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Mizoribine inhibits hepatitis C virus RNA replication: Effect of combination with interferon-α

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Abstract

Interferon (IFN)-α monotherapy, as well as the more effective combination therapy of IFN-α and ribavirin, are currently used for patients with chronic hepatitis C caused by hepatitis C virus (HCV) infection, although the mechanisms of the antiviral effects of these reagents on HCV remain ambiguous, and side effects such as anemia due to the administration of ribavirin present a problem for patients who are advanced in years. Using a recently developed reporter assay system in which genome-length dicistronic HCV RNA encoding *Renilla* luciferase gene was found to replicate efficiently, we found that mizoribine, an imidazole nucleoside, inhibited HCV RNA replication. The anti-HCV activity of mizoribine (IC_{50}: approximately 100 μM) was similar to that of ribavirin. Using this genome-length HCV RNA replication monitor system, we were the first to demonstrate that the combination of IFN-α and ribavirin exhibited more effective anti-HCV activity than the use of IFN-α alone. Moreover, we found that the anti-HCV activity of mizoribine in co-treatment with IFN-α was at least equivalent to that of ribavirin. This effect was apparent in the presence of at least 5 μM mizoribine. Since mizoribine is currently used in several clinical applications and has not been associated with severe side effects, mizoribine is considered to be of potential use as a new anti-HCV reagent in combination with IFN-α.

Keywords: Hepatitis C virus; HuH-7; HCV RNA replication system; Ribavirin; Mizoribine; Interferon

Hepatitis C virus (HCV) is the major causative agent of chronic hepatitis (CH) [1,2], which progresses to liver cirrhosis and hepatocellular carcinoma [3,4]. Since at least 170 million people are currently infected with HCV worldwide, this infection is a global health problem [5]. HCV is an enveloped RNA virus belonging to *Flaviviridae*, the genome of which consists of a positive-stranded 9.6-kilobase (kb) RNA encoding an approximately 3000 amino acid polyprotein precursor [6,7]. This precursor protein is cleaved by the host and viral proteinases to generate at least 10 proteins in the following order: core, envelope 1 (E1), E2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B [8–10].

To date, interferon (IFN)-α (majority) and IFN-β (minority) are used as effective anti-HCV reagents in clinical therapy for patients with CH C; however, the effectiveness of IFN is limited to about 30% of the reported cases [11]. In 1998, combined treatment with IFN-α and ribavirin, a nucleoside analogue, has been shown to be more effective (although the effectiveness remains at less than 50%) than treatment with IFN alone [12,13]; nonetheless, it has been shown that ribavirin alone does not induce a decrease in HCV levels in patients with CH C. Furthermore, it has been reported that the combination of pegylated IFN with ribavirin led to significant improvements in terms of a sustained virological response, when compared to standard IFN and ribavirin combination therapy [14]. However, a sustained virological response is still not induced in approximately half of patients treated with these reagents.

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Moreover, the side effects of these reagents are also in some cases severe enough to lead to treatment cessation. Although the development of new effective anti-HCV reagents is urgently needed for the elimination of HCV from the human body, the lack of reproducible and efficient HCV proliferation in a cell culture has been a serious obstacle to the development of anti-HCV reagents [15].

As an efficient replication system of the HCV RNA genome in cell culture, an HCV replicon system carrying autonomously replicating HCV subgenomic RNA containing the NS3-NS5B regions derived from the strain Con-1 was first established in 1999 using a human hepatoma cell line, HuH-7 [16]. Since then, several additional replicon systems derived from the N, H77, 1B-1, and JFH-1 strains have been developed [17–20]. In addition, genome-length HCV RNA replication systems derived from the Con-1, N, and H77 strains have been also developed [17,21,22]. Moreover, in order to easily monitor the replication of HCV subgenomic RNA, several HCV replicons expressing the firefly luciferase reporter [23,24], β-lactamase reporter [25], or secreted alkaline phosphatase [26] have also been developed, although no convenient system of monitoring genome-length HCV RNA replication has been established to date. Therefore, the HCV subgenomic and genome-length RNA replication systems established thus far have been become powerful tools for the screening and evaluation of candidates for new anti-HCV reagents, including IFN and ribavirin [22–25,27].

We also previously established an HCV subgenomic replicon derived from the O strain (an older designation for this strain is 1B-2) [28]; we also recently developed a genome-length HCV RNA replication system derived from the O strain [29]. The characterization of genome-length HCV RNA replicating cells revealed the presence of an adaptive mutation (K1609E) in the NS3 helicase region [29]. Using this adaptive mutation, we established the first cell line (ORN/C-5B/KE) in which genome-length HCV RNA encoding the *Renilla* luciferase reporter gene replicated efficiently [29], and we developed a new convenient reporter assay system using ORN/C-5B/KE cells monitoring the replication of HCV RNA [29]. This reporter assay system demonstrated the usefulness of IFN-α's anti-HCV effect, since the values of *Renilla* luciferase correlated well with the level of HCV RNA after IFN treatment [29]. Therefore, this assay system is expected to become more useful for various studies of HCV than the HCV subgenomic replicon-based reporter assay systems [23–26] developed to date, because the older systems lack the core-NS2 regions containing structural proteins likely to be involved in the events that take place in the HCV-infected human liver.

