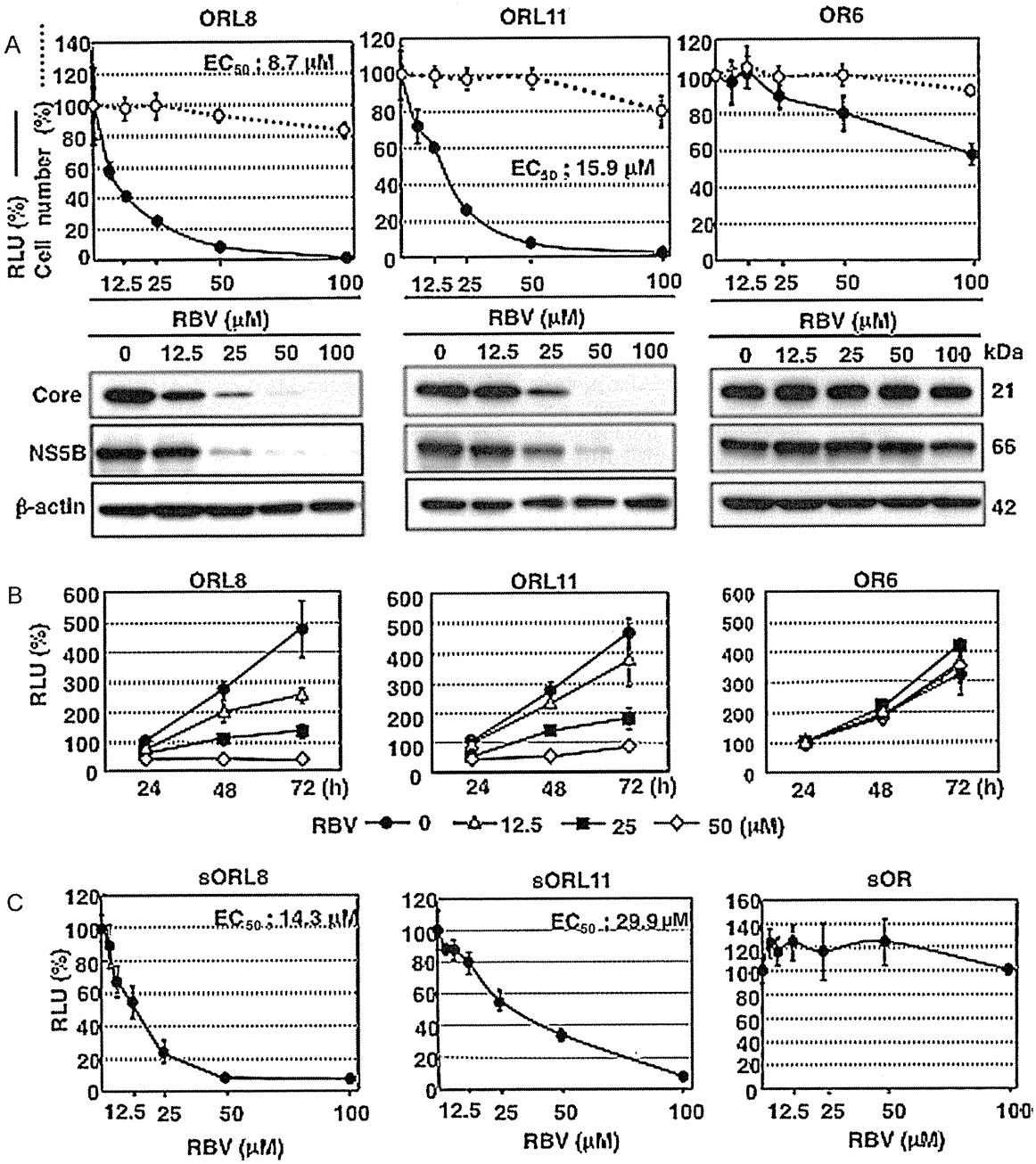

Fig. 1. Anti-HCV activity of RBV detected in the ORL8 and ORL11 system. (A) RBV sensitivities on genome-length HCV RNA replication in ORL8, ORL11, and OR6. The ORL8, ORL11 and OR6 cells were treated with RBV for 72 h, and then an RL assay (bold line in the upper panel) was performed. The relative luciferase activity (RLU) (%) calculated at each point, when the level of luciferase activity in non-treated cells was assigned to be 100% is presented here. The cell number (dotted line in the upper panel) at each concentration was determined as described in Section 2. Western blot analysis of RBV-treated ORL8, ORL11, and OR6 cells for HCV proteins, Core and NS5B, was also performed (lower panel). (B) Time-dependent anti-HCV activity of RBV. The ORL8, ORL11, and OR6 cells were treated with RBV, and an RL assay was performed at 24, 48, and 72 h after the treatment. The RLU (%) calculated at each time point, when the luciferase activity of non-treated cells at 24 h was assigned to be 100%, is shown. (C) Anti-HCV activity of RBV was observed in Li23-derived replicon assay systems (sORL8 and sORL11), but not in HuH-7-derived replicon assay system (sOR). RBV treatment and RL assay were performed as described for panel A.

two parts (6.0 kb covering 5′-UTR to NS3 and 6.1 kb covering NS3 to NS5B) and the two PCR products were subcloned for the sequence analysis as described above.

2.9. RNA interference and quantitative RT-PCR

SiRNA duplexes targeting the coding regions of human IMPDH1 (Dhamacon; catalog no. M-009687-01) and IMPDH2 (Dharmacon; catalog no. M-004330-02) were chemically synthesized. SiRNA duplex non-targeting (Dharmacon; catalog no. D-001206-13) was also used as a control. ORL8 cells were transfected with the indicated siRNA duplexes using Oligofectamine (Invitrogen) (Dansako et al., 2007). Extraction of total RNA and quantitative RT-PCR analysis for HCV RNA were performed by real-time LightCycler PCR as described previously (Ikeda et al., 2005).

2.10. Statistical analysis

Statistical comparison of the luciferase activities between the treatment groups and controls was performed using the Student's

t-test. *P* values of less than 0.05 were considered statistically significant.

3. Results

3.1. Anti-HCV activity of RBV was clearly observed in the Li23-derived assay systems, but not in the HuH-7-derived assay system

Recently we demonstrated that Li23-derived assay systems (ORL8 and ORL11), in which genome-length HCV RNA (O strain of genotype 1b) encoding RL robustly replicates, were frequently more sensitive to anti-HCV reagents such as IFNs and statins than the corresponding HuH-7-derived assay system (OR6) (Kato et al., 2009). Since we had observed a marginal anti-HCV activity of RBV in OR6 system, we assumed that the anti-HCV activity of RBV might also be illuminated by ORL8 or ORL11 system. Indeed, marked differences were observed between OR6 and both of the other assay systems: RBV at clinically achievable concentrations effectively inhibited HCV RNA replication in both ORL8 and ORL11, but not in OR6 (Fig. 1A). The EC_{50} values of RBV in ORL8, ORL11, and OR6 were 8.7, 15.9, and >100 μM, respectively, without suppression of cell growth (upper panels in Fig. 1A). These pronounced differences in the anti-HCV activity of RBV were confirmed by Western blot analysis (lower panels in Fig. 1A). In addition, time course assays revealed that the anti-HCV activity of RBV was dose- and time-dependent in ORL8 and ORL11, but not in OR6 (Fig. 1B). We next examined the activity of RBV using polyclonal cell-based assay systems (sORL8, sORL11, and sOR (Ikeda et al., 2005)) harboring HCV replicon RNA. The results revealed that the EC_{50} values of RBV in sORL8 and sORL11 were 14.3 and 29.9 μM, respectively, whereas RBV showed no anti-HCV activity in sOR (Fig. 1C), suggesting that the anti-HCV activity of RBV was not either a clone-specific or genome-length HCV RNA-specific phenomenon. Moreover, we demonstrated by Western blot (upper panel of Fig. 2) and quantitative RT-PCR (lower panel of Fig. 2) analyses that RBV suppressed HCV RNA replication in HCV-JFH1-infected ORL8c cells, but not in HCV-JFH1-infected RSc cells, which HCV could infect and efficiently replicate within (Ariumi et al., 2007; Kato et al., 2009). These results also indicate that only the Li23-derived assay system can illuminate the anti-HCV activity of RBV.

3.2. An ENT inhibitor cancelled anti-HCV activity of RBV

As one possible explanation for the pronounced differences in RBV activity between the Li23- and HuH-7-derived assay systems, we considered that the efficiencies in the cellular uptake of RBV might have differed between the two types of cells. To date, two families of nucleoside transporter proteins have been identified: the ENT family (ENT1, ENT2, and ENT3) and the concentrative nucleotide transporter (CNT) family (CNT1, CNT2, and CNT3) (Pastor-Anglada et al., 2005). Two recent reports showed that ENT1 and CNT3 might be responsible for RBV uptake in HuH-7 cells (Ibarra and Pfeiffer, 2009), and that ENT1, but not ENT2 or CNTs, is a major RBV uptake transporter in human hepatocytes (Fukuchi et al., 2010). To test these points, we first examined the effects of an ENT inhibitor, NBMPR, and a CNT inhibitor, phloridzin dihydrate, on the anti-HCV activity of RBV (50 μM; 90% effective concentration [EC_{90}]) in ORL8 system. The results revealed that 5 μM NBMPR partially attenuated the anti-HCV activity of RBV in ORL8 (Fig. 3A). The marginal activity of RBV was also not changed in OR6 system treated with these transporter inhibitors (data not shown). A significant dose-dependency of the cancellation by NBMPR was also observed in ORL8 (Fig. 3B). Since we observed a lack of expression of CNT family members in ORL8 cells (data not shown), these

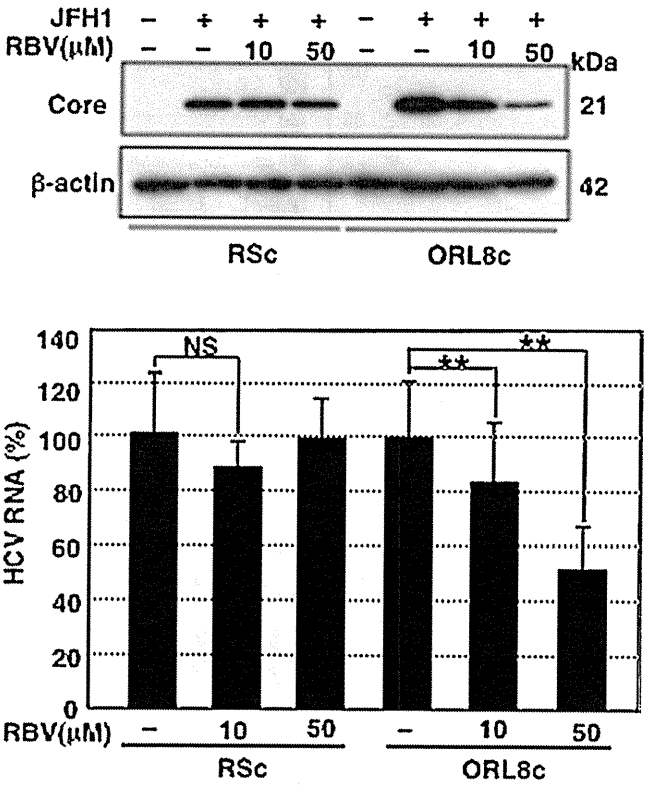

Fig. 2. RBV inhibited HCV production in JFH1-infected ORL8c cells, but not in JFH1-infected RSc cells. JFH1-infected ORL8c and RSc cells were treated with RBV for 72 h, and subjected to Western blot analysis using anti-Core or β-actin antibody (upper panel), and to quantitative RT-PCR analysis (lower panel). Asterisks indicate significant differences compared to the control treatment. $^{**}P < 0.01$; NS, not significant.

results suggest that cellular uptake of RBV is mediated by ENT member(s). Accordingly, we next examined the levels of ENT mRNAs in ORL8 and OR6 cells. However, the expression levels of ENT1, ENT2, and ENT3 mRNAs were comparable between ORL8 and OR6 cells (Fig. 3C). In addition, sequence analysis of ENT1, ENT2, and ENT3 mRNAs (data not shown) and Western blot analysis of ENT1 protein (Fig. 3D) revealed no differences between the two cell lines. These results suggest that the expression levels of ENT members are not associated with the differences in RBV activity.

