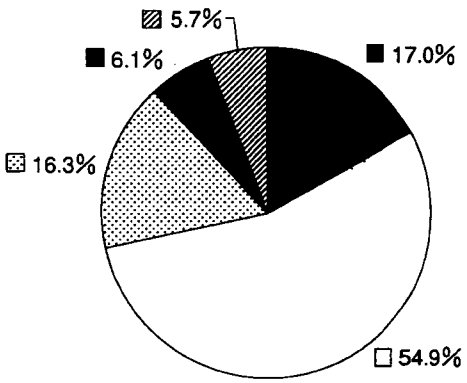
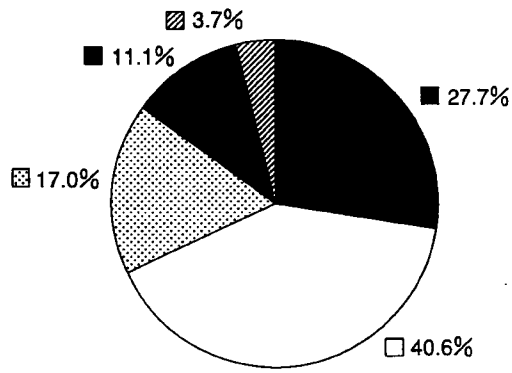
いては，有効回答数2131件中「血中動態パラメータ(tmax, Cmax, $t_{1/2}$, AUC)」(222件)，「代謝・排泄経路」(218件)，「肝障害・腎障害における情報」(215件)，「食事の影響」(208件)が多かったが，「代謝・輸送に関連する分子種の遺伝子多型の情報」は75件と少なかった．その他の意見として「薬効の持続時間」,「妊婦，産婦，授乳婦等への投与」「吸収部位」「妊産婦への投与」「水，オクタノール係数・尿中未変化体排泄率」などがあった．「薬物動態の添付文書での記載について，後発医薬品は先発医薬品と比べて十分ですか(複数回答可)」(図15)では，有効回答数526件中「先発医薬品との同等性比較データがない」(155件)が最も多く，「動態パラメータが記載されていない」(94件)，「血中濃度推移のグラフがない」(91件)となった．「その他」の意見としては，「後発医薬品



■ 1. 相互作用の項の冒頭に書いてあればよい
□ 2. 相互作用の項の一覧表の中で、機序・危険因子欄に記載
⊠ 3. 上記1及び2の両方に記載が必要
■ 4. 相互作用の項には記載不要(薬物動態の項にあればよい)
■ 5. 添付文書に記載する必要はない
▨ 5-1 インタビューフォームに記載
■ 5-2 提供する必要はない

図12. 相互作用に関与する薬物代謝・輸送分子種が判明している場合，添付文書の相互作用の項での分子種の記載方法はどれが適切ですか(複数回答可，有効回答数264件)



■ 1. 十分である
□ 2-1 必要な情報が欠けている
⊠ 2-2 改訂すべき事項が反映されていない
■ 2-3 その他
▨ 0. 無回答(無選択)

図13. 後発医薬品の添付文書の相互作用欄の記載は先発医薬品と比べて十分ですか(有効回答数261件)

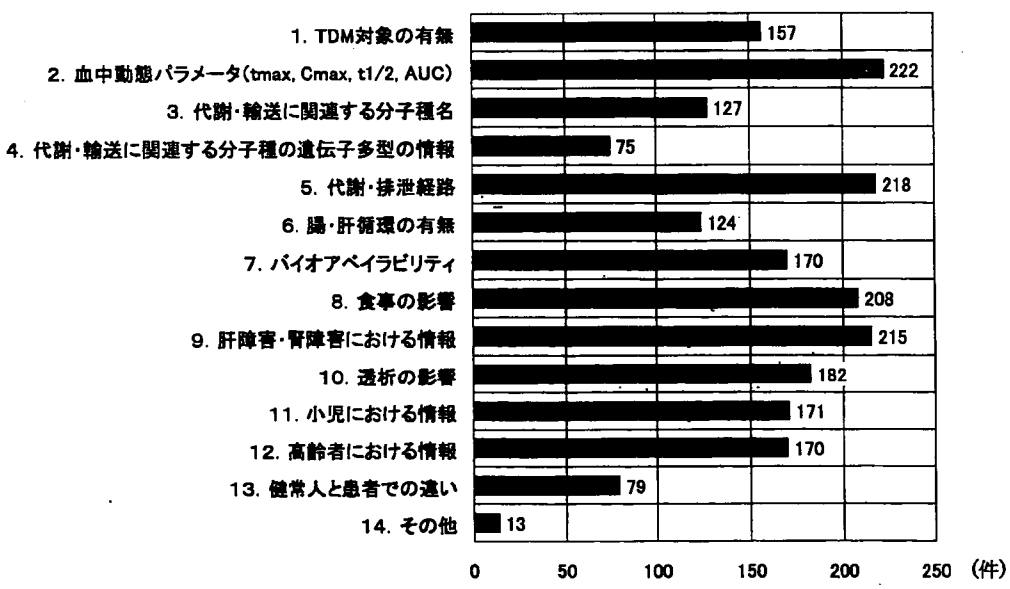


図14. 薬物動態について，情報がある場合には添付文書の薬物動態欄に記載する必要がある事項(複数回答可，有効回答数2131件)

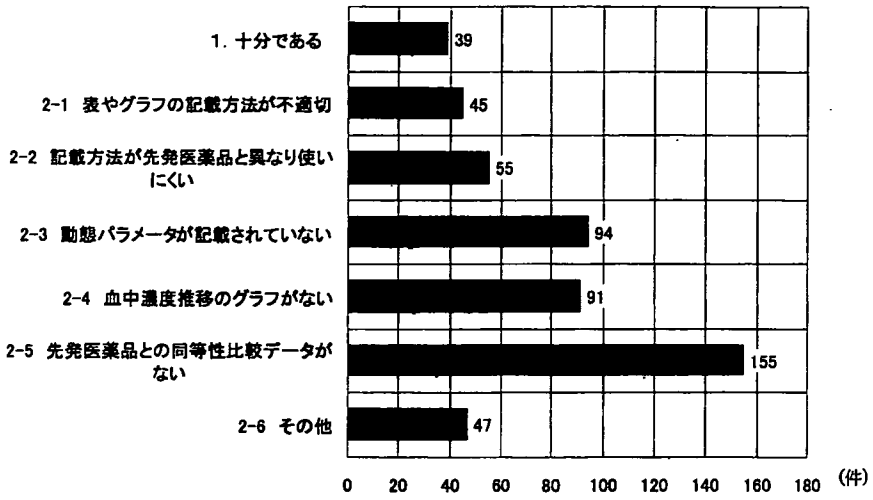


図 15. 薬物動態の添付文書での記載について，後発医薬品は先発医薬品と比べて十分ですか(複数回答可，有効回答数 526 件)

の採用が少なく判断できない」(16 件)，「製薬会社により差がある」(5 件)，「薬物動態の項目がないなど，基本的に問題」(4 件)などがあった．

考　察

今回のアンケート調査により，平成 9 年の改訂時に導入された全体の記載順序および相互作用欄の一覧表形式については，ほとんどの回答者により支持されていることが判明したが，相互作用欄での記載順や薬剤名の記載方法については，重要度を反映するよう，また，具体的な薬剤名を記載するよう改善を求める意見が多かった．ドイツの一般医に対するアンケート調査では半数以上が，現在の情報提供については重大さ，機序，用量調節について不満足としていること[4]，米国での薬局薬剤師を対象とした調査では，相互作用検出のためのコンピュータ警告システムでは重要な相互作用とそれ以外の相互作用が区別できない点に不満が多かったこと[5]は，これらの情報の必要性の高さを示していると考えられる．

一方，ソリブジン事件直後の旧様式の時期に，フルオロウラシル系薬剤を用いたがん補助療法の市販後試験参加施設の 477 人の医師を対象に行われたアンケート調査[6]では，ソリブジンとフルオロウラシル系抗がん剤との相互作用について添付文書から知ったのは 2.5% にすぎず，また，添付文書上，「両剤の併用を避けること」とされている場合の相互作用の解釈に意識の違いがみられ，相互作用による薬禍防止のために有用な手段として，添付文書における警告や禁忌の明確化を必要とする回答が多数を占めるなど，今回の調査結果と類似の結果が得られている．この原因として，短期間に一斉に行われた添付文書様式改訂では，すでに市販されていた医薬品については記載事項の並べ替えなどの表面的な変更にとどまり，必要な情報の追加など，質の面では，十分に改善が行われなかった可能性が考えられる．実際，記載要領の改訂後も，一部の添付文書では依然として，薬物相互作用の重篤度，機序，根拠が不明確であること，相手方薬剤が不明確であること[7-9]が報告されている．このように十分な内容の改善がなされなかった要因として，規制当局は様式の変更の指示を行うのみで，個々の添付文書の記載内容の変更については，各企業の自主的な判断に任せられていたことが考えられる．

薬物動態データ(AUC，C_{max})の変化率および投与条件，相互作用に関与する酵素分子種や，サプリメント，嗜好品を含む食品との臨床的な相互作用について，相互作用欄に記載すべきとの意見が多かった．グレープフルーツジュースをはじめとする食品との相互作用については，大きな薬物動態学的な影響がある場合が知られているが[10-13]，医療従事者の意識は一様ではないとの報告もあり，今後も十分な情報提供が必要と考えられる[14]．わが国の添付文書の問題として，特に相互作用に関しては，定量的な変化率に関する文献情報が，反映されていないこと，また，類薬での結果を引用した曖昧な表現がなされており，特に，米国の添付文書との比較では，定量的数値について，十分な情報が記載されていないことが確認されている[15,16]．

また，相互作用の主要な原因の一つである CYP に関する記載について，全添付文書を対象とした調査では，CYP の記載率が全体の 1 割程度と非常に少なく，一方，最近承認された医薬品では，承認申請時に CYP に関する試験が実施され，CYP に関する記載率も高いことが示されている[17]．これらの事項については，当該医薬品の市販後に承認取得者とは独立に研究が実施され，明ら

かにされることも多いが，出版後，添付文書改訂への反映まで5年以上かかる場合も多いことも報告されており[18]，添付文書への適切な情報の反映が望まれる．

後発医薬品の薬物動態欄の記載については，平山ら[19]および中村ら[20]からも同様の報告があるが，後発医薬品の承認申請に当たっては，先発医薬品との生物学的同等性試験の実施が求められ，製剤間の同等性を確認しているが，血中濃度は試験毎に測定されるため，特に，生物学的利用率が低い医薬品については，食後か空腹時か，また，測定タイミング，測定手法の違いなどの試験条件や，試験間のばらつきにより，各試験で標準製剤(先発医薬品)との生物学的同等性が示されていても，試験間で血中動態パラメータの数値に違いが生じることがある．なお，最近，厚生労働省から，後発医薬品についても，生物学的同等性に関する薬物動態データの記載を徹底するよう通知が出されている[21]．

今回のアンケート調査から，理解しやすさを目的として導入された相互作用の表形式については，強く支持されていたが，内容については，不十分との意見が多いことが判明した．今回の調査対象は，比較的大規模な病院であり，薬物動態に関する使用頻度，関心が特に高い可能性があり，また，本調査では1施設1回答としたが，回答者の業務内容，職位等の属性について特定していないため，調剤業務担当者，モニタリング担当者，DI担当者などで，関心事項が異なり，アンケート回答者の属性により回答内容が変わる可能性はあるが，相互作用の形式や追記すべきデータについては明快な回答が得られており，今回の回答結果を一般に外挿することは妥当と考えられる．表形式の利点を維持しつつ，関与する代謝酵素分子種や，変化率などの必要な事項について追加記載することで，添付文書は，より使いやすく，臨床上有用なものになると考えられる．

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Species Difference in the Inhibitory Effect of Nonsteroidal Anti-Inflammatory Drugs on the Uptake of Methotrexate by Human Kidney Slices

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ABSTRACT

Simultaneous use of nonsteroidal anti-inflammatory drugs (NSAIDs), probenecid, and other drugs has been reported to delay the plasma elimination of methotrexate in patients. Previously, we have reported that inhibition of the uptake process cannot explain such drug-drug interactions using rats. The present study quantitatively evaluated the possible role of the transporters in such drug-drug interactions using human kidney slices and membrane vesicles expressing human ATP-binding cassette (ABC) transporters. The uptake of methotrexate by human kidney slices was saturable with a K_m of 45 to 49 μM. Saturable uptake of methotrexate by human kidney slices was markedly inhibited by *p*-aminohippurate and benzylpenicillin, but only weakly by 5-methyltetrahydrofolate. These transport characteristics are similar to those of a basolateral organic anion transporter (OAT) 3/*SLC22A8*. NSAIDs and probenecid inhibited the uptake of methotrexate by human kidney slices, and, in particular, salicylate, indomethacin, phenylbutazone, and probenecid were predicted to exhibit significant inhibition at clinically observed plasma concentrations. Among ABC transporters, such as BCRP/*ABCG2*, multidrug resistance-associated protein (MRP) 2/*ABCC2*, and MRP4/*ABCC4*, which are candidates for the luminal efflux of methotrexate, ATP-dependent uptake of methotrexate by MRP4-expressing membrane vesicles was most potently inhibited by NSAIDs. Salicylate and indomethacin were predicted to inhibit MRP4 at clinical plasma concentrations. Diclofenac-glucuronide significantly inhibited MRP2-mediated transport of methotrexate in a concentration-dependent manner, whereas naproxen-glucuronide had no effect. Inhibition of renal uptake (via OAT3) and efflux processes (via MRP2 and MRP4) explains the possible sites of drug-drug interaction for methotrexate with probenecid and some NSAIDs, including their glucuronides.

