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# Novel Anticytomegalovirus Activity of Immunosuppressant Mizoribine and Its Synergism with Ganciclovir

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## ABSTRACT

Cytomegalovirus (CMV) infection is a prominent infection in transplant recipients. The immunosuppressive drug mizoribine was shown to have anti-CMV activity in vitro and was reported to have an anti-CMV effect in renal transplantation. This study characterized the anti-CMV activity of mizoribine in vitro and its synergistic activity with ganciclovir. Mizoribine suppressed replication and at the $EC_{50}$ for plaque inhibition of 12.0 $\mu$g/ml. Mizoribine and ganciclovir exerted a strong synergism in anti-CMV activity. Mizoribine depletes guanosine nucleotides by inhibiting inosine monophosphate dehydrogenase and may increase the ratio of ganciclovir to guanosine in treated cells, resulting in a strong synergistic augmentation of the anti-CMV activity of ganciclovir. Two clinical isolates with UL97 mutations were less susceptible to mizoribine than the Towne strain but were equally susceptible in the presence of guanine. Two mizoribine-resistant strains were isolated after culture for 3 months with 100 $\mu$g/ml mizoribine, but they were as sensitive to ganciclovir as the parent Towne strain. The anti-CMV activity of mizoribine was antagonized by 2′-deoxyguanosine. Mizoribine inhibited CMV replication directly, and the sequence of mizoribine-resistant mutants of UL97 and UL54 was identical to that of the parent Towne strain, indicating the different anti-CMV action from ganciclovir, foscarnet, and maribavir. Mizoribine as an immunosuppressive and anti-CMV drug in the clinical regimen was suggested to suppress replication of CMV in vivo and control CMV infection in transplant recipients in combination with ganciclovir.

Mycophenolic acid and mizoribine (Bredinin; Asahi Kasei Pharma, Tokyo, Japan) are immunosuppressants that specifically inhibit the rate-limiting enzyme inosine monophosphate dehydrogenase (IMPDH) in the de novo pathway of purine biosynthesis, thereby depleting guanosine nucleotides (Allison, 2000). Because T and B cells use the de novo pathway almost exclusively, these drugs specifically inhibit the action of these lymphocytes and have been used as immunosuppressants. Mycophenolic acid (Fig. 1) was isolated by Gosio (1896) from corn broth cultures containing *Penicillium* species and was shown to inhibit IMPDH efficaciously in the treatment of psoriasis (Epinette et al., 1987). A prodrug of mycophenolic acid, mycophenolate mofetil (CellCept; F. Hoffmann-La Roche AG, Basel, Switzerland), is now approved for use in many countries to prevent allograft rejection and treat ongoing rejection (European Mycophenolate Mofetil Cooperative Study Group, 1995; Sollinger, 1995; Takahashi et al., 1995; Gonwa, 1996; The Tricontinental Mycophenolate Mofetil Renal Transplantation Study Group, 1996; Mele and Halloran, 2000). Mizoribine (Fig. 1) was isolated from the culture medium of *Eupenicillium brefeldianum* and phosphorylated by adenosine kinase to convert it to its active form, mizoribine-5′-phosphate. Both mizoribine and mycophenolic acid specifically inhibit IMPDH in the de novo pathway of guanosine biosynthesis and thereby deplete guanosine nucleotides in lymphocytes, resulting in inhibition of the actions of T and B lymphocytes and immunosuppression (Koyama and Tsuji, 1983; Turka et al., 1991; Dayton et al., 1992; Catapano et al., 1995; Meredith et al., 1997; Metz et al., 2001).

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**ABBREVIATIONS:** IMPDH, inosine monophosphate dehydrogenase; CMV, cytomegalovirus; HEL, human embryonic lung; HSV, herpes simplex virus; rGMP, guanosine monophosphate.

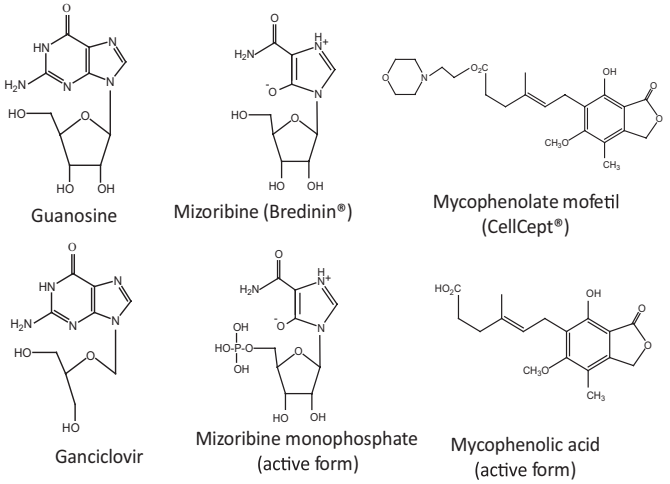


**Fig. 1.** Structure of guanosine, ganciclovir, mizoribine, and its monophosphate, mycophenolate mofetil, and mycophenolic acid.

