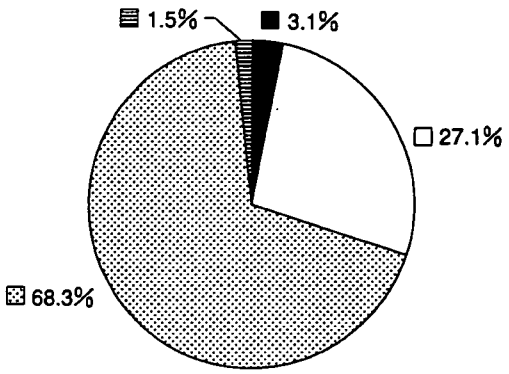
れた場合(臨床薬物動態データはなく，有害事象は未知)，どのように情報提供するのが適切と思いますか」(図9)については，「使用上の注意の「その他の注意」」と「インタビューフォームに記載」がおのおの35.1%となり，「添付文書の相互作用欄(一覧表)」は21.5%であった．同様に，「臨床における有害事象の発生の十分な情報はないが，動物試験等で重大な有害事象(薬物動態，薬理作用を含む)が生じる場合，どのように情報提供するのが適切と思いますか」(図10)については，「使用上の注意のその他の注意」(41.9%)，「インタビューフォームに記載」(29.8%)，「添付文書の相互作用欄(一覧表)」(21.9%)の順であり，*in vitro* 試験と同様に，相互作用欄以外で提供すべきとの意見であった．また，「臨床における有害事象の発生の十分な情報はないが，類薬で臨床上の相互作用が知られており，メカニズムに照らして同様の相互作用が否定できない場合，どのように情報提供するのが適切と思いますか」(図11)については，「添付文書の相互作用欄(一覧表)」が69.5%を占め，21.4%は添付文書ではなく「インタビューフォームに記載」との回答であった．「その他の回答」は「使用上の注意のその他の注意」(2件)であった．

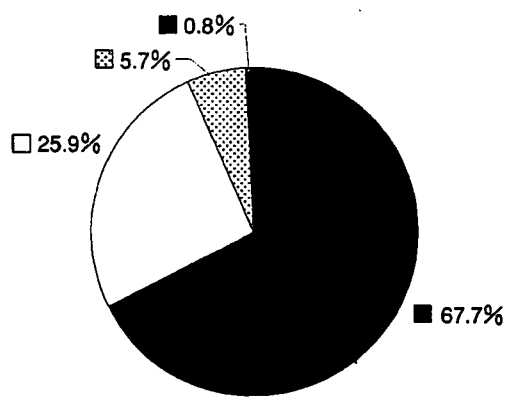
「相互作用欄で臨床薬物動態データ(AUC，C_{max})について，どのように情報提供するのが適切と思いますか(複数回答可)」の設問に対しては，有効回答数287件中，「変化率と投与条件を記載」(159件)が最も多く，「投与条件が承認された用法・用量と同一または近い場合のみ変化率を記載」(70件)とする意見はその半数以下であった．

食品類(嗜好品，サプリメント，健康食品等を含む)と



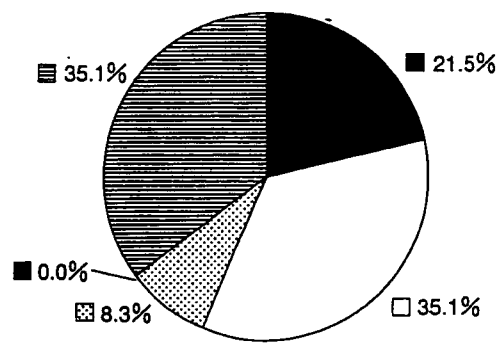
■ 1. グループ記載(個別薬剤名は記載しない)がよい
□ 2. グループ記載(代表的薬剤名のみ記載)がよい
▨ 3. グループ名と個別薬剤名を併記
▤ 4. グループ記載とせず個別薬剤名を記載

図7. 添付文書の相互作用に関する薬剤の記載について，クラスエフェクトと考えられる相互作用に関して，グループ化(「アゾール系抗真菌薬」，「フィブラート系薬剤」など)して記載されているものがありますが，どのようにお考えですか(有効回答数262件)



■ 1. 添付文書の相互作用欄(一覧表)に記載すべき
□ 2-1 インタビューフォームに記載
▨ 2-2 個別企業からではなく、共通のデータベースとして情報提供されればよい
■ 2-3 ヒトでの有害事象との関連が不明なため、提供する必要はない

図8. 臨床における有害事象の発生の十分な情報はないが，健常人に対する薬物動態試験などにより併用による薬物動態の変動が大きいことが示されている場合，どのように情報提供するのが適切と思いますか(有効回答数263件)