Drug-drug interactions involving metabolism and/or excretion processes prolong the plasma elimination half-lives leading to the accumulation of drugs in the body and potentiate pharmacological/adverse effects. Recent progress in molecular biological research has shown that many types of transporters play important roles in the tissue uptake and/or subsequent secretion of drugs in the liver and kidney, and such transporters exhibit a broad substrate specificity with a degree of overlap, suggesting the possibility of transporter-mediated drug-drug interactions with other substrates (Shitara et al., 2005; Li et al., 2006).

Methotrexate (MTX) is an analog of natural folate and has been widely and successfully used for the treatment of neoplastic diseases and autoimmune diseases, including rheu-

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ABBREVIATIONS: MTX, methotrexate; NSAID, nonsteroidal anti-inflammatory drug; BBM, brush border membrane; Oat/OAT, organic anion transporter; r, rat; h, human; RFC, reduced folate carrier; MRP, multidrug resistance-associated protein; BCRP, breast cancer resistance protein; PAH, *p*-aminohippurate; DHEAS, dehydroepiandrosterone sulfate; PCG, benzylpenicillin; 2,4-D, 2,4-dichlorophenoxyacetate; 5-MTHF, 5-methyltetrahydrofolate; HEK, human embryonic kidney; MOI, multiplicity of infection; TS, Tris-sucrose.

matoid arthritis and psoriasis. However, when administered concomitantly with nonsteroidal anti-inflammatory drugs (NSAIDs) (Liegler et al., 1969; Ellison and Servi, 1985; Maiche, 1986; Thyss et al., 1986; Ng et al., 1987; Tracy et al., 1992), penicillin antibiotics (Ronchera et al., 1993; Yamamoto et al., 1997; Titier et al., 2002), probenecid (Aherne et al., 1978), and ciprofloxacin (Dalle et al., 2002), the elimination of MTX from the systemic circulation was delayed or its pharmacokinetics was affected, sometimes resulting in severe adverse effects. Considering that MTX is largely excreted into the urine in unchanged form, the inhibition of renal tubular secretion has been considered as a site of drug-drug interactions.

Renal secretion of drugs is achieved by vectorial transport via the kidney epithelium of the proximal tubules, which consists of the uptake from blood via the basolateral membrane and the subsequent efflux into the lumen via the brush border membrane (BBM). MTX has been shown to be a substrate of basolateral organic anion transporters rOat1/hOAT1 (*Slc22a6/SLC22A6*) (Sekine et al., 1997; Hosoyamada et al., 1999; Nozaki et al., 2004) and rOat3/hOAT3 (*Slc22a8/SLC22A8*) (Cha et al., 2001; Nozaki et al., 2004). NSAIDs are inhibitors of rOat3 and exhibit significant inhibition of rOat3-mediated uptake at clinical plasma concentrations. We quantitatively investigated drug-drug interactions between MTX and NSAIDs using rat kidney slices (Nozaki et al., 2004). Unexpectedly, the MTX uptake was not markedly inhibited by NSAIDs in rat kidney slices because of the involvement of the NSAIDs-insensitive uptake mechanism, presumably reduced folate carrier (RFC)-1 (*Slc19a1*), a transporter of reduced folate and its derivatives (Nozaki et al., 2004). However, the possibility of interspecies differences could not be excluded. Indeed, the drug-drug interaction between famotidine and probenecid could not be reproduced in rodents because of an interspecies difference in the tissue distribution of OCT1 and the transport activity exhibited by OAT3 (Tahara et al., 2005). Recently, we were able to obtain kidney slices from human intact renal cortical tissues removed from surgically nephrectomized patients with renal cell carcinoma and have demonstrated that they retain the transport activities of OAT1 and OAT3 (Nozaki et al., 2007). In the present study, the inhibitory effects of NSAIDs on the uptake of MTX by human kidney slices were examined to evaluate their clinical relevance.

In addition to the uptake process, it is also possible that NSAIDs and other inhibitors accumulate in the renal tubular cells by basolateral organic anion transporter(s) and inhibit the excretion of MTX across the BBM. To date, many kinds of transporters of organic anions have been identified on the apical side of the human kidney epithelium, including multidrug resistance-associated protein (MRP) 2, MRP4, breast cancer resistance protein (BCRP), OAT4, URAT1, and NPT1 (for review, see Russel et al., 2002). Hulot et al. (2005) identified a heterozygous mutation, which results in a loss of function of MRP2, in a patient who exhibited delayed MTX elimination. In addition, the pharmacokinetics of MTX was analyzed in *Bcrp1* knockout mice. The area under the curve of the plasma concentration-time curve of MTX was approximately 2-fold higher in *Bcrp1* knockout mice than in wild-type mice, whereas the amount of MTX excreted into the urine was unaltered (Breedveld et al., 2004). Therefore, the renal clearance of MTX, which is calculated by dividing the amount of MTX excreted into the urine by the area under the curve, was reduced in *Bcrp1* knockout mice by approximately 50%. MRP4 is also expressed in the BBM of kidney proximal tubules and involved in the renal secretion of organic anions (Hasegawa et al., 2007; Imaoka et al., 2007). The present study examined the effect of NSAIDs and their glucuronide conjugates on the ATP-dependent uptake of MTX by MRP2-, BCRP-, and MRP4-expressing membrane vesicles.

Materials and Methods

Materials. [^{3}H]MTX (25–29 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). [^{3}H]*p*-Aminohippurate (PAH; 4.1 Ci/mmol) and [^{3}H]dehydroepiandrosterone sulfate (DHEAS; 60 Ci/mmol) were purchased from PerkinElmer Life Science (Boston, MA), and [^{14}C]benzylpenicillin (PCG; 59 mCi/mmol) and [^{3}H]2,4-dichlorophenoxyacetate (2,4-D; 20 Ci/mmol) were obtained from GE Healthcare BioSciences (Waukesha, WI). Unlabeled MTX and 5-methyltetrahydrofolate (5-MTHF) were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals used in the present study were of analytical grade and commercially available.

Preparation of Human Kidney Slices and Uptake of [^{3}H]MTX by Human Kidney Slices. This study protocol was approved by the Ethics Review Boards at the Graduate School of Pharmaceutical Sciences, The University of Tokyo (Tokyo, Japan) and Tokyo Women's Medical University (Tokyo, Japan). All participants provided written informed consent.

Intact renal cortical tissues were obtained from five surgically nephrectomized patients with renal cell carcinoma at Tokyo Women's Medical University between November, 2005 and January, 2006. Human kidney slices were prepared from kidney subjects; subsequently, the uptake of MTX and other substances by these human kidney slices was examined as described previously (Nozaki et al., 2007). The uptake of typical hOAT1 substrates (PAH and 2,4-D) and hOAT3 substrates (PCG and DHEAS) by human kidney slices was examined as positive controls and found to be comparable with previous results (Nozaki et al., 2007). Because the uptake of MTX by kidney slices apparently lasts for at least for 30 min (Fleck et al., 2002), the accumulation of MTX in human kidney slices for 15 min was used for the subsequent analyses.

Transport Studies in hOAT1- and hOAT3-Transfected HEK293 Cells. hOAT1- and hOAT3-transfected HEK293 cells were established as described previously (Tahara et al., 2005). HEK293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin at 37°C with 5% CO_2 and 95% humidity. HEK293 cells were seeded on 12-well plates at a density of 1.2×10^5 cells/well. Cells were cultured for 48 h with the above-mentioned medium and for an additional 24 h with culture medium supplemented with 5 mM sodium butyrate before the transport studies.

Transport studies were carried out as described previously (Tahara et al., 2005). Uptake was initiated by adding Krebs-Henseleit 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 $CaCl_2$, pH 7.4) containing radiolabeled compounds in the presence or absence of inhibitors after cells had been washed twice and preincubated with buffer. The uptake was terminated at designated times by aspirating the incubation buffer and adding ice-cold buffer. Cells were washed twice with ice-cold buffer and dissolved in 500 μl of 0.2 N NaOH. The aliquots neutralized with 2 N HCl were transferred to scintillation vials containing 2 ml of scintillation cocktail (Clearsol I; Nacalai Tesque Inc., Kyoto, Japan), and the radioactivities associated with the specimens were determined in a liquid scintillation counter.

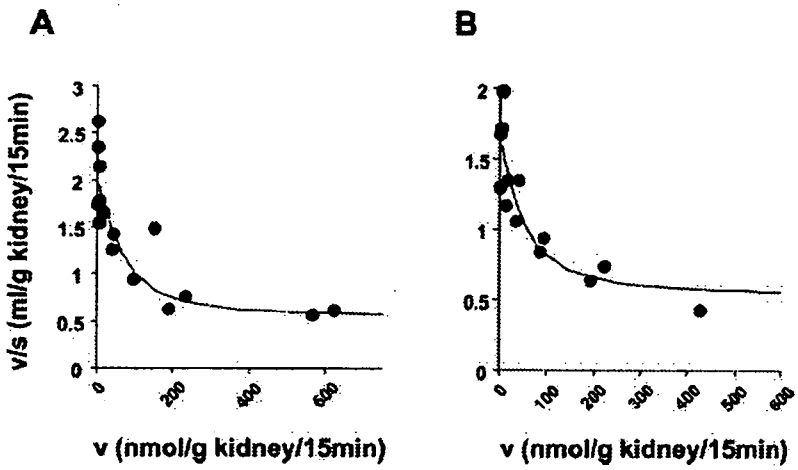

Fig. 1. Concentration dependence of the uptake of MTX by human kidney slices. The concentration dependence of the uptake of MTX is shown as an Eadie-Hofstee plot. The uptake of MTX was measured at concentrations between 0.1 and 10,000 μM for 15 min at 37°C. A and B, data for human kidney slices prepared from subjects 1 and 2, respectively. Each point represents the results from one slice. Solid lines represent the fitted lines obtained by nonlinear regression analysis.

The remaining 50-μl aliquot of cell lysate was used to determine the protein concentration by the method of Lowry with bovine serum albumin as a standard.

