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Inhibitory effects of *p*-aminohippurate and probenecid on the renal clearance of adefovir and benzylpenicillin as probe drugs for organic anion transporter (OAT) 1 and OAT3 in humans

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ABSTRACT

Probe substrates for, and inhibitors of, specific transporters are desired to evaluate quantitatively the in vivo functions of transporters in humans. Based on published data, adefovir and benzylpenicillin were selected as organic anion transporter (OAT) 1- and OAT3-selective probe substrates, respectively. In human kidney slices, probenecid potently inhibited the uptake of both adefovir and benzylpenicillin with inhibition constant (K_i) values of 18.6 ± 5.1 and 12.6 ± 4.2 μM, respectively, whereas *p*-aminohippurate (PAH) preferentially inhibited adefovir uptake. A clinical drug-interaction study involving healthy subjects was performed to investigate the dose-dependent inhibition potencies of probenecid and PAH on the renal clearance of the probe substrates. Adefovir or benzylpenicillin was coadministered with different oral doses of probenecid (500, 750, or 1500 mg) or intravenous PAH infusion rates (70, 120, or 210 mg/min/person) to the same subject using a crossover design.

The renal clearance of adefovir was reduced by 45% and 46% in the subjects treated with the maximum dose of probenecid and PAH, respectively, which was in accordance with the results of in vitro inhibition study. On the other hand, renal clearance of benzylpenicillin was reduced by 78% in the subjects treated with the maximum dose of probenecid (1500 mg), which could be explained by its in vitro K_i values. However, PAH unexpectedly increased the renal clearance of benzylpenicillin by 47%. These results suggest that adefovir and benzylpenicillin can be used as probe drugs for OAT1 and OAT3, respectively, and that PAH can be used to investigate the role of OAT1 in the urinary excretion of drugs in humans, whereas it may modulate other transport processes in the kidney.

1. Introduction

Drug–drug interactions (DDIs) involving the inhibition of metabolism or excretion increase systemic exposure to victim drugs, leading to their accumulation in the body, and consequently potentiate their pharmacological/adverse effects. To avoid life-threatening events caused by DDIs in the postmarketing stage, US and EU regulatory agencies recently published new draft guidance and guideline on the appropriate risk evaluation of DDIs in the process of drug development (EMA, 2012; FDA, 2012). One of the major revisions in these documents includes the addition of descriptions with decision trees for the risk assessment of transporter-mediated DDIs. These changes were made because various human drug transporters have been characterized extensively and several clinical studies have shown the in vivo significance of these transporters to the effects of coadministered inhibitor drugs and genetic polymorphisms of certain transporters on the pharmacokinetics of clinically used substrate drugs (Konig et al., 2013; Yoshida et al., 2013).

To quantify the DDI risk of an investigational drug as either a victim or perpetrator in humans, it is convenient to use in vivo-selective substrates for, and inhibitors of, metabolic enzymes/transporters in clinical DDI studies. In the field of metabolic enzymes, in vivo probe substrates and inhibitors are well established, and cocktail dosing of

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probe substrates enables one to determine the in vivo activity of multiple enzymes simultaneously. However, few validated probe drugs that can sensitively detect alterations in the transport activity of a specific transporter have been found.

The urinary excretion mechanism for most anionic drugs comprises both active tubular secretion and glomerular filtration in the kidney (Ito et al., 2013). We have demonstrated previously that the transporters that mediate the uptake process from the blood circulation act as the rate-determining process in the overall tubular secretion of anionic drugs in the kidney (Watanabe et al., 2011, 2009). Because of their broad substrate specificities, organic anion transporter 1 (OAT1/*SLC22A6*) and OAT3/*SLC22A8* are considered to be the major transporters in the kidney responsible for the basolateral uptake of various organic anions including drugs and uremic toxins. OAT1 plays a major role in the renal uptake of hydrophilic and small organic anions such as *p*-aminohippurate (PAH), 2,4-dichlorophenoxyacetate, and acyclic nucleotide phosphonates, whereas OAT3 has a broader substrate specificity than OAT1 and accepts amphipathic and hydrophobic organic anions, and even some organic cations (cimetidine) and zwitterions (fexofenadine) (Burckhardt, 2012; Kusuhara and Sugiyama, 2009). Probenecid has been used to characterize the urinary excretion mechanisms of drugs. Probenecid is a potent inhibitor of both OAT1 and OAT3, and coadministration of probenecid at a therapeutic dose causes a marked inhibition of the tubular secretion of OAT1 and OAT3 substrate drugs (Kusuhara et al., 2013; Shitara et al., 2005). Thus, both the European Medicines Agency (EMA) and US Food and Drug Administration (FDA) selected these transporters as important transporters for drug disposition in their DDI guideline and draft guidance (EMA, 2012; FDA, 2012). However, the selective inhibitors and selective substrates for OAT1 and OAT3 that can be used in clinical situations and the appropriate clinical protocols for these probe drugs have not been established in humans.