3.3. RBV did not act as a mutagen in HCV RNA replication

Since the suppressive effect of RBV on HCV RNA replication was clearly observed in ORL8 system, we expected that ORL8 cells would be suitable for analysis of the anti-HCV mechanism of RBV. In regard to the anti-HCV mechanism of RBV, several groups have proposed that RBV (50–400 μM) acts as an RNA mutagen and induces error catastrophe in HCV RNA replication (Contreras et al., 2002; Zhou et al., 2003). Therefore, we first examined whether or not error catastrophe theory is involved in the anti-HCV activity of RBV observed in ORL8 system. To test the mutagenic effect of RBV, ORL8 cells were treated with or without RBV (50 μM; EC_{90} level in ORL8 system) for 72 h, and then genome-length HCV RNA from the ORL8 cells was amplified by RT-PCR. We performed HCV quasispecies analysis by sequencing of RL to the Neo^R, NS5A, and NS5B regions using at least 10 independent clones for each region. To estimate the mutation rate, the total number of mutations and the ratio of nonsynonymous to synonymous mutations in each region were determined by comparison with the parental HCV sequences (Kato et al., 2009). The results revealed that the overall mutation rate and the ratio of nonsynonymous to synonymous mutations in each

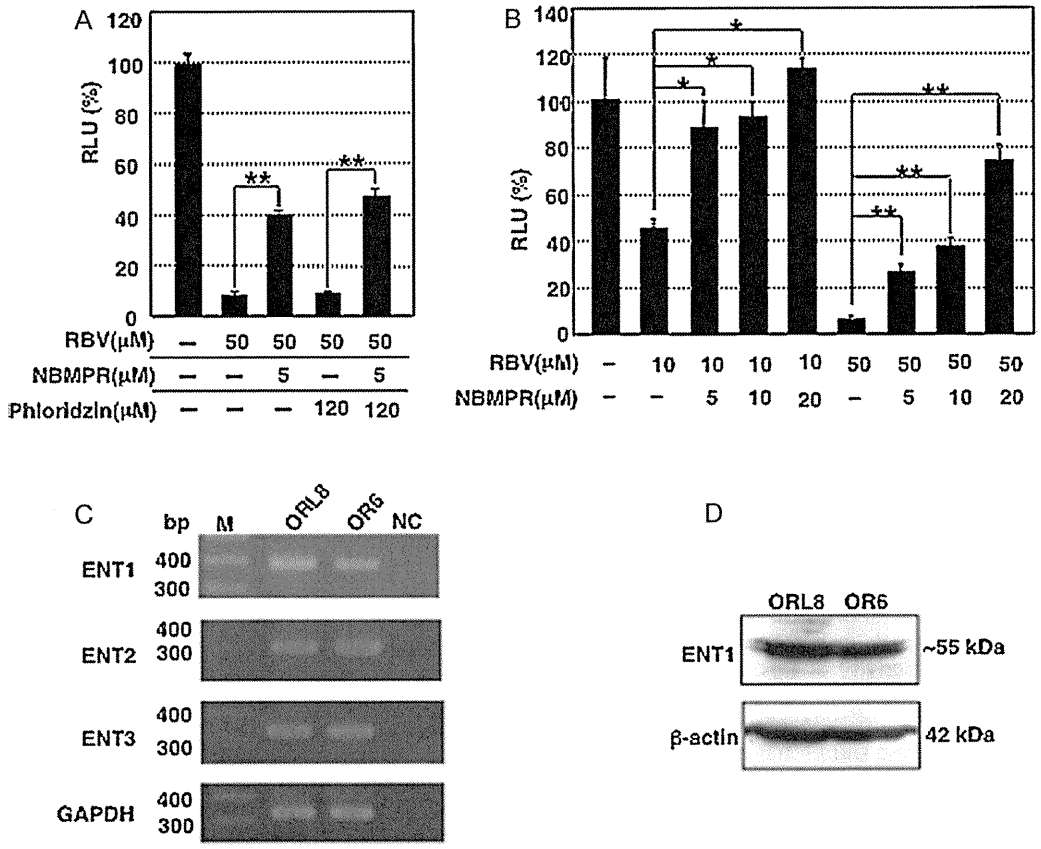

Fig. 3. An ENT inhibitor abolished anti-HCV activity of RBV. (A) An ENT inhibitor, NBMPR, canceled the anti-HCV activity of RBV in ORL8. ORL8 cells were pretreated with NBMPR and/or phloridzin dihydrate for 30 min, and then treated with RBV for 72 h, after which an RL assay was performed. Asterisks indicate significant differences compared to the control treatment. $^{**}P < 0.01$. (B) Dose-dependent cancellation by NBMPR of the activity of RBV. ORL8 cells were pretreated with NBMPR for 30 min, and then treated with RBV for 72 h, after which an RL assay was performed. Asterisks indicate significant differences compared to the control treatment. $^{*}P < 0.05$; $^{**}P < 0.01$. (C) RT-PCR analysis of ENTs. Total RNAs prepared from ORL8 and OR6 cells were subjected to RT-PCR using the primer sets for ENT1, ENT2, ENT3, and GAPDH as described in Section 2. RT-PCR products were detected by staining with ethidium bromide after 3% agarose gel electrophoresis. (D) Western blot analysis of ORL8 and OR6 cells for ENT1. The primary antibody used was ENT1. β-actin was used as a control for the amount of protein loaded per lane.

region were not increased irrespective of the presence or absence of RBV treatment (Table 1). To confirm that mutation frequencies given in Table 1 are overwhelmingly above the error level associated with the PCR, we sequenced independent five clones (6.0 kb covering 5′-UTR to NS3 and 6.1 kb covering NS3 to NS5B) obtained by PCR using KOD-plus DNA polymerase and a plasmid containing the parental HCV sequences (Kato et al., 2009) as a template. No mutations were detected in these sequenced clones, indicating that KOD-plus DNA polymerase possesses extremely high fidelity, and suggesting that the mutations obtained in the present study are not produced by the errors associated with the PCR. Therefore, these results indicate that RBV does not act as a mutagen in HCV RNA replication in ORL8 cells, and suggest that the anti-HCV activity of RBV (EC_{50}; 8.7 μM) observed in ORL8 system is not due to the induction of error catastrophe in the HCV RNA genome.

3.4. RBV did not activate the IFN-signaling pathway

Regarding HCV, Liu et al. (Liu et al., 2007) have reported that RBV (40–500 μM) enhances the IFN-signaling pathway in in vitro cell culture systems. Furthermore, a recent report showed that RBV improved early responses to PEG-IFN through enhanced IFN signaling in the treatment of patients with chronic hepatitis (Feld et al., 2010). In that study, it was shown that the RBV concentration in patients at day 3 was correlated with IP-10 induction at 12 h, but only in patients with an adequate first phase viral decline (Feld et al., 2010). Therefore, we expected that RBV would enhance the IFN-signaling pathway in our new cell culture system. Accordingly, we first examined the effect of RBV in combination with IFN-α on HCV RNA replication using ORL8 system. OR6 system was also used for purpose of comparison. The results showed that RBV had an additive effect in decreasing HCV RNA replica-

Table 1
Mutation frequencies in RL-Neo^R, NS5A, and NS5B regions.

Region	Condition	Total no. of clones	Total no. of mutations	Nonsynonymous/synonymous substitutions (ratio)
RL-Neo^R (1953 nts)	Control	12	59	39/20 (1.95)
	RBV (50 μM)	12	49	31/18 (1.72)
NS5A (1341 nts)	Control	10	35	24/11 (2.18)
	RBV (50 μM)	10	36	24/12 (2.00)
NS5B (1773 nts)	Control	10	10	3/7 (0.43)
	RBV (50 μM)	10	9	2/7 (0.29)

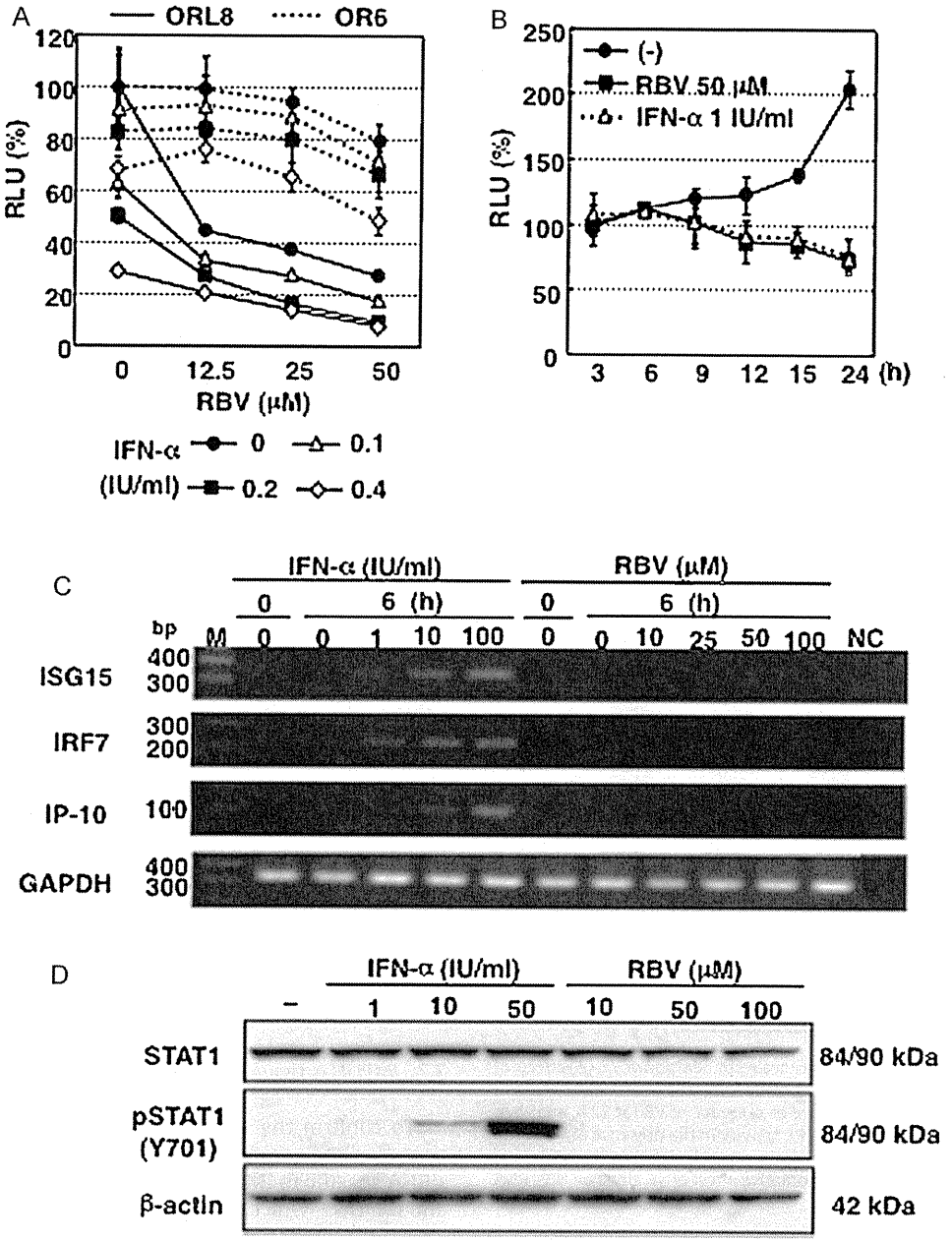

Fig. 4. RBV did not activate the IFN-signaling pathway in ORL8 cells. (A) Additive effect of RBV in combination with IFN-α. ORL8 and OR6 cells were treated with a combination of IFN-α and RBV for 72 h, after which the RL assay was performed. (B) Time course assay of the anti-HCV activity of RBV or IFN-α. ORL8 cells were treated with RBV or IFN-α, and an RL assay was performed at 3, 6, 9, 12, 15, and 24 h after treatment. Presented here is the RLU (%) calculated at each point, when the RL activity of non-treated cells at 3 h was assigned to be 100%. (C) ISGs were not induced by RBV treatment. ORL8 cells were treated with IFN-α or RBV for 6 h, and then the total RNAs extracted from the cells were subjected to RT-PCR using the primer sets for ISG15, IRF7, IP-10, and GAPDH as described in Section 2. RT-PCR products were detected by staining with ethidium bromide after 3% agarose gel electrophoresis. (D) Phosphorylation of STAT1 was not induced by RBV treatment. ORL8 cells were treated with IFN-α or RBV for 30 min, and subjected to Western blot analysis using anti-STAT1, anti-phospho-STAT1(Y701), and anti-β-actin antibodies.

tion in both assay systems, but its activity was greater in ORL8 than in OR6 (Fig. 4A). A comparative time course assay using RBV or IFN-α demonstrated that RBV- and IFN-α-treated ORL8 cells had the same anti-HCV kinetics, leading to decreased RL activity at 9 h after treatment (Fig. 4B). These results suggest that RBV induces some anti-HCV signaling pathway, such as an IFN-signaling pathway, rather than inducing IFN or directly inhibiting RNA replication.

We next examined the ability of RBV to activate ISGs. RT-PCR analysis revealed that RBV treatment (6 h) did not cause an induction of representative ISGs, ISG15, IRF7, and IP-10, in ORL8 cells, although even treatment (6 h) with 1 IU/ml (ISG15 and IRF7) or 10 IU/ml (IP-10) of IFN-α could induce these ISGs (Fig. 4C). Similar results were also obtained in OR6 cells and Huh7.5 cells (data not shown). In addition, enhancement of these ISGs was also not observed in the ORL8 cells co-treated with IFN-α and RBV (data not shown). Furthermore, we examined the phosphorylation status of STAT1 after RBV treatment. The results revealed that RBV treatment (up to 100 μM for 30 min) did not induce the phosphorylation of STAT1 in ORL8 cells, although phosphorylation of STAT1 was observed even after the treatment with 10 IU/ml of IFN-α (Fig. 4D). Together, these results indicate that RBV does not activate the IFN-signaling pathway.

