, 1b and 2a after intrahepatic injection of in vitro-transcribed RNA as well as intravenous injection of HCVcc (Hiraga et al., 2007; Kimura et al., 2008). In this study, we demonstrated the rapid turnover of serum HCV RNA and the pharmacokinetics (PK) and pharmacodynamics (PD) of telaprevir treatment. We concluded after quantitative estimation and the use of a mathematical model that HCV production equivalent to that in the human liver is possible in engrafted hepatocytes in this mouse model.

**RESULTS**

**Preliminary dose-finding study**

At the beginning of this study, we attempted to determine an effective dose regimen for telaprevir in this mouse model. Nine mice were randomized and treated with telaprevir over three time periods (Table 1). The lifetime kinetics of serum HCV RNA and of human serum albumin (HSA) in blood are represented in Fig. 1. One mouse (A07) exhibited a rapid reduction in HSA in the blood, which indicated the instability of human hepatocyte grafts. As a rapid reduction in HSA levels was not observed in subsequent experiments, this mouse was excluded from the mean analysis. After 7 days of twice daily (BID) dosing in period 1, the mean log<sub>10</sub> changes in HCV RNA from baseline (± SEM) after the 100 and 10 mg telaprevir kg<sup>−1</sup> doses were −0.49 ± 0.094 and −0.53 ± 0.039, respectively, and no dose-dependent reduction was observed. During period 2, the dose frequency was changed from BID to three times daily (TID), and the time of serum sampling was also changed from 1 to 4 h after the last dose. After the 3-day treatment, the mean log<sub>10</sub> changes of HCV RNA in 100 and 10 mg telaprevir kg<sup>−1</sup> TID groups were −1.00 ± 0.166 and −0.28 ± 0.056, respectively, and the difference between the two groups was significant. To test the reproducibility of results, mice were treated with 10 or 100 mg telaprevir kg<sup>−1</sup> TID for 10 days and then sacrificed 5 h after the administration of the last dose. The mean log<sub>10</sub> changes in serum HCV RNA were −1.46 ± 0.265 and −0.27 ± 0.073 in the 100 and 10 mg kg<sup>−1</sup> TID groups, respectively, and the difference between the means was significant.

**Evaluation of HCV turnover in this mouse model**

Because of the SCID nature of this mouse model, the virion clearance mechanism was of interest. Six mice with steady-state and high viral loads (9.7 × 10<sup>5</sup>–1.2 × 10<sup>6</sup> copies ml<sup>−1</sup>) were administered 200 mg telaprevir kg<sup>−1</sup> TID for 4 days, with 5 h intervals between doses and a 14 h intermission from drug treatment each day. Because the log<sub>10</sub> reduction in HCV RNA appeared to depend on the time of serum collection during the day (Fig. 2a), the mean log<sub>10</sub> changes in HCV RNA were plotted against time and fitted to a linear regression model (Fig. 2b). The estimated slopes (i.e. log<sub>10</sub> HCV reduction per hour) and 95% confidence intervals (CI) on days 1, 2 and 3 were −0.165 (−0.268 to 0.0616), −0.115 (−0.131 to 0.0990) and −0.153, respectively. These regression lines also suggested that extrapolated HCV loads at the actual times of the daily first doses were 0.0530, −0.220 and −0.0948 log<sub>10</sub> copies ml<sup>−1</sup>, respectively. Therefore, it appeared that the viral load

**Table 1. Telaprevir dose-finding experiment**

<table>
<thead>
<tr>
<th>Period</th>
<th>Duration (days)</th>
<th>Frequency of dose (per day)</th>
<th>Dose (mg kg&lt;sup&gt;−1&lt;/sup&gt;)</th>
<th>No. of mice</th>
<th>Mean log&lt;sub&gt;10&lt;/sub&gt; changes ± SEM</th>
<th>P value (t test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7</td>
<td>2</td>
<td>100</td>
<td>4</td>
<td>−0.49 ± 0.094 ± 0.039</td>
<td>0.7806</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10</td>
<td>3*</td>
<td>−0.53 ± 0.039 ± 0.047</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td>1</td>
<td>−0.47</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>3</td>
<td>100</td>
<td>4*</td>
<td>−1.00 ± 0.166 ± 0.28 ± 0.056</td>
<td>0.0064</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td>1</td>
<td>−0.47</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>3</td>
<td>100</td>
<td>3</td>
<td>−1.46 ± 0.265 ± 0.27 ± 0.073</td>
<td>0.0125</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10</td>
<td>3</td>
<td>−0.27 ± 0.073 ± 0.28 ± 0.056</td>
<td></td>
</tr>
</tbody>
</table>