Vesicle Transport Studies. Membrane vesicles were prepared from HEK293 cells, which were infected with human BCRP-, MRP2-, and MRP4-recombinant adenoviruses, as described previously (Hasegawa et al., 2007; Imaoka et al., 2007). In brief, HEK293 cells were infected with recombinant adenovirus containing human MRP4 [10 multiplicity of infection (MOI)] and BCRP (2 MOI). As negative controls, cells were infected with a virus containing green fluorescence protein cDNA (10 MOI). Cells were harvested 48 h after infection, and membrane vesicles were isolated by the hypotonic method (Hasegawa et al., 2007; Imaoka et al., 2007). Cells were diluted 40-fold with hypotonic buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 7.4, at 4°C) and stirred gently for 1 h on ice in the presence of 2 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 1 μg/ml pepstatin, and 5 μg/ml aprotinin. The cell lysate was centrifuged at 100,000*g* for 30 min at 4°C, and the resulting pellet was suspended in 10 ml of isotonic TS buffer (10 mM Tris-HCl, 250 mM sucrose, pH 7.4 at 4°C) and homogenized in a Dounce B homogenizer (glass/glass, tight pestle, 30 strokes). The crude membrane fraction was layered on top on a 38% (w/v) sucrose solution in 5 mM Tris-HEPES, pH 7.4, at 4°C and centrifuged in a Beckman SW41 rotor centrifuge at 280,000*g* for 45 min at 4°C. The turbid layer at the interface was collected, diluted to 23 ml with TS buffer, and centrifuged at 100,000*g* for 30 min at 4°C. The resulting pellet was suspended in 400 ml of TS buffer. Vesicles were formed by passing the suspension 30 times through a 27-gauge needle using a syringe. They were finally frozen in liquid nitrogen and stored at −80°C until required.

Vesicle transport studies were carried out as described in a previous report. In brief, the transport buffer (10 mM Tris, 250 mM sucrose, and 10 mM $MgCl_2$, pH 7.4) contained the ligands, 5 mM ATP or AMP, and an ATP-regenerating system (10 mM creatine phosphate and 100 mg/l creatine phosphokinase). An aliquot of transport medium (15 μl) was mixed rapidly with vesicle suspension (5 μg of protein/5 μl), incubated at 37°C for designated times, and the transport reaction was stopped by the addition of 1 ml of ice-cold stop solution (10 mM Tris, 250 mM sucrose, and 0.1 M $NaCl_2$, pH 7.4)., The stopped reaction mixture (900 μl) then was passed through a 0.45-μm HA filter (Millipore Corp., Bedford, MA), and the filter was washed twice with 5 ml of ice-cold stop solution. The radioactivity retained on the filter was measured in a liquid scintillation counter. The ATP-dependent uptake of ligands was calculated by subtracting the ligand uptake in the presence of AMP from that in the presence of ATP.

Preparation of Diclofenac- and Naproxen-Glucuronides. β-1-*O*-Glucuronides of diclofenac and *S*-naproxen were prepared biosynthetically in vitro from the respective parent drugs using rat liver microsomes according to published methods (Iwaki et al., 1995) with slight modifications. In brief, a mixture containing 10 mg/ml microsomal protein, 0.1 M Tris-HCl buffer, pH 6.9, 10 mM $MgCl_2$, 20 mM D-glucaric acid-1,4-lactone, 2 mM phenylmethylsulfonyl fluoride, 0.2% Triton X-100, 1 mM diclofenac or naproxen, and 10 mM UDP-glucuronic acid was incubated for 1.5 h at 37°C. The reaction was terminated by the addition of 5 volumes of acetonitrile, acidified immediately with acetic acid, and then centrifuged. The obtained supernatant was evaporated to remove organic solvent under reduced pressure at 30°C, and the residual aqueous phase was freeze-dried. The residue was redissolved in a minimal volume of acetonitrile per 50 mM acetic acid (10:90, v/v). The glucuronides in this solution were purified by liquid chromatography (30 × 1.5-cm i.d., Cosmosil $75C_{18}$-PREP; Nacalai Tesque) using a stepwise gradient (acetonitrile per 50 mM acetic acid, 10:90, 20:80, 30:70, and 50:50). Eluted glucuronide fractions were collected and freeze-dried. The identities of the glucuronides were confirmed by cleavage to the respective parent drugs with β-1-glucuronidase and 1 N NaOH. The purity of the glucuronides obtained was determined by analytical high-performance liquid chromatography and found to be homogeneous (>96%) at a UV wavelength of 254 nm, with the remaining fraction consisting of polar impurities that did not yield the respective parent drugs.

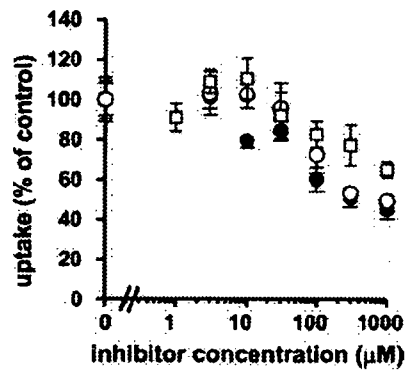

Fig. 2. Inhibitory effect of PAH, PCG, and 5-MTHF on the uptake of MTX by human kidney slices. The uptake of [^{3}H]MTX (0.1 μM) was determined in the presence and absence of unlabeled PAH (open circles), PCG (closed circles), and 5-MTHF (open squares) for 15 min at 37°C. The values are shown as a percentage of the uptake in the absence of inhibitors. The present data were taken from those of subjects 3 and 4. Each point represents the mean ± S.E. (n = 6 slices).

Kinetic Analyses. Kinetic parameters were obtained using the following Michaelis-Menten equations:

$$\text{One saturable component,} \quad v = \frac{V_{max} \times S}{K_m + S} \qquad (1)$$

one saturable, and one nonsaturable component,

$$v = \frac{V_{max} \times S}{K_m + S} + P_{dif} \times S \quad (2)$$

where v is the uptake velocity of the substrate (nanomoles per gram of kidney per 15 min or picomoles per milligram of protein per minute), S is the substrate concentration of medium (micromolar), K_m is the Michaelis constant (micromolar), V_{max} is the maximal uptake velocity (nanomoles per gram of kidney per 15 min or picomoles per milligram of protein per minute), and P_{dif} is the nonsaturable uptake clearance (milliliters per gram of kidney per 15 min).

The degree of inhibition (R) is expressed by the following equation:

$$R = \frac{CL_{+\ inhibitor}}{CL} = \frac{1}{1 + I/K_i} \quad (3)$$

where CL represents the uptake clearance and $CL_{+inhibitor}$ represents the uptake clearance in the presence of inhibitor. I represents the concentration of inhibitor (micromolar). The substrate concentration was low compared with its K_m in the inhibition studies. Fitting was performed by the nonlinear least-squares method using the MULTI program (Yamaoka et al., 1981). The input data were weighted as the reciprocals of the observed values, and the Damping Gauss Newton Method algorithm was used for fitting.

Statistical Analysis. Statistical differences were determined using a one-way analysis of variance with Dunnett's post hoc test. Differences were considered to be significant at $P < 0.05$.

Results

The Uptake of Typical hOAT1 and hOAT3 Substrates by Human Kidney Slices. The saturable uptake clearance

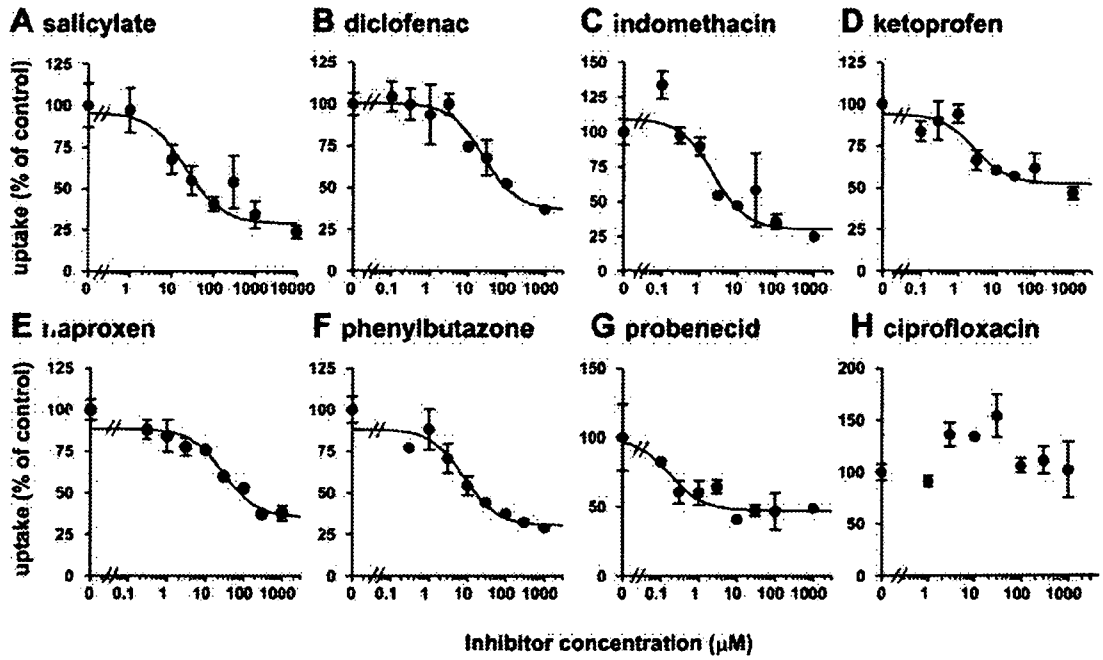

Fig. 3. Inhibitory effect of NSAIDs and other drugs on the uptake of MTX by human kidney slices. The uptake of MTX (0.1 μM) was determined in the presence and absence of unlabeled salicylate (A), diclofenac (B), indomethacin (C), ketoprofen (D), naproxen (E), phenylbutazone (F), probenecid (G), and ciprofloxacin (H) for 15 min at 37°C. The data of salicylate, indomethacin, probenecid, and ciprofloxacin were taken from subject 1, that of ketoprofen was from subject 3, that of diclofenac was from subject 4, and those of naproxen and phenylbutazone were from subject 5. Values are shown as a percentage of the uptake in the absence of inhibitors. Solid lines represent the fitted lines obtained by nonlinear regression analysis. Each point represents the mean ± S.E. (n = 3).

TABLE 1

Quantitative evaluation of drug-drug interactions with MTX using human kidney slices

Human kidney slices were incubated with buffer containing [^{3}H]MTX in the presence or absence of inhibitors, and K_i values were determined by nonlinear regression analysis (Fig. 3). Plasma unbound concentrations of the inhibitors (I_u) were calculated from the total plasma concentrations and unbound fractions.

Inhibitors	Clinical Concentration		K_i	R Value
	Total	I_u		
		μM		
Salicylate	1100–2200[a]	55–440	18.4 ± 8.6	0.040–0.25
Diclofenac	3.6[b]	<0.018	26.6 ± 5.2	1.0
Indomethacin	0.84–84[a]	0.084–8.4	3.11 ± 1.58	0.27–0.97
Ketoprofen	12[a]	0.0096	1.85 ± 0.96	1.0
Naproxen	>217[a]	0.651	14.3 ± 5.3	0.96
Phenylbutazone	162–786[c]	6.3–19.0	4.87 ± 1.4	0.20–0.44
Probenecid	170[a]	18.7	0.171 ± 0.8	0.009
Ciprofloxacin	7.6[c]	4.5	>1000	1.0

[a] Takeda et al. (2002).
[b] Riess et al. (1978).
[c] Brunton et al. (2006).

(milliliter per gram of kidney per 15 min, mean of duplicate determinations) of the typical substrates in subject 1 (PAH, 2.88; 2,4-D, 8.28; PCG, 3.87; DHEAS, 8.21), subject 2 (PAH, 1.69; 2,4-D, 6.19; PCG, 1.97; DHEAS, 5.10), subject 3 (PAH, 1.98; 2,4-D, 6.10; PCG, 1.95; DHEAS, 7.78), subject 4 (PAH, 1.25; 2,4-D, 9.11; PCG, 2.31; DHEAS, 5.18), and subject 5 (PAH, 1.48; 2,4-D, 5.83; PCG, 2.40; DHEAS, 7.55) was comparable with those in a previous report (Nozaki et al., 2007).

Characterization of the Uptake of MTX by Human Kidney Slices. The concentration dependence of the uptake of MTX was examined using human kidney slices, which were prepared from two different batches (Fig. 1, A and B). The uptake of MTX by two batches of human kidney slices consists of one saturable and one nonsaturable component, with K_m values of 48.9 ± 17.3 and 44.6 ± 23.4 μM, V_{max} of 70.2 ± 23.1 and 48.5 ± 24.1 μM, and P_{dif} of 0.514 ± 0.048 and 0.515 ± 0.065 ml/g kidney per 15 min, respectively (mean ± S.D.).