Use of mizoribine has been approved in Japan for induction and maintenance of immunosuppressive therapy after renal transplantation (Takahashi et al., 1995; Tsuzuki, 2002; Akiyama et al., 2005). Mycophenolate mofetil and mizoribine are used in combination with calcineurin inhibitors (cyclosporine or tacrolimus) and glucocorticosteroids after renal transplantation.

Cytomegalovirus (CMV) infection is one of the major complications after renal transplantation, ranging from asymptomatic viral shedding to life-threatening disease, and it increases patient morbidity and mortality. Mizoribine has anti-CMV activity in vitro (Shiraki et al., 1990). Clinical observation of CMV infection in renal transplant patients maintained on mizoribine (40 recipients) or mycophenolic mofetil (38 patients) showed that recipients maintained on mizoribine had significantly fewer incidences of CMV disease (0 versus 18.4%) than those maintained on mycophenolic mofetil without affecting graft survival (N. Yoshimura, H. Ushigome, K. Akioka, S. Nobori, M. Fujiki, K. Kozaki, T. Suzuki, K. Sakai, and M. Okamoto, submitted for publication).

Based on the clinical observations, we further characterized anti-CMV activity by isolating two mizoribine-resistant viruses and analyzing the interaction of mizoribine and ganciclovir (Cytovene; F. Hoffmann-La Roche AG) or the effect of guanine and guanosine on anti-CMV activity and found that mizoribine inhibited CMV directly in a novel manner and exhibited strong synergism in combination with ganciclovir.

## Materials and Methods

**Cells and Viruses.** Human embryonic lung (HEL) cells were grown and maintained in Eagle's minimum essential medium supplemented with 10 and 2% fetal bovine serum, respectively. A Towne strain (Plotkin et al., 1975) was provided by Dr. Furukawa (Kanazawa Medical University, Kanazawa, Japan) and used as a parent strain for isolating mizoribine-resistant mutants. Two clinical isolates resistant to ganciclovir were used. A patient with myelodysplastic syndrome developed persistent CMV antigenemia after hematopoietic stem cell transplantation, and CMV was isolated after treatment with ganciclovir and foscarnet. CMV was resistant to ganciclovir and foscarnet but sensitive to cidofovir and had mutations in UL97 (A594V) and UL54 (Q578H) (Oshima et al., 2008). This UL97 mutant was used as UL97 mutant A. A patient with pulmonary lymphangiomyomatosis developed persistent CMV antigenemia after lung transplantation, and CMV was isolated from the peripheral blood mononuclear cells after treatment with ganciclovir. This isolate was resistant to ganciclovir and used as UL97 mutant B with a mutation in UL97 (L595S). These UL97 mutants were used to examine the susceptibility to mizoribine. Mizoribine was supplied by Asahi Kasei Pharma and dissolved in water at the concentration of 5 mg/ml as a stock solution. The procedures used to determine the effects of drugs on CMV growth were reported previously (Shiraki et al., 1990, 1991a,b,c; Yukawa et al., 1996).

Intracellular virus production was assessed by one-step growth in cells treated with drugs as follows. HEL cells in 25-$cm^2$ plastic flasks were infected with 2 plaque-forming units/cell of CMV for 1 h. The cells were washed three times with maintenance medium and incubated in this medium containing the indicated concentrations of drugs for 3 days. Then the cells were washed three times, changed to 5 ml of fresh maintenance medium, frozen and thawed three times, and centrifuged at 3000 rpm for 10 min. Serially diluted samples of the supernatants were inoculated onto HEL monolayers in 60-mm plastic dishes and overlaid with nutrient methylcellulose medium. After incubation for 10 days, the cells were fixed with 5% neutral formalin and stained with methylene blue. The number of plaques was counted under a binocular microscope.

