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Interaction between rhein acyl glucuronide and methotrexate based on human organic anion transporters

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ABSTRACT

Rhein, a major bioactive compound of many medicinal herbs and the prodrug of diacerein, is often used with low dose of methotrexate as drug combination to treat rheumatoid arthritis. In this study, potential drug-drug interaction between methotrexate and rhein was investigated based on organic anion transporters (OAT). Our study demonstrated that rhein acyl glucuronide (RAG), the major metabolite of rhein in the human blood circulation, significantly inhibited the uptake of *p*-aminohippurate in hOAT1 transfected cells with IC_{50} value of 691 nM and estrone sulfate uptake in hOAT3 transfected cells with IC_{50} value of 78.5 nM. As the substrate of both hOAT1 and hOAT3, the methotrexate transport was significantly inhibited by RAG in hOAT1 transfected cells at 50 μM and hOAT3 transfected cells at 1 μM by 69% and 87%, respectively. Further *in vivo* study showed that after co-administrated with RAG in rats the $AUC_{0\text{-}24}$ values of methotrexate increased from 3109 to 5370 ng/mL*hr and the $t_{1/2}$ was prolonged by 40.5% (from 7.4 to 10.4 h), demonstrating the inhibitory effect of RAG on methotrexate excretion. In conclusion, rhein acyl glucuronide could significantly decrease the transport of methotrexate by both hOAT1 and hOAT3. The combination use of rhein, diacerein or other rhein-containing herbs with methotrexate may cause obvious drug-drug interaction and require close monitoring for potential drug interaction in clinical practice.

1. Introduction

Rhein (4, 5-dihydroxyanthraquinone-2-carboxylic acid) is a major bioactive compound of many medicinal herbs, such as *Rheum palmatum*, *Polygonum multiflorum*, *Cassia angustifolia* and *Cassia occidentalis*. Recent studies demonstrate that rhein possesses many pharmacological effects, including anti-inflammatory [1], antioxidant [2], anticancer [3], and antimicrobial [4], hepatoprotective [5] and nephroprotective activities [6]. Recently, its prodrug diacerein (diacetylrhein), which is completely converted into rhein before reaching the systemic circulation, has been developed to treat osteoarthritis and demonstrated significant effects on pain relief and function improvement [7–9]. Diacerein-containing medicines have been authorized in European Union Member States including Austria, Czech Republic, France, Greece, Italy, Portugal, Slovakia and Spain.

Methotrexate, an antifolate drug with a narrow therapeutic window, has been widely used in the treatment of rheumatoid arthritis at relatively low doses and acute lymphocytic leukemia at high doses [10,11]. It is reported that human organic anion transporter (OAT) family play important role in methotrexate transport [12–14]. Recently, a phase II clinical trial with respect to safety and efficacy of the combination of diacerein and methotrexate in the treatment of early rheumatoid arthritis was investigated in Thailand (http://clinicaltrials.gov/ct2/show/NCT01264211).

However, recent studies demonstrated that rhein possessed potent inhibitory effect on the drug transport mediated by hOAT1 and hOAT3 [15]. After oral administration, the major circulating

Abbreviations: AUC, area under the plasma time-concentration curve; ES, estrone sulfate; HBSS, Hank's balanced salt solution; HEK293, human embryonic kidney 293; IS, internal standard; OAT, organic anion transporter; PAH, *p*-aminohippuric acid; RAG, rhein acyl glucuronide.

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form of rhein in the body is the glucuronide conjugates (60%), followed by unchanged form (20%) and sulfate conjugates (20%) in human beings [16,17]. In addition, our previous study further confirmed that rhein was mainly metabolized to rhein acyl glucuronide (RAG) in human liver microsomes and RAG was chemically reactive [18]. As a result, RAG might affect the drug transport mediated by hOAT1 and hOAT3, and ultimately induce severe drug-drug interaction with methotrexate.

In order to fully understand the potential interaction of RAG with methotrexate, the inhibitory effect of RAG on hOAT1 and hOAT3 was examined in cell lines stably expressing each transporter independently. More importantly, the interference of RAG with the transport of methotrexate was investigated, both *in vivo* and *in vitro*.

2. Materials and methods

2.1. Chemicals

Rhein was obtained from Shanghai Jiukun International Trade Co., Ltd. (Shanghai, China). RAG was synthesized in our laboratory as described previously [18]. *p*-aminohippuric acid (PAH), estrone sulfate (ES) and probenecid were purchased from Sigma–Aldrich (St. Louis, MO, USA). Methotrexate was purchased from Aladdin Industrial Corporation (Shanghai, China). Fetal bovine serum, Dulbecco's modified Eagle's medium and trypsin were from Hyclone (Logan, UT, USA). The bicinchoninic acid protein assay kit was purchased from Alpha Pharmaceutical Co. (Jiangsu, China).