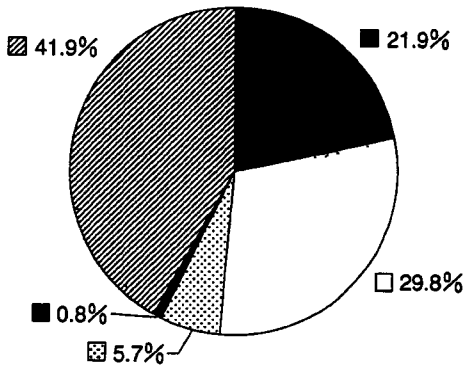


■ 1. 添付文書の相互作用欄(一覧表)に記載すべき
□ 2-1 インタビューフォームに記載
▨ 2-2 個別企業からではなく、共通のデータベースとして情報提供されればよい
■ 2-3 ヒトでの有害事象との関連が不明なため、提供する必要はない
▤ 3. 使用上の注意の「その他の注意」として記載する

図9. 臨床における有害事象の発生の十分な情報はないが，*in vitro* 試験で強い相互作用が認められた場合(臨床薬物動態データはなく，有害事象は未知)，どのように情報提供するのが適切と思いますか(有効回答数265件)

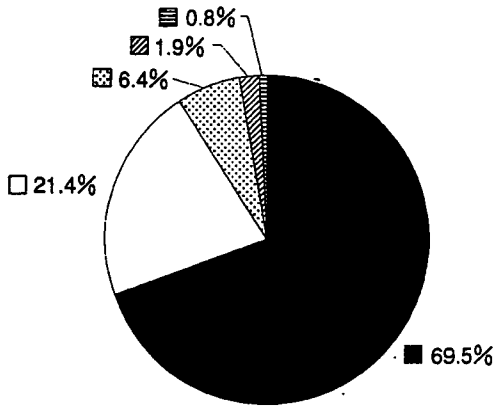
の相互作用に関して，「食品類による薬剤の血中レベルの変動(ヒトのデータ)が大きい場合，どのように情報提供するのが適切と思いますか(複数回答可)」については，有効回答数384件中，「有害事象または効果の減弱の報告がある場合」(223件)が多く，次いで，「有害事象との関連が示唆されている場合」(119件)であった．また，「食品成分に薬剤との薬理学的な相互作用の可能性が示されている場合(例：抗凝固剤と，血液凝固に影響を与える食品成分)，どのように情報提供するのが適切と思いますか(複数回答可)」についても同様に有効回答数412件中，「有害事象または効果の減弱の報告がある場合」(227件)の回答が多く，次いで，「有害事象との関連が示唆されている場合」(112件)であった．一方，「食品類と薬剤を併用した場合の有害事象報告があるが，作用メカニズムが不明な場合(例：抗凝固剤服用者がある食品を摂取して出血)，どのように情報提供するのが適切と思いますか(複数回答可)」では，有効回答数383件中「重篤な結果を引き起こす可能性がある場合」(176件)，「因果関係が明らかな場合」(167件)と，添付文書への記載を求める意見が多く，「ヒトでの有害事象との関連が不明なため提供する必要はない」を選択したものはなかった．同様に，「日本国内では通常利用されていないか，摂取方法が大きく異なる食品類と薬剤を併用した場合の有害事象報告がある場合(例：ハーブ濃縮物をサプリメントとして摂取)，どのように情報提供するのが適切と思いますか(複数回答可)」については有効回答数362件中，「因果関係が明らかな場合」(152件)，「重篤な結果を引き起こす可能性がある場合」(141件)に相互作用欄への記載を求めるとの回答が多く，「日本では使用される可能性が低いので，提供する必要はない」とする意見は2件のみであった．

「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)」(図12)については，「相互作用の項の一覧表の中で，機序・危険因子欄に記載」が約半数(54.9%)を占め，「相互作用の項の冒頭」(17.0%)，「上記の両方」(16.3%)を含めると，代謝に関与する分子種名を相互作用欄に記載すべきとの意見が，88.2%を占めた．一方，「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」(図13)については，「十分である」としたのは27.7%にすぎず，「必要な情報が欠けている」(40.6%)，「改訂すべき情報が反映されていない」(17.0%)との意見があった．「その他」(11.1%)には「後発医薬品を使用していない」，「メーカーにより異なる」などの意見があった．「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)」(図14)につ



■ 1. 添付文書の相互作用欄(一覧表)に記載すべき
□ 2-1 インタビューフォームに記載
⊠ 2-2 個別企業からではなく、共通のデータベースとして情報提供されればよい
■ 2-3 ヒトでの有害事象との関連が不明なため、提供する必要はない
▨ 3. 使用上の注意の「その他の注意」として記載する