**Susceptibility Assay.** To evaluate the antiviral efficacies of mizoribine, mycophenolic acid, and ganciclovir on CMV infection, a plaque reduction assay was performed as described previously (Cockley et al., 1988; Shiraki et al., 1990, 1991a,b; Yukawa et al., 1996). In brief, confluent HEL cells in 60-mm dishes were infected with 100 plaque-forming units of CMV in 0.2 ml for 1 h. Then, 1% methylcellulose nutrient culture medium was prepared just before use by adjusting the concentrations of ganciclovir alone (0, 0.1, 0.2, 0.5, 1, 2, 5, and 10 μg/ml) or mizoribine or mycophenolic acid alone (0, 1, 2, 5, 10, 20, 50, and 100 μg/ml) and overlaid on the infected cells. Susceptibility to mizoribine in the plaque reduction assay was assessed in the presence of 10 μg/ml guanine or 2′-deoxyguanosine. The cells were incubated at 37°C for 10 to 14 days and fixed with 5% neutral formalin, followed by staining with 0.03% methylene blue. The number of plaques was counted under a dissecting microscope. The 50% effective concentration for plaque inhibition ($EC_{50}$) was defined as the concentration at which the plaque number decreased to half of that in cells cultured without the addition of antiviral drugs. The $EC_{50}$ was determined by using the computer program Microplate Manager III (Bio-Rad Laboratories, Hercules, CA).

Cytotoxicity of mizoribine and ganciclovir to HEL cells was assessed by the CellTiter 96 Aqueous Nonradioactive Cell Proliferation Assay from Promega (Madison, WI) according to the recommendations of the manufacturer, and the 50% cytotoxic concentration ($CC_{50}$) was determined as the concentration that reduced the absorbance of untreated cells at 490 nm by 50% (Sasivimolphan et al., 2009). In brief, HEL cells were seeded in 96-well plates and incubated for 24 h, and mizoribine and ganciclovir were added into the wells. Final concentrations of mizoribine ranged from 10 to 1000 μg/ml and ganciclovir ranged from 0.3 to 300 μg/ml. Ninety-six hours later, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium and phenazine methosulfate were added into the well, and the absorbance was measured using a microplate reader.

**Interaction of Mizoribine with Ganciclovir on Anti-CMV Action.** To analyze the interaction of ganciclovir and mizoribine in the plaque reduction assay of CMV graphically, infected cells in 60-mm Petri dishes were overlaid with methylcellulose containing a mixture of ganciclovir (0, 0.1, 0.2, 0.5, 1, 2, 5, and 10 μg/ml) and mizoribine (0, 1, 2, 5, 10, 20, 50, and 100 μg/ml), and the $EC_{50}$ values of these agents in their various concentrations were plotted as an isobologram (Kurokawa et al., 2001; Tallarida, 2001; Suzuki et al., 2006). Synergy and antagonism were defined as deviations from dose-wise additivity, which occurs when two drugs interact as though they were the same drug. Curves falling below the line of

additivity indicate synergy; curves on the line indicate an additive reaction; and curves above the line indicate an antagonistic reaction.

**Isolation and Characterization of Mizoribine-Resistant Viruses.** One hundred plaque-forming units of the plaque-purified Towne strain in 0.2 ml was inoculated into HEL cells in four 25-cm$^2$ plastic flasks and cultured in the presence of 100 μg/ml mizoribine. When extensive cytopathology developed, 0.2 ml of 100 times-diluted culture supernatant was inoculated and cultured in the presence of 100 μg/ml mizoribine. Four independent sets of cultures were treated with 100 μg/ml mizoribine for 3 months by repeating these cycles. Then the four independent cultures were frozen and thawed three times, followed by centrifugation at 3000 rpm for 10 min, and serially diluted supernatants were inoculated into HEL cells in 60-mm Petri dishes overlaid with nutrient methylcellulose medium containing 100 μg/ml mizoribine. Fifty-seven clones from four independent cultures were isolated from plaques in Petri dishes containing three or four plaques to avoid cloning from an overlapping plaque. Isolated clones were inoculated and propagated in HEL cells in 25-cm$^2$ plastic flasks with 100 μg/ml mizoribine and harvested by three cycles of freezing and thawing followed by centrifugation at 3000 rpm for 10 min. The parent Towne strain and 57 clones were inoculated into HEL cells in two 25-cm$^2$ plastic flasks each and cultured for 3 days in the presence and absence of 100 μg/ml mizoribine. Virus yields of each clone in the presence and absence of 100 μg/ml mizoribine were compared, and the two clones showing the greatest virus yields in the presence of 100 μg/ml mizoribine were selected for further characterization after confirmation of resistance to mizoribine by the plaque reduction assay.