*One mouse was excluded because of instability of human hepatocyte grafts.
reverted back towards baseline levels during the 14 h intermission from drug treatment.

**PK analysis**

To assess drug exposure after repeated dosing in this mouse model, mice were administered 100 or 300 mg telaprevir kg\(^{-1}\) BID for 4 days. The mice receiving 300 mg kg\(^{-1}\) BID for 4 days had a mean 2 log\(_{10}\) fold HCV reduction, whereas those receiving 100 mg kg\(^{-1}\) BID had up to a 1.5 log\(_{10}\) fold reduction by day 3 (Fig. 3a). Plasma telaprevir concentrations after administration of the final dose are indicated in Fig. 3(b). The estimated half-life of telaprevir in the 100 and 300 mg kg\(^{-1}\) groups was 2.4 and 3.8 h, respectively.

**PK/PD analysis and the dose-dependent reduction in HCV RNA**

To evaluate the correlation between telaprevir concentration and HCV reductions in this mouse model, we used another cohort of 12 HCV-infected mice with high viral loads (1.6 \(\times\) 10\(^6\)–3.9 \(\times\) 10\(^8\) copies ml\(^{-1}\)). In this crossover study, mice were randomized into three groups (n=4 each), each of which underwent two periods of dosing for 5 days separated by a 1-week washout period. Serum and plasma samples were collected once daily 5 h after dosing. The mean log\(_{10}\) changes in HCV RNA (\(\pm\) SEM) at different dose levels were calculated from the combined results of both periods (Fig. 4a). The mean log\(_{10}\) reductions from baseline in the 100 and 300 mg kg\(^{-1}\) groups were approximately 1 log\(_{10}\) and 1.5–2 log\(_{10}\), respectively, and the difference between the two groups was statistically significant. The means calculated in each period separately are also shown in Fig. 4(b). The plasma telaprevir concentration was positively correlated with the log\(_{10}\) HCV RNA reduction level in each period (Fig. 4c).

**Drug concentrations and HCV levels in blood correlate with those in the liver**

The correlation between telaprevir concentrations in the plasma and liver was analysed in a double logarithmic plot 5 (dose-finding cohort) or 8 h (PK and PK/PD cohorts) after the last dose (Fig. 5). The linear regression lines suggested that telaprevir concentrations in the liver were 5–
human quantified by duplex real-time RT-PCR analysis using levels in the total cellular RNA extract were relatively extracted from two, one and four discrete small sections (approx. 50 mg) of the liver in the preliminary dose-extracted from two, one and four discrete small sections of the chimeric chimeric human livers (data not shown). This result indicated that occupancy rates of human cells varied indicated that occupancy rates of human cells varied individually and/or among small sections of the chimeric human liver. Therefore, the mean difference in \( \Delta C_v = C_{vHCV} - C_{vE2m} \) in each mouse was calculated and plotted against the viral load in serum (Fig. 6). After treatment with telaprevir for up to 10 days, mean \( \Delta C_v \) values ranged between 11 (HCV RNA content: \( 2^{11} = 2 \times 10^3 \)-fold lower than \( h_2m \) expression) and 17 (1 \times 10^5 \)-fold lower) among the HCV-infected mice and correlated linearly with log_{10} serum HCV RNA levels.

