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Inhibition of bovine viral diarrhea virus (BVDV) by mizoribine: synergistic effect of combination with interferon-α

Koichiro Yanagida[a,b], Chiaki Baba[a,c], Masanori Baba[a,*]

[a] *Division of Antiviral Chemotherapy, Center for Chronic Viral Diseases, Graduate School of Medical and Dental Sciences, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima 890-8544, Japan*
[b] *Planova Division, Asahi Kasei Pharma Corporation, Miyazaki 882-0847, Japan*
[c] *Department of Dermatology, Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima 890-8544, Japan*

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Abstract

Bovine viral diarrhea virus (BVDV) is a well-characterized member of the Flaviviridae family. BVDV may be a surrogate model for human hepatitis C virus (HCV), since HCV does not replicate efficiently in cell cultures and animals. Mizoribine, a nucleoside analog clinically used as an immunosuppressant, was found to be active against the replication of BVDV in cell culture. We further investigated the combination of mizoribine and interferon (IFN)-α for antiviral activity and found that the combination synergistically inhibited BVDV replication in bovine kidney cells, as monitored by the inhibition of virus-induced cytopathicity. The combination of ribavirin and IFN-α was also synergistic in inhibiting BVDV replication. Treatment of infected cells with a combination of mizoribine and IFN-α at the concentrations, at which the respective compounds proved to be inactive, markedly reduced viral infectivity in culture supernatants. These results indicate that mizoribine in combination with IFN-α may have potential for the treatment of HCV infection.

Keywords: BVDV; HCV; Mizoribine; Interferon; Combination

1. Introduction

Human hepatitis C virus (HCV) is an enveloped virus with a positive-sense RNA genome of approximately 9.6 kb. At present, at least 170 million people are chronically infected with HCV. HCV infection frequently causes chronic hepatitis, which often progresses to cirrhosis and hepatocellular carcinoma (Liang et al., 2000). Since a reliable cell culture system has not (yet) been established, the life cycle of HCV is largely unknown (Moriishi and Matsuura, 2003). No vaccine is available for HCV infection. Currently standard treatment for chronic hepatitis C consists of pegylated interferon (IFN)-α in combination with the nucleoside analogue ribavirin (1-β-D-ribo-furanosyl-1,2,4-triaxole-3-carboxamide). However, the virus cannot be eliminated from approximately half of the infected patients treated with these agents (McHutchison et al., 1998). In addition, side effects of these agents are sometimes serious problems. Therefore, alternative agents for the treatment of HCV infection are mandatory, yet the discovery and development of new anti-HCV agents seems to be extremely difficult due to the lack of an appropriate cell culture system.

HCV belongs to the family Flaviviridae, which encompasses three genera: hepacivirus, flavivirus, and pestivirus. Bovine viral diarrhea virus (BVDV), a member of the pestivirus genus and is associates with mucosal diseases in cattle. The virus contains a positive-sense RNA genome of approximately 12.6 kb. All members of the Flaviviridae share similarities in virion structure, genome organization, and their replication machinery. Since BVDV shares many important features with HCV, BVDV provides a surrogate model for HCV (Buckwold et al., 2003a,b), in particular, for molecular studies of viral proteins (Nam et al., 2001) and evaluation of antiviral compounds (Ouzounov et al., 2002; Stuyver et al., 2003; Buckwold et al., 2003a,b). Both HCV and BVDV

* Corresponding author. Tel.: +81 99 275 5930; fax: +81 99 275 5932.
E-mail address: baba@m.kufm.kagoshima-u.ac.jp (M. Baba).

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utilize an internal ribosomal entry site (IRES) within the 5′ nontranslated region (5′NTR) for translation of the viral polyprotein and express similar nonstructural proteins, including NS2, NS3, NS4A, NS4B, NS5A, and NS5B. Thus, antiviral agents active against BVDV would likely inhibit HCV replication.