**Sequencing of Mizoribine-Resistant Mutants.** DNA sequences of the mizoribine-resistant mutant clones 14 and 46 were determined and deposited in the Data Bank of Japan/GenBank/European Molecular Biology Laboratory as follows: UL27 (UL27 mutations confer maribavir resistance) (Hantz et al., 2009), UL44 (DNA polymerase-associated protein), UL45 (ribonucleotide reductase), UL54 (DNA polymerase catalytic domain), UL57 (single-stranded DNA binding protein), UL97 (protein kinase), UL112/113, and UL114 (uracil DNA glycosylase). Clone 46 of the mizoribine-resistant mutant was further cloned, and the UL97 and UL54 of its eight clones were sequenced to rule out the heterogeneity of the clone.

## Results

**Susceptibility of CMV to Mizoribine.** Figure 2a shows the profile of the concentration-dependent suppression of plaque formation by mizoribine, whereas a similar immunosuppressant, mycophenolic acid, had no effect on CMV plaque formation. The $EC_{50}$ of mizoribine of the Towne strain was 12.0 μg/ml, and its $CC_{50}$ was more than 1000 μg/ml, indicating the inhibition of plaque formation was anti-CMV activity and not cytotoxicity. The serum concentration of mizoribine was 1 to 3 μg/ml in renal transplant recipients (N. Yoshimura, H. Ushigome, K. Akioka, S. Nobori, M. Fujiki, K. Kozaki, T. Suzuki, K. Sakai, and M. Okamoto, submitted for publication), and mizoribine at this serum concentration of 1 to 3 μg/ml reduced plaque formation to 60 to 70% of the untreated culture as shown in Fig. 2a.

The dose-dependent inhibition of intracellular virus growth is shown in Fig. 2b. The intracellular growth and plaque formation were dose-dependently inhibited by mizoribine. Thus, mizoribine inhibited plaque formation and intracellular virus growth.

**Synergistic Anti-CMV Activity of Mizoribine with Ganciclovir.** Figure 3 shows the strongly synergistic anti-CMV activity of mizoribine with ganciclovir. The synergistic activity of ganciclovir and mizoribine indicates that their

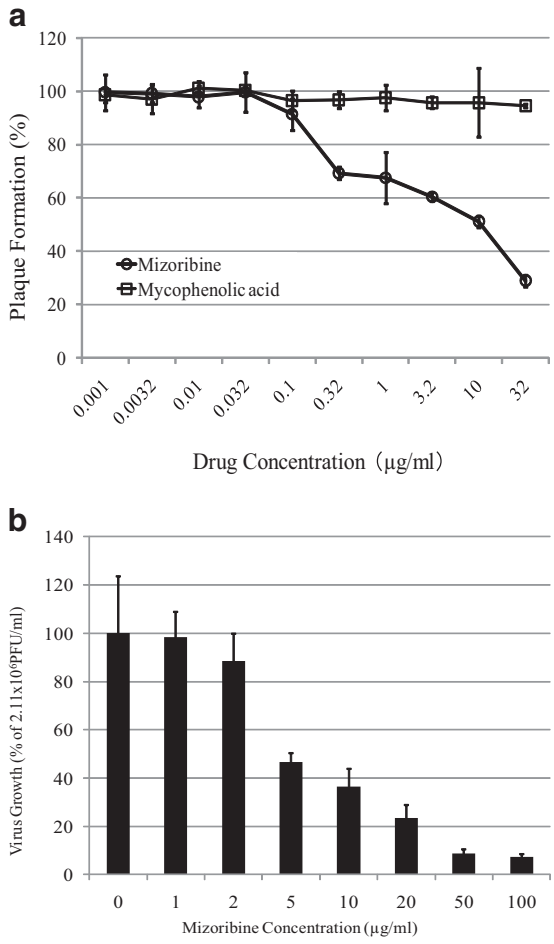


**Fig. 2.** a, contrasting susceptibilities of the Towne strain to mizoribine (○) and mycophenolate mofetil (□) assessed by plaque formation. Dose-dependent effect of mizoribine (○) and mycophenolate mofetil (□) on plaque formation was expressed by the mean ± S.D. ($n$ = 3). Mizoribine suppressed plaque formation, and its $EC_{50}$ was 12.0 ± 2.2 μg/ml ($n$ = 3). b, dose-dependent inhibition of intracellular virus growth of CMV assessed by one-step growth. Dose-dependent inhibition of mizoribine on virus growth was expressed by the mean ± S.D. ($n$ = 4).