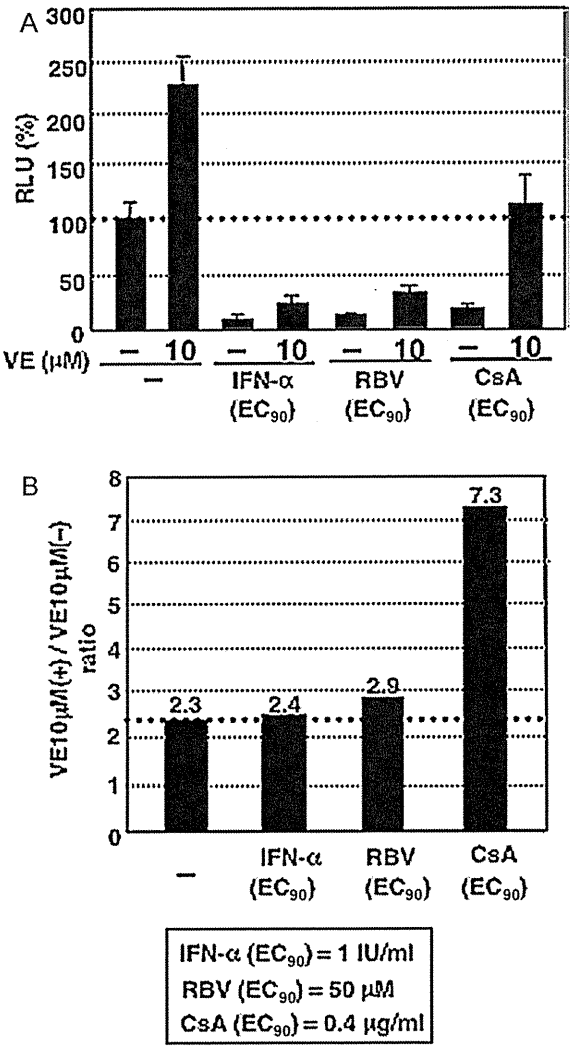

Fig. 5. The anti-HCV activity of RBV was not canceled by addition of VE. (A) Effects of VE on IFN-α, RBV, and CsA at the EC_{90}. ORL8 cells were treated with control medium (–), IFN-α, RBV, or CsA in either the absence or presence of VE for 72 h, and then an RL assay was performed. (B) The ratio of RL activity in the presence of VE to the RL activity in the absence of VE. The above ratio was calculated from the data of panel A. The horizontal line indicates the promotive effect of VE alone on HCV RNA replication as a baseline.

3.5. RBV did not induce the oxidative stress or subsequent anti-HCV status

Recently we reported that the antioxidant VE negated the antiviral activities of a broad range of anti-HCV reagents, including CsA, and demonstrated the involvement of the MEK-ERK1/2-signaling pathway in the anti-HCV status induced by oxidative stress (Yano et al., 2007, 2009). Therefore, we next expected that RBV induces oxidative stress. Accordingly, we examined the effect of VE on RBV, IFN-α, or CsA at the EC_{90} level in ORL8 system. Although the anti-HCV activity of CsA was canceled to a significant level by VE, the inhibitory effects of RBV and IFN-α were hardly influenced by co-treatment with VE (Fig. 5A). We normalized these results by dividing the RL value obtained in the presence of VE by that in the absence of VE as described previously (Yano et al., 2007) (Fig. 5B). The value of RBV was almost the same as that of IFN-α or control, although the value of CsA was somewhat higher (7.3) which was consistent with previous findings (Yano et al., 2007). These results suggest that induction of oxidative stress is not associated with the activity of RBV detected in ORL8 system.

3.6. Guanosine dose-dependently attenuated the anti-HCV activity of RBV

Previously, using a qualitative colony-forming efficiency (CFE) assay of an HCV RNA replicon, Zhou et al. (2003) showed that RBV (50 μM) reduced the CFE by 2-fold in HuH-7 cells, although 10 μM RBV did not result in a significant change in CFE. In that study, when exogenous guanosine, but not adenosine, which would replenish GTP pools via the salvage pathway, was co-administered with RBV, the RBV-induced CFE reduction was partially cancelled (Zhou et al., 2003). From this result, the authors suggested that IMPDH inhibition and subsequent lowering of GTP pools contribute to the observed reduction in CFE. However, they failed to observe the any suppressive effects of the IMPDH inhibitors MPA and Merimepodib (MMPD)/VX-497 on HCV RNA replication (Zhou et al., 2003). Conversely, Henry et al. showed that MPA exerted anti-HCV activity on HCV RNA replication in HuH-7-derived cells (Henry et al., 2006). Therefore, in order to resolve these controversial results, we initially examined the anti-HCV activity of MPA in ORL8 and OR6 systems. The results revealed that MPA strongly inhibited HCV RNA replication in both systems without suppression of cell growth. The EC_{50} values of MPA in the ORL8 and OR6 were 0.29 and 0.57 μM, respectively (Fig. 6A). Dose-dependent cancellation by guanosine, but not by adenosine, of the activity of MPA, was observed in both systems (Fig. 6B and data not shown for OR6 system). These results suggest that the depression of GTP induced by inhibition of IMPDH decreases the level of HCV RNA replication. From these results, we expected that anti-HCV activity of RBV observed in ORL8 might also have been associated with the inhibition of IMPDH. Indeed, significant dose-dependent cancellation by guanosine, but not by adenosine, of the anti-HCV activity of RBV (10 μM) was observed in ORL8 (Fig. 6C). ORL11 also showed a similar cancellation by guanosine (data not shown). The suppressive effect of guanosine on the activity of RBV in ORL8 was confirmed by Western blot analysis (Fig. 6D). These results suggest that the anti-HCV activity of RBV at clinically achievable concentrations in ORL8 is mediated through the inhibition of IMPDH by RBV.

3.7. IMPDH is required for HCV RNA replication

To confirm the involvement of IMPDH on HCV RNA replication, the endogenous expression of IMPDH was suppressed by siRNA specific to IMPDH. Since IMPDH has two isoforms, IMPDH1 and IMPDH2, which share 84% amino-acid homology (Wang et al., 2008), we prepared IMPDH1- and/or IMPDH2-knockdown ORL8 cells. The effective knockdown of IMPDH1 and/or IMPDH2 in ORL8 cells was confirmed by quantitative RT-PCR (Fig. 7A). We observed that the levels of HCV RNA replication in these knockdown cells were notably reduced compared with the control cells without suppression of cell growth (Fig. 7B). These results suggest that IMPDH is crucial for the maintenance of HCV RNA replication. Taken together, these results indicate that the inhibitory activity of RBV on HCV RNA replication in Li23-derived cells is mediated through the inhibition of IMPDH by RBV.

4. Discussion

In this study, using novel Li23-derived cell culture assay systems, we demonstrated for the first time that RBV at clinically achievable concentrations efficiently inhibited HCV RNA replication, and clarified that its anti-HCV activity was mediated by the inhibition of IMPDH.

To date, several mechanisms as described above have been proposed based on the results of studies using an HuH-7-derived cell culture system (Feld and Hoofnagle, 2005; Feld et al., 2010; Lau

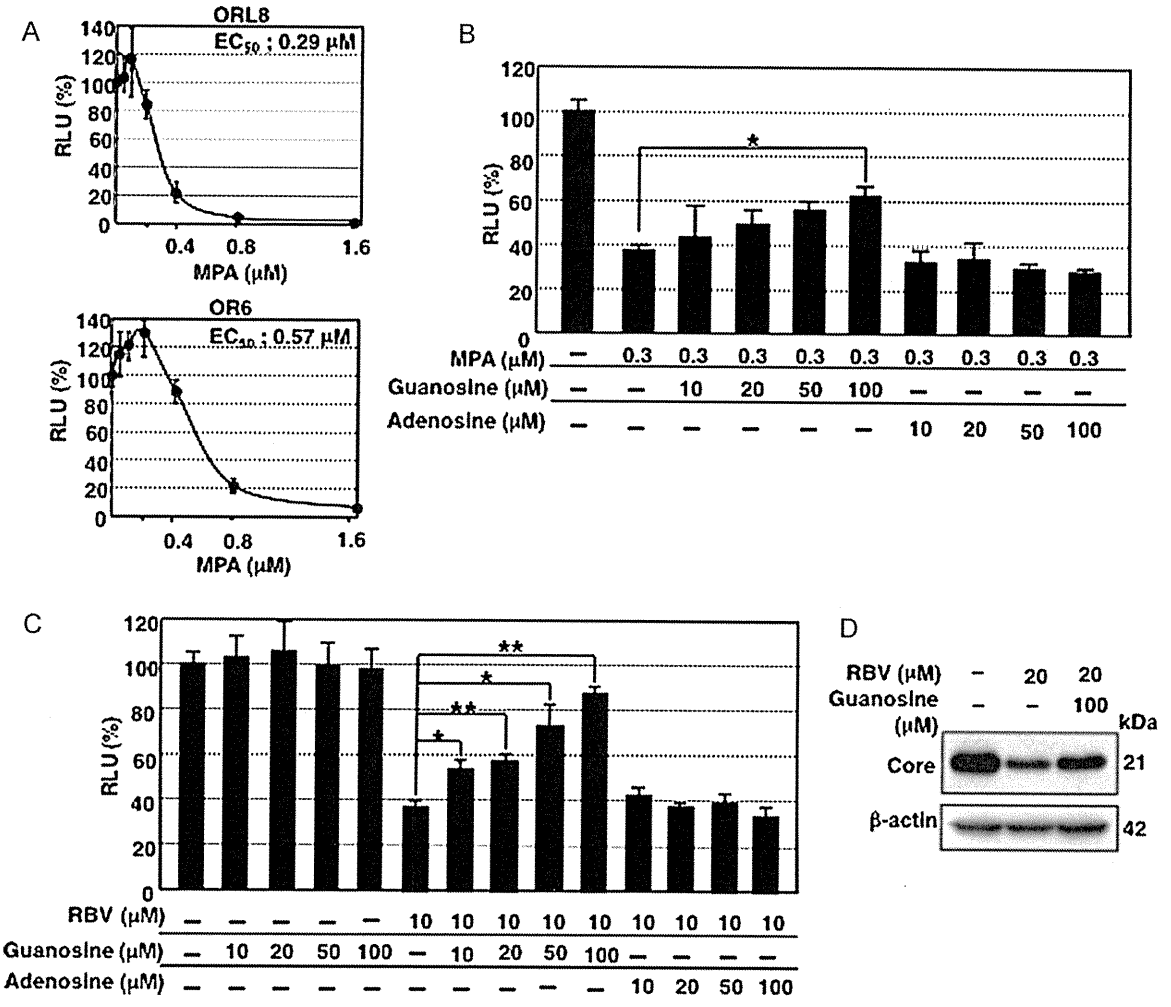

Fig. 6. Guanosine canceled the anti-HCV activity of RBV in ORL8 system. (A) Anti-HCV activity of MPA in ORL8 and OR6. The ORL8 and OR6 cells were treated with MPA for 72 h, and then RL assay was performed. (B) Effect of guanosine or adenosine on MPA in ORL8 system. ORL8 cells were treated with MPA alone or in combination with guanosine or adenosine for 72 h, and then RL assay was performed. Asterisk indicates a significant difference compared to the control treatment. *$P<0.05$. (C) Effect of guanosine or adenosine on RBV in ORL8 system. ORL8 cells were treated with RBV alone or in combination with guanosine or adenosine for 72 h, and then the RL assay was performed. Asterisks indicate significant differences compared to the control treatment. *$P<0.05$; **$P<0.01$. (D) Effect of guanosine on RBV in ORL8 system. ORL8 cells were treated with RBV alone or in combination with guanosine for 72 h, and subjected to Western blot analysis using anti-Core and β-actin antibodies.

et al., 2002; Thomas et al., 2011; Zhou et al., 2003). Although the effective concentrations (50–1000 μM) of RBV in those studies were much higher than the clinically achievable concentrations (5–14 μM) (Feld et al., 2010; Pawlotsky et al., 2004; Tanabe et al., 2004), the effective concentration of RBV in this study was close to the clinically achievable concentrations. Furthermore, it is noteworthy that the replication of a different HCV strain (JFH1 of genotype 2a) in the Li23-derived cell culture system, but not in the HuH-7-derived cell culture system, was also suppressed with RBV at the concentration of 10 μM (Fig. 1C). These results demonstrate that the Li23 cell-derived assay system is a more sensitive biosensor of RBV than the HuH-7 cell-derived assay system.