Mizoribine is an imidazole nucleoside, which is isolated from culture medium of the mold *Eupenicillium brefeldianum* M-2166, and is structurally similar to ribavirin and acts as an immunosuppressant which exerts its effects without severe side effects [30].

In 1984, mizoribine was authorized by the Japanese Government as an immunosuppressive drug for renal transplantation, thereafter lupus nephritis, rheumatoid arthritis, and nephritic syndrome were also added to the list in 1990, 1992, and 1995, respectively [30,31]. Single use or combinatorial use of mizoribine with other immunosuppressive drugs including steroid, azathioprine, methotrexate, or cyclosporin has been accepted in clinical practice, because of good synergistic effects among them without any adverse effects [31]. On the other hand, mizoribine has been known to possess antiviral activities against influenza virus types A and B as in vitro effects [32]. Since it has been recently reported that mizoribine inhibited the replication of bovine viral diarrhea virus that shares a similar structural organization with HCV [33], we speculated that mizoribine possesses similar anti-HCV activity to that of ribavirin, as reported using HCV subgenomic replicon cells [24,34].

To evaluate whether or not mizoribine possesses anti-HCV activity, our monitoring system of genome-length HCV RNA replication was used. Here, we report the findings that not only ribavirin, but also mizoribine, inhibits HCV RNA replication and increases the anti-HCV activity of IFN-α.

Materials and methods

Cell cultures. ORN/C-5B/KE6 cells (designated as OR6 cells; Ikeda et al., in preparation), a cell line cloned from ORN/C-5B/KE cells [29] that supports genome-length HCV RNA encoding the luciferase reporter gene, were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum in the presence of G418 (300 μg/ml; Geneticine, Invitrogen). The OR6 cells were known to possess the G418-resistant phenotype, because neomycin phosphotransferase (Neo) was produced by the efficient intracellular replication of HCV RNA. Therefore, when HCV RNA was excluded from the cells, or when HCV RNA levels decreased, the cells were killed in the presence of G418.

Compounds. Mizoribine (4-carbamoyl-1-β-D-ribofuranosylimidazolium-5-olate) was kindly provided by the Asahi Kasei Pharma (Tokyo, Japan). Ribavirin (1-β-D-ribofuranosyl-1*H*-1,2,4-triazole-3-carboxiamide) was also kindly provided by the Yamasa (Chiba, Japan). The purities of both reagents exceeded 99%. Human IFN-α was purchased from Sigma–Aldrich (I-2396).

Northern blot analysis. Total RNAs from the cultured cells were extracted with the RNeasy extraction kit (Qiagen) and were quantified by spectrophotometry at 260 nm. Four micrograms of RNA was used for the detection of HCV RNA and β-actin with reagents included in the Northern Max kit (Ambion). Northern blotting and hybridization were performed as described previously [18,29]. An RNA Ladder (Invitrogen) was used to mark molecular length.

Western blot analysis. The preparation of cell lysates, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and immunoblotting analysis with a polyvinylidene difluoride membrane were performed as previously described [9]. The antibodies used to examine the expression levels of HCV proteins were those against the core (Institute of Immunology, Tokyo), E1 (a generous gift from

M. Kohara, Tokyo Metropolitan Institute of Medical Science), E2 [35], NS3 (Novocastra Laboratories, UK), and NS5B (a generous gift from M. Kohara, Tokyo Metropolitan Institute of Medical Science). Anti-β-actin antibody (AC-15, Sigma–Aldrich) was also used to detect β-actin as the internal control. Immunocomplexes on the membranes were detected by enhanced chemiluminescence assay (Renaissance; Perkin-Elmer Life Sciences, Wellesley, MA).

Antiviral assays. To monitor the antiviral effect of IFN-α, ribavirin, or mizoribine, OR6 cells were plated onto 24-well plates (1.5×10^4 cells per well) and cultured for 24 h. Then, the cells were treated with human IFN-α, ribavirin, or mizoribine at several concentrations for 24, 48, and 72 h, and the cells were also treated with combination of IFN-α and ribavirin or IFN-α and mizoribine at several concentrations for 72 h. After treatment, the cells were subjected to luciferase assay using the *Renilla* luciferase assay system (Promega). Briefly, after removal of the medium, the cells were washed twice with PBS. The cells were extracted with 100 μl of *Renilla* lysis reagent, and the relative luciferase unit value in 10 μl of lysates was measured by adding 50 μl of *Renilla* luciferase assay reagent according to the manufacturer's protocol. A manual Lumat LB 9501/16 luminometer (EG&G Berthold, Bad Wildbad, Germany) was used for the detection of luciferase activity.