sites of action may be different. The viability of HEL cells treated with 1000 μg/ml mizoribine and 10 μg/ml ganciclovir was 73% and not toxic to HEL cells, and their synergistic activity was not caused by cytotoxicity. Low concentrations of mizoribine reduced the ganciclovir concentration required for 50% plaque reduction, and this indicated that even low concentrations (less than 1 μg/ml) of mizoribine, a concentration that is attained in the sera of transplant recipients, efficiently increased the anti-CMV activity of ganciclovir. Strong synergism is characterized by the distance of the curve from the line indicating the additivity of drug action, and the curve was nearer to the axes than to the additivity line.

**Isolation and Characterization of Mizoribine-Resistant Viruses.** The Towne strain was inoculated into HEL cells in four separate T-25 flasks and cultured in the presence of 100 μg/ml mizoribine. When the cell sheet showed extensive cytopathology, the supernatant was diluted and inoculated for another cycle of mizoribine treatment. Fifty-seven clones were selected, propagated, and selected again in the presence of 100 μg/ml mizoribine. Two candidate clones (clones 14 and 46) were derived from the different sets among four independent sets of mizoribine-treated cultures and

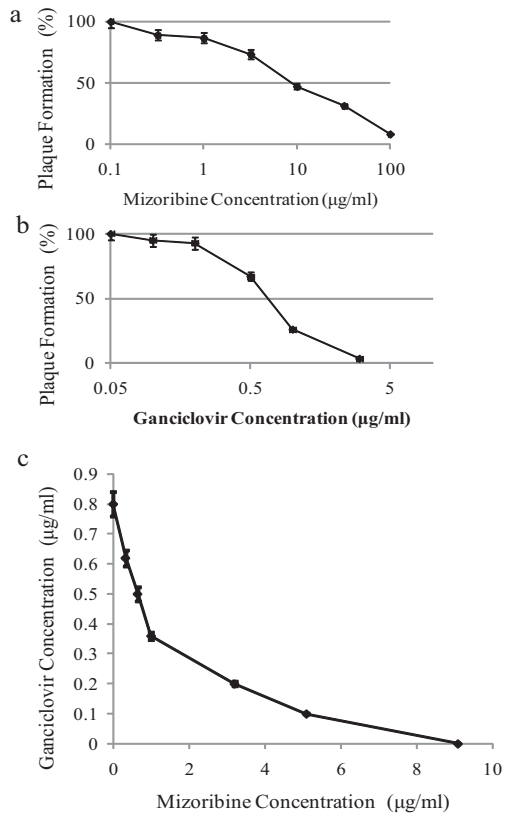


**Fig. 3.** Anti-CMV activity of mizoribine (a), ganciclovir (b), and a combination of both (c) was assessed by the plaque reduction assay. The activity was expressed as the mean ± S.D. ($n$ = 4) of the $EC_{50}$ values. Isobologram shows synergistic anti-CMV activity by combination of mizoribine with ganciclovir. Interaction of mizoribine and ganciclovir on CMV plaque formation was analyzed by isobologram. The dotted lines indicate the theoretical additive activity, and the plotted line overlays the additive line. The cell viability was 73% in the treatment of the cells with the combination of 1000 μg/ml mizoribine and 10 μg/ml ganciclovir.

were significantly more resistant to mizoribine than the parent Towne strain.