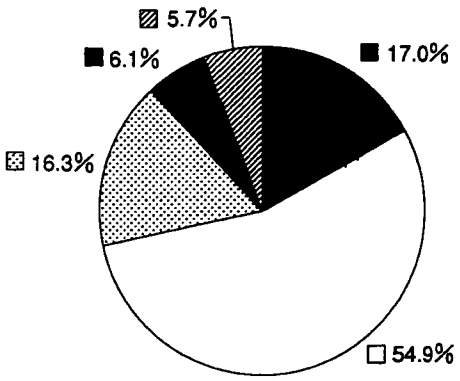
図10. 臨床における有害事象の発生の十分な情報はないが，動物試験等で重大な有害事象(薬物動態，薬理作用を含む)が生じる場合，どのように情報提供するのが適切と思いますか(有効回答数265件)



■ 1. 添付文書の相互作用欄(一覧表)に記載すべき
□ 2-1 インタビューフォームに記載
⊠ 2-2 個別企業からではなく、共通のデータベースとして情報提供されればよい
▨ 2-3 ヒトでの有害事象との関連が不明なため、提供する必要はない
▤ 3. その他の回答

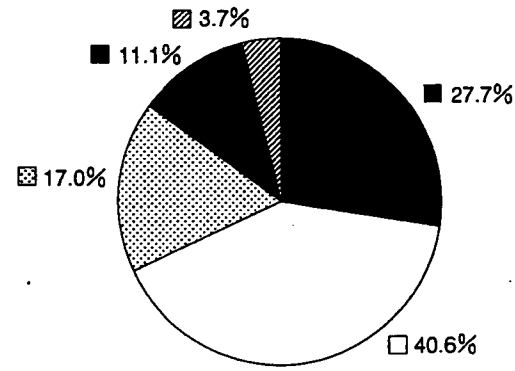
図11. 臨床における有害事象の発生の十分な情報はないが，類薬で臨床上の相互作用が知られており，メカニズムに照らして同様の相互作用が否定できない場合，どのように情報提供するのが適切と思いますか(有効回答数264件)

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)

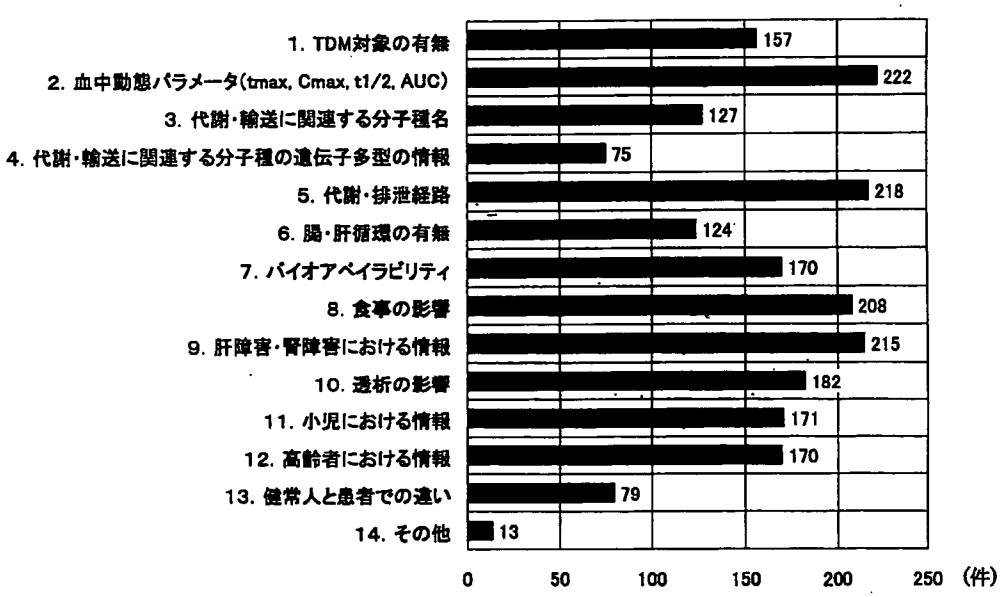


図14. 薬物動態について，情報がある場合には添付文書の薬物動態欄に記載する必要がある事項(複数回答可，有効回答数2131件)