Viral dynamics model analysis
To evaluate time-dependent reductions in HCV with BID dosing, 12 HCV-infected elderly mice, which maintained high and steady-state viral loads (1.2 \times 10^6–8.5 \times 10^7 copies ml^{-1}) for more than 6 months, were treated with 200 mg telaprevir kg^{-1} BID for 3 days. The mice were divided into two groups, and serum samples were collected just before the second dose and 4 \((n=6)\) or 8 \((n=6)\) h after every two administrations. The single administration of telaprevir resulted in a mean 0.8–1.0 log_{10}-fold reduction in HCV RNA in both groups. After the second dose, the pattern of viral kinetics appeared to depend on the time of serum collection, and the mean HCV RNA reduction level was higher in the 8 h group than in the 4 h group and plateaued at approximately a 2 log_{10}-fold reduction in both groups after treatment for 3 days (Fig. 7). Finally, we attempted to estimate parameters of efficacy (\( e \)) and virus clearance (\( c \)) per hour in this mouse model for comparison with estimates derived from human studies. Because the mean viral kinetics of the 8 h group was biphasic, the values in the 8 h group were used together for the mathematical model analysis. The estimated \( e \) and \( c \) values were 0.992 (95 % CI 0.982–1.00) and 0.200 (95 % CI 0.110–0.291), respectively.

DISCUSSION
Using a mouse model with a chimeric human liver for HCV infection, we analysed the PK/PD of telaprevir treatment and investigated HCV dynamics during the initial phase of protease inhibitor treatment. All the mice in this study were expected to have more than half of their livers repopulated by human hepatocytes (Tateno et al., 2004), which simulates a human drug metabolism profile (Katoh et al., 2007; Reesink et al., 2006). After the infection with HCV genotype 1b, high viral loads were maintained in the mice for more than 6 months. Recent studies have indicated the utility of a human/mouse chimera model for HCV infection to evaluate antiviral efficacy (Kneteman et al., 2006, 2009) and preclinical safety (Vanwolleghem et al., 2007). However, PK/PD studies and estimations of virus clearance rate have rarely been performed in this mouse model. HCV production, including intracellular replication in engrafted hepatocytes, has also not yet been elucidated. Despite the SCID nature of this mouse model, a 2 log_{10}-fold HCV RNA reduction was observed within 0.5 days, as has been observed previously in CHC patients (Forrestier et al., 2007; Reesink et al., 2006). In this mouse model, the rapid rebound in HCV load during the intermission from drug exposure indicated the rapid production and release of HCV into the circulation. This finding indicates that a virion-clearing compartment, which does not depend on T- and B-cell responses, may exist in this mouse model.
One possible explanation is that viral kinetics after liver transplantation in humans may play a role in HCV clearance under immunosuppressed conditions (Dahari et al., 2005; Powers et al., 2006; Schiano et al., 2005). This observation suggests that this mouse model is capable of evaluating ‘first-phase’ HCV clearance after drug treatment.

In a clinical trial of telaprevir, CHC patients who exhibited a continuous decline in viral kinetics had mean plasma trough levels above 1000 ng ml$^{-1}$; therefore, a dose of 750 mg TID was selected for further clinical studies (Sarrazin et al., 2007). When HCV-infected mice were administered 100 or 300 mg telaprevir kg$^{-1}$, a plasma concentration above 1000 ng ml$^{-1}$ was maintained beyond 8 h in mice treated with 300 mg kg$^{-1}$ but not in those treated with 100 mg kg$^{-1}$. This result suggests that the extrapolation of telaprevir doses from this mouse model to human studies depends on body surface area, i.e. approximately 15th of a dose in this mouse model may be equivalent to a dose in humans. In another cohort of mice treated with 100 and 300 mg telaprevir kg$^{-1}$ BID, a dose-dependent reduction in HCV was observed and the plasma telaprevir concentration correlated significantly with the HCV reduction level. Therefore, the PK/PD results in this mouse model may be able to indicate a targeted dose range in clinical studies.

