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Uptake and metabolism of mizoribine, an immunosuppressant, in L5178Y-R mouse lymphoma cells in vitro and peripheral blood mononuclear cells of rats and kidney transplant recipients in vivo

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ABSTRACT

The cellular uptake of mizoribine (MZR), an immunosuppressant, and metabolism of MZR to MZR-5′-monophosphate (MZRP), an active metabolite, were evaluated in L5178Y-R mouse lymphoma cells and peripheral blood mononuclear cells (PBMCs) of rats and kidney transplant recipients (KTRs, n = 22). Real-time PCR analysis revealed the expression of ENT1 and ENT2 mRNAs, but not of CNTs, in L5178Y-R cells and rat's PBMCs. In L5178Y-R cells, the uptake of MZR was suppressed by adenosine, a substrate for ENT1 and ENT2, but not by 5-(4-nitrobenzyl)-6-thioinosine (0.1 μM), an ENT1 inhibitor. Saturable metabolism of MZR to MZRP was observed. In rats, peak plasma concentrations of MZR and peak concentrations of MZR and MZRP in PBMCs were observed 3 h after oral administration. MZR disappeared from PBMCs in parallel with plasma MZR, but the disappearance of MZRP from PBMCs appeared to be slow. In KTRs, the mean plasma concentration of MZR 3–4 h after ingestion was 3.14 μg/ml and the mean MZRP concentration in PBMCs was 16.8% of MZR, reflecting the involvement of ENT in the uptake of MZR. A linear relationship was observed between plasma MZR concentrations ranging from 1 to 6 μg/ml and PBMC's MZRP concentrations ranging from 90 to 200 ng/ml.

1. Introduction

Mizoribine (MZR), an immunosuppressive agent, was approved as a drug to prevent rejection in kidney transplants in 1984 in Japan [1–3]. Currently, MZR is also used for the treatments of lupus nephritis, rheumatoid arthritis, primary nephritic syndrome, and so on, mostly in combination with other immunosuppressants such as cyclosporine or tacrolimus, and corticosteroids [4–6]. MZR, an imidazole nucleoside, is thought to be metabolized to MZR-5′-monophosphate (MZRP), an active metabolite, by adenosine kinase and MZRP inhibits inosine monophosphate (IMP) dehydrogenase and guanosine monophosphate (GMP) synthetase in lymphocyte cells, resulting in the inhibition of lymphocyte proliferation [3,7–10]. The key role of MZRP in immunosuppressive action of MZR is now well recognized. So far, however, the direct analysis of MZR and MZRP in lymphocytes or in peripheral blood mononuclear cells (PBMCs) rich in lymphocytes after application of MZR is not yet reported.

Transporters for nucleosides involve Na^+-independent equilibrative nucleoside transporters (ENTs), a facilitated diffusion system, and Na^+-dependent concentrative nucleoside transporters (CNTs), an active transport system. Previously, we reported that the

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intestinal absorption of MZR in rats was mediated by CNT1 and CNT2 [11–13]. The decrease in oral bioavailability of MZR due to the polymorphism of CNT1 in humans was also reported [14,15]. Although the expression of a variety of transporters including solute carrier (SLC) and ATP-binding cassette (ABC) transporters in lymphocytes and/or PBMCs has been reported, the expression of nucleoside transporters is not yet reported [16–18].

In the present study, the uptake of MZR and metabolism of MZR to MZRP were examined in L5178Y-R mouse lymphoma cells in vitro and in PBMCs of rats and kidney transplant recipients (KTRs) in vivo. L5178Y-R cells, a metastatic cell strain of mouse lymphoma L5178Y cells, have been widely used in toxicity and radiation studies and are known to be more radioresistant and more sensitive to camptothecin than L5178Y cells [19]. To examine the uptake mechanism of MZR, the expression of mRNAs for ENT1, ENT2, CNT1, CNT2 and CNT3 was determined in L5178Y-R cells and PBMCs of rats using corresponding primers for ENTs and CNTs [20–23]. Also, to compare the concentrations of MZR directly between the extracellular (incubation medium or plasma) and intracellular fluids, the volumes of intracellular fluids of L5178Y-R cells and rat's PBMCs were estimated. Finally, Relationships among MZR concentrations in plasma, MZR concentrations in PBMCs and MZRP concentrations in PBMCs were also examined.

2. Materials and methods

2.1. Materials

MZR, or Bredinin® (Asahi Kasei Pharma Corporation, Tokyo, Japan), was used as provided. MZRP was synthesized in the same manner as reported previously [24]. RPMI medium 1640, a cell culture medium, was purchased from Life Technologies Japan Ltd. (Tokyo, Japan). Fetal bovine serum (FBS) was purchased from Moregate Biotech (Bulinba, Australia). TriPure Isolation Reagent was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Sulfanylamide (SA), 5-(4-nitrobenzyl)-6-thioinosine (NBMPR) and adenosine were purchased from Wako Pure Chemicals (Osaka, Japan). L5178Y-R cells were provided by Asahi Kasei Pharma Corporation (Tokyo, Japan). ReverTra Ace® qPCR Master Mix with gDNA Remover was purchased from Toyobo (Osaka, Japan). Fast SYBR™ Green Master Mix was purchased from Applied Biosystems Inc. (CA, USA). All other chemicals used were of the highest purity available.