Cell viability. To examine the cytotoxic effects of ribavirin and mizoribine on OR6 cells, the cells were seeded at a density of 4×10^5 cells per dish onto dishes with a diameter of 95 mm. After 24-h culture, the cells were treated with or without ribavirin or mizoribine at final concentrations of 50 and 100 μM for 72 h in the absence of G418. Then, the number of viable cells was counted in an improved Neubauer-type hemotocytometer after trypan blue dye (Invitrogen) treatment. In addition, in order to examine the β-actin levels in OR6 cells treated with ribavirin or mizoribine, the cells were seeded at a density of 1×10^5 cells per well onto six-well plates. After 24-h culture, the cells were treated with or without ribavirin or mizoribine at final concentrations of 12.5, 25, 50, and 100 μM for 72 h. Then, Western blot analysis was performed using anti-β-actin antibody as described above.

Results

Establishment of cloned cells in which genome-length HCV RNA encoding Renilla luciferase reporter gene replicates efficiently

Recently, we developed a dicistronic genome-length HCV RNA (O strain) replication system that stably expresses *Renilla* luciferase as a reporter in order to facilitate the monitoring of HCV replication [29]. The schematic organization of the genes of genome-length HCV RNA encoding the *Renilla* luciferase gene (ORN/C-5B/KE) is shown in Fig. 1A. Since this replication system consists of a polyclonal cell line in which ORN/C-5B/KE RNA replicates efficiently, we attempted to obtain a cloned cell line which exhibited more efficient and stable replication of ORN/C-5B/KE RNA. We thus obtained several cloned ORN/C-5B/KE cell lines supporting the efficient replication of genome-length HCV RNA, and we characterized these stable cell lines (Ikeda et al., in preparation). In this study, the OR6 cell line, one of the cloned cell lines, was used for the evaluation of the antiviral activity of IFN-α, ribavirin, and mizoribine, as described below. We first confirmed the presence of HCV RNA and HCV proteins in OR6 cells by Northern and Western blot analyses,

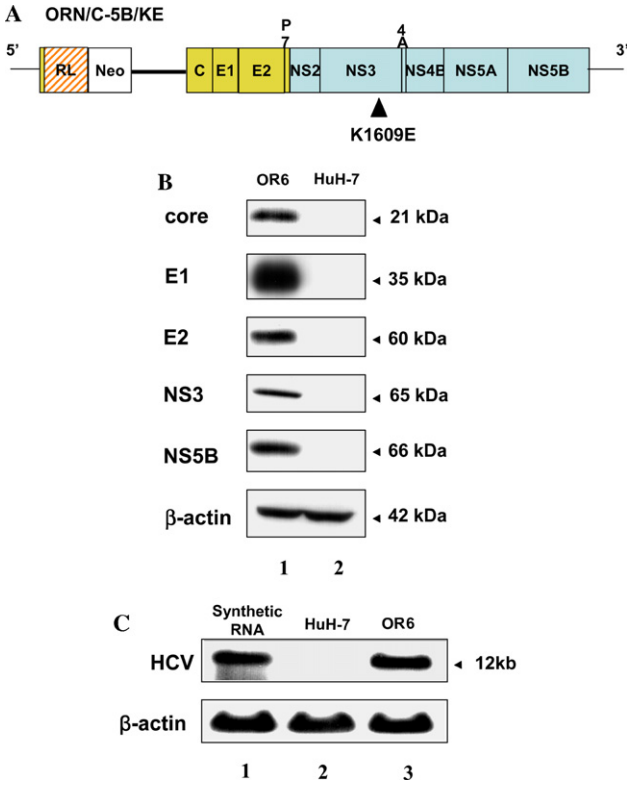

Fig. 1. Characterization of genome-length HCV RNA encoding *Renilla* luciferase gene as a reporter. (A) Schematic gene organization of genome-length HCV RNA encoding *Renilla* luciferase gene. *Renilla* luciferase gene (RL) is depicted as a striped box and is expressed as a fusion protein with Neo. The position of an adaptive mutation, K1609E, is indicated by a triangle. (B) Western blot analysis. Production of core, E1, E2, NS3, and NS5B in OR6 cells (lane 1) and HuH-7 cells (lane 2) were analyzed by immunoblotting using anti-core, anti-E1, anti-E2, anti-NS3, and anti-NS5B antibodies, respectively. β-Actin was used as a control for the amount of protein loaded per lane. (C) Northern blot analysis. Total RNAs from HuH-7 cells (lane 2) and OR6 cells (lane 3) were analyzed by Northern blot analysis using a positive-stranded HCV RNA-specific RNA probe (upper panel) and a β-actin-specific RNA probe (lower panel), respectively. In vitro-synthesized transcript of ORN/C-5B/KE (lane 1; 10^8 genome equivalents spiked into normal cellular RNA) was used for the comparison of expression levels.

respectively. Twelve kilobases of HCV-specific RNA (Fig. 1B), and core, E1, E2, NS3, and NS5B proteins (Fig. 1C) was clearly detected, indicating that genome-length HCV RNA efficiently replicates in OR6 cells.