Whereas a telaprevir concentration in plasma equivalent to its dosage in clinical trials was achieved in this mouse model, the serum HCV RNA level plateaued at a decrease of approximately 2 log$_{10}$-fold within several days of treatment. A saturated reduction of approximately 2 log$_{10}$-fold after treatments with BILN 2061 and IFN was also reported in an analogous mouse model (Kneteman et al., 2006; Vanwolleghem et al., 2007). These observations led us to examine HCV replication in the chimeric human liver. In the relative quantification of HCV RNA against human-specific endogenous gene expression, we observed a correlation between the serum HCV RNA level and the mean $\Delta$Ct value in the liver, despite no correlation between the total RNA concentration and each Ct value of two target genes in the liver RNA extracts. This result can be interpreted to indicate that HCV replicated only in...
engrafted human hepatocytes, and the observed HCV reduction in serum might reflect virus replication in the human hepatocyte grafts. Moreover, the relative content of HCV RNA was $2 \times 10^{3} - 1 \times 10^{5}$-fold lower than h\textbeta \textsubscript{2m} expression, whereas an HCV replicon cell line, which had approximately 1000 replicon genomes per cell (Quinkert et al., 2005), contained nearly equal amounts of both genes (data not shown). HCV replication was much lower in the engrafted human hepatocytes than in an HCV replicon cell line, and HCV infected only a small portion of the engrafted human hepatocytes. It has been reported that 4–25 % of hepatocytes in a CHC patient were positive for replicative-intermediate RNA, and the mean number of viral genomes per productively infected hepatocyte ranged from 7 to 64 molecules (Chang et al., 2003). Also, a more recent report suggested that the percentage of HCV antigen-positive hepatocytes in patients varied from 0 to 40 %, and the HCV content in 2000 microdissected HCV-positive cells ranged from 40 to 1800 international units using a branched DNA assay (Vona et al., 2004). Therefore, we suggest that HCV replication efficiency in engrafted human hepatocytes is equivalent to that in CHC patients.

The differences observed between the engrafted human hepatocytes and the HCV replicon cell line can be explained by the following assumptions: approximately 10 % of engrafted human hepatocytes are productively infected.

Fig. 5. Correlation between telaprevir concentrations in the liver and plasma. Telaprevir concentrations in the liver and plasma were determined at the end of the three different experiments indicated in Fig. 1 (dose-finding), Fig. 3 (PK) and Fig. 4 (PK/PD). Telaprevir concentrations in the liver were plotted against those in plasma 5 (a) or 8 (b) h after the last dose. Linear regressions (solid lines) and 95 % CI (dashed lines) are indicated.

Fig. 6. Correlation between HCV content in the liver and serum. Relative quantification of HCV RNA levels in the liver was determined by the difference between threshold cycles (\Delta Ct) of HCV RNA and h\textbeta \textsubscript{2m} in a duplex real-time RT-PCR analysis. Linear regressions (solid line) and 95 % CI (dashed lines) are indicated.

Fig. 7. Viral dynamics under BID telaprevir treatment. Mice were administered 200 mg telaprevir kg\textsuperscript{-1} BID at the times indicated by arrows. Serum samples were collected just before the second dose was administered and 4 (n=6) or 8 (n=6) h after every two doses were administered. Mean log\textsubscript{10} changes (± SEM) in serum HCV RNA are plotted. The solved equation described in Methods was fitted to the values in the 8 h group (solid line), and the estimated efficacy and virion clearance rates were 0.992 (95 % CI 0.982–1.00) and 0.200 (95 % CI 0.110–0.291), respectively.
infected and harbour approximately ten HCV genomes per cell at baseline steady state and a 2-log10-fold reduction is achieved with drug treatment.

Mathematical models have proven valuable in understanding the in vivo dynamics of HCV, and very rapid dynamic processes occur on timescales of hours to days, and slower processes occur on timescales of weeks to months (Perelson & Ribeiro, 2008). In the last experiment, we observed a biphasic decline in the HCV RNA level after BID dosing for 5 days. During the first 2 days of the treatment, a discrepancy in viral kinetics between the serum-sampling time points was noted. Similarly, fluctuations in viral kinetics during the first-phase slope were observed in patients who received IFN three times a week (Pavlotsky et al., 2004). Variable efficacy rate determined by PK parameters can explain fluctuations during the first-phase slope in mathematical model analysis (Talal et al., 2004).