2.2. Cell cultures

Stably transfected human embryonic kidney 293 (HEK293) cells overexpressing human OAT1 and OAT3 as well as vectors were provided by professor Dafang Zhong, Shanghai Institute of Materia Medica, Chinese Academy of Sciences (Shanghai, China). The cell lines expressing a single transporter gene were used to distinguish roles of various transporters individually, while corresponding control cells were prepared with vectors that did not contain target gene. All cell lines were maintained at 37 °C with 5% CO_2 and 95% humidity in high-glucose Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin.

2.3. Cellular uptake assay

2.5×10^5 cells/well were seeded in 24-well culture plates and grown in high dulbecco's modified Eagle's medium for two days. On the day of the experiment, cells were washed twice with pre-warmed Hank's balanced salt solution (HBSS) and equilibrated with HBSS for 30 min at 37 °C. Uptake experiments were initiated by addition of 500 μL defined concentration of substrates with or without inhibitors in HBSS.

2.4. Effect of RAG on PAH and ES transport

PAH (40 μM) was used as the substrate of hOAT1 and incubated with RAG for 6 min in inhibition study. ES (5 μM) was used as a substrate of hOAT3 and incubated with RAG for 5 min in hOAT3 inhibition study. The inhibition effect of RAG ranged from 5 to 50000 nM and 0.01–10000 nM was investigated in hOAT1 and hOAT3 expressed cells, respectively. At the end of incubation, cells were quickly rinsed three times with ice-cold HBSS, and lysed with 0.2 M NaOH for 1 h at 4 °C. After neutralized with 0.2 M HCl, the samples were analyzed by LC-MS/MS. Protein assay was carried out by using the Pierce™ BCA Protein Assay Reagent Kit (Thermo Scientific, Rockford, USA) with albumin as the standard.

2.5. Effect of RAG on methotrexate transport

Before addition of methotrexate, cells were washed twice with pre-warmed HBSS (PH 6.5) and pre-incubated with different concentration of RAG in HBSS (1 μM, 5 μM and 50 μM) for 30 min at 37 °C. The uptake experiment was initiated by the addition of methotrexate (50 μM for hOAT1, 25 μM for hOAT3). The incubation was terminated by washing cells with ice-cold HBSS. The cells were lysed with 250 μL of 0.2 M NaOH solution for 1 h at 4 °C and then neutralized with 250 μL of 0.2 M HCl solution. The intracellular methotrexate concentrations were quantified by LC-MS/MS.

2.6. In vivo pharmacokinetic studies

Experimental protocols, handling and treatment of rats was approved by the University Ethics Committee (approval number: BK20150349) and conducted according to the regulations for the Use and Care of Experimental Animals at Soochow University. Male Sprague–Dawley rats (obtained from Suzhou Supusi Biological Technology Co., Ltd) were used for the study (200–250 g body weight). The animals were kept in a room at 22–24 °C with 55–60% relative humidity and a light cycle (12 h light and 12 h dark). They had free access to standard rodent chow and clean water. On the day of experiments, methotrexate (2 mg/kg) was administered intravenously to rats with or without pretreatment with RAG (5 mg/kg) intravenously 1 min prior to methotrexate administration. Blood samples were collected from the jugular vein at 0, 0.033, 0.083, 0.167, 0.333, 0.5, 0.75, 1, 1.5, 3, 6, 12, 24 h postdose. Plasma samples were obtained by centrifugation at 13,000 rpm. All samples were stored at -80 °C until analyzed.

2.7. LC-MS/MS analysis

150 μL acetonitrile containing internal standard (IS) were added to 50 μL of cell homogenate, and samples were immediately vortexed for 2 min followed by centrifugation at 13,000 rpm for 10 min. Aliquots of 10 μL of the supernatants were collected for analysis.

All samples were analyzed by a LC-MS/MS system consisting of an API 4000 Qtrap mass spectrometer equipped with two LC-20AD pumps with a CBM-20 A controller, DGU-20 A solvent degasser, a SIL-20 A autosampler (Shimadzu, Columbia, MD, USA) and a turbo-V ionization source (Applied Biosystems, Foster City, CA, USA). The mobile phase consisted of 0.03% acetic acid water solution (A) and acetonitrile (B). Samples were analyzed on an Agela Venusil XBP C_{18} column (50 × 2.1 mm, 5 μm) adopting a gradient elution as follows: 0–1.0 min, 5% B; 1.0–1.5 min, 5-40% B; 1.5–3.5 min, 40% B; 3.5–3.6 min, 40-5% B; 3.6–5.0 min, 5% B. For MS/MS quantitation of methotrexate, the API 4000 Qtrap mass spectrometer was operated in the ESI positive mode with multiple reaction-monitoring model. The ion transitions and collision energy were set as *m/z* 455.1 (M + H) → 308.1 and + 31 V, respectively.

2.8. Data analysis

All data are reported as mean ± SD from three independent experiments as indicated. Pharmacokinetic parameters were calculated using a non-compartmental analysis by using Phoenix WinNonlin 6.1 software (Certara, Princeton, NJ). The comparisons of pharmacokinetic parameters were conducted using standard student's t-test. Differences between groups were assumed statistically significant for P values < 0.05.