IFN-α efficiently inhibited the replication of genome-length HCV RNA

Since it is well known that the HCV replicon [28,34,36,37] and replicable genome-length HCV RNA [29] are both highly sensitive to IFN-α, the extent of IFN sensitivity of genome-length HCV RNA (O strain) replication was first characterized by a luciferase assay system using OR6 cells. IFN-α treatments of several doses (final concentration: 1–40 IU/ml) were performed

using OR6 cells, and *Renilla* luciferase activity was measured as described under Materials and methods. The results clearly demonstrated that the luciferase activity had decreased in a dose- and time-dependent manner, when the cells were treated with more than 10 IU/ml IFN-α (Fig. 2A). The relative luciferase activity decreased less than 1% at 72 h after treatment with 20 IU/ml IFN-α. These results indicate that the replication of genome-length HCV RNA in the OR6 cells was also highly sensitive to IFN-α. However, interestingly, when the cells were treated with less than 4 IU/ml IFN-α, an IFN-α dose-dependent recovery of luciferase activity was observed at 48 or 72 h after IFN treatment (Fig. 2A), suggesting that such doses of IFN-α were insufficient for the complete abolishment of HCV RNA replication in OR6 cells. This phenomenon may be similar to recurrence in patients with CH C who receive IFN therapy. However, from another perspective, it was of note that this reporter assay was able to distinguish between effects caused by small differences in doses

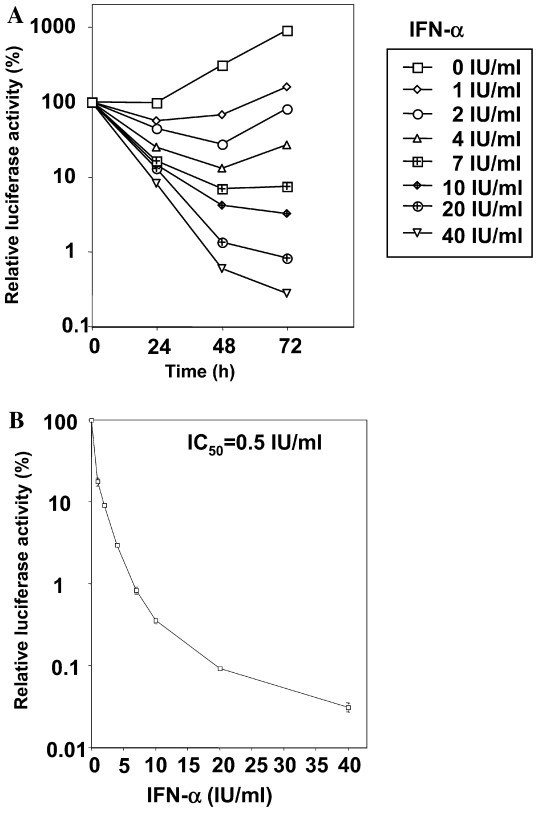

Fig. 2. Inhibition of HCV RNA replication in OR6 cells treated with IFN-α. (A) IFN-α sensitivity of HCV RNA replication in OR6 cells. The cells were treated with IFN-α (0, 1, 2, 4, 7, 10, 20, and 40 IU/ml), and at 24, 48, and 72 h after the treatment, and the *Renilla* luciferase assay was performed as described under Materials and methods. The relative luciferase activity (%) calculated at each point, when the luciferase activity of non-treated cells at 0 h was assigned to be 100%, is presented here. The data indicate means ± SD of triplicates from three independent experiments. (B) Dose–response curve of IFN-α. At 72 h after IFN-α treatment, a *Renilla* luciferase assay was performed.

of IFN (e.g., 1 and 2 IU/ml). Based on the dose–response curve at 72 h after treatment with IFN-α, the concentration of IFN-α required for a 50% reduction of luciferase activity (IC_{50}) was calculated to be approximately 0.5 IU/ml (Fig. 2B). This value was almost equal to the previous finding obtained using a HCV subgenomic replicon (N strain)-based luciferase reporter system [24], although the IC_{50} value in that study was obtained at 48 h after IFN treatment.

Ribavirin alone showed inhibitory effects on genome-length HCV RNA replication

Since OR6 cells were considered to be a reliable system for monitoring HCV RNA replication, we then evaluated whether or not ribavirin alone could inhibit the replication of genome-length HCV RNA in OR6 cells. First, luciferase activity was measured over a time course (up to 72 h) following treatment with or without ribavirin (25 and 50 μM). The results revealed a significant decrease in luciferase activity starting 48 h after treatment with ribavirin (Fig. 3A). The dose–response curve measured at 72 h after treatment with ribavirin (up to 200 μM) estimated that the IC_{50} value of ribavirin was 76 μM (Fig. 3B). We confirmed that ribavirin (up to 200 μM) did not inhibit *Renilla* luciferase activity in the reporter assay using HuH-7 cells transfected with pRL-CMV [38], which expresses *Renilla* luciferase under the control of cytomegalovirus promoter (data not shown). These results suggest that ribavirin alone can exert inhibitory effects against the replication of HCV RNA, although its effects were much weaker than those of IFN-α. The IC_{50} value of ribavirin in this study was slightly lower than that ($IC_{50} = 126$ μM) of a previous study using an HCV replicon (N strain)-based luciferase reporter system [24], although the IC_{50} value in that study was obtained at 48 h after treatment.