The estimated clearance rates in this mouse model basically agreed with estimates determined in humans infected with HCV genotype 1 and undergoing IFN-based therapies (Herrmann et al., 2003; Neumann et al., 1998; Pavlotsky et al., 2004) or large-volume plasma apheresis (Ramratnam et al., 1999). Total virion production during steady-state viral kinetics in this mouse model was calculated by multiplying c by the initial viral load ($V_0$) and then normalizing the extracellular fluid volume. From previous studies, it was determined that $10^{11}-10^{13}$ virions are produced daily in patients with high HCV loads (Neumann et al., 1998; Ramratnam et al., 1999). In this mouse model, the volume of extracellular fluid and weight of the liver were approximately 20 and 9% of the body weight (data not shown), and the mean log$_{10}$ $V_0$ ($\pm$ SEM) among the mice with mean clearance rates of 4.8 and 8.0 per day was $6.96 \pm 0.26$ and $7.00 \pm 0.33$, respectively. The results of the calculations indicated that 1 g of the chimeric human liver produced $1 \times 10^{8}-2 \times 10^{8}$ virions per day. The typical weight of the human liver is 1–2 kg; thus, the capacity of human hepatocytes to produce HCV in this mouse model may be equivalent to that in CHC patients. In conclusion, a mouse model with a chimeric human liver can simulate HCV replication in human patients quantitatively and dynamically, and this mouse model may be suitable for preclinical evaluations of novel HCV-specific agents and other therapeutic strategies, including combination regimens.

METHODS

Generation of mice with chimeric human livers and HCV infection. The generation of iPA$^{-/+}$/SCID$^{-/+}$ mice and transplantation of frozen human hepatocytes was performed at PhoenixBio. Graft function was monitored on the basis of HSA levels in blood (Tsuge et al., 2005). All the mice had high HSA levels, which suggested that nearly half of their livers were repopulated by human hepatocytes (Tateno et al., 2004). After obtaining written informed consent, we collected sera periodically from patients who were chronically infected with HCV genotype 1b and failed to respond to PEG-IFN and RBV therapy. The mice were inoculated with the serum samples via the orbital vein after anaesthetization. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of the Graduate School of Biomedical Sciences, Hiroshima University.

Compound preparation and experimental designs. A telaprevir suspension was prepared as described previously (Perni et al., 2006) and used in experiments 1 and 2. In the other experiments, a telaprevir suspension was prepared daily as in the tablet formulation (Forestier et al., 2007; Hézode et al., 2009; McHutchison et al., 2009). A suspension of telaprevir was administered via oral gavage.

Experiment 1: preliminary dose-finding study. Ten out of 11 mice developed serum HCV loads greater than $10^6$ copies ml$^{-1}$. Nine mice with high viral loads ($>10^7$ copies ml$^{-1}$) were randomized and administered $10$ or $100$ mg telaprevir kg$^{-1}$ BID or TID over three periods. During period 1, the mice were administered $100$ ($n=4$) or $10$ ($n=4$) mg telaprevir kg$^{-1}$ or vehicle ($n=1$) BID at 18:00 and 10:00 h for 7 days, and serum samples were collected before treatment and 1 h after administration in the morning on the third and/or seventh day. During period 2, the mice were administered $100$ ($n=5$) or $10$ ($n=4$) mg telaprevir kg$^{-1}$ TID for 3 days, and serum samples were collected before treatment and 4 h after administration of the last dose. Three mice died between periods 2 and 3. During period 3, the mice were administered $100$ ($n=3$) or $10$ ($n=3$) mg telaprevir kg$^{-1}$ TID for 10 days. The mice were sacrificed 5 h after administration of the last dose, and plasma, serum and liver samples were collected.