The subgenomic HCV replicon cell culture system has recently been recognized as an available method for discovery of new anti-HCV agents (Larkin et al., 2003). For instance, the immunosuppressant cyclosporine A was found to have a strong inhibitory effect on HCV replicons in Huh-7 cells (Watashi et al., 2003). However, the replicons do not produce infectious virions. Therefore, they cannot be used for the identification and characterization of antiviral agents acting at early or late stages of the viral replication cycle, such as virion attachment, entry, uncoating, maturation, and release. Similarly, antiviral agents that reduce the infectivity of progeny virions cannot be identified in the replicon system (Buckwold et al., 2003a,b). Therefore, various systems including the anti-BVDV assay, are helpful in assessing promising candidates for the treatment of HCV infection.

Combination chemotherapy is often more effective than monotherapy in treating persistent and chronic viral infections. For instance, treatment of HCV infection using combinations of IFN-α and ribavirin generated a sustained response in 31–38% of the patients as determined by serum HCV RNA levels after 24 weeks (Reichard et al., 1998; Davis et al., 1998). In contrast, IFN-α monotherapy produced a similar response in only 18% of the patients. Ribavirin monotherapy showed only transient reduction of hepatic enzyme levels and no effect on serum HCV RNA levels (Bodenheimer et al., 1997; Reichard et al., 1998). It has recently been reported that the combination of IFN-α and the immunosuppressant cyclosporine A is more effective than IFN-α monotherapy (Inoue et al., 2003), which is based on the finding that cyclosporine A inhibited HCV protein expression in a subgenomic replicon system (Watashi et al., 2003). Furthermore, several antiviral studies in vitro using BVDV replication and HCV replicon systems have been carried out. The combination of IFN-α with *n*-butyl deoxynojirimycin showed synergistic inhibition of infectious BVDV production in vitro (Ouzounov et al., 2002). Combination of multiple drugs having synergistic antiviral activity would allow us to reduce the dose of each drug to achieve the same efficacy, expecting that their side effects could also be diminished.

Mizoribine is a nucleoside analogue that acts as an immunosuppressant without significant side effect. The compound has an antiproliferative activity against lymphocytes, yet it does not interfere with purine synthesis in other cell lines (Ishikawa, 1999). Mizoribine is known to have inhibitory effects on the replication of some DNA and RNA viruses, such as vaccinia virus (Mizuno et al., 1974), influenza virus types A and B (Hosoya et al., 1993), and herpes viruses in combination with acyclovir (Pancheva et al., 2002). However, mizoribine has previously been reported to have no detectable anti-BVDV activity (Stuyver et al., 2002). Although the antiviral mode of action of mizoribine remains to be elucidated, as for ribavirin, inhibition of inosine monophosphate dehydrogenase (IMPDH) could well be involved in the antiviral activity.

In this study, we have examined mizoribine alone or in the combination with IFN-α for their inhibitory effects on BVDV replication in cell cultures.

2. Materials and methods

2.1. Cell and virus

Madin–Darby bovine kidney (MDBK) cells (NBK-1, JCRB9028) were purchased from the Japan Health Sciences Foundation (Osaka, Japan). The cells were grown in Dulbecco's modified Eagle's medium (Gibco/BRL, Grand Island, NY) supplemented with 10% heat-inactivated horse serum (Gibco/BRL), 100 U/ml penicillin G, and 100 μg/ml streptomycin (culture medium). BVDV (nose strain) was purchased from Kyoto Biken (Kyoto, Japan). The virus was propagated in MDBK cells, and their culture supernatants were used as virus stocks. The stocks were titrated in MDBK cells and stored at −80 °C until use. The virus titer was expressed as a 50% cell culture infectious dose per ml ($CCID_{50}$/ml).

