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Methotrexate-bestatin interaction: Involvement of P-glycoprotein and organic anion transporters in rats

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ABSTRACT

To clarify the pharmacokinetic interaction and its possible mechanism, mutual effects between methotrexate (MTX) and bestatin in oral absorption and renal excretion in rats were examined *in vivo* and *in vitro*. A sensitive, quick and high performance method (LC–MS/MS) was used to determine concentrations of MTX and bestatin in biological samples. Plasma concentrations of MTX and bestatin markedly increased following oral and intravenous administration of MTX in combination with bestatin. The cumulative urinary excretion and renal clearance of the two drugs significantly decreased when MTX and bestatin were co-administered intravenously. Uptake of the two drugs in *in situ* single-pass intestinal perfusion studies and *in vitro* everted intestinal sac preparations significantly increased when co-administered, while uptake in rat kidney slices and hOAT1- or hOAT3-HEK 293 cells significantly decreased. Transport rates of bestatin and MTX from basolateral-to-apical transepithelial transport in MDR1-MDCK cells significantly decreased following co-administration. Additionally, intracellular concentrations increased, and the efflux transport of the two drugs was inhibited when given together. The IC_{50} values of MTX and bestatin in K562 and K562/ADR cells decreased when the two were co-administered. These findings indicate that the pharmacokinetic mechanism of interaction between MTX and bestatin occurs through co-transport by P-gp in the intestinal mucosa and OATs within the kidneys.

1. Introduction

The treatment of cancer with chemotherapeutic drugs is frequently impaired or ineffective as a result of either de novo or acquired resistance of tumor cells (Mistry et al., 2001). Tumor resistance can occur not only to a single cytotoxic drug used, but also occurs as a cross-resistance to a whole range of drugs with different structures and cellular targets. This phenomenon is called multiple drug resistance (MDR). Once MDR develops, the use of high doses to overcome resistance is ineffective, toxic effects appear and resistance is further stimulated (Ozben, 2006).

Although the etiology of MDR is multifactorial, overexpression of P-glycoprotein (P-gp; 170–180 kDa) has been shown to be involved in MDR (Choi, 2005). The overexpression of P-gp has been linked to the development of MDR in human cancers such as leukemias, lymphomas, multiple myelomas, neuroblastomas and soft tissues sarcomas (Tulpule et al., 2002). One strategy for reversing MDR in cells expressing P-gp is the combined use of anticancer drugs with chemosensitizers. Inhibitors of P-gp can be used to enhance oral bioavailability. Down-regulating P-gp or circumventing other MDR mechanisms is other approaches to overcoming MDR (Choi, 2005; Liscovitch and Lavie, 2002). Several compounds, including 20(S)-Rh2 (Zhang et al., 2010a,b), zosuquidar (LY335979), pantoprazole (Breedveld et al., 2005) and rotenone (Molnár et al., 2010) have been investigated to reverse P-gp-mediated multidrug resistance and are capable of effectively

Abbreviations: MTX, methotrexate; DDI, drug–drug interaction; P-gp, P-glycoprotein; OAT, organic anion transporter; MRP, multidrug resistance-associated protein; BCRP, breast cancer resistance protein; OATP, organic anion transporting polypeptide; KRB, Krebs–Ringer buffer; AUC, area under the plasma concentration–time curve; CL_P, plasma clearance rate; CL_R, renal clearance rate; MDR, multidrug resistance; K562, doxorubicin-sensitive erythroleukemic cells; K562/ADR, doxorubicin-resistant erythroleukemic cells (K562/adriamycin); ADR, adriamycin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphe-nyltetrazolium bromide.

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reversing the MDR phenotype *in vitro* (Krishna and Mayer, 2001). However, because of the unacceptable toxicity and side effects of these agents, their clinical use has been hampered. Therefore, many of the current efforts are directed toward the search for new compounds capable of inhibiting P-gp at nontoxic doses in cancer chemotherapy (Arora et al., 2005). In order to enhance the beneficial effects or reduce the toxicity of the drugs in question (Guo et al., 2012), it is helpful to find a drug that is a hypotoxic drug that can also affect the function of P-gp. Methotrexate (MTX), an anti-folate and anti-cancer agent, is commonly used for cancer chemotherapy, rheumatoid arthritis, and severe psoriasis, but with a narrow therapeutic window (Lin et al., 2009). Since MTX is a substrate of the organic anion transporters 1 (OAT1) and OAT3, these transporters have been suggested to contribute to the renal excretion of MTX (Sekine et al., 1997). MTX is also known as a substrate for P-gp (Choi, 2005). In recent years, there have been reports indicating that MTX resistance and drug interaction is associated with P-gp and OATs (Takeda et al., 2002; Yokooji et al., 2007).