Mizoribine possessed similar anti-HCV activity to that of ribavirin

Since our assay system using OR6 cells demonstrated the anti-HCV activity of ribavirin alone, we next used our assay system to evaluate the effects of mizoribine, which is an imidazole nucleoside and is currently used to treat several diseases, but has not yet been applied for the treatment of patients with CH C. We found that luciferase activity clearly decreased starting 48 h after treatment with mizoribine (25 and 50 μM) (Fig. 4A). These findings were similar to those observed with ribavirin treatment. The dose–response curve measured at 72 h after treatment with mizoribine (up to 200 μM) estimated that the IC_{50} value of mizoribine was 99 μM (Fig. 4B). This value was slightly higher than that (76 μM) of ribavirin, as obtained by our assay system. We confirmed that mizoribine (up to 200 μM) also did not

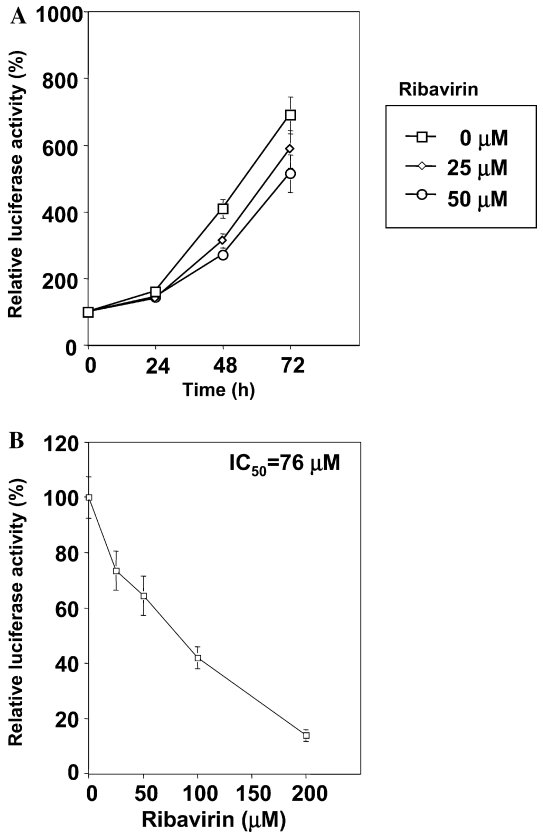

Fig. 3. Ribavirin alone inhibits HCV RNA replication in OR6 cells. (A) Inhibitory effect of ribavirin against HCV RNA replication. OR6 cells were treated with ribavirin (0, 25, and 50 μM), and at 24, 48, and 72 h after treatment, a *Renilla* luciferase assay was performed and the relative luciferase activity was calculated, as shown in Fig. 2. (B) Dose–response curve of ribavirin. At 72 h after ribavirin treatment, a *Renilla* luciferase assay was performed.

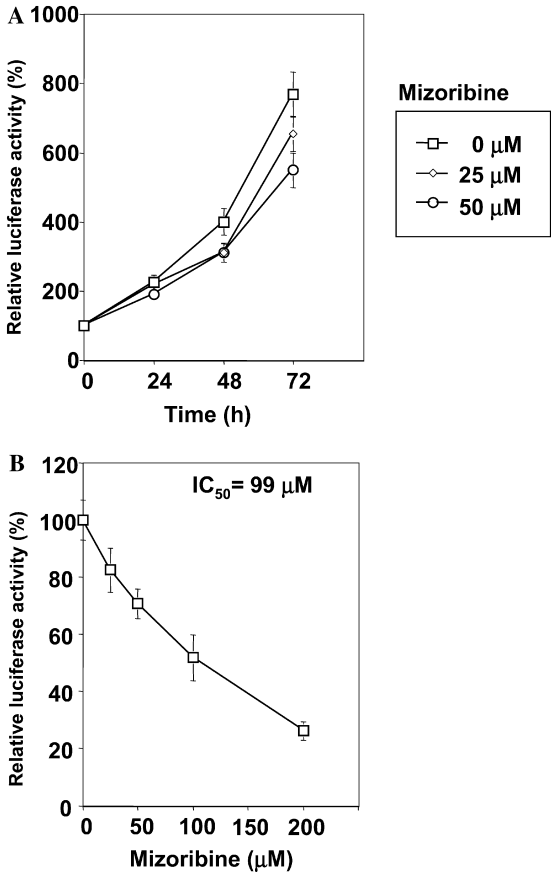

Fig. 4. Mizoribine alone also inhibits HCV RNA replication in OR6 cells. (A) Inhibitory effect of mizoribine against HCV RNA replication. OR6 cells were treated with mizoribine (0, 25, and 50 μM), and at 24, 48, and 72 h after treatment, a *Renilla* luciferase assay was performed and the relative luciferase activity was calculated, as shown in Fig. 2. (B) Dose–response curve of mizoribine. At 72 h after mizoribine treatment, a *Renilla* luciferase assay was performed.

inhibit *Renilla* luciferase activity in the reporter assay using HuH-7 cells transfected with pRL-CMV [38]. In summary, these results suggest that mizoribine alone also possesses the potential to suppress HCV RNA replication.