**Susceptibility of Ganciclovir-Resistant Clinical Isolates and Mizoribine-Resistant Viruses to Mizoribine.** Table 1 shows the susceptibility of the Towne strain, two ganciclovir-resistant clinical isolates with the UL97 mutation, and two mizoribine-resistant strains to ganciclovir, foscarnet, and mizoribine in the absence or presence of guanine and 2′-deoxyguanosine. A clinical isolate resistant to ganciclovir and foscarnet with UL54 and UL97 mutations and one with a UL97 mutation were less susceptible to mizoribine at $EC_{50}$ of 31.9 to 33.7 μg/ml than the Towne strain (12.0 μg/ml), which retains susceptibility to mizoribine. However, this relatively lower susceptibility to mizoribine of the two UL97 mutants was abolished by addition of guanine to the overlay medium, and they were as sensitive to mizoribine as the Towne strain in the presence of guanine, with an $EC_{50}$ of approximately 90 μg/ml. Two mizoribine-resistant clones (14 and 46) were resistant to mizoribine but as sensitive to ganciclovir and foscarnet as the parent Towne strain. They were resistant to mizoribine in the absence or presence of guanine than the original Towne strain and the two UL97 mutants despite the changes in the $EC_{50}$ values (12.0–33.7 to 78.3–90.7 μg/ml). Thus, guanine reduced the susceptibility of CMV to mizoribine, but the $EC_{50}$s to mizoribine of mizoribine-resistant mutants were >200 μg/ml and higher than those of the parent Towne strain and two UL97 mutants (78.3–90.7 μg/ml). The lower susceptibility to mizoribine of the two UL97 mutants was eliminated by guanine, whereas the relative resistance to mizoribine of the two mizoribine-resistant mutants (clones 14 and 46) was conserved in the presence of guanine compared with the Towne strain and two UL97 mutants. Addition of 2′-deoxyguanosine inactivated anti-CMV activity of mizoribine in all the viruses examined, and their $EC_{50}$s were more than 200 μg/ml. Thus, the sensitivity of CMV strains to mizoribine was affected by guanine or 2′-deoxyguanosine, indicating that guanosine nucleotides are highly associated with the anti-CMV activity of mizoribine. The mizoribine-resistant clones were resistant to mizoribine than the original Towne strain and ganciclovir-resistant strains, even when the presence of guanine modified the susceptibility ($EC_{50}$) of ganciclovir-resistant mutants to a level of susceptibility similar to that of the Towne strain. Mizoribine inhibited plaque formation of the Towne strain and the ganciclovir-resistant isolates, and isolation of mizoribine-resistant mutants indicated that mizoribine worked directly and inhibited CMV.

**Analysis of Nucleotide Sequences of Mizoribine-Resistant Mutants.** The nucleotide sequences of mizoribine-resistant mutants were compared with those of the parent Towne strain. There was no difference between them in the nucleotide sequences of the genes specific to the resistance to ganciclovir, foscarnet, and maribavir (Prichard, 2009) such as UL97 protein kinase, UL54 DNA polymerase catalytic subunit, and UL27 (maribavir resistance) (Hantz et al., 2009). Clone 46 of the mizoribine-resistant mutant was further cloned, and the UL97 and UL54 of its eight clones were sequenced. The UL97 and UL54 of eight clones were identical to those of the parent Towne strain; thus, mizoribine resis-



TABLE 1

Susceptibility of CMV strains to antiviral agents and effects of guanine and deoxyguanosine on mizoribine sensitivity

$EC_{50}$ values were determined from the three or four independent experiments and expressed as the mean ± S.D. micrograms per milliliter. $CC_{50}$ values of mizoribine and ganciclovir were more than 1000 and 15.5 ± 1.92 μg/ml ($n$ = 6), respectively.

| | Towne | Clone 14[a] | Clone 46[a] | UL97 Mutant A[b] | UL97 Mutant B[b] |
|---|---|---|---|---|---|
| Ganciclovir | 1.2 ± 0.25 | 1.2 ± 0.20 | 1.6 ± 0.48 | 4.54 ± 1.03 | 3.6 ± 0.5 |
| Phosphonoformic acid | 13.9 ± 3.1 | 12.9 ± 1.7 | 17.1 ± 3.3 | >100 | N.D. |
| Mizoribine | 12.0 ± 2.2 | 53.0 ± 24.1 | >100 | 33.7 ± 5.7 | 31.9 ± 13.9 |
| Guanine-mizoribine[c] | 78.3 ± 18.9 | >200 | >200 | 90.7 ± 10.1 | 87.0 ± 7.2 |
| Guanosine-mizoribine[c] | >200 | >200 | >200 | >200 | >200 |

N.D., not determined.

[a] Clones 14 and 46 were mizoribine-resistant clones isolated after culture for 3 months in the presence of 100 μg/ml mizoribine.

[b] UL97 mutants A and B were clinical isolates resistant to ganciclovir and had mutations in UL97 (A594V) and UL54 (Q578H) (Oshima et al., 2008) and UL97 (L595S), respectively.

