Novel anti-cytomegalovirus activity of immunosuppressant mizoribine and its synergism with ganciclovir

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Non-standard abbreviations:

CMV (cytomegalovirus); IMP (inosine monophosphate); IMPDH (IMP dehydrogenase); HEL (human embryonic lung); PFU (plaque-forming units); HSV (herpes simplex virus); GCV-MP (ganciclovir-monophosphate); GCV-DP (ganciclovir-diphosphate); GCV-TP (ganciclovir triphosphate)

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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 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 EC₅₀ for plaque inhibition of 12.0 µg/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 three months’ culture with 100 µg/ml of 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.
Introduction

Mycophenolic acid and mizoribine (Bredinin®) are immunosuppressants that specifically inhibit the rate-limiting enzyme inosine monophosphate (IMP) 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®), is now approved for use in many countries to prevent allograft rejection and to treat ongoing rejection (Group, 1995; Sollinger, 1995; Takahashi et al., 1995; Gonwa, 1996; Group, 1996; Mele and Halloran, 2000). Mizoribine (Fig. 1) was isolated from the culture medium of *Eupenicillium brefeldianum* and is 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).

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.

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®) 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.

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 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 Co. 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; Shiraki et al., 1991a; Shiraki et al., 1991b; Shiraki et al., 1991c; Yukawa et al., 1996).

Intracellular virus production was assessed by one-step growth in cells treated with drugs as follows. HEL cells in 25 cm² plastic flasks were infected with 2 plaque-forming units (PFU)/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 3,000 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; Shiraki et al., 1991a; Shiraki et al., 1991b; Yukawa et al., 1996). Briefly, confluent HEL cells in 60 mm dishes were infected with 100 PFU of CMV in 0.2 ml for 1 h. Then 1% methylcellulose nutrient culture medium were 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 of guanine or 2’-deoxyguanosine. The cells were incubated at 37°C for
10–14 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 (BioRad, Hercules, CA).

Cytotoxicity of mizoribine and ganciclovir to HEL cells was assessed by the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay from Promega (Madison, WI) according to the recommendations of the manufacturer and the 50% cytotoxic concentration (CC_{50}) was determined the concentration that reduced the absorbance of untreated cells at 490 nm by 50% (Sasivimolphan et al., 2009). Briefly, HEL cells were seeded in 96-well 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)-2H-tetrazolium and phenazine methasulfate were added into the well and the absorbance was measured using a microplate reader.

**Interaction of mizoribine with ganciclovir on anti-CMV action** In order 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 if 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 PFU 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 of 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 of mizoribine. Four independent sets of cultures were treated with 100 µg/ml of mizoribine for three months by repeating these cycles. Then the four independent cultures were frozen and thawed three times, followed by centrifugation at 3,000 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 of 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 of 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 three days in the presence and absence of 100 µg/ml of mizoribine. Virus yields of each clone in the presence and absence of 100 µg/ml of mizoribine were compared, and the two clones showing the greatest virus yields in the presence of 100 µg/ml of 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 mutants
Clone 14 and 46 were determined and deposited in DDBJ/GENBANK/EMBL were 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
Fig. 2a shows the profile of the concentration-dependent suppression of plaque formation by mizoribine, while a similar immunosuppressant, mycophenolic acid, had no effect on CMV plaque formation. The EC₅₀ of mizoribine of the Towne strain was 12.0 µg/ml and its CC₅₀ was more than 1,000 µg/ml, indicating the inhibition of plaque formation was anti-CMV activity and not by 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 60-70% 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
Fig. 3 shows the strongly synergistic anti-CMV activity of mizoribine with ganciclovir. The synergistic activity of ganciclovir and mizoribine indicates that their sites of action may differ. The viability of HEL
cells treated with 1,000 µg/ml of mizoribine and 10 µg/ml of ganciclovir was 73% and not toxic to HEL cells and their synergistic activity was not due to 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 augmented 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 of 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 of mizoribine. Two candidate clones (clones 14 and 46) were derived from the different sets among four independent sets of mizoribine-treated cultures and 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 EC50 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 about 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 in spite of 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, while the relative resistance to mizoribine of the two mizoribine-resistant mutants (clones 14 and 46) was conserved in the presence of guanine compared to the Towne strain and two UL97 mutants. Addition of 2’-deoxyguanosine inactivated anti-CMV activity of mizoribine in all 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 54 of its eight clones were sequenced. The UL97 and UL54 of eight clones were identical to those of the parent Towne strain and thus mizoribine resistance was not related to the target proteins, UL97 and UL54, of ganciclovir, foscarnet, and maribavir. Furthermore, the mutation was not found though 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\textsubscript{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, demonstrating that the principal mechanism of action is inhibition of de novo guanosine nucleotide synthesis. This 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; Neyts et al., 1998; Neyts and De Clercq, 1998; Pancheva et al., 1999; 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 demonstrated.