The finding that RBV remarkably inhibited HCV RNA replication in our new assay systems led us to analyze the anti-HCV mechanism of RBV. In this study, we evaluated several possible anti-HCV mechanisms of RBV, as described above. Regarding the induction of error catastrophe by RBV, we obtained no evidence that RBV (even at 50 μM) acted as a mutagen in HCV RNA replication. Therefore, we could not explain the mechanism underlying the suppression of HCV RNA replication by RBV according to the theory of error catastrophe. In addition, no increasing mutation rate of HCV RNA in patients receiving RBV monotherapy or a combination of RBV plus IFN-α was observed in a previous clinical study (Chevaliez and Pawlotsky, 2007). In consideration of all these findings, we suggest that the clinically achievable concentrations of RBV do not act as a mutagen in HCV RNA replication. Indeed, our previous study using the replicon cell culture system demonstrated that RBV treatment (6 months at 5 and 25 μM) did not accelerate the mutation rate or increase the genetic diversity of the HCV replicon (Kato et al., 2005). In regard to the effect of RBV on the IFN system, we obtained no evidence that RBV (even at 50 μM) induced ISGs (ISG15, IRF7, and IP-10) or phosphorylation of STAT1 even in the cells co-treated with IFN-α and RBV (data not shown). On the other hand, very recently Thomas et al. (Thomas et al., 2011) reported that RBV treatment (500 μM) resulted in the induction of a distinct set of ISGs including ISG15, IRF7, and IRF9, using HuH-7-derived cell line Huh7.5.1. In that study, they demonstrated that the induction of these ISGs was mediated by a novel mechanism different from those associated with IFN signaling and double stranded RNA sensing pathway, and concluded that the effect of RBV on ISG regulation is IFN-independent. However, in our cell culture system, which is highly sensitive to RBV, the induction of ISG15 and IRF7 by RBV was not observed (Fig. 4C). This kind of controversial results may be dependent on the difference of cell lines used in both studies, since recent microarray analysis revealed that the expression profiles of Li23 and HuH-7 cells, both of which possess an environment

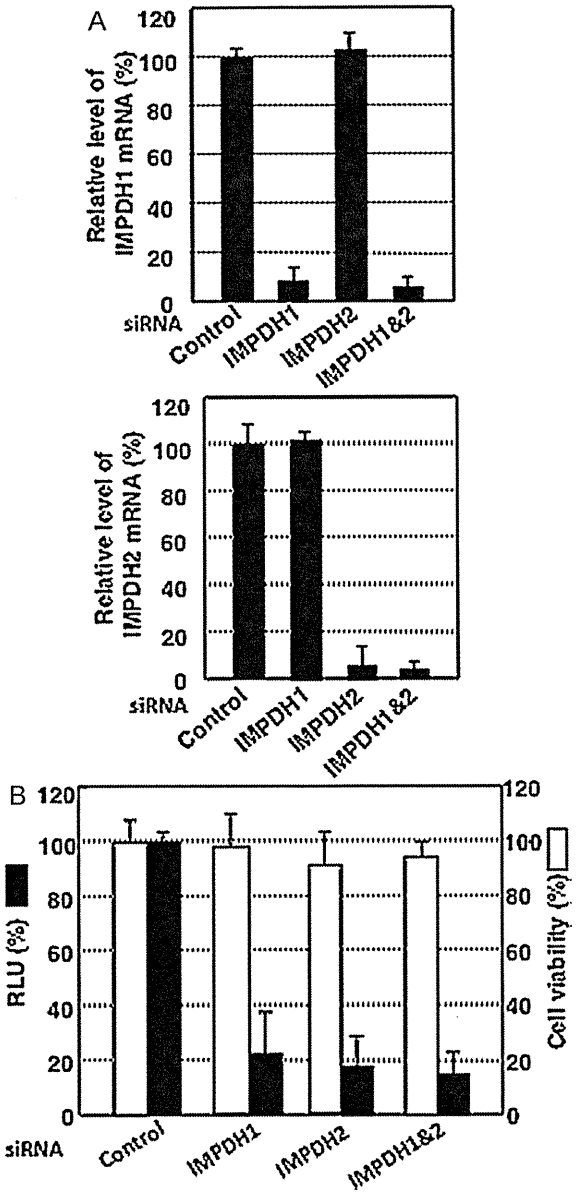

Fig. 7. IMPDH is required for HCV RNA replication. (A) Inhibition of IMPDH1 and IMPDH2 expression by siRNA in ORL8 cells. ORL8 cells were transfected with 8 nM siRNA targeting for IMPDH1 and/or IMPDH2. After 72 h, the expression levels of IMPDH1 and IMPDH2 mRNAs were determined by the quantitative RT-PCR. Experiments were done in triplicate. (B) Suppression of HCV RNA replication in IMPDH1- and/or IMPDH2-knockdown ORL8 cells. The RLU (%) calculated, when the luciferase activity of the cells treated with control siRNA was assigned to be 100%, is shown. The cell viability was determined as described in Section 2.

for robust HCV replication, differed considerably (Kato et al., 2009; Mori et al., 2010). However, Thomas et al. (2011) observed that the addition of guanosine to the medium could block RBV-induced ISGs induction. Therefore, further additional studies would be needed to resolve the differences of results obtained from both studies.

The highlight in this study is that a Li23-derived cell culture system clearly demonstrated an association between the suppression of HCV RNA replication by RBV and IMPDH inhibition by RBV. Although RBV is known to be an IMPDH inhibitor (Lau et al., 2002), it had been considered that such inhibitory activity would not contribute to the anti-HCV activity of RBV, because of the marginal antiviral effect of RBV in HuH-7-derived HCV RNA replicating cells (Naka et al., 2005; Tanabe et al., 2004; Zhou et al., 2003). Although Zhou et al. (2003) previously showed that exogenous guanosine cancelled the RBV-induced CFE reduction using an HuH-7-based HCV replicon system, they did not observe any dose-dependent reversion of the adverse effect of RBV by the addition of guanosine. However, in our Li23-based HCV replication assay system, we observed a near complete cancellation of the activity of RBV in the dose-dependent manner of guanosine (Fig. 6C). This finding indicated that anti-HCV activity of RBV might be mediated through the inhibition of IMPDH by RBV. Indeed, we could demonstrate that HCV RNA replication was notably suppressed in IMPDH-knockdown ORL8 cells (Fig. 7B). Taken together, these results revealed that the Li23-derived assay system was superior to HuH-7-derived assay system in order to clarify the anti-HCV mechanism of RBV.

The remarkable effect of RBV observed in this study was considered to be due to the difference in the cell lines used, because Li23-derived cells possessed rather different gene expression profiles from those in HuH-7-derived cells (Kato et al., 2009; Mori et al., 2010). As one of the possibilities, we examined the expression status of nucleoside transporters (ENT family) involved in cellular uptake of RBV or ATP-binding cassette transporters, including multidrug resistance 1, which is involved in cellular excretion. However, the mRNA levels of these transporters were almost the same in both types of cells (Fig. 3C). Although unfortunately we failed to clarify the mechanism underlying the remarkable differences in the activity of RBV in both types of cells, we observed that the anti-HCV activity of RBV was completely canceled by NBMPR, an ENT inhibitor, suggesting that RBV is taken by ENT member(s) at least in ORL8 cells. This finding supports the recent report describing the involvement of ENT1 on cellular uptake of RBV (Fukuchi et al., 2010; Ibarra and Pfeiffer, 2009). Therefore, a comparative analysis regarding the functions of ENT member(s) derived from both types of cells will be needed. As the other possibility, the differences of activities or expression levels of IMPDH in OR6 and ORL8 cells may contribute to the remarkable effect of RBV observed in ORL8 cells.

On the other hand, it has been known that rapid reduction of the intracellular level of GTP occurs when RBV inhibits IMPDH (Feld and Hoofnagle, 2005). Therefore, it is assumed that the decrease of GTP would lead to a suppression of HCV replication. To date, several studies (Lohmann et al., 1999; Luo et al., 2000; Simister et al., 2009) have shown that high concentration of GTP (approximately 500 μM corresponding to the intracellular concentration) is required for the efficient de novo initiation of RNA synthesis by HCV NS5B RdRp. In addition, Simister et al. (2009) showed that change from 500 μM to 100 μM of GTP concentration decreased a log of the NS5B RdRp activity. From these studies, we expect that the inhibition of IMPDH by RBV may cause rapid decrease of intracellular GTP concentration, resulting in the suppression of de novo RNA synthesis by NS5B. Before our assumption, MMPD/VX-497 has developed as an inhibitor of IMPDH, and it has been shown to exert anti-HCV activity (EC_{50}; 0.39 μM) in an HCV replicon system (Marcellin et al., 2007). However, MMPD/VX-497 monotherapy of patients with chronic hepatitis C had no effect on HCV RNA levels (Marcellin et al., 2007) just as, in another study, RBV monotherapy had no effect on HCV RNA levels in patients with chronic hepatitis C (Di Bisceglie et al., 1995). Although we showed that the EC_{50} value of RBV in this study was equivalent to the clinically achievable concentrations (Feld et al., 2010; Pawlotsky et al., 2004; Tanabe et al., 2004), we considered that the effective concentration for a reduction of HCV RNA levels in monotherapy would be less than the EC_{90} value. However, an IMPDH inhibitor at EC_{50} would be effective in combination with IFN-α as an adjuvant. Indeed, combination therapy with IFN-α and MMPD/VX-497 was effective in previously untreated patients with chronic hepatitis C (McHutchison et al., 2005). However, a recent study (Rustgi et al., 2009) showed that the addition of MMPD/VX-497 to PEG-IFN-α and RBV combination

therapy in patients who had been nonrespondent to PEG-IFN-α and RBV combination therapy did not increase the proportion of patients who achieved an SVR. Since we showed that RBV also acted as an IMPDH inhibitor in the present study, it would seem to be a reasonable result that MMPD/VX-497 had no significant effect on patients who were nonresponsive to combination therapy with PEG-IFN-α and RBV.

In conclusion, we clarified the anti-HCV mechanism of RBV in a new HCV cell culture system. The fact that anti-HCV activity of RBV was mediated by the inhibition of IMPDH would provide a clue to the mechanism of the increase of SVR by the current standard combination therapy with PEG-IFN-α and RBV. In addition, our findings should also be useful for the screening and development of new anti-HCV drugs, which inhibit IMPDH, with reduced side effects, including anemia.

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BASIC STUDIES

Anti-ulcer agent teprenone inhibits hepatitis C virus replication: potential treatment for hepatitis C

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Abstract

Background: Previously we reported that 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, statins, inhibited hepatitis C virus (HCV) RNA replication. Furthermore, recent reports revealed that the statins are associated with a reduced risk of hepatocellular carcinoma and lower portal pressure in patients with cirrhosis. The statins exhibited anti-HCV activity by inhibiting geranylgeranylation of host proteins essential for HCV RNA replication. Geranylgeranyl pyrophosphate (GGPP) is a substrate for geranylgeranyltransferase. Therefore, we examined the potential of geranyl compounds with chemical structures similar to those of GGPP to inhibit HCV RNA replication. *Methods:* We tested geranyl compounds [geranylgeraniol, geranylgeranoic acid, vitamin K_2 and teprenone (Selbex)] for their effects on HCV RNA replication using genome-length HCV RNA-replicating cells (the OR6 assay system) and a JFH-1 infection cell culture system. Teprenone is the major component of the anti-ulcer agent, Selbex. We also examined the anti-HCV activities of the geranyl compounds in combination with interferon (IFN)-α or statins. *Results:* Among the geranyl compounds tested, only teprenone exhibited anti-HCV activity at a clinically achievable concentration. However, other anti-ulcer agents tested had no inhibitory effect on HCV RNA replication. The combination of teprenone and IFN-α exhibited a strong inhibitory effect on HCV RNA replication. Although teprenone alone did not inhibit geranylgeranylation, surprisingly, statins' inhibitory action against geranylgeranylation was enhanced by cotreatment with teprenone. *Conclusions:* The anti-ulcer agent teprenone inhibited HCV RNA replication and enhanced statins' inhibitory action against geranylgeranylation. This newly discovered function of teprenone may improve the treatment of HCV-associated liver diseases as an adjuvant to statins.

Hepatitis C virus (HCV) infection frequently causes persistent hepatitis and leads to cirrhosis and hepatocellular carcinoma (HCC). Currently, the combination therapy of pegylated interferon (IFN) with ribavirin is available for patients with chronic hepatitis C (CH C) and yields a sustained virological response rate of about 50% (1). However, about half of CH C patients are still susceptible to the progression of the disease to fatal cirrhosis and HCC. Therefore, the development of more effective reagents for the treatment of HCV infection is urgent.

To overcome this problem, we developed a genome-length HCV RNA (strain O of genotype 1b) replication system (OR6) with luciferase as a reporter, which facilitated the prompt and precise monitoring of HCV RNA replication in hepatoma cells (HuH-7-derived OR6 cells) (2). Using this OR6 system, we recently reported that 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, statins, inhibited HCV RNA replication efficiently (3–5). Among five statins – fluvastatin (FLV), atorvastatin (ATV), simvastatin (SIV), pravastatin (PRV) and lovastatin (LOV) – FLV exhibited the strongest anti-HCV activity, while PRV had no effect on HCV RNA replication (3, 6). More recently, Bader *et al.* (7) demonstrated that FLV inhibited HCV RNA replication

*Contributed equally.

in humans. Furthermore, recent reports revealed that the statins were associated with a reduced risk of HCC (8) and lower portal pressure in patients with cirrhosis (9).

Statins targeted the mevalonate pathway. This pathway is branched after farnesyl pyrophosphate (FPP) into cholesterol and geranylgeranyl pyrophosphate (GGPP) biosynthesis pathways. The inhibition of GGPP but not of cholesterol is essential for HCV RNA replication in the inhibitory activity of statins (3, 10, 11). To date, one of the proteins, FBL2, was reported as the host protein essential for HCV RNA replication. HCV RNA replication requires geranylgeranylation of FBL2 by geranylgeranyltransferase with GGPP (12).

We have attempted to examine the effects of geranyl compounds [geranylgeraniol (GGOH), geranylgeranoic acid, vitamin K_2 (VK2) and teprenone] on HCV RNA replication using the OR6 assay system and the JFH-1 infection cell culture system, because their chemical formulas are similar to that of the GGPP, a substrate for geranylgeranyltransferase in geranylgeranylation (13–15). The anti-ulcer agent teprenone (also called geranylgeranylacetone) is reported to block the function of GGPP by the competitive inhibition of the mevalonate pathway (16). Teprenone is the major component of the clinically used anti-ulcer reagent, Selbex.

Here, we reported the inhibitory activity of teprenone on HCV RNA replication and the effect of teprenone in combination with statins on their inhibitory action against geranylgeranylation.