The anti-HCV activity of ribavirin and mizoribine was not found to be due to cytotoxicity

Since it has been reported that the proliferation of the HCV subgenomic replicon is dependent on host-cell growth [39], it remained to be clarified whether or not the inhibitory effects of ribavirin and mizoribine on HCV RNA replication were caused by their respective cytotoxicities. To examine this possibility, we investigated the cytotoxicities of ribavirin and mizoribine with respect to OR6 cells using two different approaches. First, we examined cell viability at 72 h after treatment with both reagents (50 and 100 μM each). When the number of cells without treatment was compared to that of cells with treatment, no significant decrease in cell number was observed following treatment with ribavirin or mizoribine (Fig. 5A). Second, we examined the amount of β-actin in OR6 cells treated with ribavirin or mizoribine (each up to 100 μM) by Western blot analysis. The results revealed that neither of these reagents led to a decrease in β-actin at concentrations up to 100 μM (Fig. 5B). These results indicated that neither ribavirin nor mizoribine (at least at concentrations $\leq$100 μM) showed cytotoxicity to the OR6 cells used in our assay system, which suggests that both reagents possess the ability to inhibit the replication of HCV RNA via specific antiviral mechanism(s).

Co-treatment of IFN-α and mizoribine effectively inhibited HCV RNA replication

Since it has been reported that the combination of IFN-α and ribavirin exhibits synergistic inhibitory effects on the HCV replicon [24], we examined the inhibitory effects of the combination of IFN-α and ribavirin

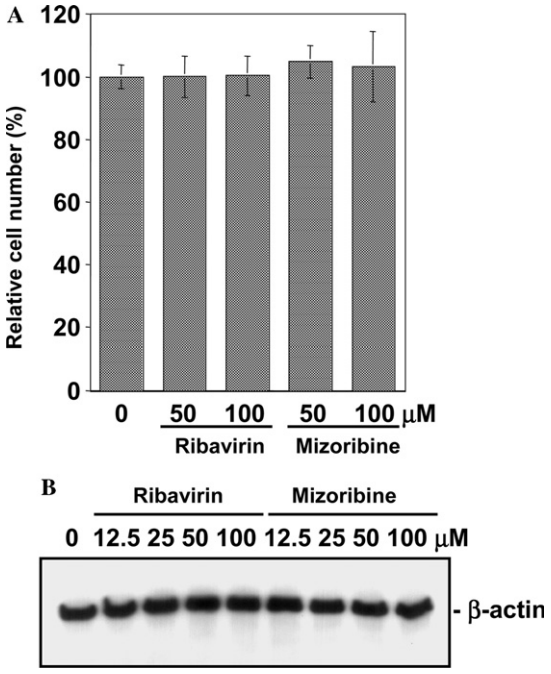

Fig. 5. No cytotoxicity of ribavirin or mizoribine in OR6 cells. (A) Cell viability after treatment with ribavirin or mizoribine. OR6 cells were cultured in the absence or presence of ribavirin or mizoribine (50 and 100 μM each) for 72 h, and then the cell number was determined as described under Materials and methods. The relative cell number (%) calculated at each point, when the cell number of non-treated cells was assigned to be 100%, is presented here. The data indicate means ± SD of triplicates from two independent experiments. (B) Western blot analysis for β-actin. OR6 cells were cultured in the absence or presence of ribavirin or mizoribine (12.5, 25, 50, and 100 μM each), and then the cells were subjected to Western blot analysis using anti-β-actin antibody.