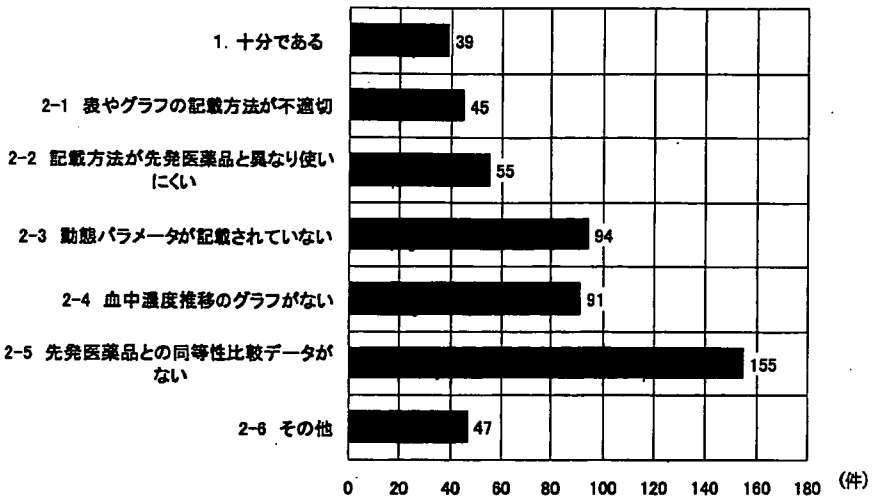


図 15. 薬物動態の添付文書での記載について，後発医薬品は先発医薬品と比べて十分ですか(複数回答可，有効回答数 526 件)

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[6]，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[7]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[8]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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Characterization of the Uptake of Organic Anion Transporter (OAT) 1 and OAT3 Substrates by Human Kidney Slices

Yoshitane Nozaki, Hiroyuki Kusuhara, Tsunenori Kondo, Maki Hasegawa, Yoshiyuki Shiroyanagi, Hayakazu Nakazawa, Teruo Okano, and Yuichi Sugiyama

Department of Molecular Pharmacokinetics, Graduate School of Pharmaceutical Sciences, University of Tokyo, Tokyo, Japan (Y.N., H.K., M.H., Y.Su.); Department of Urology, Kidney Center (T.K., Y.Sh.), and Institute of Advanced Biomedical Engineering and Science (T.O., Y.Sh), Tokyo Women's Medical University, Tokyo, Japan; and Department of Urology, Tokyo Women's Medical University, Medical Center, East, Tokyo, Japan (H.N.)

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ABSTRACT

The activities of renal multispecific organic anion transporters (OATs) 1 and 3 have not been fully evaluated in human kidneys. In the present study, the uptake of some organic anions was characterized in kidney slices from human intact renal cortical tissues: hOAT1 and hOAT3 substrates [*p*-aminohippurate (PAH) and 2,4-dichlorophenoxyacetate (2,4-D)] and hOAT3 substrates [benzylpenicillin (PCG), dehydroepiandrosterone sulfate (DHEAS), and estrone sulfate (ES)]. Despite large inter-batch differences, hOAT1 and hOAT3 mRNA levels correlated well, and there was a good correlation between the uptake of PAH and PCG by kidney slices. The uptake of organic anions by kidney slices was saturable with K_m values of 31 to 48 μM for PAH, 0.73 to 4.9 μM for 2,4-D, 14 to 90 μM for PCG, and 9.2 to 11 μM for ES. These parameters were comparable with those for hOAT1 and/or hOAT3. The uptake of DHEAS consists of two saturable components with K_m values of 2.2 to 3.9 and 1300 μM, and the K_m value of the high-affinity component was close to that for hOAT3. Furthermore, PAH more potently inhibited the uptake of 2,4-D than that of PCG and DHEAS. PCG had a weaker effect on the uptake of PAH and 2,4-D than expected from its K_m value. Taken together, it is likely that the uptake of PAH and 2,4-D is due to OAT1, and the uptake of PCG and ES and part of DHEAS uptake are due to OAT3 in human kidney slices. Human kidney slices are useful tools for characterizing the renal uptake of drugs.

The kidney plays an important role in the urinary excretion of endogenous wastes and xenobiotics, including drugs and their metabolites. Urinary excretion of drugs is mediated by glomerular filtration and tubular secretion and selective and passive reabsorption. Various kinds of membrane transporters involved in the renal tubular secretion have been identified in the proximal tubules. In particular, organic anion transporter (OAT) 1 (*SLC22A6*) and OAT3 (*SLC22A8*) are exclusively expressed on the basolateral membrane of the proximal tubules (Hosoyamada et al., 1999; Tojo et al., 1999; Cha et al., 2001; Hasegawa et al., 2002; Motohashi et al., 2002) and play major roles in the uptake of a variety of organic anions on the basolateral membrane of kidney proximal tubule epithelial cells (Wright and Dantzler, 2004; Sweet, 2005; Sekine et al., 2006).