Materials and methods

Reagents and antibodies

Teprenone (Selbex), geranylgeranoic acid, ecabet sodium and sofalcon, gefarnate were purchased from Eisai Co. Ltd (Tokyo, Japan), BIOMOL (Plymouth Meeting, PA, USA), Mitsubishi Tanabe Pharma (Osaka, Japan), Taisho Pharmaceutical Co. (Tokyo, Japan) and Dainippon Sumitomo Pharma Co. Ltd (Osaka, Japan) respectively. GGPP, GGOH, VK2, IFN-α, vitamin E, linoleic acid and mevalonate were purchased from Sigma (St Louis, MO, USA). Cyclosporine A, FLV, LOV and PRV were purchased from Calbiochem (Los Angeles, CA, USA). ATV, SIV and pitavastatin (PTV) were purchased from Astellas Pharma Inc. (Tokyo, Japan), Banyu Pharmaceutical Co. Ltd (Tokyo, Japan), and Kowa Co. Ltd (Nagoya, Japan) respectively.

The antibodies used in this study were those specific to the Core (CP11, Institute of Immunology, Tokyo), NS5A (a generous gift from Dr A. Takamizawa, Research Foundation for Microbial Diseases, Osaka University), NS5B (a generous gift from Dr M. Kohara, Tokyo Metropolitan Institute of Medical Science) and β-actin (Sigma). Anti-heat shock protein (HSP) 90 and anti-HSP70 antibodies were purchased from BD Bioscience (San Jose, CA, USA). Anti-Rap1A (sc-1482) and anti-Rap1 (sc-65) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell cultures

OR6 is a cell line cloned from ORN/C-5B/KE RNA-replicating HuH-7 cells as described previously (2) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin, streptomycin and G418 (300 μg/ml; Geneticin, Invitrogen, Carlsbad, CA, USA). ORN/C-5B/KE RNA is derived from HCV-O, and OR6c cells are cured OR6 cells from which HCV RNA was eliminated by IFN-α treatment as described previously (2). HCV-O/RLGE is the authentic HCV RNA containing adaptive mutations of Q1112R, P1115L, E1203G and K1609E in the NS3 region and replicates efficiently in OR6c cells.

OR6 reporter assay

For the *Renilla* luciferase (RL) assay, $1.0–1.5 \times 10^4$ OR6 cells were plated onto 24-well plates in triplicate and precultured for 24 h. The cells were treated with each compound for 72 h. Then, the cells were harvested and subjected to an RL assay according to the manufacturer's protocol (2).

Western blot analysis

For western blot analysis, $4–4.5 \times 10^4$ OR6 or OR6c cells harbouring HCV-O/RLGE RNA were plated onto six-well plates and cultured for 24 h, and were then treated with each compound for 72 h. Preparation of the cell lysates, sodium dodecyl sulphate-polyacrylamide gel electrophoresis and immunoblotting were then performed as described previously (17).

Cell growth assay

To examine the effect of each reagent on OR6 cell growth, $6.0–6.5 \times 10^4$ OR6 cells were plated onto six-well plates in triplicate and were precultured for 24 h. The cells were treated with or without each compound for 72 h, and then the viable cells were counted after trypan blue dye treatment as described previously (18).

WST-1 cell proliferation assay

The OR6 cells (2×10^3 cells) were plated onto a 96-well plate in triplicate at 24 h before treatment with each reagent. The cells at 24, 48 and 72 h after treatment were subjected to a WST-1 cell proliferation assay (Takara Bio, Otsu, Japan) according to the manufacturer's protocol.

Reverse transcription-polymerase chain reaction

Reverse transcription-polymerase chain reaction (RT-PCR) for HMG-CoA reductase and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed by a method described previously (19). Briefly, using cellular total RNAs (2 μg), cDNA was synthesized using Superscript II with the oligo dT primer. One-tenth of the synthesized cDNA was subjected to PCR with the

following primer pairs: HMG-CoA reductase, 5′-ATGCC ATCCCTGTTGGAGTG-3′ and 5′-TGTTCATCCCCATG GCATCCC-3′; and GAPDH, 5′-GACTCATGACCACAG TCCATGC-3′ and 5′-GAGGAGACCACCTGGTGCTCA G-3′.

Hepatitis C virus infection experiment

For the infection experiment with the JFH-1 virus, HuH-7-derived RSc cells (1×10^5 cells) were plated onto six-well plates and cultured for 24 h (20). Then, the cells were infected with 100 μl (equivalent to a multiple of infection of 0.1–0.2) of inoculum and cultured for 24 h. The cells were treated with each reagent for 72 h. The culture supernatants and cells were collected for quantification of the Core by an enzyme-linked immunosorbent assay (ELISA) (Mitsubishi Kagaku Bio-Clinical Laboratories, Tokyo, Japan) and for western blot analysis respectively.

Statistical analysis

The luciferase activities were statistically compared between the various treatment groups using Student's *t*-test. *P* values of < 0.05 were considered statistically significant. The mean ± standard deviation is determined from at least three independent experiments.

Results

Anti-hepatitis C virus activity of teprenone is a unique feature not only among geranyl compounds but also among anti-ulcer agents

The mevalonate pathway is divided into two branches: cholesterol synthesis and GGPP synthesis pathways (Fig. 1). The statins exhibited anti-HCV activity via inhibition of geranylgeranylation of host proteins essential for HCV RNA replication. Therefore, we examined the effects of geranyl compounds [GGOH, geranylgeranoic acid, VK2 and teprenone (Selbex)] on HCV RNA replication using the OR6 assay system, because their chemical structures are similar to that of the GGPP (Fig. 2A) (16). Teprenone inhibited HCV RNA replication in a dose-dependent manner without affecting OR6 cell growth up to a concentration of 20 μg/ml (Fig. 2B). The 50% effective concentration (EC_{50}) of teprenone is 5.3 μg/ml. On the other hand, GGOH, geranylgeranoic acid and VK2 did not inhibit HCV RNA replication at the concentration without cytotoxicity (Fig. 2C–E). We also demonstrated that teprenone did not affect cell proliferation within this concentration (supporting information, Fig. S1A). These results suggest that anti-HCV activity of teprenone was not a common feature among geranyl compounds.

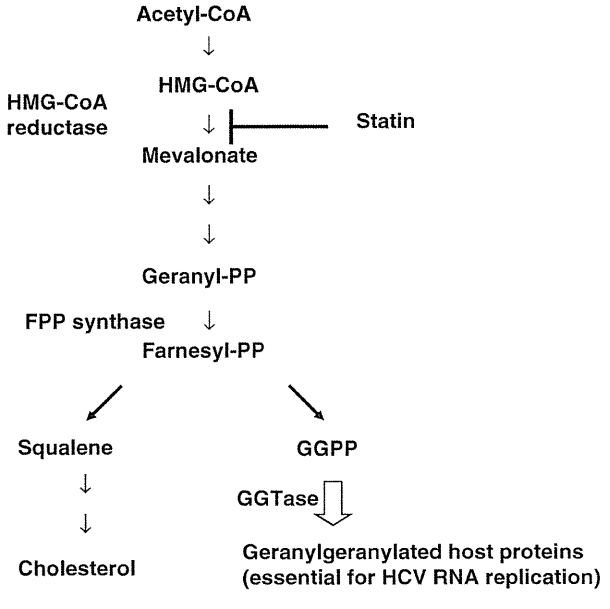

Fig. 1. Schema of the mevalonate pathway.

Teprenone is used for patients with gastritis and gastric ulcers. Therefore, we examined anti-ulcer agents for their inhibitory effects against HCV RNA replication. The chemical structures of three anti-ulcer agents – ecabet sodium, sofalcon and gefarnate – are shown in supporting information, Figure S1B. None of these agents exhibited inhibitory effects on HCV RNA replication (supporting information, Fig. S1C–E). These results indicate that the anti-HCV activity of teprenone may not be a common feature among anti-ulcer agents.

Teprenone inhibited authentic hepatitis C virus RNA replication

The genome-length HCV RNA replicating in the OR6 cells contained three non-natural elements – RL, neomycin phosphotransferase and encephalomyocarditis virus internal ribosomal entry site. To further confirm that the anti-HCV activity of teprenone was not because of the inhibition of these three exogenous genes or their products, we used authentic 9.6 kb HCV RNA-replicating cells. We introduced *in vitro* synthesized HCV-O/RLGE RNA into cured OR6c cells (Fig. 3A). As shown in Figure 3B, teprenone inhibited Core expression in HCV-O/RLGE-replicating OR6c cells in a dose-dependent manner. These results indicate that the anti-HCV activity of teprenone was because of the inhibition of HCV RNA itself, but not exogenous genes or their products.

Teprenone enhanced anti-hepatitis C virus activity of interferon-α

We examined whether or not teprenone would enhance the anti-HCV activity of IFN-α. We did this by studying the inhibitory effects of combinations of IFN-α (0, 2.5, 5 and 10 IU/ml) and teprenone (0, 10 and 20 μg/ml) using the OR6 assay system. Teprenone enhanced the anti-HCV activity of IFN-α in a dose-dependent manner (Fig. 4). Teprenone with IFN-α also inhibited Core expression (Fig. 4). We also demonstrated that teprenone did not

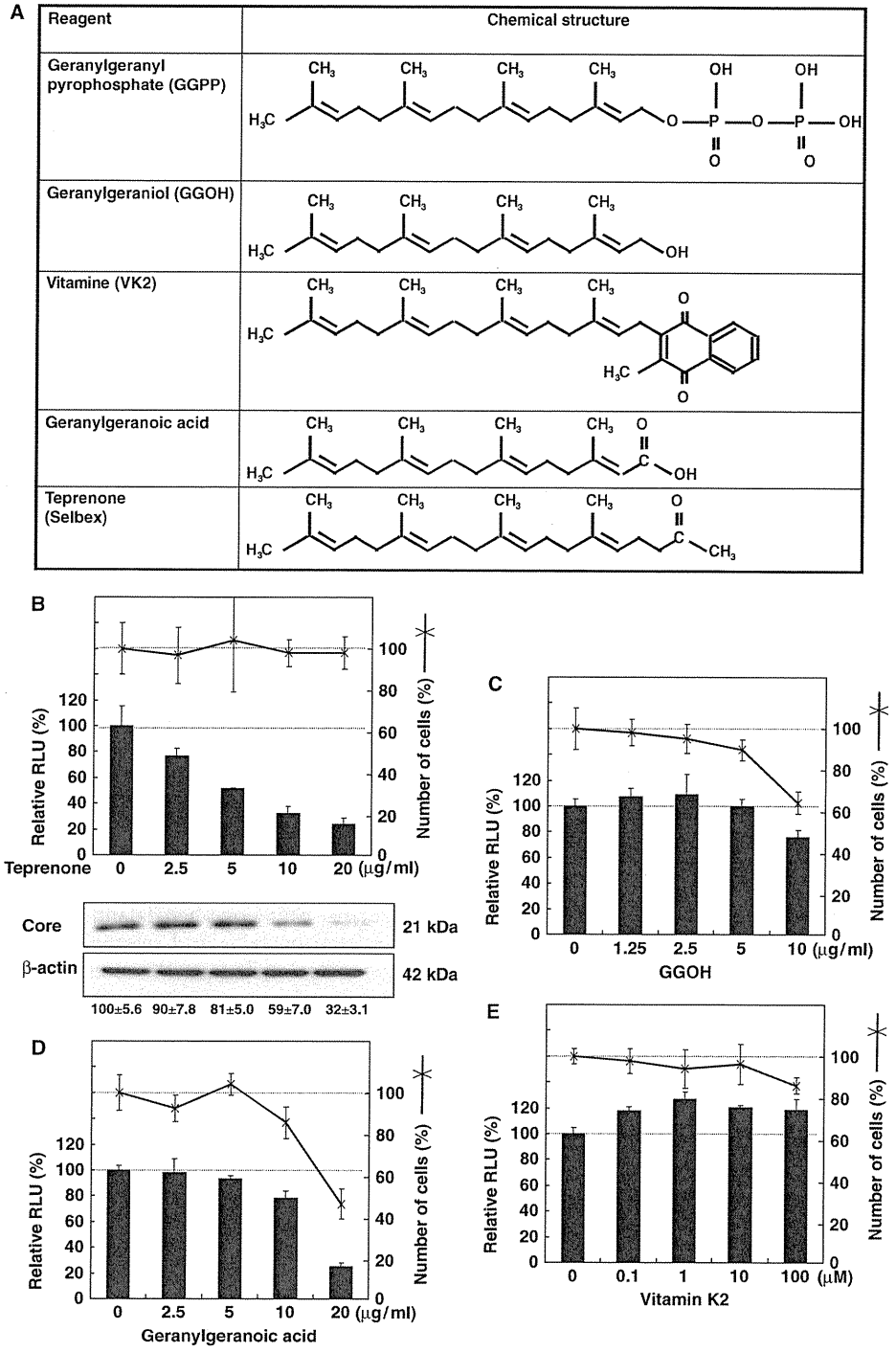

Fig. 2. The effects of geranyl compounds and anti-ulcer agents on hepatitis C virus (HCV) RNA replication. (A) Structures of geranyl compounds. (B) Anti-HCV activity of teprenone on HCV RNA replication in OR6 cells. OR6 cells were treated with teprenone (0, 2.5, 5, 10 and 20 μg/ml) for 72 h. *Renilla* luciferase (RL) activity for HCV RNA replication is shown as a percentage of control. Each bar represents the average with standard deviations of triplicate data points. Cell viability was also shown as a percentage of control. After 72-h treatment, the production of the Core was analysed by immunoblotting using anti-Core antibody (lower panel). β-actin was used as a control for the amount of protein loaded per lane. The signal intensities of Core from three independent assays were quantified by densitometry and normalized by that of β-actin. Each of the mean ± standard deviation is under the lower panel. (C to E) OR6 cells were treated with geranylgeraniol (0, 1.25, 2.5, 5 and 10 μg/ml) (C), geranylgeranoic acid (0, 2.5, 5, 10 and 20 μg/ml) (D) and VK2 (0, 0.1, 1, 10 and 100 μM) (E) for 72 h. RL activity and cell viability after treatment were determined as shown in (B).