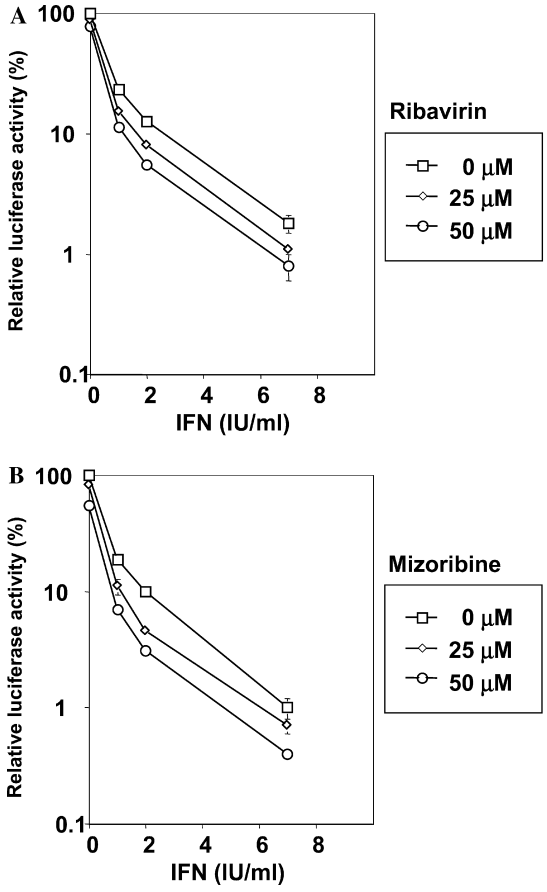

Fig. 6. Both ribavirin and mizoribine enhance the inhibition of HCV RNA replication due to IFN-α. (A) Effect of ribavirin in combination with IFN-α. OR6 cells were co-treated with IFN-α (0, 1, 2, and 7 IU/ml) and ribavirin (0, 25, and 50 μM), and at 72 h after treatment, a *Renilla* luciferase assay was performed and the relative luciferase activity was calculated, as shown in Fig. 2. The data indicate means ± SD of triplicates from two independent experiments. (B) Effect of mizoribine in combination with IFN-α. *Renilla* luciferase assay was performed as described in (A).

or mizoribine on genome-length HCV RNA replication. The dose–response curves of IFN-α (until 7 IU/ml) were obtained under each of the following fixed concentrations of ribavirin or mizoribine: 0, 25, and 50 μM. The results revealed that the curves shifted to the left with increasing concentrations of ribavirin (Fig. 6A) or mizoribine (Fig. 6B) treatment, indicating that co-treatment was more effective than treatment with IFN-α alone. Although the precise mechanism of such a clear effect of co-treatment remains unclear at present, the inhibitory effect of mizoribine (50 μM) appeared to be slightly stronger than that of ribavirin (Fig. 6). These results indicate that anti-HCV activity of mizoribine in co-treatment with IFN-α is at least equivalent to that of ribavirin, suggesting that mizoribine could be useful as a new anti-HCV reagent when in combination therapy with IFN-α.

Although we demonstrated that a 25-μM dose of ribavirin or mizoribine was effective for the inhibition of HCV RNA replication, the clinically achievable concentration of ribavirin has been estimated to be 10–14 μM [24,40]. To examine whether or not concentrations of less than 25 μM of ribavirin or mizoribine exert inhibitory effects on HCV RNA replication, we next obtained a low-dose (5–25 μM) response curve of ribavirin or mizoribine under the condition of a fixed concentration (2 IU/ml) of IFN-α. The results revealed a clear decrease in relative luciferase activity, even in the cells co-treated with 5 μM of ribavirin (Fig. 7A) or mizoribine (Fig. 7B), as compared with that of the cells treated with IFN-α alone. The inhibitory effect of mizoribine at concentration of less than 25 μM also appeared to be slightly stronger than that of ribavirin. It was of note that co-treatment with mizoribine at a dose of 25 μM showed a twofold enhancement of anti-HCV activity, compared with the effects of treatment with IFN-α alone (Fig. 7B); however, it should also be noted that only 20% of the inhibition was observed in the case of solo treatment with mizoribine at a dose of 25 μM (Fig. 4B).

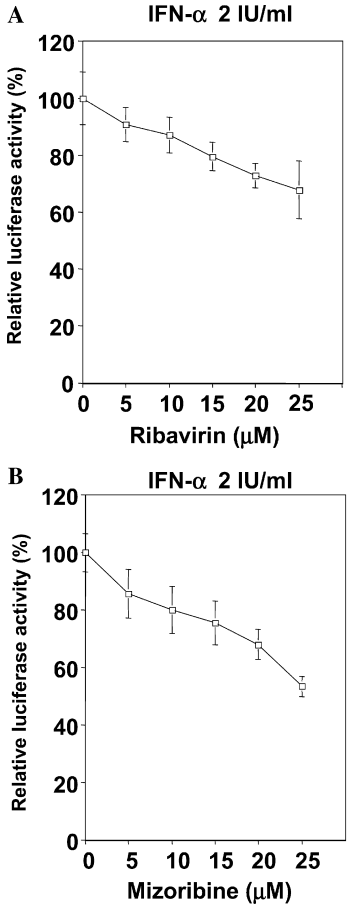

Fig. 7. Low-dose treatment of ribavirin or mizoribine is also effective for increasing the IFN-α inhibition of HCV RNA replication. (A) Effect of ribavirin in combination with IFN-α. OR6 cells were co-treated with ribavirin (0, 5, 10, 15, 20, and 25 μM) and a fixed concentration (2 IU/ml) of IFN-α, and a *Renilla* luciferase assay was performed at 72 h after treatment. The relative luciferase activity calculated at each point, when the luciferase activity of cells treated with IFN-α alone was assigned to be 100%, is presented here. The data indicate means ± SD of triplicates from three independent experiments. (B) Effect of mizoribine in combination with IFN-α. *Renilla* luciferase assay was performed as described in (A).