Experiment 2: evaluation of HCV turnover. Eleven mice were infected with HCV and eight mice survived for more than 15 weeks with steady-state and high viral loads ($10^5-10^6$ copies ml$^{-1}$). Six of the mice were administered $200$ mg telaprevir kg$^{-1}$ TID at 9:00, 14:00 and 19:00 h for 4 days. On day 1, serum samples were collected before dose administration, 4 h after the first and second doses were administered, and 2 h after the third dose was administered ($n=2$ each). On day 2, serum samples were collected 1 h after each of the three doses was administered ($n=2$ each). Serum samples were also collected 4 h after the first and second doses were administered on day 3 ($n=3$ each) and 4 h after the second dose was administered on day 4.

Experiment 3: PK analysis. After a washout period, six mice from experiment 2 were administered $100$ or $300$ mg telaprevir kg$^{-1}$ ($n=3$ each) BID at 19:00 and 9:00 h for 4 days. Serum samples were collected before dose administration, 4 ($n=1$) or 8 ($n=2$) h after administration of the second dose, and 5 h after every two doses were administered. After the final dose was administered, plasma for PK analysis was collected at 1 and 4 h. The mice were sacrificed at 8 h, and serum, plasma and liver samples were collected.

Experiment 4: dose dependence and PK/PD analysis. Thirty-six mice were infected with HCV and 15 survived for more than 13 weeks. The median survival time of this cohort was 81 days after infection. Twelve HCV-infected mice were randomized into three groups (A–C; $n=4$ each) and underwent two periods of BID dosing for 5 days, which were separated by 1-week washout periods. During the first period, the mice in groups A, B and C were administered $300$ mg telaprevir kg$^{-1}$, $100$ mg telaprevir kg$^{-1}$ and vehicle,
respectively. Because two mice in group A and two mice in group C died before the second period, two remaining mice in group C and one back-up mouse were assigned to group A (n=2) and group B (n=1). During the second period, mice that received high or low doses were crossed over to the alternative treatment. Serum samples were collected before the first dose was administered and 5 h after every two doses were administered. Plasma samples were also collected at the same time on days 1, 3 and 5 in the first period and days 1, 3 and 4 in the second period. The mice were sacrificed 8 h after administration of the final dose, and serum, plasma and liver samples were collected.

Experiment 5: viral kinetics with BID dosing After infection of 45 mice, 12 HCV-infected mice maintained steady-state and high viral loads (1.2×10^5–8.5×10^7 copies ml⁻¹) for more than 6 months. The median survival time of this cohort was 131 days after infection. These mice were treated with 200 mg telaprevir kg⁻¹ BID at 19:00 and 9:00 h for 3 days. The mice were divided into two groups and serum samples were collected just before the second dose was administered and 4 (n=6) or 8 (n=6) h after every two doses were administered.

Serum RNA extraction and HCV RNA quantification. HCV RNA was isolated from 10 μl serum under denaturing conditions using a SepaGene RV-R kit (Sanko Junyaku). The dried precipitates were dissolved in 10 μl diethylpyrocarbonate-treated water. Extracts were duplicated and assayed by quantitative real-time RT-PCR using TaqMan EZ RT-PCR core reagents (Applied Biosystems). Nucleotide positions of the probe and primer sets refer to HCV H77 strain (GenBank accession no. AF009606). The TaqMan probe 5’-6-FAM-CTGCGGAACCGGTGAGTACAC-BHQ-1-3’ (nt 148–168) was purchased from Biosearch Technologies, and the forward (5’-AGACATGTAAGCAGCATCAT-BHQ-1-3’; nt 379–399) and reverse (5’-9AGTACCAACAGGCTTCTGC-3’; nt 272–290) primers were purchased from Sigma-Aldrich. The 25 μl RT-PCR mixture contained 0.2 nmol forward and reverse primers ml⁻¹, 0.3 nmol TaqMan probe ml⁻¹ and 5 μl extracted RNA, and was monitored using a PRISM 7900HT sequence detection system (Applied Biosystems). The thermal profile was 2 min at 50 °C, 30 min at 60 °C for reverse transcription and 5 min at 95 °C, followed by 45 cycles of 20 s at 95 °C and 1 min at 62 °C. The HCV replicon RNA was transcribed in vitro using a T7 RiboMax Express Large Scale RNA Production System (Promega) and purified twice using gel filtration. The concentration of this transcription was determined by absorbance at 260 nm and serially diluted 10-fold to prepare a standard curve for each assay.

