Possible Involvement of Organic Anion Transporter 2 on the Interaction of Theophylline with Erythromycin in the Human Liver

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Abbreviations: Cytochrome P450, CYP; hOat, human organic anion transporter; MRPs, multidrug resistance associated proteins; OATP/oatp, organic anion transporting polypeptide.
ABSTRACT:

Organic anion transporter 2 (Oat2 [SLC22A7]) is a multispecific organic anion transporter. Although several substrates of human Oat2 (hOat2) have been elucidated, a possible involvement of hOat2 on the drug interaction is less defined. The purpose of this study was to investigate the interaction of theophylline with erythromycin mediated by hOat2 using a Xenopus laevis oocyte expression system. When expressed in X. oocytes, hOat2 mediated the transport of theophylline and erythromycin. The finding indicates that the two compounds are novel substrates for hOat2. The apparent $K_m$ values for the uptake of hOat2 which mediated the transport of theophylline and erythromycin were 18.5 $\mu$M and 12.6 $\mu$M, respectively. The hOat2-mediated uptake of [14C]theophylline and [14C]erythromycin were cis-inhibited by adding erythromycin and theophylline, respectively. Our present findings suggest that hOat2 may, at least in part, be involved in the theophylline-erythromycin interaction in the human liver.
Introduction

The liver is a central organ for the detoxification and elimination of a wide variety of organic compounds. Several distinct liver-predominant multispecific organic anion transporters such as MRPs (MRP1-5[ABCC1-5]), organic anion transporting polypeptides (OATP1A2[SLCO1A2], 1B1[SLCO1B1], 1B3[SLCO1B3], 2B1[SLCO2B1]), and Oat5[SLC22A10]) have been isolated and well characterized (Cole and Deeley, 1993; Simonson et al., 1994; Allikmets et al., 1996; Ito et al., 1998; Sun et al., 2001; Hagenbuch and Meier, 2004). To date, five different human Oat isoforms (hOat1 - 5) have been isolated (Sun et al., 2001). Although the role of hOat5 has not been characterized yet, hOat2 is considered to be one of the key molecules in hepatic handling of organic anions because this isoform is highly expressed in the human liver (Sun et al., 2001).

Theophylline, 1,3-dimethylxanthine, has been widely used as a bronchodilatory drug for the treatment of neonatal apnea in premature newborns and patients with chronic obstructive airway disease such as asthma and bronchitis. Theophylline is metabolized by N-demethylation to 1-methylxanthine and 3-methylxanthine and by 8-hydroxylation to 1,3-dimethyluric acid in the liver (Fuhr et al., 1992). It is well known that many drugs increase or decrease the clearance of theophylline, probably by interaction with one or more of the cytochrome P450 species (CYPs). It has been reported that theophylline is metabolized by CYP1A2, 1B1, 2E1, and 3A4 (Sarkar et al., 1992; Ha et al., 1995; Rasmussen et al., 1995; Shimada et al., 1997).
Since the frequency and severity of toxicity from theophylline increases as serum concentrations exceed 20 µg/mL and efficacy is diminished as levels decline below 10 µg/mL, the administration of a second drug that alters the elimination of theophylline has potential clinical relevance (Jenne et al., 1972; Mangione et al., 1978; Aslaksen et al., 1981).

Macrolide antibiotics such as erythromycin have been used for treatment of a variety of infections and are often combined with other drug therapies. For example, patients with chronic asthma receiving continuous therapy with theophylline may require short courses of erythromycin for unrelated pyogenic infections. It has been reported that serum theophylline concentrations increased in patients with concomitant administration of erythromycin by inhibiting hepatic CYPs (Cummins et al., 1976; Kozak et al., 1977; Pfeifer et al., 1979). However, it is not clear at this time whether such a drug interaction could be mediated by drug transporters.

In the present study, therefore, we investigated the possible involvement of hOat2 on the interaction of theophylline with erythromycin.
Experimental Procedures

Chemicals. [14C]Theophylline (52 mCi/mmol) and [14C]erythromycin (55 mCi/mmol) were purchased from American Radiolabeled Chemicals, Inc (St. Louis, MO, U.S.A.). All other chemicals were of the highest grade commercially available.