2.2. Compounds

Human IFN-α2b was purchased from PBL Biomedical Laboratories (New Brunswick, NJ). Mizoribine (4-carbamoyl-1-β-D-ribofuranosylimidazolium-5-olate) and ribavirin (1-β-D-ribofuranosyl-1*H*-1,2,4-triazole-3-carboxiamide) (Fig. 1) were synthesized in Asahi Kasei Pharma Corporation (Tokyo, Japan). The compounds were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 20 mM to avoid any antiviral and cytotoxic effects of DMSO. The stock solution was stored at −20 °C until use. IFN-α was stored at −80 °C.

2.3. Antiviral assays

MDBK cells (5×10^5 cells/well) were seeded in a six-well culture plates. After a 24-h incubation at 37 °C, the cells were

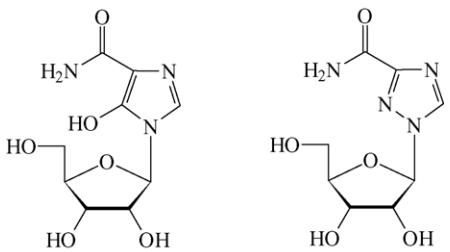

Fig. 1. Chemical structures of mizoribine and ribavirin.

infected with BVDV at a multiplicity of infection (MOI) of 0.03 and further incubated in fresh culture medium containing either IFN-α, mizoribine, or ribavirin for 24 h. After incubation, the medium containing the virus and compounds was removed. The cells were washed with phosphate-buffered saline (PBS) and incubated with culture medium containing 0.6% agar (overlay medium). Viral plaques were counted microscopically at 48 h after virus infection.

The antiviral activities of the compounds were also determined by the inhibition of virus-induced cytopathicity in MDBK cells. Briefly, the cells (2×10^4 cells/well) were infected with BVDV at a MOI of 0.03 and placed into a microtiter plate. Culture medium containing either mizoribine or ribavirin alone or in combination with IFN-α was added to each well of the plate and incubated at 37 °C. After a 72-h incubation, the number of viable cells was determined by a dye method using a water-soluble tetrazolium (TetraColor ONE^{TM}, Seikagaku Corporation, Tokyo, Japan), according to the Manufacturer's instructions. The cytotoxicity of the test compounds was evaluated in parallel with their antiviral activity. The assay was based on the inhibition of proliferation and viability of mock-infected MDBK cells, as determined by the tetrazolium method.

2.4. Viral yield reduction assay

MDBK cells (2×10^4 cells/well) were infected with BVDV at a MOI of 0.03 and placed into a microtiter plate. Culture medium containing either mizoribine or ribavirin alone or in combination with IFN-α was added to each well of the plate and incubated at 37 °C. After a 24-h incubation, culture supernatants were collected and examined for their infectivity. The infectivity was determined in MDBK cells and expressed as $CCID_{50}$/ml.

2.5. Synergy calculation

The multiple-drug effect was evaluated by the median-effect principle and the isobologram method (Belen'kii and Schinazi, 1994; Chou and Talalay, 1984). This method involves the conversion of dose–effect curves for each compound and for multiple diluted fixed-ratio combinations of compounds into the median effect plot. The slope and χ-intercept of the plot were used for calculation of the combination index (CI). CIs of <1, 1, and >1 indicate synergism, additive effect, and antagonism, respectively. All experiments were carried out in triplicate, and each experiment was repeated six times for determination of antiviral activities and CIs.

3. Results

3.1. Inhibition of plaque formation

Plaque reduction assays were carried out to evaluate the inhibitory effects of IFN-α, mizoribine, and ribavirin on BVDV replication. When MDBK cells (5×10^5 cells) were inoculated into each well of a six-well plate, the cells became 50–80% confluent monolayer after a 24-h incubation. This cell density appeared to be optimal for BVDV plaque formation, since the efficiency of plaque formation was found to be much lower in confluent monolayer cells (data not shown). Furthermore, neutral red used for staining cells was cytotoxic to MDBK cells. Therefore, the number of plaques was determined microscopically without neutral read staining in the present assay. As shown in Fig. 2, dose-dependent inhibition of plaque formation was observed with all compounds. The 50% effective concentration (EC_{50}) of IFN-α, mizoribine, and ribavirin in this experiment was 1.7 U/ml, 18 μM, and 0.69 μM, respectively. The 50% cytotoxic concentration (CC_{50}) of all compounds was greater than the highest concentrations tested for their antiviral activities. Although representative results are shown in Fig. 2, the assays were