[c] $EC_{50}$ to mizoribine was determined in the presence of 10 μg/ml guanine and 2′-deoxyguanosine.

tance was not related to the target proteins, UL97 and UL54, of ganciclovir, foscarnet, and maribavir. Furthermore, the mutation was not found, although the nucleotide sequences of UL44 DNA polymerase associate protein, UL45 ribonucleotide reductase, UL57 single-stranded DNA binding protein, UL112/113, and UL114 uracil-DNA glycosylase were analyzed. Thus, the target of anti-CMV action of mizoribine might be different from the current anti-CMV agents and suggested a new mechanism of anti-CMV action.

## Discussion

Mycophenolic acid and mizoribine interrupt the S phase of the cell cycle by specifically inhibiting the rate-limiting enzyme IMPDH in the de novo pathway of purine biosynthesis. Mizoribine alone suppressed CMV plaque formation dose-dependently, and its $EC_{50}$ was 12.0 μg/ml. Mizoribine is phosphorylated in cells to mizoribine-5′-phosphate (Fig. 1), a potent inhibitor of IMPDH ($K_i = 10^{-8}$ M). The suppression of lymphocyte proliferation by mizoribine is reversed by guanosine, evidence that the principal mechanism of action is inhibition of de novo guanosine nucleotide synthesis. This activity indicates that cells treated with mizoribine had lower amounts of guanosine nucleotides than untreated cells. Mycophenolic acid, which inhibits IMPDH, synergistically inhibited herpes simplex virus (HSV) in vitro and in vivo with acyclovir (Pancheva et al., 1997, 1999; Neyts and De Clercq, 1998; Neyts et al., 1998; Mayer et al., 2003) and CMV in vitro and in vivo with ganciclovir (Neyts et al., 1998), although the anti-CMV activity of mycophenolic acid was not shown.

The isobologram showed that mizoribine and ganciclovir together inhibited CMV plaque formation strongly and synergistically (Tallarida, 2001). The ratio of ganciclovir over 2′-deoxyguanosine was increased via the inhibition of endogenous guanosine synthesis by IMPDH with mizoribine, and ganciclovir may be incorporated into CMV DNA more efficiently in mizoribine-treated infected cells than untreated infected cells. Similar potentiation of acyclovir action has been reported in HSV-infected cells treated with a ribonucleotide reductase inhibitor (Spector et al., 1985, 1989; Reardon and Spector, 1991). Reduction in the concentration of de novo synthesized deoxynucleotides facilitates incorporation of acyclovir and potentiates acyclovir action in HSV-infected cells.

The synergism observed in the isobologram was exaggerated by the depletion of guanosine nucleotides in addition to the interaction of mizoribine and ganciclovir on CMV. Although both mycophenolic acid and mizoribine potentiated anti-CMV activity of ganciclovir by depleting guanosine nucleotides, mizoribine had its own anti-CMV activity in contrast to mycophenolic acid. Thus, a mizoribine concentration of less than 1 μg/ml efficiently reduced to almost half the ganciclovir concentration required to inhibit CMV plaque formation by 50%.

Mizoribine did not inhibit varicella-zoster virus plaque formation, but the number of infected cells in the plaques was dose-dependently reduced (data not shown). HSV plaque formation was not affected by mizoribine. Thus, mizoribine specifically inhibited CMV plaque formation.

The two ganciclovir-resistant clinical isolates with the UL97 mutation were less susceptible to mizoribine, but the addition of guanine decreased the susceptibility of the Towne strain to mizoribine. Guanine is conjugated with phosphoribosyl pyrophosphate and converted to guanosine monophosphate (rGMP) by hypoxanthine guanine phosphoribosyltransferase as illustrated in Fig. 4. Therefore, inhibition of IMPDH by mizoribine caused rGMP depletion (Koyama and Tsuji, 1983; Turka et al., 1991; Dayton et al., 1992; Catapano et al., 1995; Meredith et al., 1997; Metz et al., 2001), but guanine supplied rGMP and rescued the lack of rGMP. Thus, mizoribine inhibits the conversion of inosine monophosphate to rGMP, and guanine would complement the deficiency of rGMP in mizoribine-treated cells. Therefore, the $EC_{50}$ of mizoribine against the Towne strain was increased from 10 to 90 μg/ml by the addition of guanine. Guanine changed UL97 mutants from less susceptible to mizoribine than the Towne strain to a similar susceptibility to the Towne strain, and the $EC_{50}$s of the Towne strain and UL97 mutants were lower than those of the two mizoribine-resistant mutants, indicating that mizoribine resistance was independent of guanine. The UL97 mutants were as susceptible to mizoribine as the Towne strain in the presence of guanine, and the lower susceptibility was caused by guanosine nucleotide metabolism rather than a direct action on UL97. In contrast, 2′-deoxyguanosine, which is phosphorylated to 2′-deoxyguanosine monophosphate for DNA synthesis, made all the CMV strains insusceptible to mizoribine at the $EC_{50}$ concentration of more than 200 μg/ml. Thus, guanosine nucleotide metabolism modified the susceptibility of CMV to mizoribine by complementing the IMPDH inhibition of mizoribine actions, whereas resistance to mizoribine was preserved in two mizoribine-resistant mutants.