Figure 2 describes the inhibitory effect of PAH, PCG, and 5-MTHF on the uptake of MTX by human kidney slices. PAH, PCG, and 5-MTHF (typical inhibitors of hOAT1, hOAT3, and RFC-1, respectively) inhibited the uptake of MTX in a concentration-dependent manner. PAH and PCG inhibited the saturable component of MTX uptake (49.5 ± 1.2 and 45.0 ± 4.8% of control at 1 mM, respectively), whereas the inhibitory effect of 5-MTHF was weak (65.0 ± 4.0% of control at 1 mM) (Fig. 2).

Inhibitory Effect of NSAIDs on the Uptake of MTX by Human Kidney Slices and hOAT1 and hOAT3. The effect of NSAIDs and other drugs was examined with regard to the uptake of MTX in human kidney slices (Fig. 3). Except for ciprofloxacin, the inhibitors inhibited MTX uptake in a concentration-dependent manner. The K_i values are summarized in Table 1. The unbound plasma concentrations (I_u) at clinical dosages are taken from the literature, and, based on the K_i values, the degree of inhibition in clinical situations (R) was predicted (Table 1). The inhibitory effect of NSAIDs on hOAT1- and hOAT3-mediated uptake was also examined, and the K_i values of NSAIDs for hOAT1 and hOAT3 are summarized in Table 2.

ATP-Dependent Uptake of MTX by Human BCRP-, MRP2-, and MRP4-Expressing Vesicles. The uptake of MTX by human BCRP-, MRP2-, and MRP4-expressing vesicles and control vesicles was examined in the presence of ATP or AMP. The ATP-dependent uptake of MTX was significantly greater in BCRP-, MRP2-, and MRP4-expressing vesicles than that in control vesicles (Fig. 4, A–C, respectively). The concentration dependence of BCRP-, MRP2-, and MRP4-mediated transport of MTX was examined (Fig. 4, D–F, respectively), and their K_m values were 5210 ± 500, 1540 ± 250, and 103 ± 5 μM, and their V_{max} values were 74.1 ± 7.6, 21.2 ± 2.8, and 1.33 ± 0.06 nmol/mg protein per 5 min, respectively.

Inhibitory Effect of NSAIDs and Other Drugs on ATP-Dependent Transport of MTX via BCRP, MRP2, and MRP4. We examined the inhibitory effect of NSAIDs and other drugs on the ATP-dependent transport of MTX via BCRP, MRP2, and MRP4 (Fig. 5, A–C, respectively). BCRP-mediated transport of MTX was partially inhibited by indomethacin, phenylbutazone, diclofenac, and probenecid (Fig. 5A). MRP2-mediated transport of MTX was inhibited only by probenecid and stimulated in the presence of 1 μM phenylbutazone (Fig. 5B). Compared with BCRP and MRP2, MRP4 was more sensitive to the tested inhibitors (Fig. 5C), and indomethacin, ketoprofen, ibuprofen, naproxen, phenylbutazone, and salicylate inhibited the MRP4-mediated transport of MTX in a concentration-dependent manner, with K_i values of 2.95 ± 0.76, 23.3 ± 6.8, 73.3 ± 20.9, 75.3 ± 19.7, 354 ± 54, and 218 ± 29 μM, respectively (mean ± S.D.) (data not shown). The clinical concentrations, plasma unbound concentrations, K_i values calculated from in vitro vesicle transport studies, and R values of inhibitors are summarized in Table 3. The inhibitory effect of diclofenac and naproxen glucuronides on BCRP-, MRP2-, and MRP4-mediated transport of MTX was also examined (Fig. 5, D–F, respectively). Diclofenac glucuronide significantly inhibited MRP2-mediated transport of MTX in a concentration-dependent manner, whereas BCRP and MRP4 were inhibited slightly or not at all by diclofenac and naproxen glucuronides.

Discussion

NSAIDs, penicillin, and other drugs have been reported to inhibit the renal tubular secretion of MTX, leading, in some cases, to lethal toxicity. The underlying mechanisms of the interactions remain to be elucidated. We previously investigated this interactions focusing on the uptake process using rat kidney slices and reported that the inhibitory effect of NSAIDs on the uptake of MTX by rat kidney slices was too weak to account for the drug-drug interactions by inhibition of the uptake process (Nozaki et al., 2004). In the present study, we re-evaluated the drug-drug interactions using human kidney slices and membrane vesicles expressing human ATP-binding cassette transporters.

The uptake of MTX by human kidney slices was saturable (Fig. 1). Nonlinear regression analysis revealed that the uptake of MTX in human kidney slices consists of one saturable component and one nonsaturable component, whereas the uptake in rat kidney slices consisted of three components (two saturable components and one nonsaturable component) (Nozaki et al., 2004). The K_m value of MTX uptake in human kidney slices was comparable with that of the low-affinity component in rat kidney slices (77 μM). To identify the candidate transporter involved, inhibition studies were carried out. Although PAH and PCG exhibited different potencies

TABLE 2

The K_i values of NSAIDs for hOAT1 and hOAT3

The inhibitory effect of NSAIDs on the uptake of PAH (0.1 μM) and PCG (1 μM) by hOAT1- and hOAT3-transfected HEK293 cells, respectively, was examined. The K_i values were determined by nonlinear regression analysis. All values represent the mean ± S.D.

Inhibitors	K_i	
	hOAT1	hOAT3
	μM	
Salicylate	407 ± 82	111 ± 28
Diclofenac	1.52 ± 0.07	6.57 ± 0.48
Sulindac	77.8 ± 11.1	6.89 ± 1.51
Indomethacin	6.72 ± 1.22	0.979 ± 0.052
Etodolac	103 ± 23	12.0 ± 3.8
Tolmetin	5.08 ± 0.48	5.32 ± 0.53
Ibuprofen	1.38 ± 0.48	5.11 ± 1.13
Ketoprofen	0.890 ± 0.400	5.04 ± 1.5
Naproxen	1.18 ± 0.60	7.15 ± 2.34
Phenylbutazone	71.6 ± 7.1	6.82 ± 1.75
Piroxicam	N.D.	4.83 ± 1.63

N.D., not determined.

with regard to the uptake of OAT1 and OAT3 substrates in human kidney slices (Nozaki et al., 2007), they inhibited the uptake of MTX with a similar potency in human kidney slices (Fig. 2). In addition, 5-MTHF weakly inhibited MTX uptake in comparison with PAH and PCG. It should be noted that PAH and PCG did not fully inhibit the saturable uptake of MTX by human kidney slices. Saturable uptake accounted for 75 and 67% of the net uptake, whereas almost 50% of the uptake remained in the presence of 1 mM of PAH or PCG.

The effect of NSAIDs and other drugs, all of which have caused drug-drug interactions with MTX in clinical situations, was examined using human kidney slices (Fig. 3). All the tested compounds, except ciprofloxacin, inhibited the uptake of MTX in human kidney slices in a concentration-dependent manner. Using K_i values determined in this study and the plasma unbound concentrations at clinical dosages, the degree of inhibition (R value) was predicted (Table 1). Among the tested compounds, the R values of salicylate, phenylbutazone, and probenecid were less than 1, suggesting that their inhibition is clinically relevant. In particular, probenecid is predicted to markedly inhibit the uptake of MTX in the kidney. Indomethacin has also the potential to inhibit the renal uptake of MTX at high clinical concentrations. It should be noted that the degree of inhibition by ketoprofen and probenecid was smaller than that by other drugs. Fifty percent of the uptake remained as the noninhibitable fraction for ketoprofen and probenecid, whereas the saturable fraction was almost completely inhibited by the other drugs (Fig. 3). Together with the partial inhibition by PAH and PCG, this suggests an involvement of multiple transporters in the uptake of MTX in human kidney slices, which exhibited different sensitivity to ketoprofen and probenecid. Because probenecid is a potent inhibitor of OAT1 and OAT3 (Tahara et al., 2005), the degree of inhibition by probenecid suggests a contribution of OAT1 and OAT3 to the net uptake. This is also supported by the fact that the inhibitable fraction by probenecid was comparable with that by PAH and PCG (Figs. 2 and 3G). Unlike the typical substrates (Nozaki et al., 2007), the inhibition profiles by PAH and PCG were similar and failed to clearly indicate the isoform involved in MTX uptake. Considering that the K_m value determined in the human kidney is similar to that for OAT3 (21 μM) rather than OAT1 (550 μM) (Takeda et al., 2002), it is likely that OAT3 makes a more significant contribution to the net uptake process.

There was an interspecies difference in the potency of inhibition by NSAIDs for the uptake of MTX in human and rat kidney slices. Unlike rodents, some drugs are predicted to inhibit significantly the renal uptake process of MTX in clinical situations. Two factors can account for this interspecies difference. Firstly, the contribution of OATs to the net uptake is greater in human than in rat kidney. Indeed, the PAH- and PCG-inhibitable fraction was greater in human kidney slices than in rat kidney slices (50 versus 30% in human and rat

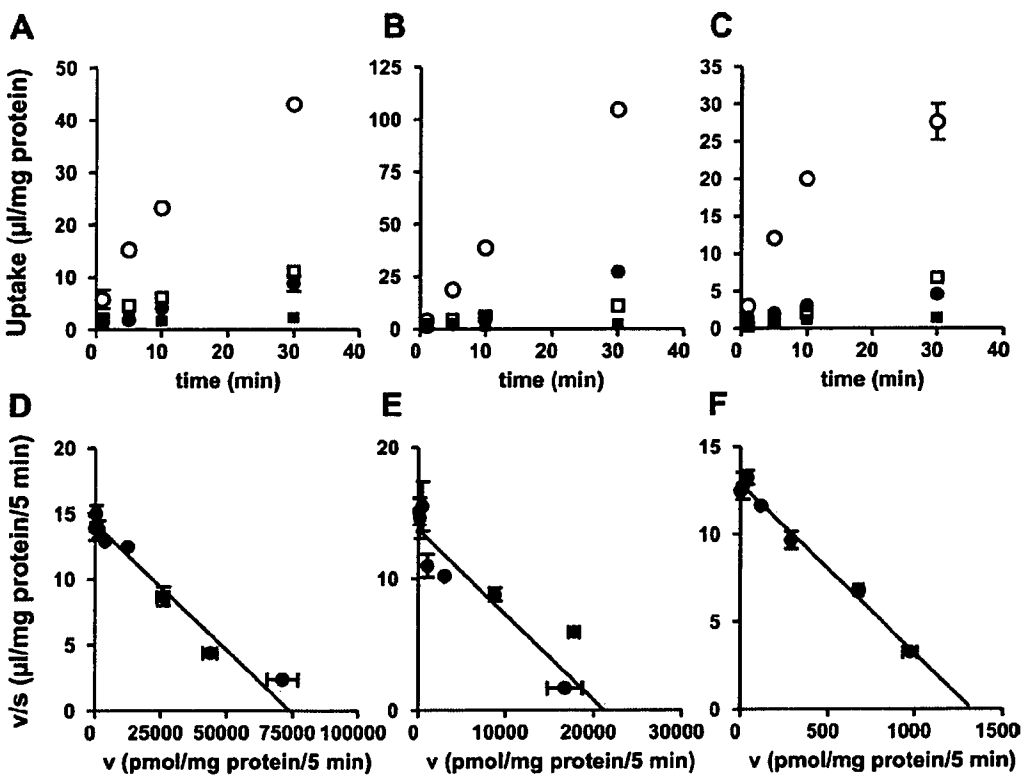

Fig. 4. The uptake of MTX by BCRP-, MRP2-, and MRP4-expressing vesicles. Time profiles of ATP-dependent uptake of MTX (A–C). Membrane vesicles (5 μg) prepared from HEK293 cells infected with BCRP (A), MRP2 (B), and MRP4 (C) adenoviruses (circles) or GFP adenoviruses (squares) were incubated at 37°C in the presence of [^{3}H]MTX (0.1 μM). Open symbols, uptake in the presence of ATP; closed symbols, uptake in the presence of AMP. Concentration dependence of the uptake of MTX (D–F). The uptake of [^{3}H]MTX (1 μM–30 mM for BCRP, 1 μM–10 mM for MRP2, 0.1 μM–3 mM for MRP4) by membrane vesicles prepared from HEK293 cells infected with BCRP (D), MRP2 (E), and MRP4 (F) adenoviruses was measured for 5 min at 37°C. Values shown are given by subtracting the uptake clearance in the presence of AMP from that in the presence of ATP. Data are shown as Eadie-Hofstee plots. Solid lines represent the fitted lines obtained by nonlinear regression analysis. Each point represents the mean ± S.E. (n = 3).

kidney slices, respectively) (Nozaki et al., 2004; this study). Secondly, the NSAIDs, except for ketoprofen, inhibited the unknown transporter more potently in human kidney slices than in rat kidney slices. In particular, the K_i value of salicylate determined in human kidney slices was smaller than that for OAT3 (Tables 1 and 2). These NSAIDs may be more potent inhibitors of this unknown transporter than OAT3. As suggested in rodents, RFC-1 is a candidate transporter. In addition, recently, proton-coupled folate transporter/heme carrier protein 1 (PCFT/HCP1) was also identified as a novel MTX transporter, which is also expressed in the kidney, at least, at the mRNA level (Qiu et al., 2006). This transporter may be another candidate transporter. Further studies are required to elucidate their importance.