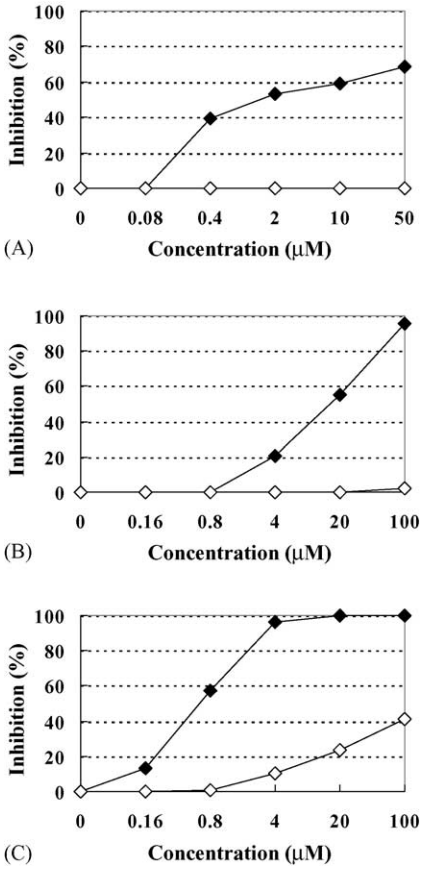

Fig. 2. Inhibition of BVDV plaque formation by IFN-α, mizoribine, and ribavirin. MDBK cells were infected with BVDV and incubated in the presence of IFN-α (A), mizoribine (B), or ribavirin (C) for another 24 h. After incubation, the medium containing the virus and compounds was removed, and the cells were washed and incubated with overlay medium. Viral plaques were counted microscopically at 48 h after virus infection. Cytotoxicity of the compounds was evaluated in parallel with their antiviral activity. It was based on the viability of mock-infected cells incubated in the presence of the compounds for 2 days. Filled and open squares represent the inhibition of BVDV replication and cell viability, respectively. Representative results are shown.

Table 1
Anti-BVDV activity and cytotoxicity of IFN-α, mizoribine and ribavirin in MDBK cells[a]

Compound	Plaque reduction assay		Cytopathicity inhibition assay	
	EC_{50}[b]	CC_{50}[c]	EC_{50}[b]	CC_{50}[c]
IFN-α (U/ml)	4.5 ± 3.7	>50	25 ± 14	>100
Mizoribine (μM)	14.2 ± 6.5	>100	5.3 ± 2.6	50.1 ± 6.9
Ribavirin (μM)	1.1 ± 0.4	>100	3.8 ± 1.6	11.8 ± 6.7

[a] All data represent means ± standard deviations for at least three separate experiments.
[b] Fifty percent effective concentration based on the reduction of BVDV plaque formation (plaque reduction assay) or the inhibition of virus-induced cytopathicity (cytopathicity inhibition assay).
[c] Fifty percent cytotoxicity concentration based on the reduction of cell viability in mock-infected cells after 2 and 3 days of compound exposure for plaque reduction assay and for cytopathicity inhibition assay, respectively.

repeated at least three times. Therefore, the means and standard deviations for the EC_{50} and CC_{50} values in the repeated experiments are shown in Table 1. These results indicate that IFN-α, mizoribine, and ribavirin are selective inhibitors of BVDV replication.