The purpose of this study was to understand quantitatively the transport functions of OAT1 and OAT3 by the use of probe substrate drugs and inhibitors in vivo in humans. Based on the published and in house information, we selected adefovir and benzylpenicillin as the probe substrate drugs for OAT1 and OAT3, respectively, because of their selective recognition by each OAT isoform and low renal clearance compared with renal blood flow rate (Cihlar et al., 1999; Nozaki et al., 2007; Vanwert et al., 2007). To evaluate OAT1- and OAT3-mediated transport in a clinical study, PAH and probenecid were selected as inhibitors. PAH is a prototypical OAT1 substrate; because its Michaelis constant (K_m) is much larger for OAT3 than for OAT1, it can discriminate OAT1-mediated uptake from the net renal uptake in vitro (Deguchi et al., 2004; Nozaki et al., 2007; Tahara et al., 2005a). Importantly, PAH has been used clinically to measure the capacity of renal secretion clearance by saturating this process (i.e., OAT1-mediated uptake (Vallon et al., 2008)), indicating that PAH can also inhibit OAT1-mediated transport in vivo. By contrast, we found no information about any OAT3-selective inhibitor that would be applicable to a clinical DDI study. Therefore, we decided to use probenecid because it inhibits both OAT1 and OAT3 (Tahara et al., 2005a; Takeda et al., 2001). This study intended to provide a rationale for the use of adefovir and benzylpenicillin as probe drugs for OAT1 and OAT3, respectively, and the use of PAH as an inhibitor to investigate the importance of OAT1 in the renal elimination of test drugs.

2. Materials and methods

2.1. Chemicals

[^{14}C]-Benzylpenicillin (52.6 mCi/mmol) and [^{3}H]-adefovir (10 Ci/mmol) were purchased from Moravek Biochemistry, Inc. (Brea, CA). [^{3}H]-*p*-Aminohippurate (PAH; 4.1 Ci/mmol), [^{14}C]-inulin (8 mCi/mmol), and [^{3}H]-dehydroepiandrosterone sulfate (DHEAS; 74 Ci/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA). Unlabeled adefovir and tenofovir were kindly provided by Gilead Science, Inc. (Forest, CA). Unlabeled PAH and DHEAS were purchased from Sigma–Aldrich (St Louis, MO), and unlabeled benzylpenicillin was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals and reagents were of analytical grade and are commercially available.

2.2. Cell culture

HEK293 cells were cultured in Dulbecco's modified Eagle's medium (low glucose; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Sigma–Aldrich) and 1% Penicillin–Streptomycin, Liquid (Invitrogen) at 37 °C in 5% CO_2 at 95% humidity.

2.3. Uptake assay with transporter-expressing cell lines

Human OAT1- and OAT3-expressing HEK293 cells were constructed as described previously (Tahara et al., 2005b). human OAT1- and human OAT3-expressing HEK293 cells and vector-transfected control cells were seeded in 12-well plates at a density of 1.5×10^5 cells/well. After 2 days of culture, the medium was replaced with fresh medium containing 5 mM sodium butyrate to induce the expression of the transporter. The transport study was performed 24 h after induction. The cells were washed twice and preincubated with transport buffer (118 mM NaCl, 23.8 mM $NaHCO_3$, 4.83 mM KCl, 0.96 mM KH_2PO_4, 1.20 mM $MgSO_4$, 12.5 mM HEPES, 5 mM glucose, and 1.53 mM $CaCl_2$, adjusted to pH 7.4 and prewarmed to 37 °C) at 37 °C for 15 min before the uptake assay.

The uptake of [^{3}H]-adefovir and [^{14}C]-benzylpenicillin (0.1 μM including unlabeled compounds) was measured for 5 min in the absence or presence of PAH or probenecid to evaluate the inhibitory effects of PAH and probenecid on the OAT1-mediated uptake of adefovir and the OAT3-mediated uptake of benzylpenicillin since the linearity of the time-dependent uptake of adefovir and benzylpenicillin was maintained up to 5 min (data not shown). The tested substrate and inhibitor are added in the incubation media simultaneously. After the designated periods, ice-cold transport buffer was added to the cells to stop the uptake, and the cells were washed three times in ice-cold transport buffer. The intracellular accumulation of radioactivity was measured at the end of the experiments by lysing the cells with 500 μL of 0.2 N NaOH and maintaining them overnight at room temperature. On the following day, the cell lysates were neutralized with 250 μL of 0.4 N HCl, and the radioactivity in the cell lysates was measured in a liquid scintillation counter (LS 6000SE; Beckman Instruments, Fullerton, CA) after the addition of a scintillation cocktail (Clear-sol I; Nacalai Tesque, Tokyo, Japan). Aliquots (50 μL) of the cell lysates were used to measure the protein concentration with bovine serum albumin as the standard (Lowry method).

2.4. Uptake assay with human kidney slices

This study protocol was approved by the ethics committees of the Faculty of Pharmaceutical Sciences of the University of Tokyo, Japan, and Tokyo Women's Medical University, Japan. All participants provided their written informed consent.

The uptake assay was performed as described previously (Nozaki et al., 2007). Intact renal cortical tissues, obtained from patients who had been surgically nephrectomized because of renal cell carcinoma at Tokyo Women's Medical University, were used as the source of the kidney slices. The kidney slices (300 μm in thickness) were prepared and placed in an ice-cold oxygenated incubation buffer before