Xenopus Laevis Oocyte Preparation, and cRNA Synthesis. Isolation and preparation of X. oocytes was performed as described elsewhere (Kobayashi et al., 2002b). The hOat2 cDNA was linearized with BamHI, and the capped cRNA was transcribed in vitro by T7 RNA polymerase. Defolliculated oocytes were microinjected with 50 ng of in vitro transcribed cRNA under a stereomicroscope using a microdispenser (Drummond scientific, Broomall, PA, U.S.A.) and incubated for two days in a modified Barth’s solution containing gentamicin (50 µg/ml) at 18°C.

Transport Assays. Uptake experiments of radiolabeled substrates were performed in an ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 5 mM HEPES, pH 7.4) at room temperature. Oocytes were incubated in an ND96 solution containing radiolabeled substrates for 1 h at room temperature. For the kinetic study, concentration-dependent uptake experiments of [14C]theophylline and [14C]erythromycin via hOat2 were performed with each compound at a final concentration ranging from 1, 2, 5, 10, 15, and 20 µM, and 1, 5, 10, 20, 40, and 60 µM, respectively. The compounds were incubated with oocytes expressing hOat2 for 1 h at room temperature. The uptake was terminated by the addition of 2 ml of an ice-cold ND96 solution, and the oocytes were washed with the same solution at
least five times. Counts in control (non-injected) oocytes were subtracted from the counts in cRNA-injected oocytes. The oocytes were solubilized with 250 µl 10% SDS, and accumulated radioactivity was determined with a liquid scintillation counter. \( K_m \) indicates the Michaelis-Menten constant (micromolar). We repeated each experiment more than 3 times to confirm the results.

**Inhibition Study.** For the inhibition study, oocytes expressing hOat2 were incubated for 1 h in an ND96 solution containing \([^{14}\text{C}]\text{theophylline} (10 \mu\text{M}) or [^{14}\text{C}]\text{erythromycin} (10 \mu\text{M}) in the presence or absence of inhibitors at a final concentration of 1 mM. Theophylline, erythromycin, clarithromycin, and valproate were directly dissolved in an ND96 solution from a stock solution. These stock solutions of the inhibitors were prepared in dimethyl sulfoxide and diluted to a final concentration as described above. The final concentration of dimethyl sulfoxide in the assay medium did not exceed 1.0%.

**Statistical Analysis.** Statistical differences were determined by the unpaired Student’s \( t \)-test. Comparisons of data measuring initial rates of uptake of radiolabeled substrates in the presence and absence of inhibitors were determined by ANOVA. The values represent the mean ± S.E.M. (*p*<0.05).
Results and Discussion

This paper describes a possible involvement of hOat2[SLC22A7] on the interaction of theophylline with erythromycin using a X. oocyte expression system.

Theophylline has been widely used for the treatment of asthma and chronic obstructive pulmonary disease (COPD). Pharmacokinetic studies have revealed that orally administered theophylline is almost completely absorbed, and the liver is responsible for biotransformation of about 90% of theophylline (Cornish and Christman, 1957; Cummins et al., 1976; Kozak et al., 1977; Pfeifer et al., 1979). It has been reported that theophylline clearance is potentially modified by many factors including hepatic microsomal CYP-mediated demethylation, xanthine oxidase, or hepatic uptake (Jonkman and Upton, 1984).

On the other hand, macrolide antibiotic erythromycin was derived from Streptomyces erythreus in 1952. When acute infectious complications arise, macrolides are frequently used, and erythromycin is often chosen in the clinical setting. In addition, erythromycin has a broad spectrum of activity similar to penicillin; therefore, this drug can be chosen for the treatment of infections in patients who are allergic to penicillin.