3.2. Combination of IFN-α with mizoribine or ribavirin

Combination antiviral activities of two compounds (IFN-α plus mizoribine or IFN-α plus ribavirin) were examined by the cytopathicity inhibition assay. Prior to the combination experiments, the optimal concentration ratio of two compounds (combination ratio) had to be determined. After preliminary experiments, three different ratios were chosen for each combination (data not shown). The ratios of IFN-α (U/ml) and mizoribine (μM) were 5:2, 5:1, and 10:1. The same ratios were also used for the combination of IFN-α and ribavirin. To exclude the cytotoxicity of test compounds in the combination experiments, the CC_{50} values of each compound alone and their combinations was determined. Here, the mock-infected cells were also exposed to the test compound for 72 h. The CC_{50} values of IFN-α alone, mizoribine alone, and ribavirin alone was >100 U/ml, 55 μM, and 13 μM, respectively. Furthermore, no cytotoxicity was observed for the combination of 40 U/ml IFN-α and 4 μM mizoribine (data not shown). On the other hand, the combination of 20 U/ml IFN-α and 4 μM ribavirin slightly reduced the viability of mock-infected MDBK cells (64% of the control). According to these observations, all combination experiments were carried out below the cytotoxic concentrations.

The EC_{50} of IFN-α alone, mizoribine alone, and ribavirin alone was 25 ± 14 U/ml, 5.3 ± 2.6 μM, and 3.8 ± 1.6 μM, respectively (Table 2). When the combinations of IFN-α and mizoribine were examined, the CIs of all combinations were less than 1.0, irrespective of the inhibition levels (Table 2). For instance, the CI of the 5:2 combination was 0.72 ± 0.24 at a levels of 50% inhibition, indicating synergism. At a level of 90% inhibition, synergism appeared to be more significant and the CI was 0.41 ± 0.25. A similar result was observed for the combination of IFN-α and mizoribine at different ratios. When the combinations of IFN-α and ribavirin were examined, the results were similar to those obtained in the combinations of IFN-α and mizoribine (Table 2). These results indicate that the combination of IFN-α and mizoribine synergistically inhibits BVDV replication and that the combination of IFN-α and ribavirin shows synergism as well.

Combination antiviral activities were also determined by virus yield reduction in culture supernatants. Since there was no significant difference in CIs among the different combination ratios in the cytopathicity inhibition assays, the ratio of IFN-α and either mizoribine or ribavirin was fixed (5:1) in the virus yield reduction assay. Mizoribine alone at 2 μM did not affect the infectivity of BVDV in culture supernatants after 24 h of viral infection (Fig. 3A). IFN-α alone at 10 U/ml reduced the infectivity to 63% of the control level. Although

Table 2
Anti-BVDV activities of IFN-α, mizoribine, ribavirin, and their combinations in MDBK cells[a]

Combination (ratio)	EC_{50}[b]	CC_{50}[c]	SI[d]	CI at the following percent inhibition[e]		
				50%	70%	90%
IFN-α (U/ml)	25 ± 14	>50	>4.0			
Mizoribine (μM)	5.3 ± 2.6	50.1 ± 6.9	9.5			
Ribavirin (μM)	3.8 ± 1.6	18.1 ± 6.7	4.8			
IFN-α + mizoribine						
(5:2)				0.72 ± 0.24	0.56 ± 0.24	0.41 ± 0.25
(5:1)				0.79 ± 0.26	0.63 ± 0.27	0.48 ± 0.28
(10:1)				0.66 ± 0.28	0.47 ± 0.21	0.29 ± 0.15
IFN-α + ribavirin						
(5:2)				0.67 ± 0.17	0.58 ± 0.21	0.50 ± 0.24
(5:1)				0.72 ± 0.30	0.61 ± 0.36	0.52 ± 0.39
(10:1)				0.81 ± 0.28	0.63 ± 0.29	0.48 ± 0.31

[a] All data represent means ± standard deviations for six separate experiments, except for cytotoxicity experiments.
[b] Fifty percent effective concentration based on the inhibition of BVDV-induced cytopathicity.
[c] Fifty percent cytotoxicity concentration based on the reduction of cell viability.
[d] Selectivity index (CC_{50}/EC_{50}).
[e] Combination index giving 50, 70, or 90% inhibition under the mutually exclusive assumption.