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; Spector et al., 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 (PRPP) 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 IMP 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 $\mu$g/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 due to 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 CMV strains insusceptible to mizoribine at the EC$_{50}$ concentration of more than 200 $\mu$g/ml. Thus, guanosine nucleotide metabolism modified the susceptibility of CMV to mizoribine by complementing the IMPDH inhibition of mizoribine actions, while 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 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, forcarnet, 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. As 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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References


61:1029-1037.


Footnotes

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Legends for Figures

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

**Figure 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).

**Figure 3.** Anti-CMV activity of mizoribine (a), ganciclovir (b), and 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 of mizoribine and 10 µg/ml of ganciclovir.

**Figure 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.
Table 1

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

<table>
<thead>
<tr>
<th></th>
<th>Towne</th>
<th>clone 14&lt;sup&gt;a&lt;/sup&gt;</th>
<th>clone 46&lt;sup&gt;a&lt;/sup&gt;</th>
<th>UL97 mutant A&lt;sup&gt;b&lt;/sup&gt;</th>
<th>UL97 mutant B&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td>Ganciclovir</td>
<td>1.2 ± 0.25</td>
<td>1.2 ± 0.20</td>
<td>1.6 ± 0.48</td>
<td>4.54 ± 1.03</td>
<td>3.6 ± 0.5</td>
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<td>phosphonoformic acid</td>
<td>13.9 ± 3.1</td>
<td>12.9 ± 1.7</td>
<td>17.1 ± 3.3</td>
<td>&gt;100</td>
<td>ND&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mizoribine</td>
<td>12.0 ± 2.2</td>
<td>53.0 ± 24.1</td>
<td>&gt;100</td>
<td>33.7 ± 5.7</td>
<td>31.9 ± 13.9</td>
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<tr>
<td>Guanine-mizoribine&lt;sup&gt;c&lt;/sup&gt;</td>
<td>78.3 ± 18.9</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>90.7 ± 10.1</td>
<td>87.0 ± 7.2</td>
</tr>
<tr>
<td>Guanosine-mizoribine&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;200</td>
</tr>
</tbody>
</table>

EC<sub>50</sub> values were determined from the three or four independent experiments and expressed as the mean ± S.D. μg/ml.

CC<sub>50</sub> values of mizoribine and ganciclovir were more than 1000μg/ml and 15.5 ± 1.92μg/ml (n=6), respectively.

<sup>a</sup> Clones 14 and 46 were mizoribine-resistant clones isolated after three months’ culture in the presence of 100μg/ml of mizoribine.

<sup>b</sup> 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 in UL97 (L595S), respectively.

<sup>c</sup> EC<sub>50</sub> to mizoribine was determined in the presence of 10 μg/ml of guanine and 2’-deoxyguanosine.

<sup>d</sup> ND: not determined.
Fig. 1

Guanosine
Mizoribine (Bredinin®)
Mycophenolate mofetil (CellCept®)

Ganciclovir
Mizoribine monophosphate (active form)
Mycophenolic acid (active form)
Fig. 2b

Virus Growth (% of 2.11x10^9 PFU/ml) vs. Mizaribine Concentration (µg/ml)

- 0 µg/ml: 100% growth
- 1 µg/ml: 120% growth
- 2 µg/ml: 80% growth
- 5 µg/ml: 50% growth
- 10 µg/ml: 20% growth
- 20 µg/ml: 10% growth
- 50 µg/ml: 5% growth
- 100 µg/ml: 0% growth
Fig. 4

Metabolic pathway of guanine, deoxyguanosine, and mizoribine

- Mizoribine
  - Adenosine kinase
  - Mycophenolic acid
  - Mizoribine monophosphate

- Guanine + PRPP
  - Hypoxanthine-guanine phosphoribosyltransferase

- Inosine MP
  - IMP dehydrogenase

- Guanosine
  - Guanosine kinase
  - Guanine monophosphate (GMP)
  - GCV-MP

- rGMP
  - rGDP
  - rGTP
  - RNA

- dGMP
  - dGDP
  - dGTP
  - DNA

UL97
- Ganciclovir (GCV)