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Short Communication

DIFFERENTIAL CYTOTOXIC EFFECTS OF MIZORIBINE AND ITS AGLYCONE ON HUMAN AND MURINE CELLS AND ON NORMAL AND ENZYME-DEFICIENT HUMAN CELLS

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Abstract—The growth inhibitory mechanisms of mizoribine, an immunosuppressive imidazole nucleoside used clinically to inhibit rejection reactions after renal transplantation and in the treatment of systemic lupus erythematosus and rheumatoid arthritis, were studied in human and murine cells. We found that (a) human cells were 20- to 60-fold more resistant than murine cells to both mizoribine and its aglycone, (b) adenine phosphoribosyltransferase (APRT)-deficient human cells were resistant to aglycone but not to mizoribine, (c) hypoxanthine phosphoribosyltransferase (HPRT)-deficient human cells were at least 100-fold more sensitive to both mizoribine and aglycone, and (d) the decrease in intracellular GTP broadly paralleled the cytotoxicity in each case. Therefore, data obtained from studies using non-human tissues should be interpreted carefully before clinical application. Results indicate that the growth inhibitory effect of the aglycone but not of mizoribine is mediated by APRT, and depletion of guanine nucleotides is responsible for the effects of both drugs. Our data also suggest that the drugs may reduce mutant HPRT-deficient somatic cells *in vivo*, and may cause enhanced adverse reactions in HPRT-deficient individuals. The drug may have altered effects in patients receiving other purine or pyrimidine analogs.

Key words: mizoribine; immunosuppressant; differential cytotoxicity; IMP dehydrogenase; adenine phosphoribosyltransferase; hypoxanthine phosphoribosyltransferase

Mizoribine (4-carbamoyl-1-B-ribofuranosylimidazolium-5-olate) is a purine nucleoside analog isolated from *Eupenicillium brefeldianum* [1], and has been shown to exhibit cytotoxic effects in mammalian cell culture studies [2–4]. In animal studies, mizoribine was found to suppress rejection reactions, prolong survival after renal allografts [5], and be effective in countering experimentally induced nephritis [6]. On the basis of those reports, mizoribine has been used clinically as an immunosuppressant after renal transplantation [7, 8] and for lupus nephritis [9], and recently for rheumatoid arthritis [10] and Behçet syndrome [11].

Studies on the mechanisms of the immunosuppressive effects of mizoribine in murine systems demonstrated that mizoribine is phosphorylated by adenosine kinase [4], and that the monophosphate form inhibits IMP dehydrogenase [4] or both IMP dehydrogenase and GMP synthase [12]. The inhibition of the enzyme(s) results in the depletion of intracellular guanine nucleotides, as shown in both murine and human systems [4, 13]. Guanine nucleotide depletion causes G_1/S block in human T cells [13]. Unlike mizoribine, mizoribine aglycone (4-carbamoylimidazolium 5-olate), the ribose-free form of mizoribine, is believed to be metabolized by APRT† (EC 2.4.2.7) [3], as shown in murine systems.

Although complete deficiencies of purine enzymes are rare, heterozygotes or partial deficiencies of these enzymes are not uncommon; the frequency of heterozygotes for APRT deficiency was estimated to be 1.2% among Japanese nationals [14]. Since mizoribine and its derivatives are metabolized through purine enzymes, persons with purine enzyme deficiencies may respond differently to the drug. The present study utilized human cells, some of which were deficient in purine metabolic enzymes, to examine the metabolism of mizoribine. We also compared the metabolism of mizoribine in human and in murine cells.

Materials and Methods

Chemicals. Mizoribine and its aglycone were obtained from the Toyo-Jozo Co. (Tokyo, Japan). Purine bases and purine nucleotides were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Cell lines. Epstein-Barr virus transformed B cell lines (the TS01 and WR110 lines from control subjects, the WR011 and WR081 lines from patients with APRT deficiencies [15], and the WR018 line from a patient with a partial HPRT (EC 2.4.2.8) deficiency [16]) were established in our laboratory [17]. They were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamine. Murine L cells and R1.1 cells from mouse T cell lymphoma used in this study were furnished by Dr. D. A. Carson (University of California, San Diego, La Jolla, CA, U.S.A.).