OAT1 and OAT3 are multispecific transporters accepting a variety of structurally unrelated compounds as substrates, including clinically important drugs, such as diuretics (Uwai et al., 2000; Hasannejad et al., 2004), β-lactam antibiotics (Jariyawat et al., 1999; Ueo et al., 2005), antiviral drugs (Cihlar et al., 1999; Wada et al., 2000), an anticancer drug (methotrexate) (Cha et al., 2001), nonsteroidal anti-inflammatory drugs (Apiwattanakul et al., 1999), HMG-CoA reductase inhibitors (Hasegawa et al., 2002; Takeda et al., 2004), and H_2 receptor antagonists (Tahara et al., 2005a). OAT3 exhibits a broader substrate specificity than OAT1 and accepts amphipathic and hydrophilic organic anions and some organic cations, although the transport activities of hydrophilic organic anions with a low molecular weight by OAT3 is

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ABBREVIATIONS: OAT, organic anion transporter; PAH, *p*-aminohippurate; 2,4-D, 2,4-dichlorophenoxyacetate; PCG, benzylpenicillin; DHEAS, dehydroepiandrosterone sulfate; ES, estrone sulfate; KG, ketoglutarate; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

much lower than those by OAT1 (Kusuhara et al., 1999; Hasegawa et al., 2003). Consistent with these transport activities, kinetic analyses using rat kidney slices suggest that OAT1 plays a major role in the renal uptake of hydrophilic organic anions with a small molecular weight, such as *p*-aminohippurate (PAH), 2,4-dichlorophenoxyacetate (2,4-D), and uremic toxins (hippurate and indole acetate), whereas OAT3 mainly accounts for the uptake of benzylpenicillin (PCG), dehydroepiandrosterone sulfate (DHEAS), uremic toxins (indoxyl sulfate and 3-carboxy-4-methyl-5-propyl-2-furanopropionate), and, in part, that of estrone sulfate (ES), but makes only a limited contribution to the uptake of hydrophilic and small organic anions (Hasegawa et al., 2002, 2003; Sweet et al., 2002; Deguchi et al., 2004; Eraly et al., 2006).

The functional importance of human OATs in drug disposition has been extensively analyzed by in vitro studies using cDNA transfectant. There is a poor correlation in the transport activities of OAT3 between rats and humans, whereas there is a good correlation for OAT1 (Deguchi et al., 2004; Tahara et al., 2005b). Therefore, the renal uptake involving OAT3 may exhibit species difference. Indeed, the effect of probenecid on the renal clearance of famotidine is species-dependent. Probenecid markedly inhibits the secretion clearance of famotidine in humans (Inotsume et al., 1990), whereas it has no effect on the renal clearance in rats, even though the plasma concentration of probenecid is similar or rather higher than that in clinical studies (Lin et al., 1988). This is accounted for partly by the species difference in the transport activity of famotidine by rat and human OAT3 (r/hOAT3), greater for hOAT3 than rOat3, and partly by the rodent-specific expression of organic cation transporter 1 (Tahara et al., 2005a). These studies prompted us to establish an in vitro experimental system to evaluate the contribution of hOAT1 and hOAT3 using human kidney.

Kidney slices have been widely used to characterize renal uptake. The extracellular marker compounds, such as methoxy-inulin and sucrose, were below the limit of detection in the luminal space of the proximal tubules, whereas they could be detected in the extracellular space (Wedeen and Weiner, 1973). Therefore, the kidney slices allow limited access of drugs from the luminal space in the kidney slices but free access from the basolateral side, thereby allowing evaluation of basolateral uptake. In vitro studies of the uptake of drugs by mammalian kidney slices have proved useful for examining uptake mechanisms. Fleck et al. (2000, 2002) prepared kidney slices from human kidney and demonstrated the active accumulation of PAH and methotrexate, suggesting that human kidney slices also retain the activities of organic anion transporters. However, transport studies using human kidney tissue have not been thoroughly investigated focusing on the contribution of different transporters. The purpose of the present study is to compare the uptake of OAT1 and OAT3 substrate drugs by human kidney slices and to establish inhibitors to evaluate the contribution of OAT1 and OAT3.

Materials and Methods

Materials. [^{3}H]PAH (4.1 Ci/mmol), [^{3}H]DHEAS (60 Ci/mmol), and [^{3}H]ES (43.1 Ci/mmol) were purchased from PerkinElmer Life Science (Boston, MA). [^{14}C]PCG (59 mCi/mmol) and [^{3}H]2,4-D (20 Ci/mmol) were purchased from GE Healthcare Bio-Sciences (Waukesha, WI) and American Radiolabeled Chemicals (St. Louis, MO), respectively. Unlabeled PAH, DHEAS, ES, and 2,4-D were purchased from Sigma-Aldrich (St. Louis, MO), and unlabeled PCG and α-ketoglutarate (KG) were from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals were of analytical grade and commercially available.

Preparation of Human Kidney Slices and Uptake of Organic Anions by Human Kidney Slices. This study protocol was approved by the Ethics Review Boards at both 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 42 surgically nephrectomized patients with renal cell carcinoma at Tokyo Women's Medical University between October, 2003 and September, 2005. Samples of human kidney from subjects were stored in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) on ice immediately after kidney removal. After 30-min transportation, kidney slices were prepared as described below.