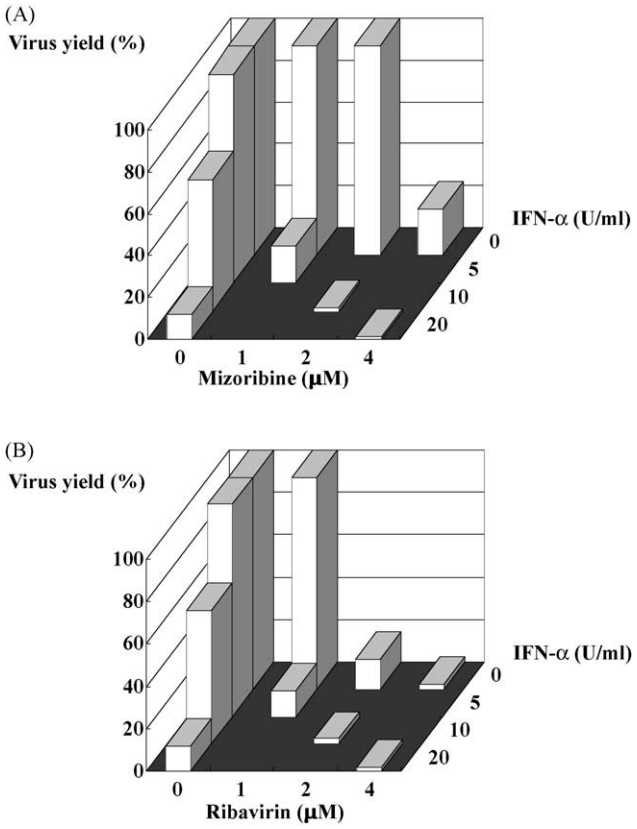

Fig. 3. Inhibition of BVDV production by IFN-α, mizoribine, ribavirin, and their combinations. MDBK cells were infected with BVDV and incubated in the presence of the test compounds. After a 24-h incubation, culture supernatants were collected and examined for their infectivity. The infectivity (virus yield) is expressed as a ratio of $CCID_{50}$ of each combination to that of the control (no compound). The infectivity of the control culture was 4.6×10^4 $CCID_{50}$/ml. Results of experiments, in which a 5:1 ratio of the compounds was used, are displayed.

the effect of IFN-α alone or mizoribine alone at these concentrations was marginal, the combination of IFN-α and mizoribine at these concentrations resulted in strong inhibition of viral production in culture supernatants. The virus titer was found to be only 1.8% of the control level (Fig. 3A). Different from mizoribine, ribavirin alone at 2 μM could reduce the viral infectivity to 14% of the control level. However, the combination of 10 U/ml IFN-α and 2 μM ribavirin achieved greater reduction of the infectivity (2.5% of the control level) than that expected (9.1%), indicating that the combination was also synergistic.

4. Discussion

In this study, we demonstrated that the combinations of IFN-α and either mizoribine or ribavirin synergistically inhibited BVDV replication. Prior to the combination experiments, a number of compounds have been screened for their inhibitory effects on BVDV replication in the plaque reduction assay, since this method has been commonly utilized. Among the compounds, only mizoribine and the reference compound ribavirin were found to be active (data not shown). Mizoribine did not show apparent cytotoxicity to mock-infected MDBK cells at the highest concentration examined. Although ribavirin appeared to be more cytotoxic than mizoribine, its CC_{50} was well above the EC_{50} for BVDV replication. These results indicate that both mizoribine and ribavirin are selective inhibitor of BVDV replication in cell culture.