Primary cell culture. Peripheral blood mononuclear cells from healthy individuals were cultured in medium containing 1% PHA (Wellcome Research Laboratories, Beckenham, U.K.) for 3 days, and then activated T cells were transferred to the medium supplemented with 20% crude IL-2 prepared from human tonsils, as described previously [18]. Murine (BALB/c) spleen cells were cultured in the medium containing 2 μg/mL concanavalin A (Sigma), 10 mM HEPES and 50 μM 2-mercaptoethanol at an initial cell density of 5×10^6/mL for 5 days

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† Abbreviations: APRT, adenine phosphoribosyltransferase; HPRT, hypoxanthine phosphoribosyltransferase; PHA, phytohemagglutinin; and IL-2, interleukin 2.

Table 1. Concentrations of mizoribine and aglycone required to achieve 50% growth inhibition of human and murine cells*

Cell	IC_{50} (μM)	
	Mizoribine	Aglycone
Mouse		
T cell	1.3	NT†
R1.1	1.7	2.3
L cell	1.7	1.8
Human		
T cell	40.0	42.0
B cell	31.0–78.0‡	41.0

* Logarithmic-phase cells (1×10^4) were cultured in 200 μL medium containing various concentrations of mizoribine or aglycone for 7 days, and the numbers of viable cells were counted by the trypan blue exclusion method. The IC_{50} was obtained from the curve of control cell growth at various drug concentrations. Each value is the average from at least three determinations.

† NT = not tested.

‡ The range of values is shown, since the results changed widely in repeated experiments using human B cells.

and then maintained in the medium supplemented with 20% crude human IL-2, 10 mM HEPES and 50 μM 2-mercaptoethanol.

Growth inhibition test. Logarithmic-phase cells were harvested and plated in duplicate at 1×10^4 viable B cells/well in 96-well microtiter plates in 200 μL medium containing various concentrations of mizoribine or aglycone. The growth inhibitory effects of the chemicals against PHA-activated human and concanavalin A-activated mouse T cells were tested by similar methods, except for the addition of 20% human IL-2 to the medium. After 7 days of culturing, the number of viable cells was counted by the trypan blue exclusion method. Cell growth was expressed as a percentage of the number of cells in the control cultures, and the IC_{50} was obtained from the curve of cell growth at various drug concentrations.

Rescue experiments. In rescue experiments, 10, 25 or 50 μM adenine, guanine, or hypoxanthine was added to the medium containing mizoribine, and 1×10^4 WR110 cells/well were cultured in each medium. The percent growth (as compared with controls) was determined after 7 days.

Quantification of nucleotides. Cells were cultured in the medium containing various concentrations of mizoribine at an initial cell density of 2×10^5 cells/mL in 10-mL flasks. After culturing for 24 hr, the cells were collected and washed 3 times in cold 10 mM PBS (pH 7.4). The cell pellets were vortexed in 300 μL of 0.4 N cold perchloric acid, placed on ice for 15 min, and centrifuged. To extract perchloric acid, 600 μL Freon-octylamine (Freon: trioctylamine, 73:27) was added to the supernatant and vortexed extensively. After centrifugation, cellular nucleotides in the upper water layer were measured by reverse-phase chromatography (μBondapak C18, Toyo Soda Manufacturing Co., Tokyo, Japan).

Results and Discussion

Sensitivities of human and murine cells to mizoribine and aglycone. Table 1 summarizes IC_{50} values of mizoribine and aglycone for the various cells. The data indicate that human cells, regardless of type, are more resistant to the compounds than are the murine cells. Differences in sensitivity were between 20- and 60-fold. Mizoribine and aglycone are metabolized by different enzymes to form nucleotides. Therefore, a difference in sensitivity of human and murine cells is likely to be dependent on a difference in sensitivity of the cells to mizoribine nucleotides rather than to differences in the pathways for forming the nucleotides. Thus, when the metabolism and the effects of this compound are considered in clinical situations, data obtained from human rather than from non-human cells should be more reliable.