Uptake studies by human kidney slices were carried out following previous reports (Hasegawa et al., 2002, 2003). Kidney slices (300 μm thick) from intact human cortical tissue were kept in ice-cold buffer before use. The buffer for the present study consists of 120 mM NaCl, 16.2 mM KCl, 1 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 10 mM NaH_2PO_4/Na_2HPO_4 adjusted to pH 7.5. One slice, weighing 3 to 10 mg, was selected and incubated at 37°C on a 12-well plate with 1 ml of oxygenated buffer in each well after preincubation of slices for 5 min at 37°C. After incubating for 15 min, slices were rapidly removed from the incubation buffer, washed twice in ice-cold buffer, blotted on filter paper, weighed, and dissolved in 1 ml of Soluene-350 (Packard Instruments, Downers Grove, IL) at 50°C for 12 h. The radioactivity in the specimens was determined in scintillation cocktail (Hionic Flour; Packard Instruments).

Quantification of mRNA of hOAT1 and hOAT3 in Human Renal Cortical Tissue. Total RNA was isolated from intact cortical tissue of human kidney using ISOGEN (NIPPON GENE, Tokyo, Japan) according to manufacturer's protocol, followed by DNase treatment (TaKaRa, Shiga, Japan). Total RNA was converted to cDNA using random 9-mers and avian myeloblastosis virus reverse transcriptase. Real-time quantitative PCR was performed using SYBR Green (TaKaRa) and a LightCycler system (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Nucleotide sequences of the primers for hOAT1 and hOAT3 and GAPDH are: hOAT1, forward, 5′-GGCACCTTGATTGGCTATGT-3′; hOAT1, reverse, 5′-AAAAGGCGCAGAGACCAGTA-3′; hOAT3, forward, 5′-GTCCATACGCTGGTGGTCTT-3′; hOAT3, reverse, 5′-GCTGAGCCTTTCTCCCTCTT-3′; GAPDH, forward, 5′-GAAGGTGAAGGTCGGAGTC-3′; and GAPDH, reverse, 5′-GAAGATGGTGATGGGATTTC-3′.

GAPDH was used as a housekeeping gene for the internal standards. An external standard curve was generated by dilution of the target PCR fragment, which was purified by agarose gel electrophoresis. The absolute concentration of external standard was measured by PicoGreen dsDNA Quantitation Reagent (Molecular Probes, Eugene, OR). Expression of hOAT1 and hOAT3 were normalized by the expression of GAPDH.

Transport Studies in hOAT1- and hOAT3-Transfected Cells. hOAT1- and hOAT3-transfected HEK293 cells were established as described previously (Deguchi et al., 2004). 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 starting the transport studies.

Transport studies were carried out as described previously (Sug-

iyama et al., 2001). Uptake was initiated by adding the medium containing the radiolabeled compounds in the presence or absence of inhibitors after cells had been washed twice and preincubated 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 mM glucose, and 1.53 mM $CaCl_2$, pH 7.4). The uptake was terminated at designated times by aspirating the incubation buffer and adding ice-cold Krebs-Henseleit 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 radioactivity associated with the specimens was determined in a liquid scintillation counter. The remaining 50-μl aliquots of cell lysate were used to determine the protein concentration by the method of Lowry with bovine serum albumin as a standard.

Kinetic Analyses. Kinetic parameters were obtained using the following Michaelis-Menten equation: one saturable component,

$$v = \frac{V_{max} \times S}{K_m + S} \quad (1)$$

one saturable and one nonsaturable component,

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

and two saturable components,

$$v = \frac{V_{max.1} \times S}{K_{m.1} + S} \times \frac{V_{max.2} \times S}{K_{m.2} \times S} \quad (3)$$

where v is the uptake velocity of the substrate (nanomoles per gram kidney per 15 min or picomoles per milligram of protein per minute), S is the substrate concentration of the medium (micromolar), K_m is the Michaelis constant (micromolar), V_{max} is the maximal uptake velocity (nanomoles per gram kidney per 15 min or picomoles per milligram of protein per minute), and P_{dif} is the nonsaturable uptake clearance. Fitting was performed by the nonlinear least-squares method using the MULTI program (Yamaoka et al., 1981). The input data were weighed as the reciprocals of the observed values, and the Damping Gauss Newton Method algorithm was used for fitting.

Results

Quantification of hOAT1 and hOAT3 mRNA Expression in Human Kidney Cortex. Figure 1A shows the relative mRNA expression levels of hOAT1 and hOAT3 in 42 human kidney cortical tissues. The expression levels of hOAT1 and hOAT3 mRNA, normalized by GAPDH, showed very large deviations: from 0.147 ($\times 10^{-2}$) to 31.1 ($\times 10^{-2}$) for hOAT1 and from 0.051 ($\times 10^{-2}$) to 40.6 ($\times 10^{-2}$) for hOAT3. The average of the relative expression of hOAT1 and hOAT3 was 8.97 ± 1.27 ($\times 10^{-2}$) and 15.0 ± 1.4 ($\times 10^{-2}$), respectively. A linear correlation between the relative expression of hOAT1 and hOAT3 was observed (Fig. 1B, $R^2 = 0.814$, $p < 0.001$).