Several reports suggest that macrolide antibiotics may alter theophylline clearance. The interaction between theophylline and erythromycin would clearly be of clinical importance because of its toxicity. The exact mechanism of interference with theophylline disposition by erythromycin is still unclear; it might be explained by
inhibition of the 1-demethylation pathway by erythromycin by inhibiting hepatic CYP1A2 and 3A4 and resulting in an increase of the blood concentration of theophylline (Adebayo et al., 1986; Periti et al., 1992). Because the therapeutic range of theophylline is very narrow, changes in theophylline clearance may result in lack of a sufficient bronchodilating effect or in the appearance of a toxic effect.

Oat2 was initially called the novel liver-specific transport protein (NLT) (Simonson et al., 1994). Oat2, as well as OATP/Oatp, is a multispecific organic anion transporter and is highly expressed at the sinusoidal membrane of the liver (Simonson et al., 1994; Sekine et al., 1998). However, as far as we know, it is not clear at this time whether the theophylline-erythromycin interaction can occur via hepatic drug transporters. Therefore, we focused on the role of hOat2 and assumed that hOat2 could transport theophylline and erythromycin.

Using X. oocytes injected with hOat2 cRNA, we firstly examined the transport of theophylline and erythromycin mediated by hOat2. Since the uptake of [14C]theophylline by un-injected oocytes was equal to the oocytes injected with 50 nL of water (data not shown), un-injected oocytes were used as the control instead of water-injected oocytes throughout this study. As shown in Fig. 1, both [14C]theophylline and [14C]erythromycin were significantly transported via hOat2 to about 5.9-fold and 33.3-fold that of the controls, respectively. These compounds are identified for the first time as hOat2 substrates. We have revealed that mouse Oat2 (mOat2) is a multispecific organic anion transporter (Kobayashi et al., 2002b).
Therefore, we assumed that mOat2, as well as hOat2, also mediates the transport of theophylline and erythromycin. As expected, both compounds were significantly transported via mOat2 (data not shown). Taken together, previous data indicate that theophylline and erythromycin are conserved substrates of h- and m-Oat2.

To determine the affinity of theophylline and erythromycin with hOat2, based on these findings, concentration dependence of the uptake of \[^{14}\text{C}]\text{theophylline}\ and \[^{14}\text{C}]\text{erythromycin}\ via \text{hOat2}\ was subsequently examined. As shown in Fig. 2, hOat2-mediated the transport of \[^{14}\text{C}]\text{theophylline}\, and \[^{14}\text{C}]\text{erythromycin}\ showed saturable kinetics and followed the Michaelis-Menten equation. Eadie-Hofstee plots of the concentration dependence of theophylline and erythromycin uptake after subtraction of the uptake by non-injected oocytes revealed that the estimated \(K_m\) values are 12.6 µM and 18.5 µM, respectively. Thus, the affinities of both compounds with hOat2 are very similar. These results indicate that theophylline and erythromycin are transported via hOat2.

The therapeutic range of plasma concentration and unbound fraction of theophylline were reported to be 55-110 µM and 40-60%, respectively (Tenenbein, 1989). Therefore, the steady-state concentration of unbound theophylline in the plasma is estimated to be approximately 41.3 µM. In addition, the maximum plasma concentration and unbound fraction of erythromycin were reported to be 4.8 µM and 84%, respectively (Periti et al., 1989); the steady-state concentration of unbound erythromycin in the plasma is estimated to be approximately 4.0 µM. Comparing the
plasma concentration and our kinetic data, hOat2 may be a responsible molecule for the transport of theophylline and the interaction of theophylline with erythromycin in the human liver.

To confirm hOat2 involvement in the theophylline-erythromycin drug interaction, cis-inhibition of hOat2-mediated drug uptake experiments were performed. The results are shown in Fig. 3. The hOat2-mediated transport of [14C]theophylline was strongly inhibited by erythromycin and slightly inhibited by clarithromycin. No inhibitory effect was observed by valproate. Similarly, transport of [14C]erythromycin via hOat2 was inhibited by unlabeled theophylline, but not by clarithromycin and valproate. Although some reports were unable to confirm any significant effects of theophylline-erythromycin drug interaction (Kelly et al., 1981; Melethil et al., 1982), our present findings together with the membrane localization suggest that theophylline-erythromycin drug interaction may occur at the sinusoidal membrane of the liver by inhibiting hOat2-mediated transport.