Human kidney slice studies also suggested that diclofenac, ketoprofen, and naproxen do not inhibit the uptake of MTX at clinical concentrations, although they have caused drug-drug interactions with MTX in clinical situations (Thyss et al., 1986; Ng et al., 1987; Tracy et al., 1992; Davies and Anderson, 1997a). Because renal tubular secretion involves excretion into the lumen through the BBM of the proximal tubules, inhibition of apical efflux transporters can also serve as an alternative interaction site. Therefore, the effect of NSAIDs was examined for the ATP binding cassette transporters, such as MRP2, BCRP, and MRP4, which accept MTX as a substrate. ATP-dependent transport of MTX was observed in BCRP-, MRP2-, and MRP4-expressing vesicles (Fig. 4, A–C). The K_m values of MTX for BCRP, MRP2, and MRP4 were consistent with previously reported values (Bakos et al., 2000; Mitomo et al., 2003; Volk and Schneider, 2003). The

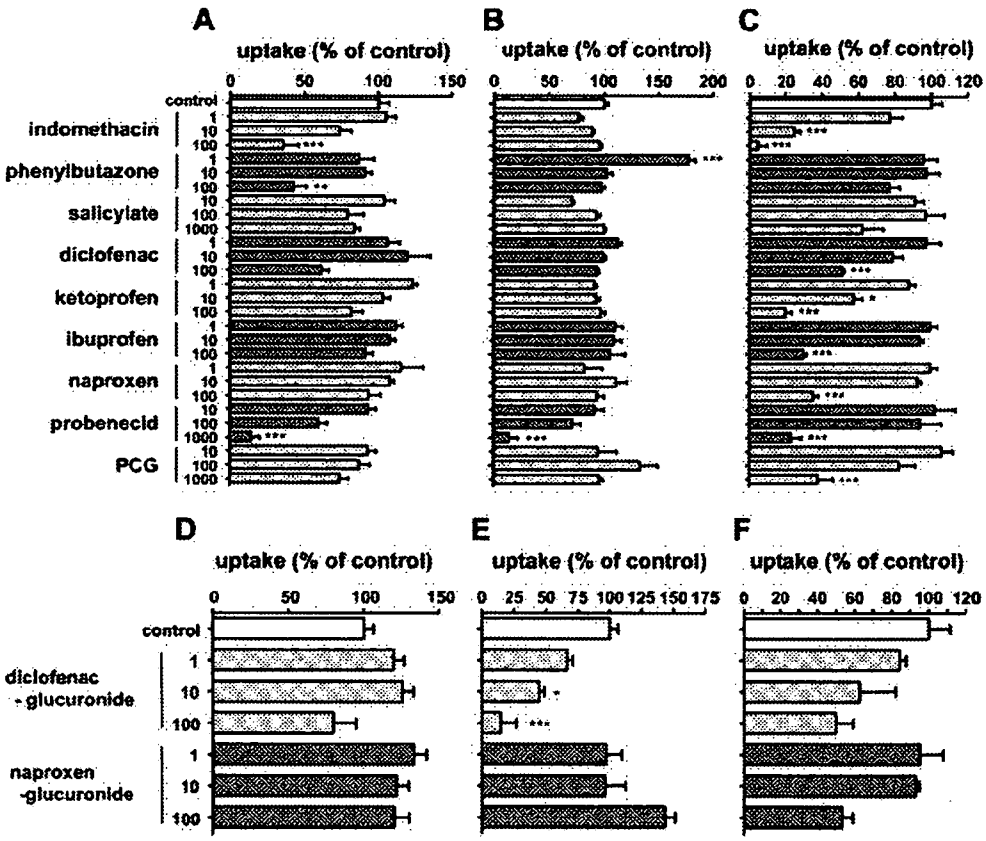

Fig. 5. Inhibitory effect of NSAIDs and other drugs on BCRP-, MRP2-, and MRP4-mediated transport of MTX. The uptake of MTX (0.1 μM) by membrane vesicles prepared from HEK293 cells infected BCRP, MRP2, and MRP4 adenoviruses was measured for 5 min at 37°C in the presence or absence of inhibitors (A–C, respectively). Values are given by subtracting the uptake clearance in the presence of AMP from that in the presence of ATP and are shown as a percentage of the uptake in the absence of inhibitors. Inhibitory effect of diclofenac- and naproxen-glucuronides on the BCRP-, MRP2-, and MRP4-mediated transport of MTX (D–F, respectively). Each value represents the mean ± S.E. (n = 3). *, P < 0.05; **, P < 0.01; ***, P < 0.001 statistically different from control.

TABLE 3

Quantitative evaluation of drug-drug interactions between MTX and NSAIDs via MRP4

Inhibitory effect of NSAIDs on MRP4-mediated transport of MTX was examined, and K_i values were determined by nonlinear regression analysis. All K_i values represent the mean ± S.D. Plasma unbound concentrations of the inhibitors (I_u) were calculated from the total plasma concentrations and unbound fractions.

Inhibitors	Clinical Concentration		K_i	R Value
	Total	I_u		
		μM		
Salicylate	1100–2200[a]	55–440	218	0.33–0.80
Diclofenac	3.6[b]	<0.018	>100	1.0
Indomethacin	0.84–84[a]	0.084–8.4	2.95	0.26–0.97
Ibuprofen	48.6[a]	<0.485	73.3	1.0
Ketoprofen	12[a]	0.0096	23.3	1.0
Naproxen	>217[a]	0.651	75.3	0.99
Phenylbutazone	162–786[a]	6.3–19.0	354	0.95–0.98

[a] Takeda et al. (2002).
[b] Riess et al. (1978).

effect of NSAIDs, probenecid, and PCG on the BCRP-, MRP2-, and MRP4-mediated transport of MTX was examined (Fig. 5, A–C, respectively). NSAIDs showed only a weak or minimal effect on MRP2 (Fig. 5B), which is consistent with a previous report (Horikawa et al., 2002). Because NSAIDs are mainly excreted into the urine as the glucuronide-conjugated form (Davies and Anderson, 1997a,b), we evaluated the inhibitory effect of diclofenac and naproxen glucuronide, which were prepared biosynthetically in vitro, on MRP2-mediated transport of MTX (Fig. 5E). Diclofenac glucuronide significantly inhibited the MRP2-mediated transport of MTX in a concentration-dependent manner (Fig. 5E). Therefore, this drug-drug interaction may involve inhibition of MRP2 by the glucuronide conjugate, but not the parent compound, although the clinical relevance of this inhibition remains unknown. BCRP-mediated transport of MTX was significantly inhibited by 100 μM indomethacin and phenylbutazone and 1000 μM probenecid (Fig. 5A). However, such inhibition was not clinically relevant considering their unbound plasma concentrations in clinical situations. MRP4 is more susceptible to NSAIDs compared with BCRP and MRP2 (Fig. 5C), which agrees with very recently published results (El-Sheikh et al., 2007). Salicylate, indomethacin, ibuprofen, ketoprofen, naproxen, and phenylbutazone inhibited MRP4-mediated transport of MTX in a concentration-dependent manner, and the K_i values of these NSAIDs for MRP4 were generally comparable with previous results with some exceptions. Salicylate exhibited no inhibition of MRP4-mediated MTX transport at a concentration of 100 μM (Fig. 5). Addition of experimental points at higher concentrations gave K_i values of 218 μM, although the K_i value was 7-fold smaller than the previously reported values for some unknown reason. Based on R values (Table 3), salicylate can be expected to inhibit MRP4-mediated transport at clinical doses, and indomethacin also has a potential to inhibit MRP4 at high clinical concentrations. Because several NSAIDs are substrates of OAT1 (Apiwattanakul et al., 1999), it is possible that NSAIDs, concentrated in the renal tubular cells by basolateral organic anion transporter(s), may exhibit a greater inhibition than expected from the plasma unbound concentrations. It must be kept in mind that the impact of the inhibition of MRP4 by salicylate and/or indomethacin on the renal elimination of MTX totally depends on the contribution of MRP4 to the net efflux across the BBM. It is required to evaluate the contribution of apical efflux transporters in the future for more reliable prediction.

In conclusion, the present study suggests that drug-drug interactions between MTX and salicylate, indomethacin, phenylbutazone, and probenecid involve inhibition of the uptake mediated by OAT3 and other unknown transporters. The transport studies using human kidney slices demonstrated an interspecies difference in the inhibition potencies of NSAIDs, indicating the importance of using human materials for the quantitative prediction of drug-drug interactions. As far as MRP4 is concerned, salicylate and indomethacin were predicted to have a significant effect in clinical situations. In addition to the parent compounds, drug-drug interactions may involve the inhibition of apical ATP-binding cassette transporters (MRP2 and MRP4) by glucuronide conjugates of NSAIDs.

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Investigation of the Inhibitory Effects of Various Drugs on the Hepatic Uptake of Fexofenadine in Humans

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ABSTRACT:

Fexofenadine (FEX), an H_1-receptor antagonist, is eliminated from the liver mainly in an unchanged form. Our previous study suggested that organic anion-transporting polypeptide (OATP) 1B3 contributes mainly to the hepatic uptake of FEX. On the other hand, a clinical report demonstrated that a T521C mutation of OATP1B1 increased its plasma area under the curve. Several compounds are reported to have a drug interaction with FEX, and some of this may be caused by the inhibition of its hepatic uptake. We determined which transporters are involved in the hepatobiliary transport of FEX by using double transfectants and examined whether clinically reported drug interactions with FEX could be explained by the inhibition of its hepatic uptake. Vectorial basal-to-apical transport of FEX was observed in double transfectants expressing OATP1B1/multidrug resistance-associated protein 2 (MRP2) and OATP1B3/MRP2, suggesting that OATP1B1 as well as OATP1B3 is involved in the hepatic uptake of FEX and that MRP2 can recognize FEX as a substrate. The inhibitory effects of compounds on FEX uptake in OATP1B3-expressing HEK293 cells were investigated, and the maximal degree of increase in plasma AUC of FEX by drug interaction in clinical situations was estimated. As a result, cyclosporin A and rifampicin were found to have the potential to interact with OATP1B3-mediated uptake at clinical concentrations. From these results, most of the reported drug interaction cannot be explained by the inhibition of hepatic uptake of FEX, and different mechanisms such as the inhibition of intestinal efflux should be considered.

Fexofenadine (FEX) is an orally active nonsedating histamine H_1 receptor antagonist for the treatment of allergic rhinitis and chronic idiopathic urticaria. After oral administration of [^{14}C]FEX to healthy volunteers, 80% of the total dose was recovered in feces and 12% in urine in an unchanged form (Lippert et al., 1995). Because the absolute oral bioavailability of FEX was reported to be 33% (product information, Hoechst Marion, Roussel, Laval, QC, Canada), about two-thirds of bioavailable FEX is estimated to be excreted into bile. Accordingly, some drug transporters in the liver are major determinants for the clearance of FEX from systemic blood.

The first step in the process of elimination from the liver is hepatic uptake across the sinusoidal membrane. Accumulated evidence has supported the idea that organic anion-transporting polypeptide (OATP) 1B1 (OATP1B1) and OATP1B3 play major roles in the hepatic uptake of a wide variety of compounds including endogenous compounds and clinically important drugs such as HMG-CoA reductase inhibitors (statins) (Hagenbuch and Meier, 2004). Shimizu et al. (2005) have demonstrated that OATP1B3 contributes mainly to the hepatic uptake of FEX using transporter-expressing HEK293 cells. On the other hand, a recent clinical report has shown that the genetic polymorphism of OATP1B1 (T521C), which was reported to decrease the transport clearance, increased the plasma AUC of FEX (Niemi et al., 2005). These results suggested that OATP1B1 as well as OATP1B3 is involved in the uptake of FEX into human liver.