Bestatin, an antibiotic of microbial origin and a small molecular weight dipeptide, is known to be a biological response modifier that demonstrates anti-tumor effects through augmentation of the host immune system (Lkhagvaa et al., 2008) and has been used in clinical trials for the treatment of leukemia. Bestatin has been confirmed as a P-gp substrate in our group (to be published), as well as a substrate of OAT1 and OAT3 (Zhu et al., 2012). It is excreted rapidly from the kidneys largely in an intact form (about 80%) (Scornik and Botbol, 1997). Both MTX and bestatin have played important roles in cancer chemotherapy; furthermore, MTX is always combined with other anti-drugs in the treatment of various human malignancies (Swierkot and Szechiński, 2006) and bestatin augments immune resistance to cancer and suppresses tumor growth. As such, bestatin is a reasonable choice for combination with MTX in the treatment of acute nonlymphocytic leukemia (Lkhagvaa et al., 2008) and chronic myelogenous leukemia (Sawafuji et al., 2003). However, the transporter-mediated interaction between MTX and bestatin is not fully understood.

The purpose of the present study is to elucidate the involvement of P-gp and OATs in the interaction between MTX and bestatin. LC–MS/MS was used to determine MTX and bestatin following *in vivo* oral administration. *In vivo* urinary excretion, *in vitro* kidney slices, *in situ* single-pass intestinal perfusion, *in vitro* everted small-intestinal sac preparations, and transfected-cell uptake and transport were analyzed. An MTT assay was used to investigate changes in cell viability when MTX and bestatin were administered together. The findings demonstrate that the pharmacokinetic mechanism of the DDI between MTX and bestatin is the result of both compounds being transported by P-gp in the intestinal mucosa, and OATs within the kidneys.

2. Materials and methods

2.1. Materials

MTX was purchased from Sigma (USA). Bestatin was provided by Pharmaceutical Co., Ltd. Shenzhen Main Luck. Cilostazol (internal standard) was purchased from Pharmaceutical Co., Ltd. Zhejiang Kinglyuan. All other chemicals were of analytical grade and were commercially available.

2.2. Cell culture

hOAT1- and hOAT3-HEK 293 cells (provided by Professor Yuichi Sugiyama, Graduate School of Pharmaceutical Sciences, University of Tokyo and Li-kun Gong, Shanghai Institute of Materia Medica, Chinese Academy of Science, Shanghai, China, respectively) and MDR1-MDCK cells (a kind gift from Professor Su Zeng, College of Pharmacy, Zhejiang University, Hangzhou, China) were routinely maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (heat-inactivated), 1% non-essential amino acid solution, 100 U/ml penicillin and 0.1 mg/ml streptomycin. ADR-sensitive erythroleukemic cells (K562) and doxorubicin-resistant erythroleukemic cells (K562/ADR) were purchased from Nanjing KeyGen Biotech. Co. Ltd (Nanjing, China) and were grown in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. All cell cultures were maintained at 37 °C and kept in a 95% humidified atmosphere containing 5% CO_2. The K562/adr cell line was cultured in RPMI 1640 medium in the presence of 1.8 μM doxorubicin. Cells were grown in RPMI 1640 medium without doxorubicin for 2 weeks before experiments.

2.3. Animals

Male Wistar rats weighing 220–250 g were obtained from the Experimental Animal Center of Dalian Medical University (Dalian, China; permit number SCXK 2008-0002). Rats were allowed free access to food and water. All rat experiments were conducted in accordance with the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised 1985). All efforts were made to minimize the number of animals used and their suffering.

2.4. Pharmacokinetic interaction in rats

Before the onset of each experiment, rats were fasted overnight, allowed free access to water prior to pharmacokinetic experiments and were anesthetized with pentobarbital (60 mg/kg, i.p.) in all cases. MTX was dissolved in 2% sodium bicarbonate and brought up to volume with normal saline. Bestatin was dissolved in normal saline.

2.4.1. In vivo absorption in rats

Rats were divided randomly into three groups: (1) MTX alone (5 mg/kg) as control; (2) bestatin alone (4 mg/kg) as control and (3) MTX (5 mg/kg) + bestatin (4 mg/kg) as the experimental group. MTX and bestatin were administered orally with a gavage needle. Blood samples (0.2 ml) were collected via jugular vein at 1, 5, 10, 15, 20, 30, 45, 60, 90, 120, 240, 360, 480 and 600 min in heparin tubes for MTX and bestatin determination, as described below.

2.4.2. In vivo plasma concentration and renal excretion in rats

Rats were anesthetized with pentobarbital administered via the jugular vein. Rats were divided randomly into three groups: (1) MTX alone (5 mg/kg) as control; (2) bestatin alone (4 mg/kg) as control and (3) MTX (5 mg/kg) + bestatin (4 mg/kg) as the experimental group. Blood samples were collected at 1, 5, 15, 30, 45, 60, 120, 240, 360, 480 and 600 min after administration. Bladders were cannulated with polyethylene tubing, the distal end of which flowed into an Eppendorf tube resting on a small pad of ice (Guo et al., 2012). Urine was collected directly from the bladder at 1, 2, 4, 6, 8, 10, 12 and 24 h after administration. The concentrations of MTX and bestatin were measured by LC–MS/MS. The cumulative urinary excretion and renal clearance (CL_R) were calculated.

2.5. In situ jejunal perfusion in rats

In situ jejunal perfusion was performed as previously described (Zhang et al., 2010a,b). A laparotomy was performed following pentobarbital anesthesia, and an inflow cannula made of silastic tubing was inserted into the jejunum approximately 1 cm below the ligament of Treitz. The bile duct was ligated to prevent possible enterohepatic circulation. The jejunal segment was then rinsed