2.2. Uptake and metabolism of MZR in L5178Y-R cells in vitro

L5178Y-R cells were cultured in RPMI medium 1640 supplemented with 10% FBS at 37 °C and 5% CO_2/95% air. On the day of experiment, cells were suspended in 10 ml of RPMI medium with 2% FBS at a density of 1×10^6 cells/ml in 25 cm^2 culture flask. Then, cells were incubated with MZR at a concentration of 3 or 10 μg/ml for 0.5, 1.0, or 3.0 h at 37 °C. In an inhibition study, 0.1 μM NBMPR or 2 mM adenosine was added to the incubation medium. At the end of the incubation, cells were washed with phosphate-buffered saline (PBS: pH 7.4, 137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4) by centrifugation at 500 × g for 3 min at 4 °C twice. The final pellet (1×10^7 cells) was suspended with 1 ml of ice-cold PBS and sonicated to extract MZR and MZRP. Sonicated suspension was centrifuged at 500 × g for 5 min and the supernatant was stored at −80 °C until analysis.

2.3. Collection of PBMCs

PBMCs were collected from the freshly collected heparinized blood according to the product information protocol of Histopaque 1083 (Sigma, Missouri, USA) for rats and Lympholyte-H (Cedarlane, Ontario, Canada) for KTRs, In the case of rat's samples, heparinized blood (6 ml) was layered onto the Histopaque 1083 surface (6 ml) in a 15 ml centrifuge tube and centrifuged at 400 × g for 30 min at room temperature. In case of KTR's samples, heparinized blood (2.5 ml) was diluted 2-fold with physiological saline and layered onto the Lympholyte-H (5 ml) in a 15 ml centrifuge tube and centrifuged at 800 × g for 30 min at 4 °C. The opaque interface containing PBMCs was collected in another centrifuge tube and mixed with 5 ml of ice-cold isotonic PBS for rat's samples, or with 15 ml saline for KTR's samples by gentle inversion. The mixture was then centrifuged at 250 × g for 10 min. The precipitates were resuspended in ice-cold PBS (1 ml) or saline (1 ml) and centrifuged again at 250 × g for 10 min at 4 °C. This washing process of PBMCs was repeated twice. Non-viable cells were identified by staining with trypan blue, and the number of viable cells was counted by microscopic observation.

2.4. Measurement of cell volume

Cell volumes of L5178Y-R cells and PBMCs of untreated rats were determined by measuring the distribution of SA in the same manner as reported previously [25] with a small modification. Briefly, PBS solution (0.5 ml) containing SA (2 mg/ml) was mixed with 0.5 ml of cell suspension (1×10^7 cells/ml) in a 1.5 ml polyethylene tube by gentle conversion and incubated for 1 h at 37 °C. The tube was placed on ice for 10 min and centrifuged at 10,000 × g and 4 °C for 5 min. The upper aqueous layer was removed carefully. To the precipitate, 1 ml of 50% acetonitrile was added and sonicated for more than 10 min to extract SA. The suspension was centrifuged at 10,000 × g for 10 min, and concentration of SA in the supernatant was measured by HPLC.

2.5. Real-time PCR analysis of nucleoside transporters in L5178Y-R cells and PBMCs of rats

L5178Y-R mouse lymphoma cells, male ICR mice (7-week-old) and male Sprague-Dawley (SD) rats (8-week-old) were used. ICR mice and SD rats were purchased from Hiroshima Jikken Dobutsu Kenkyujo (or Hiroshima Laboratory Animals Laboratory, Hiroshima, Japan). Total RNA was extracted from L5178Y-R cells, the intestine (jejunum) and kidney of mice and rats, and rat's PBMCs using TriPure Isolation Reagent according to the manufacturing instructions (Roche Diagnostics GmbH, Mannheim, Germany). Total RNA was reverse transcribed into cDNA using ReverTra Ace. Real-time PCR analysis of mRNAs of ENTs and CNTs was performed on a Step One PCR system (Applied Biosystems Inc, Lincoln Centre Drive Foster City, CA, USA) using reported primers and Fast SYBR™ Green [20–23]. The forward (5′-3′) and reverse (5′-3′) primer sequences of mouse ENT (mENT), mouse CNT (mCNT) and mouse GAPDH (mGAPDH) used for the real-time PCR analysis of L5178Y-R cells are as follows [21,22]: for mENT1, CTTGGAATTCAGGGTCAGAA and ATC AGGTCACACGACACCAA; for mENT2, CATGGAAACTGAGGGGAAGA and GTTCCAAAGGCCTCACAGAG; for mCNT1, GGATGTCTTTGCCTTTCAGGT and CAATCCCAAAGGAGATGTAGG; for mCNT2, GGAAGAGT GACTTGTGCAA and GTGCTGGTATAGAGGTCACAGC; mCNT3, CTGTC TTTTGGGGAATTGGA and CAGTAGTGGAGACTCTGTTT; for mGAPDH TGACGTGCCGCCTGGAGAAA and AGTGTAGCCCAAGATGCCCTTCAG, respectively. The forward (5′-3′) and reverse (5′-3′) primer sequences of rat ENT (rENT), rat CNT (rCNT) and rat GAPDH (rGAPDH) used for real-time PCR analysis of rat's PBMCs and tissues are as follows [20,23]: for rENT1, GCCGGCCAACTACACA and TATGGCCA-GAATGACAACTGC; for rENT2, CTGCTCTTCAACGTCATGGA and ATCC TGCCAGAAGATGATGG; for rCNT1, CAACACACAGAGGCAAAGAGAGT C and CCACACCAGCAGCAAGGGCTAG; for rCNT2, GGAAGAGTGACT TGTGCAAGCTTG and GTGCTGGTATAGAGGTCACAGCA; for rGAPDH,

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