Discussion

In this study, a newly developed reporter assay system was employed in which genome-length HCV RNA efficiently replicates. Using this system, we demonstrated that ribavirin alone exerts a weak inhibitory effect on HCV RNA replication; however, this inhibitory effect was increased when ribavirin was used in combination with IFN-α. Furthermore, we found that mizoribine inhibited HCV RNA replication at a level equal to that achieved with ribavirin. Since mizoribine is currently used in several clinical treatments without inducing severe side effects, our findings suggest that mizoribine might not only be useful in combination therapy with IFN-α, it might even be used to replace ribavirin in combination therapy with IFN-α.

Since mizoribine, which is structurally similar to ribavirin, showed similar inhibitory effects to those of ribavirin with respect to HCV RNA replication, the anti-HCV activities of ribavirin and mizoribine are expected to be due to similar mechanism(s). However, the mechanism of ribavirin activity in patients with CH C remains poorly understood. To date, four possibilities have been proposed [41]: ribavirin (1) acts as an RNA mutagen that causes mutations of the HCV RNA genome and induces a so-called "error catastrophe"; (2) directly inhibits NS5B-encoded RNA-dependent RNA polymerase (RdRp); (3) enhances host T-cell mediated immunity by switching the T-cell phenotype from type 2 to type 1; and/or (4) inhibits the host enzyme inosine monophosphate dehydrogenase (IMPDH).

As regards the first possibility, several groups have demonstrated that ribavirin was able to induce an "error catastrophe" of the HCV genome [24,42–44]; however, controversial results have been reported to date [45,46]. This discrepancy between results may be due to differences in the concentrations of ribavirin used in these studies, i.e., ribavirin was used at concentrations of more than 100 μM in the former in vitro studies using HCV replicon systems, but in the latter in vitro study using an HCV replicon system [46], ribavirin was used at a concentration of 25 μM, and in the latter clinical study [45], the plasma concentration of ribavirin was estimated to be 10–14 μM [24,40]. In addition, we recently examined the inhibitory effects of mizoribine (25 μM) on the HCV replicon, but no signs of an "error catastrophe" were observed [46]. Taken together, these results suggest that an "error catastrophe" caused by ribavirin or mizoribine may not have contributed to the clearance of HCV RNA following combined treatment with IFN and ribavirin or mizoribine.

The second possibility, namely, that ribavirin or mizoribine directly inhibits the RdRp activity of NS5B, appears to be unlikely, because it has been previously demonstrated that ribavirin triphosphates at concentrations of up to 40 μM did not inhibit the RdRp activity of NS5B purified after expression in insect cells [47]. However, at higher concentrations (several hundreds of μM) of ribavirin triphosphates, NS5B catalyzed the incorporation of ribavirin opposite cytidine or uridine, and substantially the elongation of nascent RNA was blocked [48]. Therefore, ribavirin may weakly affect NS5B RdRp activity, although it is thought that this mechanism does not contribute to the antiviral activity of ribavirin observed in this study.

As regards the third possibility, i.e., that ribavirin can modulate cellular immunity by switching from type 2 to type 1, the possibility of the induction of IFN-γ in HCV RNA replicating cells treated with ribavirin or mizoribine is reasonable to consider, because the replication of HCV RNA has been reported to be very sensitive to IFN-γ [28,49]. However, since the production of

IFN-γ is well known to be restricted to T-cells and large granular lymphocytes alone [50], it is unlikely that IFN-γ is produced by ribavirin or mizoribine in hepatocyte-based HCV RNA replicating cells.

Since ribavirin and mizoribine are competitive inhibitors of IMPDH, the last possibility considered here would indicate that the inhibition of IMPDH is involved in the suppression of HCV RNA replication. However, it has been reported that other IMPDH inhibitors, namely, mycophenolic acid (MPA) and VX-497, showed only marginal antiviral effects on the HCV replicon system [43], although combination treatment with ribavirin and MPA or VX-497 enhanced anti-HCV replicon activity, and this enhancement was canceled by the addition of guanosine [43]. In addition, an additional observation that a 1,4,5-triazole derivative of ribavirin, an IMPDH inhibitor, was devoid of antiviral activity, has also been reported [41]. Although the present results suggest that IMPDH inhibition plays an important role in the anti-HCV activity of ribavirin, this antiviral activity is not completely accounted for by IMPDH inhibition. Therefore, further analysis will be necessary to clarify this point.

Although ribavirin and mizoribine showed apparent dose-dependent inhibitory effects against HCV RNA replication, their effective concentrations (IC_{50}: 76 and 99 μM for ribavirin and mizoribine, respectively) were higher than the clinically achievable ribavirin concentration (10–14 μM) reported previously [24,40]. However, when administered in combination with IFN-α, we demonstrated that a low dose (at least 5 μM) of mizoribine or ribavirin was able to enhance the anti-HCV activity of IFN-α; however, the precise mechanism of mizoribine or ribavirin activity in combination with IFN-α remains unclear. Therefore, in conclusion, the results of the present study suggest that mizoribine is a good reagent for combination therapy with IFN-α, and that mizoribine could be used to replace ribavirin when used in combination therapy with IFN-α.

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