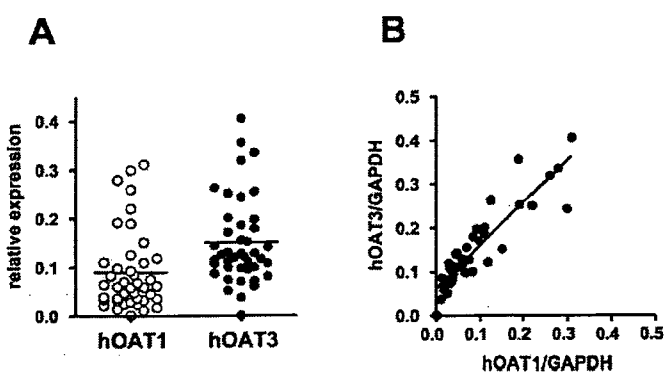

Fig. 1. Expression levels of hOAT1 and hOAT3 mRNA in human renal cortical tissues. A, relative expression levels of hOAT1 and hOAT3 mRNA in human kidney cortical tissues were measured by real-time PCR. Expression levels of hOAT1 and hOAT3 were normalized by that of GAPDH. B, correlation between the relative expression levels of hOAT1 and hOAT3 mRNA in human kidney cortical tissues. Solid line, linear regression line. Each point represents the results of one human kidney subject.

Uptake of [^{3}H]DHEAS by hOAT1- and hOAT3-Transfected HEK293 Cells. The uptake of [^{3}H]DHEAS by hOAT3-transfected cells was significantly greater than that by vector-transfected cells, which was saturable with K_m (micromolar) and V_{max} (picomoles per milligram of protein per minute) values of 12.9 ± 2.0 and 249 ± 29, respectively. The uptake of [^{3}H]DHEAS by hOAT1-transfected cells was similar to that by vector-transfected cells, whereas the uptake of [^{3}H]PAH by hOAT1- and vector-transfected cells was 38.8 ± 1.4 and 1.39 ± 0.17 μl/mg protein/min, respectively.

Interbatch Differences in the Uptake of PAH, 2,4-D, PCG, and DHEAS by Human Kidney Slices. The uptake of typical hOAT1 substrates (PAH and 2,4-D) and hOAT3 substrates (PCG and DHEAS) was examined in human kidney slices prepared from 42 subjects, and the saturable transport activities of PAH, 2,4-D, PCG, and DHEAS in human kidney slices are shown in Fig. 2. There were very large interbatch differences in their transport activities. The saturable uptake of PAH was also compared with that of PCG in each human kidney batch, and a linear correlation between PAH and PCG was observed ($R^2 = 0.715$, $p < 0.001$) (Fig. 3).

Transport Properties of the Uptake of PAH, 2,4-D, PCG, DHEAS, and ES by Human Kidney Slices. The uptake of PAH, 2,4-D, PCG, and DHEAS by human kidney slices was examined in the presence of KG in external buffer. Uptake of PAH was slightly stimulated in the presence of 10 to 30 μM KG (Fig. 4A) followed by an inhibition at greater extracellular KG concentration. KG slightly stimulated DHEAS uptake at 300 to 1000 μM, although the effect depended on the subjects (Fig. 4D). The uptake of 2,4-D and PCG was not stimulated by extracellular KG but rather inhibited at high KG concentrations. The concentration dependence of the uptake of PAH, 2,4-D, PCG, DHEAS, and ES by human kidney slices was examined (Fig. 5). The uptake of PAH, 2,4-D, PCG, and DHEAS was determined using three different human kidney batches and that of ES was determined using two different batches. Nonlinear regression analysis showed that the uptake of PAH, 2,4-D, PCG, and ES by human kidney slices consists of one saturable and one nonsaturable component, whereas that of DHEAS consists of two saturable components. The kinetic parameters are summarized in Table 1.