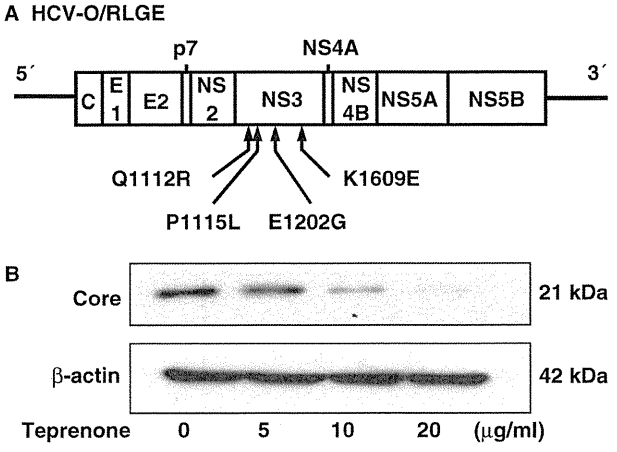

Fig. 3. Teprenone inhibited authentic hepatitis C virus (HCV) RNA replication. (A) Schematic gene organization of genome-length HCV-O/RLGE RNA. The positions of four adaptive mutations – Q1112R, P1115L, E1203G and K1609E – are indicated by arrows. (B) HCV-O/RLGE RNA was introduced into OR6c cells by electroporation as described previously (5). The cells were treated with teprenone (0, 5, 10 and 20 μg/ml) for 72 h and then the production of the Core was analysed by immunoblotting using anti-Core antibody.

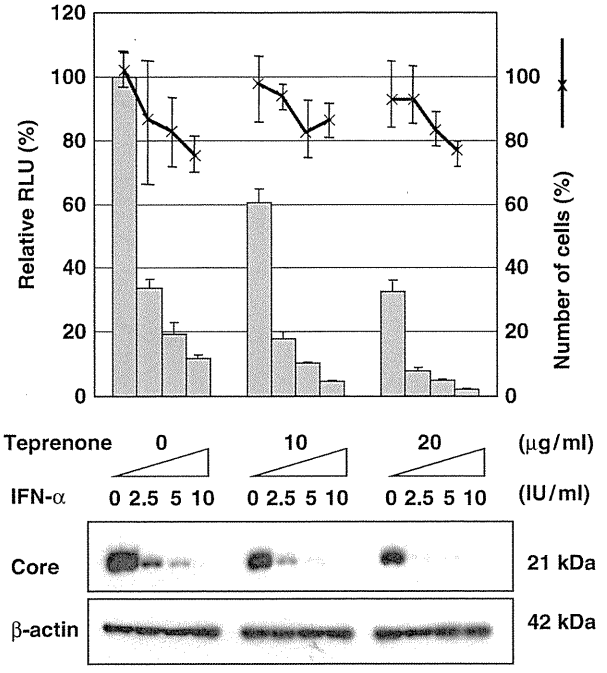

Fig. 4. Teprenone enhanced the anti-hepatitis C virus activity of interferon (IFN)-α. OR6 cells were cotreated with IFN-α (0, 2.5, 5 and 10 IU/ml) and teprenone (0, 10 and 20 μg/ml) for 72 h. *Renilla* luciferase assay was performed as described in Figure 2B. Production of the Core was analysed by immunoblotting using anti-Core antibody. The cells at 24, 48 and 72 h after treatment were subjected to a WST-1 cell proliferation assay.

affect cell proliferation within this concentration (Fig. 4). These results suggest that teprenone may be a new candidate as a complement to IFN therapy.

Teprenone exhibited anti-hepatitis C virus activity in the JFH-1 infection system

We examined the anti-HCV activity of teprenone in the JFH-1 infection system (13–15). We treated the cells with teprenone (0, 5, 10 and 20 μg/ml) at 24-h post-JFH-1 infection and cultured them for 72 h. The culture supernatants and cells were subjected to quantification of the Core by ELISA and western blot analysis respectively. Teprenone decreased the HCV Core in the supernatant (upper panel in Fig. 5A) and in the cells (lower panel in Fig. 5A) in a dose-dependent manner.

We next tested whether or not teprenone (0, 10 and 20 μg/ml) enhanced IFN-α's (0, 2.5 and 5 IU/ml) anti-HCV activity in the JFH-1 infection system. As shown in Figure 5B, teprenone enhanced the anti-HCV activity of IFN-α in a dose-dependent manner. These results suggest that teprenone also possessed anti-HCV activity in the JFH-1 infection system.

Teprenone did not inhibit geranylgeranylation

As shown in Figure 2A, the chemical structure of teprenone is similar to that of GGPP. Therefore, we examined the possibility that teprenone inhibits geranylgeranylation. Geranylgeranyl proteins possessed the C-A-A-X motif at the C-terminal of the protein: C is cystein; A is aliphatic amino acid; and X is typically leucine (or rarely isoleucine, valine or phenylalanine). Rap1A is one of the Ras-related proteins and selected to monitor the status of geranylgeranylation. We used anti-Rap1A antibody (sc-1482), which recognized only nongeranylgeranylated Rap1A (21, 22). Therefore, geranylgeranylated Rap1A is not recognized with this antibody. On the other hand, anti-Rap1 antibody (sc-65) recognizes Rap1A and Rap1B independent of the state of geranylgeranylation (22). In the following experiments, we used anti-Rap1A antibody (sc-1482) to monitor the state of geranylgeranylation.

OR6 cells were treated with PTV (1.25 μM) or teprenone (20 μg/ml) or neither. The cells were collected after treatment and subjected to luciferase assay and western blot analysis. In the untreated cells, nongeranylgeranylated Rap1A bands were not detected (Fig. 6A). PTV inhibited geranylgeranylation at 3 h and reached a plateau 12 h after treatment along with nongeranylgeranylated Rap1A bands (Fig. 6A). On the other hand, geranylgeranylation was not inhibited in the cells with teprenone treatment (Fig. 6A).

We then tested the effect of mevalonate cotreatment with PTV or teprenone. Mevalonate negated PTV's inhibitory action against geranylgeranylation and led to the loss of PTV's anti-HCV activity (Fig. 6B). However, mevalonate did not affect the anti-HCV activity of teprenone (Fig. 6B). These results indicate that teprenone

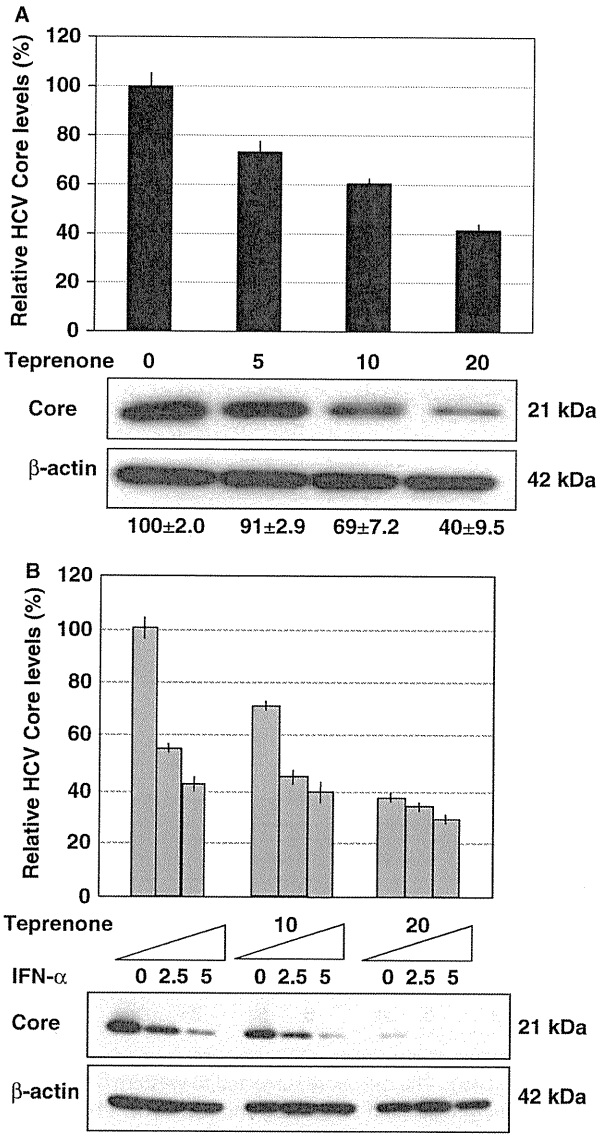

Fig. 5. Teprenone exhibited anti-hepatitis C virus (HCV) activity in the JFH-1 infection system. (A) Teprenone inhibited JFH-1 replication. HuH-7-derived RSc cells were infected with the JFH-1 virus for 24 h and were then treated with teprenone (0, 5, 10 and 20 μg/ml) for 72 h. The supernatant and the cells were subjected to quantification of the Core by ELISA and western blot analysis respectively. The signal intensities of Core were quantified by densitometry and the mean ± standard deviation is under the lower panel as shown in Figure 2B. (B) Teprenone enhanced interferon (IFN)-α's anti-HCV activity in the JFH-1 infection system. JFH-1 virus-infected cells were treated with teprenone (0, 10 and 20 μg/ml) and IFN-α (0, 2.5 and 5 IU/ml) for 72 h and then subjected to Core quantification by ELISA and western blot analysis as shown in (A).

inhibits HCV RNA replication without the inhibition of geranylgeranylation.

Statin's inhibition of HMG-CoA reductase decreased cholesterol synthesis and led to the increase of HMG-CoA reductase expression by positive feedback (3). The mRNA of HMG-CoA reductase was increased with PTV treatment but not with teprenone treatment (supporting information, Fig. S3A and B). This result suggests that teprenone, unlike PTV, did not lower the cholesterol synthesis.

The chemical structure of teprenone, which is the major component of Selbex, is similar to that of GGPP, a substrate for geranylgeranyltransferase. Therefore, we ruled out the possibility that teprenone was incorporated into host proteins instead of GGPP and led to the loss of function of the host proteins, when endogenous GGPP was depleted by PTV in OR6 cells. The nongeranylgeranylated Rap1A was detected when OR6 cells were treated with PTV (lane 3; Fig. 6C). However, exogenous GGPP decreased nongeranylgeranylated Rap1A in PTV-treated OR6 cells (lane 4; Fig. 6C). If teprenone was incorporated into Rap1A instead of GGPP and formed a psuedo-geranylgeranylation, Rap1A blotted with anti-Rap1A (sc-1482) would be decreased. Surprisingly, non-geranylgeranylated Rap1A increased in OR6 cells after treatment with PTV and teprenone (compare lanes 3 and 7 in Fig. 6C). Furthermore, it is noteworthy that the total amount of Rap1 was decreased when OR6 cells were treated with PTV and teprenone. These results suggest that teprenone was not incorporated into host protein and unexpectedly enhanced the statin's inhibitory action against geranylgeranylation.