Regarding the interaction between theophylline and clarithromycin, von Rosensteli and Adam et al. have reported that there is a slight increase in serum concentration of theophylline AUC and a 16.4% decrease in theophylline clearance with concurrent administration of both drugs (1995). Supportive evidence has been reported by Gillum et al. (1996). Taking their reports (von Rosensteli and Adam et al., 1995; Gillum et al., 1996) and our inhibition experiment into consideration, theophylline-clarithromycin interaction would be rare or if occurred, such interaction
may not be mediated by hOat2. Further study is required.

The expression of Oat isoforms (Oat1-Oat3) exhibits a sex difference in rats and mice (Buist et al., 2002; Kobayashi et al., 2002a; Kobayashi et al., 2002b; Buist and Klaassen, 2004). Rat Oat2 (rOat2) in the liver is expressed at a higher level in male rats than in females (Buist et al., 2002; Kobayashi et al., 2002a), whereas mOat2 mRNA of the liver is detected predominantly in females than in males (Kobayashi et al., 2002b). Recently, Buist and Klaassen have extensively investigated that there is no sex-related differential gene expression of mOat2 in the liver using branched DNA (bDNA) analysis (Buist and Klaassen, 2004). Although there are no reports concerning the gender-related differential expression of hOat2 mRNA in the liver of men and women, Nafziger and Bertino have reported that theophylline is cleared significantly higher in women than in men (Nafziger and Bertino, 1989), suggesting that such sex-dependent theophylline clearance might be caused by the differential gene expression of hOat2 between men and women. In this respect, additional study is needed.

In conclusion, our paper is the first report concerning the possible involvement of hOat2 on theophylline-erythromycin interaction. Our present findings, therefore, indicate that hOat2 may, at least partly, be involved in the interaction of theophylline with erythromycin at the sinusoidal membrane of the human liver. It would be interesting to elucidate whether other transporters such as OATP1B1, 1B3, and 2B1 (Kool et al., 1999; Hagenbuch and Meier, 2004) are also involved in the
theophylline-erythromycin interaction.
References


excretion of theophylline and its metabolites in the presence of erythromycin.


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Figure Legends

Figure 1. Uptake of [14C]theophylline and [14C]erythromycin by hOat2-expressing oocytes. After two days incubation, uptake experiments were performed in a solution containing Na+ for 1 h. The uptake rates of radiolabeled compounds ([14C]theophylline, 10 μM; [14C]erythromycin, 10 μM) by the control or hOat2 expressed oocytes were measured. Values are mean ± S.E.M. of 8-13 oocyte determinations. The significance between control and hOat2-cRNA-injected oocytes was determined by the unpaired t test (*p<0.05). Other experimental conditions and methods are described under Experimental Procedures.

Figure 2. Concentration-dependence of hOat2-mediated uptake of [14C]theophylline (A) and [14C]erythromycin (B). The uptake rates of theophylline and erythromycin by control or hOat2-expressing oocytes for 1 h were measured at variable concentrations. hOat2-mediated transport was determined by subtracting the transport velocity in control oocytes from that in hOat2-expressing oocytes. v/s, velocity per concentration of substrates. Values are mean ± S.E.M. of 5-11 oocyte determinations. Other experimental conditions and methods are described under Experimental Procedures.

Figure 3. Inhibition of hOat2-mediated [14C]theophylline uptake by various organic compounds. The uptake rates of 10 μM [14C]theophylline (upper panel) and 10 μM [14C]erythromycin (lower panel) by hOat2-expressing oocytes or non-injected oocytes were determined in the absence or presence of 1 mM inhibitors (theophylline,
erythromycin, clarithromycin, and valproate). Values are mean ± S.E.M. of 9-13 oocyte determinations. The values were expressed as a percentage of $[^{14}\text{C}]$theophylline or $[^{14}\text{C}]$erythromycin uptake. Statistical differences were evaluated using ANOVA ($^*p<0.05$).
Fig. 2.

A

pmol/oocyte/h vs Concentration (μM)

B

pmol/oocyte/h vs Concentration (μM)
Fig. 3.