Many clinical reports have indicated the interaction between FEX and several drugs. Among them, itraconazole (Shon et al., 2005; Shimizu et al., 2006a,b; Uno et al., 2006), ketoconazole (Common Technical Document for the Registration of Pharmaceuticals for Human Use), azithromycin (Gupta et al., 2001), erythromycin (Common Technical Document), ritonavir, lopinavir (van Heeswijk et al., 2006), verapamil, and probenecid (Yasui-Furukori et al., 2005) increased the plasma AUC of FEX, whereas rifampicin (Hamman et al., 2001) decreased it. One of the possible mechanisms for the increase of its AUC is the inhibition of multidrug resistance 1 (MDR1/ABCB1) in the small intestine by the concomitantly administered compounds. Vectorial transport was reported to be observed in MDR1-expressing LLC-PK1 cells but not in parent cells (Cvetkovic et al., 1999). Furthermore, after oral administration of FEX, the plasma AUC in Mdr1a/1b$^{-/-}$ mice was approximately 6 times greater than that in FVB mice, whereas after i.v. administration of FEX, there was no

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ABBREVIATIONS: FEX, fexofenadine; OATP organic anion-transporting polypeptide; AUC, area under the curve; MDR/Mdr, multidrug resistance; MRP, multidrug resistance-associated protein; EG, estradiol-17β-D-glucuronide; MDCK, Madin-Darby canine kidney; LC/MS, liquid chromatography/mass spectrometry.

difference in the pharmacokinetics of FEX between FVB and Mdr1a/1b$^{-/-}$ mice, suggesting that Mdr1a/1b in the small intestine limits the absorption of FEX (Tahara et al., 2005). Because most of the compounds that clinically increase the AUC of FEX have the ability to inhibit MDR1 function, these are likely to increase the intestinal absorption of FEX by the inhibition of MDR1 in the small intestine.

However, considering that the main elimination pathway of FEX is biliary excretion of the unchanged form (Lippert et al., 1995), it is also possible that drug interaction with FEX is caused by the inhibition of its hepatic uptake. Hirano et al. (2006) performed detailed investigations to see whether the inhibitory effects of various compounds on OATP1B1-mediated uptake of pitavastatin were clinically relevant. However, the inhibition potencies of several compounds for OATP1B3-mediated uptake have not yet been clarified.

Therefore, the purpose of this study was to determine which transporters are involved in the hepatobiliary transport of FEX and explore which instances can be explained by the inhibition of its hepatic uptake among clinically reported drug interactions. We identified the transporters that can transport FEX by using double transfectants expressing OATP1B1/multidrug resistance-associated protein (MRP) 2 and OATP1B3/MRP2 (Matsushima et al., 2005). It has often been found that the transcellular transport assay using double transfectants is more sensitive in detecting transporter-mediated transport than the uptake assay in single transporter-expressing cells. Thus, we analyzed the inhibitory effects of several compounds that are reported to interact with FEX in clinical situations on FEX uptake in OATP1B3-expressing HEK293 cells and compared the in vitro inhibition constant (K_i) for OATP1B3 with that for OATP1B1 obtained from a previous study (Hirano et al., 2006). After that, to determine whether the inhibition of FEX uptake by several compounds is clinically relevant, we also estimated the maximal degree of increase in the plasma AUC of FEX by considering the maximal unbound concentration of inhibitors at the inlet to the liver estimated by an established method for this calculation (Ito et al., 1998).

Materials and Methods

Materials. [^{3}H]Estradiol-17β-D-glucuronide (EG) (1.6 TBq/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). FEX hydrochloride was purchased from Toronto Research Chemicals (North York, ON, Canada). All other chemicals and reagents were of analytical grade and commercially available.

Cell Culture. MDCKII cells expressing OATP1B1/MRP2, OATP1B3/MRP2, OATP1B1, OATP1B3, and MRP2 and vector-transfected control MDCKII cells have been constructed previously (Matsushima et al., 2005, Ishiguro et al., submitted for publication). OATP1B3-expressing HEK293 cells and vector-transfected control cells were also constructed previously (Hirano et al., 2004). Transporter-expressing or vector-transfected MDCKII and HEK293 cells were grown in Dulbecco's modified Eagle's medium (low glucose) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO) and 1% antibiotic-antimycotic solution (Sigma-Aldrich) at 37°C under 5% CO_2 and 95% humidity.

Transcellular Transport Study. The transcellular transport study was performed as reported previously (Matsushima et al., 2005). Briefly, MDCKII cells were grown on Transwell membrane inserts (6.5 mm diameter, 0.4 μm pore size; Corning Coster, Bodenheim, Germany) at confluence for 7 days, and the medium was replaced with Dulbecco's modified Eagle's medium supplemented with 5 mM sodium butyrate 2 days before the transport study to induce the expression of exogenous transporter. In the transport assay, cells were first washed with Krebs-Henseleit 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, 5.0 mM glucose, and 1.53 mM $CaCl_2$ adjusted to pH 7.4) at 37°C and preincubated with Krebs-Henseleit buffer for 10 min. Subsequently, substrates were added in Krebs-Henseleit buffer either to the apical compartment (250 μl) or to the basolateral compartment (1 ml). After a designated period, 50 or 100 μl of medium was taken from the opposite side to the added substrate. Using FEX as a substrate, 50-μl aliquots were used for LC/MS quantification as described below. At the end of the experiments, cells were washed with ice-cold Krebs-Henseleit buffer and solubilized in 500 μl of 0.2 N NaOH. After addition of 100 μl of 1 N HCl, 50-μl aliquots were used to determine protein concentrations by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

Uptake Study Using OATP1B3-Expressing HEK293 Cells. Cells were seeded in 12-well plates coated with poly-L-lysine/poly-L-ornithine (Sigma-Aldrich) at a density of 1.5×10^5 cells/well. After 2 days, the cell culture medium was replaced with culture medium supplemented with 5 mM sodium butyrate 24 h before the transport assay to induce the expression of exogenous transporters. The transport study was carried out as described previously (Sugiyama et al., 2001). Uptake was initiated by adding Krebs-Henseleit buffer containing FEX. All of the procedures were performed at 37°C. The uptake was terminated at a designated time by adding ice-cold Krebs-Henseleit buffer after removal of the incubation buffer. Then, cells were washed twice with 1 ml of ice-cold buffer, solubilized in 500 μl of 0.2 N NaOH, and kept overnight at room temperature. Using EG as a substrate, aliquots (300 μl) were transferred to vials after addition of 100 μl of 1 N HCl. Using FEX as a substrate, aliquots (240 μl) were used for LC/MS quantification as described below. The remaining 50 μl of the aliquots of cell lysate were used to determine the protein concentration by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

Quantification of FEX by LC/MS. The aliquots (50 μl) obtained from the transcellular transport study were precipitated with 200 μl of methanol containing 10 nM midazolam as an internal standard, whereas the aliquots (240 μl) obtained from the uptake study were precipitated with 480 μl of methanol containing 50 nM midazolam as an internal standard. After centrifugation (15,000g for 10 min at 4°C) of the mixture, 50 μl of 0.05% formic acid was added to 50 μl of supernatant. The samples obtained were subjected to LC/MS analysis to determine the concentration of FEX. An LC/MS-2010 EV equipped with a Prominence LC system (Shimadzu, Kyoto, Japan) was used for the analysis. The samples were separated on a CAPCELL PAK C18 MG column (3 μm, 4.6 mm i.d., 75 mm; Shiseido, Tokyo, Japan) in binary gradient mode. For the mobile phase, 0.05% formic acid and methanol were used. The methanol concentration was initially 48%, which was then linearly increased up to 61.5% over 4.5 min. Finally, the column was reequilibrated in a methanol concentration of 48% for 3 min. The total run time was 7.5 min. By using this method, FEX was eluted at 4.1 min and midazolam at 2.8 min. In the mass analysis, FEX and midazolam were detected at mass-to-charge ratios of 502.3 and 326.1 under positive ionization conditions. The interface voltage was 3.5 kV, and the nebulizer gas (N_2) flow was 1.5 liters/min. The heat block and curved desolvation line temperatures were 200 and 150°C, respectively.

Kinetic Analyses. Ligand uptake in transporter-expressing cells was expressed as the uptake volume (microliters per milligram of protein), given as the amount associated with the cells divided by its initial concentration in the incubation medium. Transporter-specific uptake was obtained by subtracting the uptake into vector-transfected cells from that into transporter-expressing cells. Inhibition constants (K_i) of a series of compounds could be calculated by the following equation, if the substrate concentration was low enough compared with its K_m value:

$$CL_{+I} = \frac{CL}{1 + I/K_i} \quad (1)$$

where CL represents the uptake clearance in the absence of inhibitor, CL_{+I} represents the uptake clearance in the presence of inhibitor, and I represents the inhibitor concentration. When the data were fitted to determine the K_i value, the input data were weighted as the reciprocal of the observed values. The Damping Gauss-Newton method algorithm was used with a MULTI software program (Yamaoka et al., 1981) to fit the data.

Prediction of Clinical Drug-Drug Interactions between FEX and Various Compounds. The degree of inhibition of uptake via OATP1B1 and OATP1B3 in humans was estimated by calculating the following R value, which represents the ratio of the uptake clearance in the absence of inhibitor to that in its presence,

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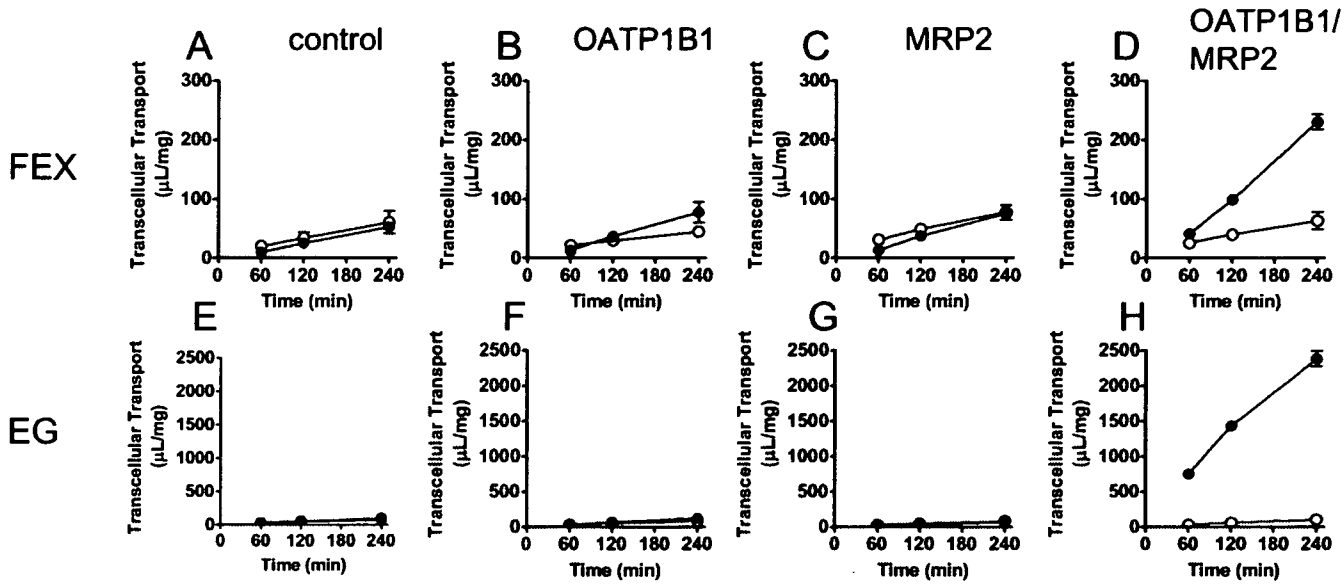

FIG. 1. Time profiles for the transcellular transport of FEX and EG across MDCKII cell monolayers expressing OATP1B1 and/or MRP2. The transcellular transport of 5 μM FEX (A–D) and 0.1 μM EG (E–H) across MDCKII cell monolayers expressing OATP1B1 (B and F), MRP2 (C and G), and both OATP1B1 and MRP2 (D and H) was compared with that across the vector-transfected control MDCKII cell monolayer (A and E). Otranscellular transport in the apical-to-basal direction; Otranscellular transport in the basal-to-apical directions. These data were obtained from three independent experiments, and each experiment was performed in triplicate. Each point with vertical bar represents the mean and S.D. Where a vertical bar is not shown, the S.D. was contained within the limits of the symbol.

$$R = 1 + \frac{f_u \cdot I_{in,max}}{K_i} \quad (2)$$

where f_u represents the protein unbound fraction of the inhibitor in blood and $I_{in,max}$ represents the estimated maximal blood concentration of the inhibitor at the inlet to the liver. The K_i value for OATP1B3 was obtained from the present in vitro study using OATP1B3-expressing HEK293 cells described above, and the K_i value for OATP1B1 is quoted from the previous reports in which pitavastatin was used as a substrate of OATP1B1 (Hirano et al., 2006). For estimation of the R value, $I_{in,max}$ was calculated by the method of Ito et al. (1998) as shown by

$$I_{in,max} = I_{max} + \frac{F_a \cdot dose \cdot k_a}{Q_h} \quad (3)$$

where I_{max} represents the reported value for the maximal blood concentration of the inhibitor in the systemic circulation in clinical situations, F_a represents the fraction absorbed from the intestine of the inhibitor, k_a is the absorption rate constant in the intestine, and Q_h represents the hepatic blood flow rate in humans (1610 ml/min) (Ito et al., 1998). To estimate the maximal $I_{in,max}$, F_a was set at 1, k_a was set at 0.1 ml^{-1} [minimum gastric emptying time (10 min)], and the blood-to-plasma concentration ratio was assumed to be 1.