Teprenone enhanced statins' inhibitory action against geranylgeranylation

To further investigate the unexpected results shown in Figure 6C, we tested the geranylgeranyl state and anti-HCV activity using the OR6 assay system. OR6 cells were treated with teprenone (0, 10 and 20 μg/ml) in combination with PTV (0, 0.25, 0.5 and 1.0 μM) for 72 h and subjected to western blot analysis for the geranylgeranyl state using anti-Rap1A (sc-1482) and anti-Rap1 (sc-65) antibodies, and for anti-HCV activity using anti-Core, anti-NS5A and anti-NS5B antibodies. Anti-HCV activity was also assessed by a luciferase reporter assay. Teprenone by itself did not inhibit geranylgeranylation (lanes 1–3; Fig. 7A). When teprenone was treated with PTV (0.25 μM), nongeranylgeranylated Rap1A increased in a dose-dependent manner (lanes 4–6; Fig. 7A). This result indicates that teprenone enhanced PTV's inhibitory action against geranylgeranylation in a dose-dependent manner. This effect of teprenone was also confirmed when PTV was treated at concentrations of 0.5 and 1.0 μM (lanes 7–12; Fig. 7A). HCV RNA replication and the expression of HCV proteins were decreased when nongeranylgeranylated Rap1As were increased. Next, we examined whether or not this function of teprenone is a common feature against statins. Teprenone enhanced the inhibitory action of ATV, SIV, FLV and LOV but not PRV against geranylgeranylation (lower panel in Fig. 7B). Teprenone also enhanced anti-HCV activity in combination with statins (upper panel in Fig. 7B). These results

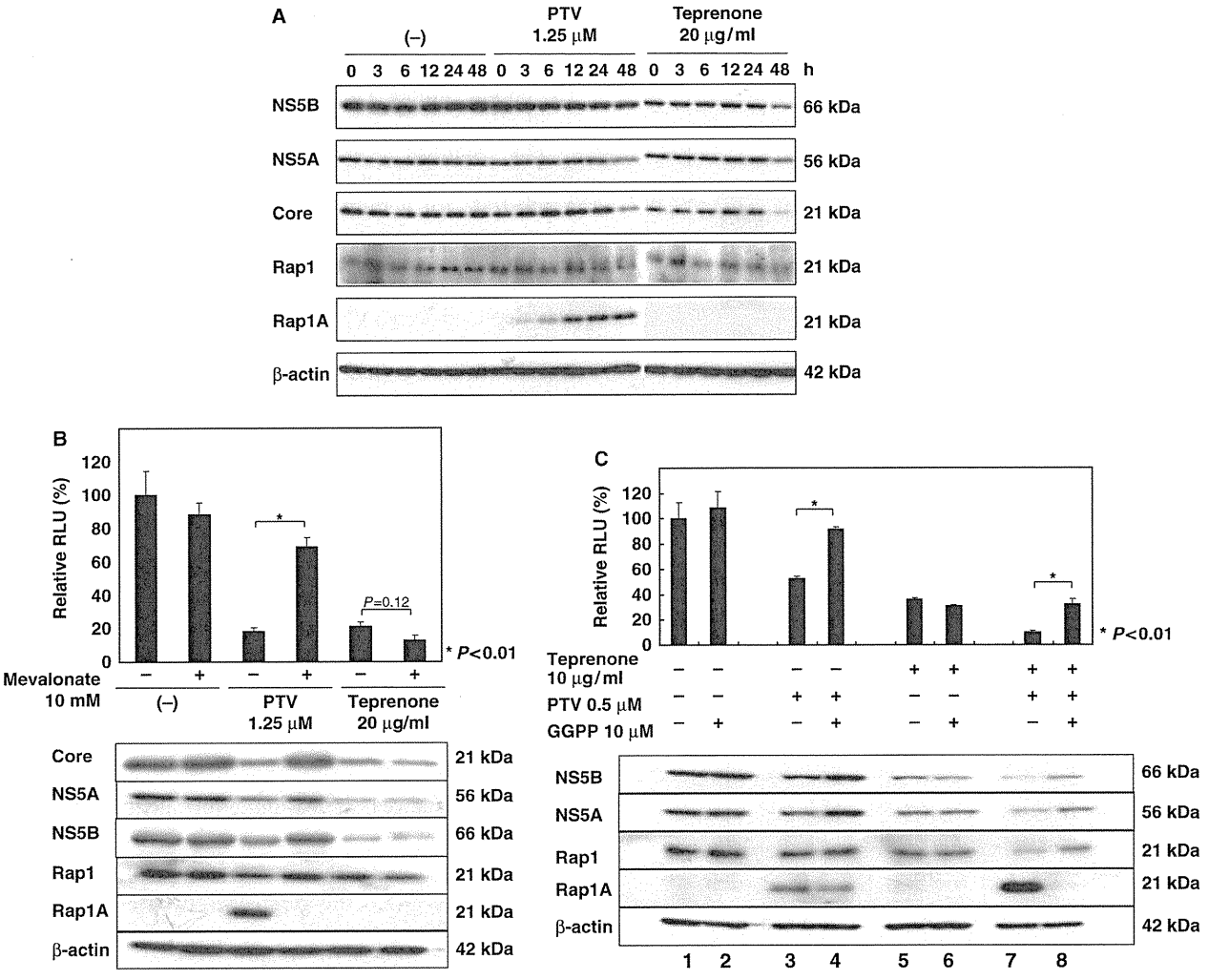

Fig. 6. Teprenone did not inhibit geranylgeranylation. (A) Teprenone did not inhibit geranylgeranylation. OR6 cells were treated with pitavastatin (PTV) (1.25 μM) or teprenone (20 μg/ml), or neither for 0, 3, 6, 12, 24 and 48 h. The cells were subjected to western blot analysis for HCV proteins using anti-NS5B, anti-NS5A and anti-Core antibodies, and for geranylgeranylation assay using anti-Rap1A (sc-1482) and anti-Rap1 (sc-65) antibodies. (B) Mevalonate did not affect the anti-HCV activity of teprenone. OR6 cells were treated with PTV (1.25 μM), teprenone (20 μg/ml) or neither in the absence or in the presence of mevalonate (10 mM) for 72 h. Then the cells were subjected to luciferase assay (upper panel) and western blot analysis using anti-Core, anti-NS5A, anti-NS5B, anti-Rap1A (sc-1482), anti-Rap1 (sc-65) and anti-β-actin antibodies (lower panel), as shown in (A). (C) Teprenone was not used as a substrate for GGT after the depletion of geranylgeranyl pyrophosphate (GGPP) by statin. OR6 cells were treated with teprenone (0 and 10 μg/ml), PTV (0 and 0.5 μM) and GGPP (0 and 10 μM) in the indicated combination for 72 h. Then the cells were subjected to luciferase assay (upper panel) and geranylgeranyl assay using anti-Rap1A (sc-1482) and anti-Rap1 (sc-65) antibodies (lower panel) as shown in (A).

suggest that teprenone enhances statins' inhibitory action against geranylgeranylation, except for PRV.

Discussion

In this study, we demonstrated that teprenone inhibited HCV RNA replication. Furthermore, teprenone exhibited anti-HCV activity in the genotype-2a JFH-1 infection system. Teprenone belongs to the geranyl compounds from its chemical structure and anti-ulcer agent from its clinical application. Therefore, we tested other geranyl compounds (GGOH and VK2, as well as geranylgeranoic acid) and other anti-ulcer agents (ecabet sodium, sofalcone and gefarnate) for their effect on HCV RNA replication. However, only teprenone exhibited anti-HCV activity among the reagents tested. Therefore, the anti-HCV activity of teprenone is a unique feature among these reagents.

The interview form from Selbex providing company Eisai reported the plasma concentration of teprenone. When 150 mg of Selbex was administered orally, its maximum plasma concentration reached 2.2 μg/ml. This is similar to the EC_{50} (5.3 μg/ml) of Selbex *in vitro*.

Ichikawa *et al.* (23) reported that teprenone induced the 2′,5′-oligoadenylate synthetases (2′5′-OAS) in

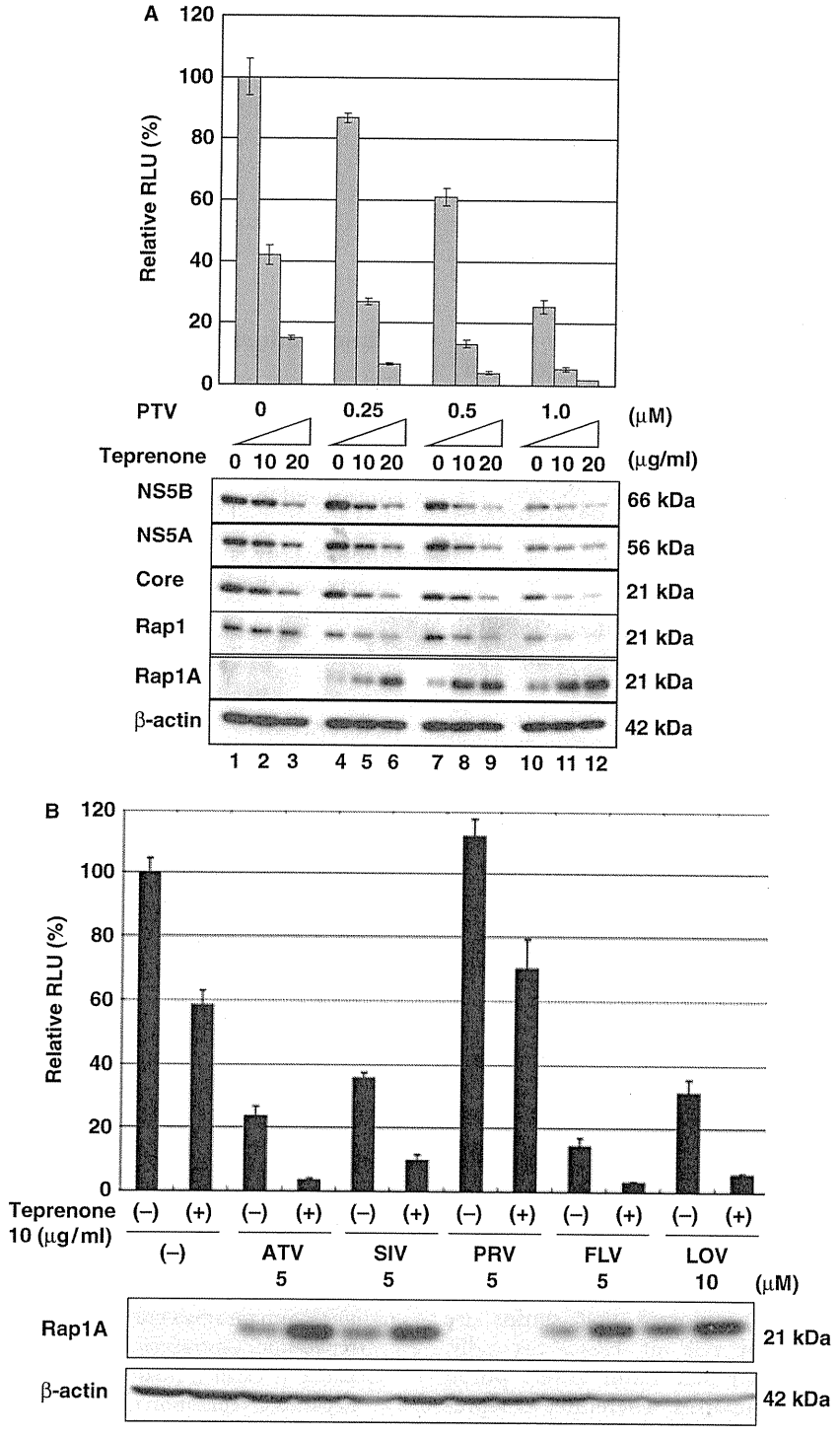

Fig. 7. Teprenone enhanced statins' inhibitory action against geranylgeranylation. (A) Teprenone enhanced pitavastatin (PTV)'s inhibitory action against geranylgeranylation. OR6 cells were treated with teprenone (0, 10 and 20 μg/ml) and PTV (0, 0.25, 0.5 and 1.0 μM) for 72 h. Then the cells were subjected to luciferase assay (upper panel) and western blot analysis using anti-NS5A, anti-Rap1A (sc-1482) and anti-Rap1 (sc-65), and anti-β-actin antibodies (lower panel), as shown in Figure 6A. (B) Teprenone enhanced statins' [except for pravastatin (PRV)] inhibitory action against geranylgeranylation. OR6 cells were treated with teprenone (0, 10 μg/ml) and atorvastatin (0, 5 μM), simvastatin (0, 5 μM), PRV (0, 5 μM), fluvastatin (0, 5 μM) and lovastatin (0, 10 μM) for 72 h. Then the cells were subjected to luciferase assay (upper panel) and western blot analysis using anti-Rap1A (sc-1482), and anti-β-actin antibodies (lower panel), as shown in Figure 6A.

human hepatoma cells. We demonstrated the activation of 2′5′-OAS and IFN-stimulated response element (ISRE) by IFN-α using the reporter assay system in our HuH-7-derived OR6 cells. However, we could not obtain evidence that teprenone activated both 2′5′-OAS and ISRE promoters (supporting information, Fig. S2A and B). Signal transducer and activator of transcription (STAT)1 and STAT2 were not phosphorylated after treatment with teprenone (supporting information, Fig. S2C). This discrepancy may have been caused by the heterogeneity of HuH-7 cells, because OR6 was selected as the clonal cell line and is highly susceptible to HCV RNA replication. Further study is needed to clarify the mechanism underlying teprenone's effect on IFN signalling.

Teprenone reportedly protects the gastric mucosa by inducing HSP (24). From this standpoint, the anti-HCV activity of teprenone was an unexpected result, because recently, it was reported that HSP90 is essential for HCV RNA replication and that an HSP90 inhibitor, geldanamycin, inhibits HCV RNA replication (25, 26). We examined whether or not teprenone induced HSP90 in hepatoma cells and found that it did not (supporting information, Fig. S4).

In this study, we monitored the geranylgeranylated state of Rap1A as a marker using nongeranylgeranylated Rap1A-detectable anti-Rap1A antibody (sc-1482). The least expected result of this sensitive geranylgeranylation assay is that teprenone enhanced statins' inhibitory action against geranylgeranylation. It is not clear in this study as to why teprenone enhanced statins' inhibitory action on geranylgeranylation. One possibility is that teprenone may cause biosynthesis from FPP to cholesterol rather than to GGPP by an unknown mechanism. To clarify this point, further study will be needed. This new function of teprenone may contribute to not only the antiviral field but also other fields, including studies on osteoporosis and on various kinds of antitumours, because geranylgeranylation and farnesylation are targets of the reagent in these fields. For example, statins interfere with the production of GGPP and FPP, which is important in the activation of small G proteins, such as K-ras and the Rho family, and disrupt the growth of malignant cells.

Recently, two important findings have been reported. Firstly, El-Serag *et al.* (8) reported that statins are associated with a reduced risk of HCC. Secondly, Abraldes *et al.* (9) reported that statin lowers portal pressure in patients with cirrhosis. Therefore, as teprenone is a strong adjuvant to statin's inhibitory action against geranylgeranylation, it may further improve portal hypertension in cirrhosis and reduce the risk of HCC in combination with statins. Although teprenone alone possesses modest anti-HCV activity, it will play a significant role in combination with IFN and/or statins in the therapy to HCV-associated liver diseases as an adjuvant like ribavirin. As teprenone is available in clinical use with a low side effect, a clinical study using teprenone in combination with IFN-α and/or statins is now underway in our institution.