Sensitivity of enzyme-deficient mutant cells. The growth inhibition curves of human B cell lines cultured with either mi-

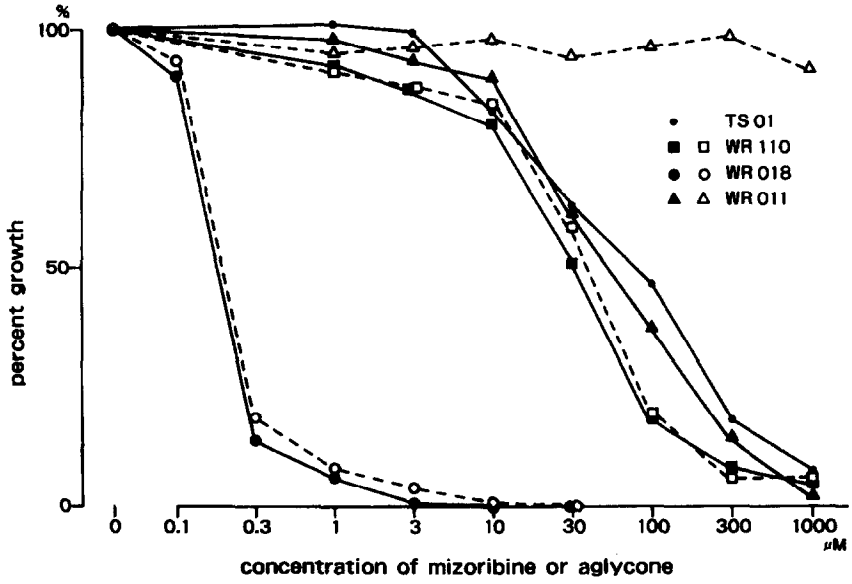

Fig. 1. Growth inhibition curves for various human B cell lines cultured with mizoribine or aglycone. Cells (1×10^4) were cultured in 200 μL medium containing various concentrations of these drugs for 7 days. The following cell lines were used: TS01 (•) and WR110 (■, □) from normal controls, WR018 (●, ○) from a patient with HPRT deficiency and WR011 (▲, △) from a patient with APRT deficiency. Closed symbols indicate the growth inhibition by mizoribine and open symbols by aglycone. The growth inhibition curves for WR081 (APRT deficiency) (data not shown) were similar to those of WR011. Each value is the average from at least two experiments.

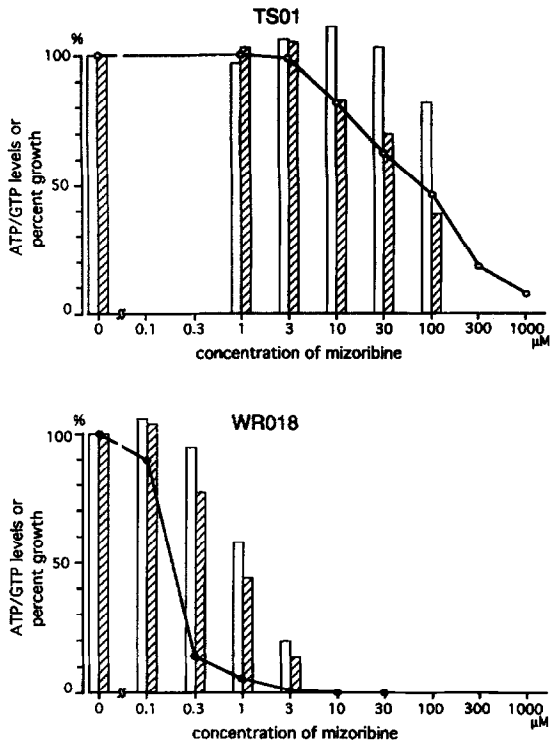

Fig. 2. Levels of cellular ATP and GTP after 24 hr and growth inhibition curves after 7 days in the cultures of human cells with various concentrations of mizoribine. Control (TS01) and HPRT-defective (WR018) human B cells (2×10^6/10 mL) were cultured in medium containing various concentrations of mizoribine for 24 hr. ATP (open bar) and GTP (shaded bar) levels were expressed as percent of control levels. Control levels of ATP and GTP in TS01 were 1840 and 549 pmol/10^6 cells, respectively, and those in WR018 were 1530 and 527 pmol/10^6 cells, respectively. The growth inhibition observed after 7 days of culturing with mizoribine is also shown for TS01 (○) and WR018 (●). The decrease in intracellular GTP broadly paralleled the cytotoxicity. Each value is the average from two determinations.

zoribine or aglycone are presented in Fig. 1. B cell lines from control subjects, TS01 and WR110, are sensitive to both mizoribine and aglycone, with IC_{50} values of 30–100 μM for each drug. APRT-deficient B cells, WR011 (Fig. 1) and WR081 (data not shown), showed marked resistance to aglycone, although both cell lines were similarly sensitive to mizoribine. These data indicate that the growth inhibitory effects of the mizoribine aglycone are dependent on APRT, but that mizoribine itself exerts its growth inhibitory effects without APRT.