Liver RNA extraction and HCV RNA quantification. A Wizard SV total RNA Isolation System (Promega) was used to obtain a DNase I-treated total RNA sample. The total RNA concentration was determined by absorbance at 260 nm. Total RNA samples were assayed by duplex real-time RT-PCR for relative quantification of HCV RNA using endogenous control gene expression of β2-microglobulin (hβ2m; GenBank accession no. NM_000408), the TaqMan probe 5’-CAG Fluor Orange 560-AGTGATTGCCAGCCAAGGATGTGTTT-3’ (nt 401–430), and the forward and reverse primer set of 5’-TTGCTCAGGCCAAAGATGTTT-3’ (nt 379–399) and 5’-TGCCGCCCTCAACAAACC-3’ (nt 434–450). To adjust the efficacy of PCR amplification of both target genes, the reaction condition was modified from the HCV single-probe assay. The temperature for extension was 60 °C, the concentration of the HCV probe was 0.24 nmol ml⁻¹ and the reaction mixture contained the TaqMan probe/primer set for hβ2m: 0.2 nmol primers ml⁻¹ and 0.12 nmol TaqMan probe ml⁻¹. Because both target genes double after one cycle of PCR, a difference in Ct between HCV and hβ2m (ΔCt = CtHCV−Cthβ2m) theoretically indicates a relative quantity of HCV RNA per control gene expression of 2−ΔCt.

Determination of drug concentration. Plasma and liver samples were analysed using chiral liquid chromatography followed by tandem mass spectrometry. After reconstitution, sample extracts were separated by normal-phase chromatography on a 2×250 mm Hypersil CPS-1 column (Thermo Hypersil-Keystone) with a mobile phase of heptane : acetone : methanol (82:17:1). Analyte concentrations were determined by turbo ion spray liquid chromatography/tandem mass spectrometry in the positive-ion mode. Analysis was performed at SRL or Mitsubishi Chemical Medience.

Statistical analysis. The HCV RNA level in serum was normalized by logarithmic conversion. Statistical analysis was performed with a mixed linear model using sas (SAS Institute). Mean differences between two groups were evaluated with Student’s t-test. The difference compared with vehicle control at each time point was evaluated by Dunnett’s multiple comparisons test. Linear and non-linear regression analyses were performed using GraphPad Prism 5 (GraphPad Software).

Viral dynamics model analysis. The basic mathematical model for the analysis of HCV infection in vivo, which is a system of three ordinary differential equations for uninfected cells (T), productively infected cells (I) and free virus (V), has been reviewed elsewhere (Perelson & Ribeiro, 2008). Briefly, one of the three equations (dV/ dt=pl−cV), where viral particles are produced at rate p per infected cell and cleared at rate c per virion, was solved. During treatment for 2–3 days, if one assumes that the number of I is approximately constant and equal to its pre-treatment value and that the viral level was at its set-point value (V₀), then pI=cV₀. Using this relationship in the equation dV/ dt=(1−ε)pI−cV, where ε is the effectiveness in blocking virion production, yields dV/ dt=(1−ε)cV₀−cV, V(0)=V₀ with the solution V(t)=V₀(1−ε+c+εt). Because the log change of viral load at time [log ΔV(t)] equals log V(t)/V₀, the solved equation [log ΔV(t)=log(1−ε+c+εt)] was fitted to the values obtained in this study via non-linear least-squares regression in order to estimate ε and c.

ACKNOWLEDGEMENTS

We thank Drs Ichimaro Yamada, Mitsubishi Tanabe Pharma Corporation, and Ann D Kwong, Gururaj Kalkeri, Susan Almquist, Steven M. Lyons and John Randle, Vertex Pharmaceuticals, for their thoughtful discussions. This work was supported in part by Grants-in-Aid for scientific research and development from the Ministry of Education, Sports, Culture and Technology and the Ministry of Health, Labour and Welfare, Japan.

REFERENCES