The two mizoribine-resistant CMV clones isolated were as susceptible to ganciclovir as the parent Towne strain. The

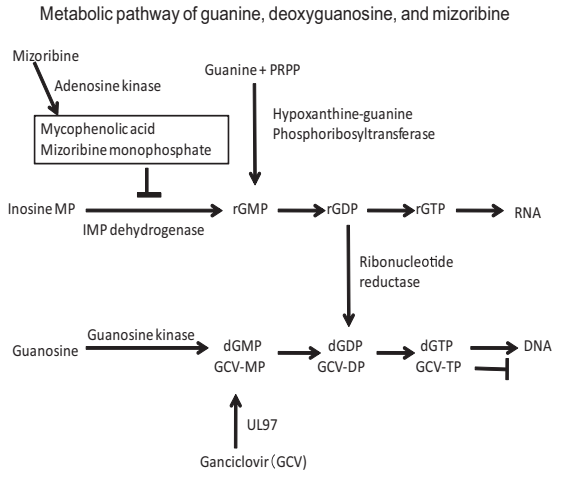


**Fig. 4.** Metabolic pathways of guanosine nucleosides and inhibition of IMPDH by mycophenolic acid and mizoribine. Mizoribine was phosphorylated by adenosine kinase and became an active form. Mycophenolic acid and mizoribine inhibit IMPDH and thereby deplete rGMP in the de novo pathway. Guanine is converted to rGMP and then to RNA or DNA after reduction of ribose of guanosine diphosphate (rGDP) to 2′-deoxyguanosine diphosphate (dGDP) by ribonucleotide reductase. Guanine is not influenced by IMPDH and rescues the lack of rGDP. 2′-Deoxyguanosine is phosphorylated to dGMP and processed to DNA. The UL97 protein kinase converts ganciclovir to ganciclovir-monophosphate (GCV-MP), which is further phosphorylated by host cell GMP kinase to ganciclovir diphosphate (GCV-DP), which is in turn further phosphorylated to ganciclovir triphosphate (GCV-TP) by cellular kinases. GCV-TP inhibits viral DNA polymerase and viral DNA synthesis. ⊣ indicates inhibition.

synergism of mizoribine with ganciclovir against CMV and the isolation of mizoribine-resistant CMV indicate that mizoribine works directly by inhibition of replication of CMV and not indirectly through inhibition of cellular metabolism. However, the target molecule of mizoribine in CMV could not be determined, and the mutation was not identified in the genes responsible for the resistance to ganciclovir, foscarnet, and maribavir, indicating the novel mechanism of anti-CMV action of mizoribine. Thus, the synergistic anti-CMV activity of mizoribine with ganciclovir and the modification of sensitivity to mizoribine by guanine or 2′-deoxyguanosine indicate that guanosine nucleotides are highly associated with the anti-CMV activity of mizoribine. Mizoribine showed anti-CMV activity and potentiated the anti-CMV activity of ganciclovir synergistically as a potent IMPDH inhibitor and anti-CMV agent.

In this study, the immunosuppressant mizoribine directly inhibited replication of CMV and potentiated the anti-CMV action of ganciclovir in infected cells in vitro. The target molecule of anti-CMV action of mizoribine was different from those of the current anti-CMV agents, and this indicated mizoribine exerted a new mechanism of anti-CMV action. Because the clinical observation in renal transplantation indicated that mizoribine significantly suppressed CMV infection without affecting graft survival (N. Yoshimura, H. Ushigome, K. Akioka, S. Nobori, M. Fujiki, K. Kozaki, T. Suzuki, K. Sakai, and M. Okamoto, submitted for publication), the anti-CMV activity of mizoribine may contribute to a lower frequency and severity of CMV infection in renal transplant recipients than in those maintained on mycophenolic acid.

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