On the other hand, HPRT-deficient WR018 B cells were extremely sensitive to both drugs, with IC_{50} values of 0.1–0.3 μM. Thus, WR018 cells were approximately 100 times more sensitive than control cells to mizoribine. In rescue experiments using enzymatically normal WR110 cells, neither adenine nor hypoxanthine at any concentration was able to reverse the growth inhibitory effects of mizoribine, whereas 10 and 25 μM guanine rescued the cells from growth inhibition at 100–1000 μM mizoribine. The combination of adenine and guanine was found to be more effective than guanine alone to reverse the growth inhibitory effects of mizoribine. As expected, 25 μM guanine failed to rescue the HPRT-deficient cells. Therefore, guanine had to be converted to nucleotides to exert the rescue effect.

Changes of cellular nucleotides. When the levels of cellular ATP and GTP were checked after 24-hr culturing of TS01 cells with mizoribine, the GTP level was found to be decreased by 10 μM mizoribine (Fig. 2). At even higher concentrations of mizoribine, ATP levels also decreased. However, in the HPRT-deficient WR018 cells, interestingly, a decrease in the level of intracellular GTP was already evident at 0.3 μM mizoribine (Fig. 2). At a concentration of 1 μM mizoribine, WR018 cells exhibited a severe decrease in the levels of both GTP and ATP, whereas normal B cells did not show detectable changes in the nucleotide levels. The decrease in GTP and the cytotoxicity began at similar concentrations of mizoribine. Together with the results of the rescue experiments in which guanine at least partially reversed the effects of mizoribine when HPRT was active, the data suggest that a decreased supply of guanine nucleotides is the main mechanism underlying the growth inhibitory effects of mizoribine. Given the fact that guanine but not hypoxanthine was able to reverse the growth inhibitory effects of mizoribine, this compound is likely to block the conversion of IMP to GMP by inhibiting IMP dehydrogenase or GMP synthase (or both).

The profound difference between normal and HPRT-deficient human B cells with respect to the growth inhibitory effects of mizoribine is of interest. Usually, mammalian cells obtain purine nucleotides from both purine salvage and *de novo* synthetic pathways. In human B cells, the *de novo* pathway is considered to be sufficient because cells that are completely deficient in either of the salvage enzymes are able to grow normally in culture medium. However, this is not true in all conditions; when mizoribine is administered, HPRT becomes an important pathway for the synthesis of GMP, and similar conditions may occur clinically. In fact, concentrations of mizoribine within body fluids have been reported to reach 5–10 μM at its peak. Considering that 1 μM mizoribine was sufficient to kill HPRT-deficient but not positive cells, this drug is likely to have differential effects on normal and HPRT-deficient somatic cells *in vivo*. Since even normal individuals possess mutant somatic cells deficient in HPRT [19, 20], the frequency of such mutant somatic cells in individuals receiving mizoribine may be low. Mizoribine may cause profound untoward effects if administered to patients with a complete deficiency of HPRT (Lesch-Nyhan syndrome). Recently, nucleoside analogs have been used widely, especially as antiviral and immunosuppressive agents. Thus, adenine arabinoside [21] and acycloguanosine [22] are effective for the treatment of herpes viruses, and both azidothymidine [23] and dideoxy nucleosides are expected to be useful for the treatment of acquired immunodeficiency syndrome. These drugs often perturb purine and pyrimidine metabolism in humans and may alter the sensitivities of other purine and pyrimidine analogs, possibly causing adverse drug interactions. For example, a drug suppressing the activity of HPRT is likely to augment the pharmacological activity of mizoribine. Therefore, an understanding of both the precise metabolism of nucleoside analogs and the mechanisms of the cytotoxic or growth inhibitory effects in relation to the purine metabolic enzymes is becoming increasingly important. In addition, we stress the importance of using human cells rather than mammalian cells of other species to study the pharmacological effects of purine and pyrimidine analogs.

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