In conclusion, we have shown that the anti-ulcer agent teprenone inhibited HCV RNA replication and enhanced statins' inhibitory action against geranylgeranylation. This newly discovered function of teprenone may contribute to improve the treatment of HCV-associated liver diseases (CH C, cirrhosis and HCC) as an adjuvant to statins.

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Supporting information

Additional supporting information may be found in the online version of this article:

Fig. S1. The effects of anti-ulcer agents on HCV RNA replication. (A) Cell proliferation assay. OR6 cells were treated with teprenone (0, 2.5, 5, 10, and 20 μg/ml), and the cells at 24, 48, and 72 hours after treatment were subjected to WST-1 cell proliferation assay. (B) Structures of ant-ulcer agents. (C-E) OR6 cells were treated with ecabet sodium (0, 2.5, 5, 10, 20 μg/ml) (C), sofalcon (0, 2.5, 5, 10, 20 μg/ml) (D), and gefarnate (0, 2.5, 5, 10, 20 μg/ml) (E) for 72 hours. Then the cells were subjected to luciferase assay (upper panel) and Western blot analysis using anti-core, and anti-β-actin antibodies (lower panel) as shown in Figure 1B.

Fig. S2. Teprenone didn't activate IFN signaling pathway. (A and B) Luciferase assays for 2'5'OAS and ISRE promoters. p2'5'OAS-luc (A) and pISRE-luc (B) transfected OR6c cells were treated with teprenone (0, 2.5, 5, and 10 μg/ml) or IFN-α (0, 2.5, 5, and 10 IU/ml) for 6 hours and then subjected to luciferase reporter assay. (C) Teprenone didn't activate STATs in OR6 cells. OR6 cells were treated with IFN-α (500 IU/ml), PTV (1.25 μM), and teprenone (20 μg/ml) for 0, 3, 6, and 12 hours. Then the cells were subjected to Western blot analysis using anti-pSTAT1 (Tyr701), anti-STAT1, anti-pSTAT2 (Tyr689), anti-core, and anti-β-actin antibodies.

Fig. S3. Teprenone treatment didn't cause positive feedback of HMG-CoA reductase (HMGCR). OR6c cells were treated with teprenone (20 μg/ml), PTV (10 μmol/L), or neither for 24 hours. The cells were subjected to RT-PCR (A) and real-time RT-quantitative PCR (B) using HMG-CoA reductase-specific primer set. H_2O was used as a negative control. GAPDH was used as an internal control.

Fig. S4. Teprenone didn't induce HSP90 or HSP70 in HuH-7 cells. OR6 cells were treated with teprenone (20 μg/ml) for 0, 3, 6, 12, 24, and 48 hours. Then the cells were subjected to Western blot analysis using anti-HSP90, anti-HSP70 anti-core, and anti-β-actin antibodies.

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RNA Polymerase Activity and Specific RNA Structure Are Required for Efficient HCV Replication in Cultured Cells

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Abstract

We have previously reported that the NS3 helicase (N3H) and NS5B-to-3′X (N5BX) regions are important for the efficient replication of hepatitis C virus (HCV) strain JFH-1 and viral production in HuH-7 cells. In the current study, we investigated the relationships between HCV genome replication, virus production, and the structure of N5BX. We found that the Q377R, A450S, S455N, R517K, and Y561F mutations in the NS5B region resulted in up-regulation of J6CF NS5B polymerase activity *in vitro*. However, the activation effects of these mutations on viral RNA replication and virus production with JFH-1 N3H appeared to differ. In the presence of the N3H region and 3′ untranslated region (UTR) of JFH-1, A450S, R517K, and Y561F together were sufficient to confer HCV genome replication activity and virus production ability to J6CF in cultured cells. Y561F was also involved in the kissing-loop interaction between SL3.2 in the NS5B region and SL2 in the 3′X region. We next analyzed the 3′ structure of HCV genome RNA. The shorter polyU/UC tracts of JFH-1 resulted in more efficient RNA replication than J6CF. Furthermore, 9458G in the JFH-1 variable region (VR) was responsible for RNA replication activity because of its RNA structures. In conclusion, N3H, high polymerase activity, enhanced kissing-loop interactions, and optimal viral RNA structure in the 3′UTR were required for J6CF replication in cultured cells.

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Introduction

Hepatitis C virus (HCV) contains a positive-stranded RNA genome and belongs to the *Flaviviridae* family [1]. Chronic HCV infection affects more than 130 million people worldwide [2]. The HCV RNA genome is approximately 9.6 kb in length and contains a long open reading frame that encodes a polyprotein of approximately 3,010 amino acids. This polyprotein is processed into at least 10 polypeptides by host and viral proteases [3,4]. The 5′-untranslated region (UTR) contains a highly conserved internal ribosome entry site (IRES) that is 341 nucleotides long [5]. The 3′UTR is known to contain a variable region (VR), a poly pyrimidine "U/C" (polyU/UC) tract, and a 98-base X-region (3′X tail) [6]. The second stem loop of the X region interacts with the NS5BSL3 cis-acting replication element (CRE) and may contribute to initiation of negative strand RNA synthesis [7].

JFH-1 belongs to genotype 2a and is the only strain that can efficiently replicate and produce virions in HuH-7 and HuH-7-derived cell lines [8,9,10]. When the structural protein-coding regions of the non-replicating HCV strains were fused to the non-structural protein-coding region and 3′UTR of JFH-1, replication was initiated and virions were produced in HuH-7-derived cells [10,11]. In order to analyze the mechanisms underlying the robust replication of JFH-1, we compared JFH-1 with J6CF. J6CF shares approximately 90% sequence homology with JFH-1 but does not replicate in HuH-7 cells. Analysis of JFH-1/J6CF chimeras demonstrated that the NS3 helicase-coding region (N3H) and the NS5B-to-3′X (N5BX) region of JFH-1 conferred replication activity to J6CF in HuH-7 cells [12]. Mutations in the N3H region are expected to affect helicase activity, while mutations in the NS5B-to-3′X region may affect polymerase and replication activity through secondary or higher order structures of the RNA. We have also previously reported that JFH-1-type mutations in the NS5B region enhanced genotype 1b RdRP activity *in vitro* [13]. Thus, JFH-1-type mutations in the NS5B region of J6CF are hypothesized to enhance J6CF RdRP activity. As mentioned above, the 3′UTR of the HCV genome consists of a VR, polyU/UC tracts of various lengths and a highly conserved 3′X tail. Deletion of the VR was reported to allow replication in both cultured cells [14] and in the chimpanzee [15]. The

Author Summary

Hepatitis C virus (HCV) is a major cause of chronic liver disease. Chronic HCV infection affects more than 130 million people worldwide. An efficient cell culture system is indispensable for HCV research and the development of antiviral strategies, including antiviral drugs and vaccines. Using one HCV strain, JFH-1, we have developed a novel cell culture system that, for the first time, has allowed for both the production of infectious HCV and the analysis of the HCV life cycle. To date, JFH-1 is the only HCV strain that replicates efficiently in cultured cells. Understanding the mechanisms underlying replication of JFH-1 in cultured cells is important and advantageous for the development of antiviral strategies. In the present study, we demonstrate that high polymerase activity, enhanced kissing-loop interactions between the NS5B and 3'X regions, and optimal viral RNA structure of the 3' UTR are required for the efficient replication of JFH-1 and viral production in cultured cells. Our data provides information that will prove essential for the establishment of replication-competent variants of HCV strains that are currently replication incompetent in cultured cells. This study also contributes to a better understanding of the mechanisms underlying persistent HCV infections.

minimum length of polyU/UC tract required for replication has also been previously determined [14,16].

In the current study, we examined RNA polymerase activity and the RNA structures of the NS5B and 3'UTR that contribute to HCV replication, and determined the essential domains required for robust HCV RNA replication in cultured cells.

Materials and Methods

Cell culture

HuH-7 cells [17] and Huh-7.5.1 cells [9] were cultured at 37°C in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum under 5% CO_2 conditions.

Construction of plasmids encoding a C-terminal 12xHis tagged HCV RdRP lacking 21 C-terminal amino acids

HCV JFH-1 and J6CF RdRP without the C-terminal 21 amino acid hydrophobic sequence were PCR amplified from pJFH1 [8] and pJ6CF (a kind gift from Jens Bukh) [15], respectively. Primer sequences for mutagenesis are listed in Table S1. Following digestion with *Xba*I and *Xho*I, DNA fragments were cloned into the *Nhe*I and *Xho*I sites of pET21b (Novagen, Madison, WI), resulting in pET21bHCVJFH-1RdRpwt and pET21bHCVJ6-CFRdRpwt. pET21bHCVJFH-1RdRpwt and pET21bHCVJ6-CFRdRpwt were then digested with *Xba*I and *Xho*I and the RdRP fragments cloned into the same restriction sites of pET28a, resulting in pET21(KM)JFH-1RdRpwt and pET21(KM)J6CFR-dRpwt, respectively.

Mutation analysis of J6CF and JFH-1 RdRP

JFH-1-type substitutions (S377R, A450S, S455N, R517K, and Y561F in the NS5B region; amino acid numbers are based on the AA relative numbering [18]) were introduced into J6CF RdRP and J6CF-like substitutions (S450A, N455S, K517R, F561Y, and F561I) and D318A were introduced into JFH-1 RdRP using the QuickChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Primer sequences for mutagenesis are listed in Table S1. Sequences were confirmed by nucleotide sequencing.

Expression, purification, and *in vitro* transcription of HCV RdRP

pET21(KM)JFH-1RdRPwt, pET21(KM)J6CFRdRPwt, and their mutants were expressed with pGEX-HSP90α [13] in *Escherichia coli* Rosetta/pLysS (Novagen). RdRP was then purified as previously described [13], with the exception that protein induction was undertaken at 18°C for 4 h. *In vitro de novo* transcription was performed as described previously [13]. Briefly, following 30 min pre-incubation without ATP, CTP, or UTP, 0.1 μM HCV RdRP was incubated in 50 mM Tris/HCl (pH 8.0), 200 mM monopotassium glutamate, 3.5 mM $MnCl_2$, 1 mM DTT, 0.5 mM GTP, 50 μM ATP, 50 μM CTP, 5 μM [α-^{32}P]UTP, 0.02 μM RNA template (SL12-1S) and 100 U/ml human placental RNase inhibitor at 29°C for 90 min. [^{32}P]-RNA products were subjected to PAGE (6% gel, 8 M urea). The resulting autoradiograph was analyzed with a Typhoon trio plus image analyzer (GE Healthcare, Piscataway, NJ). The radio isotope count of 184 nt RNA product of each mutant RdRPs was measured and compared to that of JFH-1 RdRP wt in the same PAGE.

Subgenomic-replicon constructs

pSGR-J6/N3H+5BSLX-JFH1/Luc was constructed by replacement of the 5BSL-to-3'X fragment (9211 to 9678 of JFH-1) generated by PCR with the corresponding fragment of pSGR-J6/N3H+3'UTR-JFH1/Luc [12]. Constructs with substitutions in NS5B region were generated as follows; mutations were introduced by PCR-based mutagenesis and *Xho*I-*Xba*I-restricted fragments were exchanged with the corresponding fragment of pSGR-J6/N3H+5BSLX-JFH1/Luc or pSGR-J6/N3H+3'UTR-JFH1/Luc [12]. To generate the constructs used for the analyses of the 3'UTR, VR fragments (9415–9479 of JFH-1 and J6CF) or polyU/UC fragments (9480–9579 of JFH-1 and 9480–9606 of J6CF) were generated by PCR and replaced with the corresponding fragment of pSGR-J6/N3H+5BSLX-JFH1/Luc. To generate the constructs with substitutions in the VR or 3'SL2, mutations were introduced by PCR-based mutagenesis and *Sgr*AI-*Xba*I-restricted fragments were exchanged with the corresponding fragment of pSGR-J6/N3H+5BSLX-JFH1/Luc. Primer sequences for mutagenesis are listed in Table S1.

Full-length genomic HCV constructs

Plasmids used in the analysis of genomic RNA replication and core production were constructed from pJ6/N3H+N5BX-JFH1 [12] and pJ6CF [15]. pJ6/N3H+5BSLX-JFH1 was constructed by replacement of the corresponding sequence with the 5BSL-to-3'X fragment (9211 to 9678 of JFH-1) generated by PCR. pJ6/N3H+3'UTR-JFH1 was constructed by using the N3H region [*Cla*I (3929) - *Eco*T22I (5293)] and 3'UTR [*Stu*I (9415) - *Xba*I (9678)] of JFH-1 to replace the corresponding sequences of pJ6CF. Mutagenesis was performed as described above.

RNA synthesis and transfection

RNA synthesis and transfection were performed as described previously [8,12]. Briefly, plasmids were linearized with *Xba*I, treated with Mung Bean Nuclease (New England Biolabs, Ipswich, MA) and purified. Linearized, purified DNA was then used as a template for *in vitro* RNA synthesis using the MEGAscript T7 kit (Ambion, Austin, TX) in accordance with the manufacturer's instructions. Synthesized RNA was treated with DNase I (Ambion) followed by purification using ISOGEN-LS (Nippon Gene, Tokyo, Japan). The quality of the synthesized RNA was examined via agarose gel electrophoresis. Ten micrograms of *in vitro*-