In the cytopathicity inhibition assay, the antiviral activity of mizoribine seemed to be different from that obtained in the plaque reduction assay (data not shown). Furthermore, Stuyver et al. (2002) reported that mizoribine had no detectable anti-BVDV activity. A possible explanation for this discrepancy may be the differences in the assay conditions for antiviral activity and cytotoxicity, such as a state of MDBK cells (confluent or not), an incubation period with compounds, the parameter of viral replication (plaque formation versus cell death), etc. It could not be excluded that the stability or phosphorylation of mizoribine would be affected by experimental conditions.

The reason that the cytopathicity inhibition assay was applied for our combination experiments is the followings. (1) The cytopathicity inhibition assay allows the testing of a considerable number of samples at the same time. (2) The plaque reduction assay needs overlay medium, which is particularly cumbersome in combination experiments. (3) Counting the number of plaques under a microscope is not sufficiently objective.

It has been well documented that IFN-α has antiviral, antiproliferative, and immunomoduratory properties (Goodbourn et al., 2000; Brassard et al., 2002). IFN-α elicits an antiviral state in infected cells through downstream mediators, such as 2′,5′-oligoadenylate synthetase, RNA-activated protein kinase (PKR), and 2′-phosphodiesterase. It also stimulates immune function through natural killer (NK) and T cell activation (indirect functions). The EC_{50} of IFN-α against BVDV in our plaque reduction assay and cytopathicity assay was 1.7 and 25 U/ml, respectively. These results coincide well with those previously reported: 3 IU/ml in plaque reduction assay (Ouzounov et al., 2002) and 16 IU/ml in cytopathicity inhibition assay (Buckwold et al., 2003a, b), although a different BVDV strain, NADL, was used in these studies. Furthermore, it was reported that IFN-α had an EC_{50} of 1.95 IU/ml in a HCV replicon system (Larkin et al., 2003), which is also close to our data in BVDV plaque reduction assay.

Ribavirin, an IMPDH inhibitor, has been shown to have antiviral activities against a number of RNA viruses, nevertheless, its activity against some flaviviruses such as Yellow fever virus and Dengue virus is weak (Leyssen et al., 2000). In addition, a recent report on the antiviral activity of ribavirin in a HCV replicon system indicated weak suppression of HCV replication (Tanabe et al., 2004). Ribavirin may be assumed to inhibit viral RNA transcription, elongation, and cap formation. It has been reported that ribavirin induces “error catastrophe” in poliovirus (Crotty et al., 2001).

Ribavirin also becomes a pseudo-substrate of HCV RNA-dependent RNA polymerase (Maag et al., 2001), increases mutations in HCV RNA in the binary T7 polymerase/HCV cDNA replication system (Contreras et al., 2002), and reduces HCV replicon colony-forming efficiency (Zhou et al., 2003). Mizoribine is a nucleoside analog isolated from the mold *Eupenicillium brefeldianum*. The compound acts as an immunosuppressant and is inhibitory to both humoral and cellular immunity. Mizoribine has been used for the prevention of rejection in renal transplantation and the treatment of lupus nephritis, rheumatoid arthritis, and nephritic syndrome (Ishikawa, 1999). In terms of antiviral activity, mizoribine is inhibitory to influenza virus types A and B. However, the EC_{50} of mizoribine against influenza viruses varied considerably from one strain to another (Hosoya et al., 1993). Although the antiviral mechanisms of action of mizoribine remain to be elucidated, its anti-BVDV activity and cytotoxicity were weakened but not completely annihilated by exogenous deoxyguanosine (data not shown), suggesting partial involvement of IMPDH.

Combination chemotherapy may be able to solve some problems in IFN-α treatment, IFN-α at a high dose is often associated with unwanted side effects, such as fatigue, malaise, and myalgias (Liang et al., 2000). The application of combination chemotherapy for HCV infection has been actively investigated in recent years. The combination of IFN-α and cyclosporin A in chronic hepatitis C patients was shown to be more effective than IFN-α monotherapy (Inoue et al., 2003). In this point of view, the present findings may suggest that, in combination with IFN-α, mizoribine may have potential for the treatment of HCV infections.

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