Results

Transcellular Transport of FEX and EG across the MDCKII Cell Monolayer. The transcellular transport of 5 μM FEX and 0.1 μM EG across the MDCKII monolayer was determined. The basal-to-apical transport of FEX was approximately 3.6 times higher than that in the opposite direction in OATP1B1/MRP2 double transfectants (Fig. 1D), whereas no difference in basal-to-apical transcellular transport of FEX could be observed in vector-transfected control cells and single transfectants expressing OATP1B1 or MRP2 (Fig. 1, A–C). On the other hand, the basal-to-apical transport of EG was approximately 22 times higher than that in the opposite direction in OATP1B1/MRP2 double transfectants (Fig. 1H), whereas we could not see any basal-to-apical transcellular transport of EG in vector-transfected control cells and single transfectants expressing OATP1B1 or MRP2 (Fig. 1, E–G). The basal-to-apical transport of FEX was approximately 2.9 times higher than that in the opposite direction in OATP1B3/MRP2 double transfectants (Fig. 2D), whereas the difference in each directional transport of EG was less than 2 times that in vector-transfected control cells and single transfectants expressing OATP1B3 or MRP2 (Fig. 2, A–C). As a positive control, the basal-to-apical transport of EG was approximately 3.0 times higher than that in the opposite direction in OATP1B3/MRP2 double transfectants (Fig. 2H), whereas no significant difference in basal-to-apical transcellular transport of EG was less than 2 times that in vector-transfected control cells and single transfectants expressing OATP1B3 or MRP2 (Fig. 2, E–G).

Inhibitory Effects of Various Drugs on OATP1B3-Mediated Uptake of FEX. The inhibitory effects of various drugs on the uptake of FEX were examined using OATP1B3-expressing HEK293 cells (Fig. 3). Some of the drugs we tested were reported to cause drug-drug interactions with FEX in clinical situations. Most of the compounds could inhibit OATP1B3-mediated FEX uptake. On the other hand, even 100 μM fluconazole, 30 μM itraconazole, and 100 μM cimetidine did not significantly inhibit FEX uptake (Fig. 3, C, D, and N). We also obtained the protein unbound fraction in blood (f_u) from the literature and calculated the estimated maximal concentration at the inlet to the liver ($I_{in,max}$) of the inhibitors. K_i values of various compounds for OATP1B3 obtained in the present study and the ratio of the uptake clearance in the absence of inhibitor to that in its presence (R value) are summarized in Table 1. Among several drugs we tested, only the R values of cyclosporin A and rifampicin for OATP1B3-mediated uptake exceeded 2.0. The K_i values of various compounds for OATP1B1 obtained in the previous study and the respective R values are also shown in Table 1 (Hirano et al., 2006). The K_i values of several compounds in the uptake mediated by OATP1B1 and OATP1B3 were not so different. However, the K_i values of clarithromycin and ritonavir for OATP1B1-mediated uptake of pitavastatin were more than 5-fold less compared with that for OATP1B3-mediated uptake of FEX.

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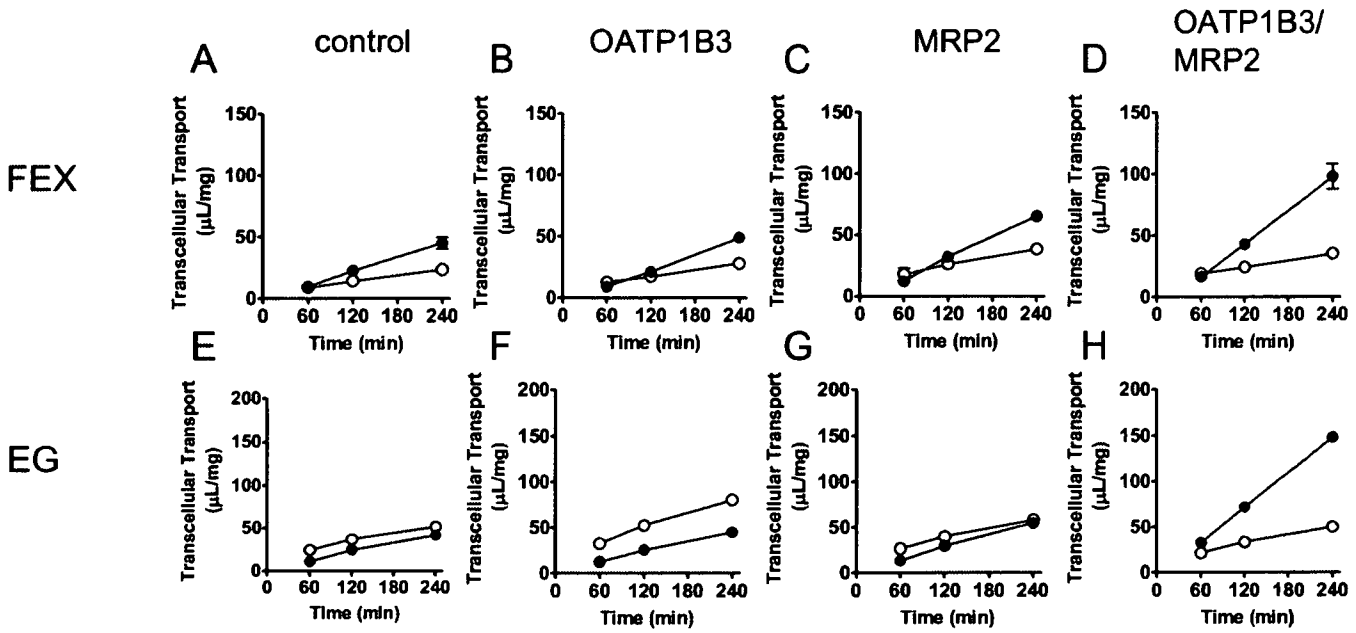

FIG. 2. Time profiles for the transcellular transport of FEX and EG across MDCKII cell monolayers expressing OATP1B3 and/or MRP2. The transcellular transport of 5 μM FEX (A–D) and 0.1 μM EG (E–H) across MDCKII cell monolayers expressing OATP1B3 (B and F), MRP2 (C and G), and both OATP1B3 and MRP2 (D and H) was compared with that across the control MDCKII cell monolayer (A and E). Otranscellular transport in the apical-to-basal direction; Otranscellular transport in the basal-to-apical directions, respectively. These data were obtained from three independent experiments, and each experiment was performed in triplicate. Each point with vertical bar represents the mean and S.D. Where a vertical bar is not shown, the S.D. was contained within the limits of the symbol.

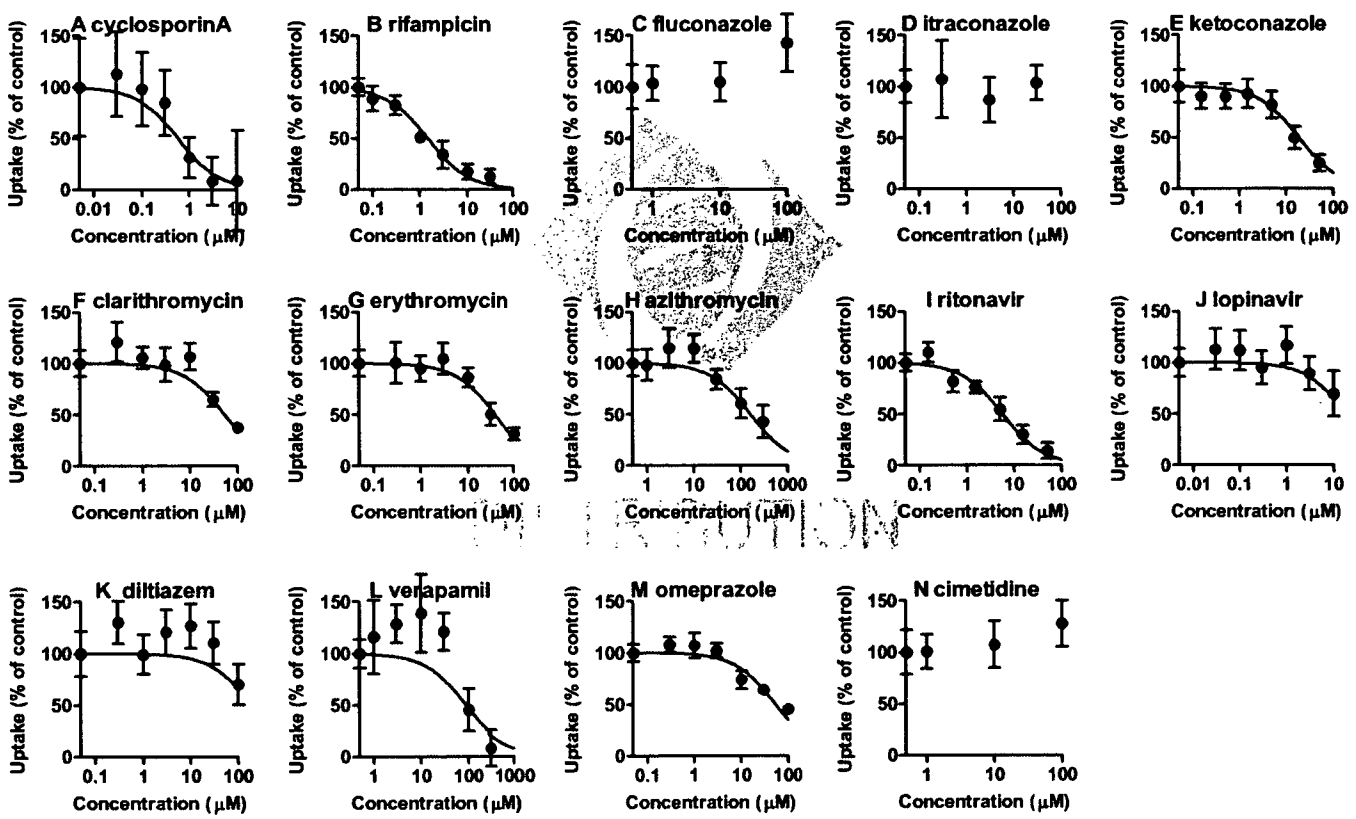

FIG. 3. Inhibitory effects of various drugs on the OATP1B3-mediated uptake of FEX. The OATP1B3-mediated uptake of FEX (10 μM) was determined in the absence or presence of inhibitors cyclosporin A (A), rifampicin (B), fluconazole (C), itraconazole (D), ketoconazole (E), clarithromycin (F), erythromycin (G), azithromycin (H), ritonavir (I), lopinavir (J), diltiazem (K), verapamil (L), omeprazole (M), and cimetidine (N) using OATP1B3-expressing HEK293 cells. These data were obtained from three independent experiments, and each experiment was performed in triplicate. Each point with vertical bar represents the mean and S.D. Where a vertical bar is not shown, the S.D. was contained within the limits of the symbol. Each solid line represents the fitting curve obtained by nonlinear regression analysis

TABLE 1

Comparison of K_i values of inhibitors for OATP1B1-mediated uptake of pitavastatin and OATP1B3-mediated uptake of FEX

K_i values are expressed as the mean ± computer-calculated S.D. The inhibitors, which are shown in boldface, increased the plasma AUC in the previous clinical studies. Data for dosages, I_{max}, and f_u are from product information of each drug except for the data with footnotes. R and $I_{in,max}$ values are calculated according to eq. 2 and eq. 3, respectively (*see Materials and Methods*).

Inhibitor	Dosage	I_{max}	$I_{in,max}$	f_u	OATP1B3		OATP1B1	
					K_i	R	K_i[a]	R
	mg	μM	μM		μM		μM	
Cyclosporin A	100	0.596	5.76	0.10[b]	0.573 ± 0.172	2.01	0.242 ± 0.029	3.55
Rifampicin	600[c]	7.90[c]	53.2	0.11[c]	1.45 ± 0.28	5.03	0.477 ± 0.030	13.3
Fluconazole	100	8.62	28.9	0.89[c]	>100	<1.26	>100	<1.26
Itraconazole	100	0.0792	8.88	0.002[c]	>30	1.00	>100	1.00
Ketoconazole	200[c]	3.20[c]	26.6	0.01[c]	18.5 ± 3.0	1.01	19.2 ± 3.9	1.01
Clarithromycin	400	1.86	35.1	0.54[c]	53.6 ± 15.9	1.42	8.26 ± 0.54	3.29
Erythromycin	200	1.12	18.0	0.16[c]	38.3 ± 7.7	1.08	11.4 ± 2.1	1.25
Azithromycin	1200	0.881	100	0.84	161 ± 7.7	1.52	N.D.	
Ritonavir	800	28.5	97.4	0.02[c]	5.64 ± 1.39	1.35	0.781 ± 0.048	3.49
Lopinavir	400	15.2	54.8	0.015	18.4 ± 7.2	1.04	N.D.	
Probenecid	2000[d]	52.0[d]	487	0.10[e]	130 ± 40[e]	1.37	76.2 ± 7.1	1.64
Diltiazem	100	0.0536	15.0	0.22[c]	193 ± 112	1.03	>100	<1.03
Verapamil	80	0.190	11.1	0.10[c]	89.5 ± 52.9	1.01	51.6 ± 15.9	1.02
Omeprazole	20	1.18	4.77	0.03	53.9 ± 14.3	1.00	N.D.	
Cimetidine	200	2.75	52.0	0.81[c]	>100	<1.42	>300	<1.14

N.D., not determined.
[a] K_i values of inhibitors for the OATP1B1-mediated uptake of pitavastatin were determined previously (Hirano et al., 2006).
[b] Data for protein unbound fraction of cyclosporin A are from a previous report (Lemaire and Tillement, 1982).
[c] These data are from Hardman and Limbard (2001).
[d] Data for dosages and I_{max} of probenecid are from a previous report (Selen et al., 1982).
[e] K_i values of probenecid were determined previously (Tahara et al., 2006).

Discussion

To determine which transporters are involved in the hepatobiliary transport of FEX, we investigated the transcellular transport of FEX using OATP1B1/MRP2 and OATP1B3/MRP2 double-transfected cells. Furthermore, to investigate whether the inhibition of FEX hepatic uptake by several drugs is clinically relevant, the inhibition constants of several drugs for OATP1B3-mediated FEX uptake obtained from in vitro analyses were determined, and the maximal degrees of increase in the plasma AUC through drug interactions were calculated using estimated maximal protein unbound concentrations of inhibitors at the inlet to the liver.

In the transcellular transport study using double-transfected cells, we observed the basal-to-apical vectorial transport of FEX not only in OATP1B3/MRP2 but also in OATP1B1/MRP2double-transfected cells (Figs. 1 and 2). Our previous report indicated that OATP1B3 contributes mainly to the hepatic uptake of FEX in humans and that OATP1B1-mediated uptake was not statistically significant although the uptake in OATP1B1-expressing cells was slightly larger than that in control cells (Shimizu et al., 2005). The involvement of OATP1B1 in FEX uptake in humans was also supported by the recent clinical report demonstrating that the genetic polymorphism of OATP1B1 (T521C) increased the plasma concentration of FEX (Niemi et al., 2005). The apparently conflicting results obtained from the present transcellular transport study and the previous uptake study may be caused by the difference in the sensitivity for the detection of the transport. We found that a transcellular transport assay using a double transfectant is more sensitive in detecting the transporter-mediated transport than an uptake assay in single transporter-expressing cells (Sasaki et al., 2002; Matsushima et al., 2005). For example, the ratio of the basal-to-apical transport of pravastatin to that in the opposite direction was 3.3, whereas it barely estimates the kinetics of pravastatin transport in OATP1B1-expressing HEK293 cells because of its small OATP1B1-mediated uptake (Matsushima et al., 2005). Therefore, these results suggest a significant contribution of OATP1B1 as well as OATP1B3 to the hepatic uptake of FEX. Further evaluation is required for the determination of the precise relative contribution of OATP1B1 and OATP1B3 to the hepatic uptake of FEX in humans.

The finding presented here is the first demonstration that human MRP2 can recognize FEX as a substrate. MRP2 is expressed in the apical membrane of the liver, kidney, and intestine. An in vivo infusion study using Eisai hyperbilirubinemic rats, which are Mrp2-deficient, revealed that Mrp2 is not important for the biliary excretion of FEX (Tahara et al., 2005). However, it is still possible that MRP2 will play an important role in the disposition of FEX in humans. This situation is very similar to that reported for pitavastatin (Hirano et al., 2005). The transcellular transport study using OATP1B1/MRP2 double-transfected cells indicated that pitavastatin could be transported by MRP2. However, the biliary excretion of pitavastatin in Eisai hyperbilirubinemic rats was not changed compared with that in control rats. Moreover, there are some reports that show species differences in the expression and function of MRP2 (Ishizuka et al., 1999; Ninomiya et al., 2005, 2006; Takekuma et al., 2007). These may also indicate the species difference in the relative contribution of efflux transporters to the biliary excretion of compounds. The methodology to determine the contribution of efflux transporters in human liver needs to be established by checking the effect of transporter-specific inhibitors on the efflux of compounds in membrane vesicles prepared from human liver or sandwich-cultured human hepatocytes.

Several reports regarding drug-drug interactions with FEX have been published. It is generally believed that one of the major mechanisms of the reported drug-drug interactions between FEX and concomitantly administered drugs is the inhibition of MDR1-mediated efflux in the small intestine, which plays an important role in limiting the entry of FEX into circulating blood (Tahara et al., 2005). However, some of the reported cases are thought to be caused by mechanisms other than the inhibition of MDR1. A regional perfusion study showed that ketoconazole and verapamil did not have a significant effect on the in vivo intestinal absorption of FEX when coadministered or given as a pretreatment despite increasing the plasma AUC of FEX (Tannergren et al., 2003). Accordingly, the involvement

of other mechanisms in addition to the inhibition of MDR1 has been supposed. One of the candidate mechanisms is considered to reduce the hepatic uptake clearance because the major route of FEX elimination is biliary excretion of the unchanged form. Because FEX is barely metabolized, the apparent intrinsic hepatic clearance is described as follows:

$$CL_{int,\,h} = CL_{uptake} \cdot \frac{CL_{excretion}}{CL_{efflux} + CL_{excretion}} \quad (4)$$

where $CL_{int,\,h}$ represents the apparent intrinsic hepatic clearance, CL_{uptake} represents the hepatic uptake clearance, $CL_{excretion}$ represents the biliary excretion clearance, and CL_{efflux} represents the backflux clearance from liver to blood. According to eq. 4, the change in the hepatic uptake clearance always directly affects the overall intrinsic hepatic clearance. The present study indicates that both OATP1B1 and OATP1B3 contribute to the hepatic uptake of FEX. Previously we reported the inhibitory effects of various drugs on OATP1B1-mediated uptake and their clinical relevance to drug-drug interaction (Hirano et al., 2006). However, this kind of systematic investigation for OATP1B3 has not been conducted. Therefore, the inhibitory effects of various drugs on OATP1B3-mediated uptake were determined. Among several compounds we tested, the R values of cyclosporin A and rifampicin for OATP1B3 as well as for OATP1B1 exceeded 2.0 (Table 1). To date, we have not been able to find a published report regarding a drug-drug interaction between FEX and cyclosporin A. Many clinical reports have indicated that cyclosporin A increases the AUC of a variety of substrates of OATP transporters, particularly HMG-CoA reductase inhibitors (Shitara et al., 2005). Although cyclosporin A is known as a clinically relevant potent OATP1B1 inhibitor (Shitara et al., 2003), we showed that cyclosporin A can also potently inhibit OATP1B3-mediated uptake. Accordingly, it is necessary to pay attention to not only the OATP1B1- but also the OATP1B3-mediated drug-drug interaction between FEX and cyclosporin A in clinical situations. Repetitive administration of rifampicin reduced the plasma AUC of FEX in a previous clinical study (Hamman et al., 2001). This report apparently conflicts with the present results in which rifampicin inhibited the OATP1B3-mediated uptake. However, rifampicin is a well known pregnane X receptor-mediated inducer and increases the expression level of MDR1 in the small intestine (Schuetz et al., 1996). Therefore, in this case, repeated dosing of rifampicin increased the expression level of MDR1 in the small intestine, which masked its inhibitory effects on the OATP1B3-mediated uptake of FEX. This concept is supported by the recent report from Lam et al. (2006) indicating that drugs should be administered 1 day after the final dose of rifampicin to minimize potential inhibitory effects of OATP transporters in the induction study (Lam et al., 2006).

When we compared K_i values for OATP1B1 with those for OATP1B3 K_i values for OATP1B1 and OATP1B3 were within the range of a 5-fold difference, except for clarithromycin and ritonavir, suggesting that the inhibitory potency of compounds for OATP1B1-mediated transport can be considered similar to that for OATP1B3-mediated transport. A specific inhibitor for each individual transporter is very useful for determining the contribution of each transporter to the overall membrane transport. Although EG and estrone-3-sulfate are recognized as selective inhibitors for OATP1B1/OATP1B3 and OATP1B1/OATP2B1, respectively (Hirano et al., 2006), unfortunately, specific inhibitors for OATP1B3 have not yet been identified. Because of the high homology and overlapping substrate specificities between OATP1B1 and OATP1B3, the use of in silico screening with a ligand-based drug design approach may be necessary to search for the selective inhibitors for OATP1B3 (Hirano et al., 2004).

Hirano et al. (2006) have indicated that cyclosporin A, rifampicin, clarithromycin, and ritonavir (R value for OATP1B1 >2.0) have a potential to interact with OATP1B1-mediated transport of pitavastatin in clinical situations (Hirano et al., 2006). Although the K_i values for OATP1B1-mediated uptake were determined by using pitavastatin as a substrate because of no significant uptake of FEX into OATP1B1-expressing HEK293 cells, if we consider the possible contribution of OATP1B1 and OATP1B3 to the hepatic uptake of FEX, these drugs may also affect the hepatic clearance of FEX. To avoid false-negative predictions of drug-drug interactions, the maximal plasma unbound concentration of inhibitors at the inlet to the liver was calculated using eq. 3. which can overestimate these concentrations (Ito et al., 1998). Therefore, in most cases, a drug-drug interaction caused by inhibition of hepatic uptake of FEX might not occur in clinical situations.

In conclusion, both OATP1B1 and OATP1B3 are involved in the hepatic uptake of FEX, and MRP2 can recognize FEX as a substrate. Among the compounds we tested, cyclosporin A and rifampicin have the potential to inhibit the OATP1B1- and OATP1B3-mediated hepatic uptake of FEX at clinically relevant concentrations. However, most of the reported clinical drug-drug interactions cannot be explained simply by the inhibition of hepatic uptake of FEX, and other mechanisms should be taken into account (e.g., inhibition of MDR1-mediated efflux in small intestine).

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