Effect of PAH and PCG on the Uptake of PAH, 2,4-D, PCG, DHEAS, and ES by Human Kidney Slices. The inhibitory effect of unlabeled PAH and PCG on the uptake of [^{3}H]PAH, [^{3}H]2,4-D, [^{14}C]PCG, and [^{3}H]DHEAS by human kidney slices was examined (Fig. 6). The uptake of [^{3}H]PAH and [^{3}H]2,4-D was more potently inhibited by unlabeled PAH than PCG (Fig. 6, A and B) and vice versa for the uptake of [^{14}C]PCG (Fig. 6, C and D). Although PCG was also a more potent inhibitor than PAH for the uptake of [^{3}H]DHEAS, the

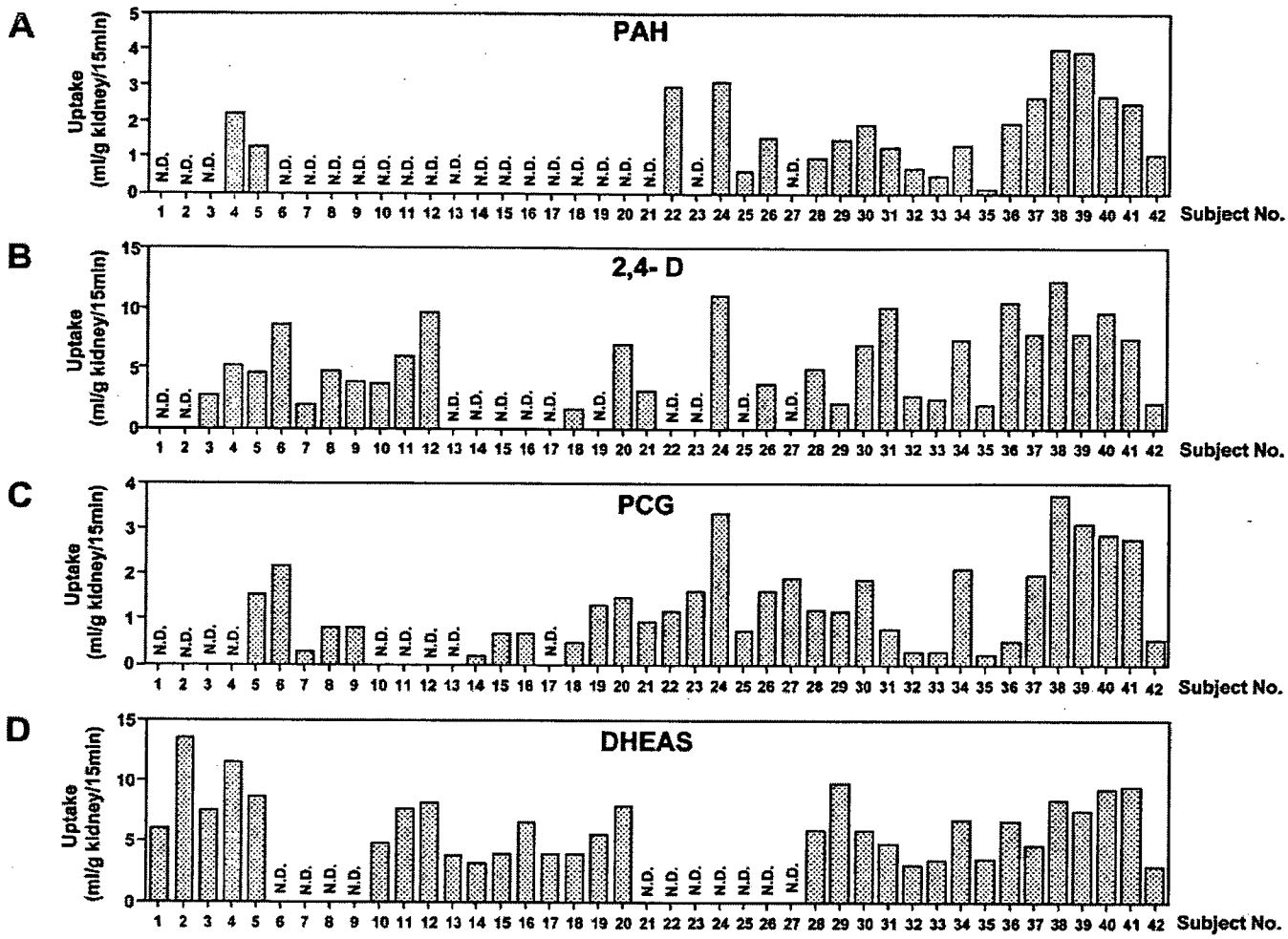

Fig. 2. Interbatch variability in the uptake of PAH, 2,4-D, PCG, and DHEAS by human kidney slices. Human kidney slices were prepared from intact renal cortical tissues donated from 42 nephrectomized patients with renal cell carcinoma, and the uptake of 0.1 μM [^{3}H]PAH (A), 0.1 μM [^{3}H]2,4-D (B), 1 μM [^{14}C]PCG (C), and 0.1 μM [^{3}H]DHEAS (D) was measured for 15 min at 37°C. The *y*-axis represents the saturable uptake clearance (milliliters per gram kidney per 15 min), which was obtained by subtracting the uptake clearance in the presence of excess amount of nonlabeled compounds (1 mM) from that under tracer conditions. All the data represent the mean (n = 2 slices). N.D., not determined due to a lack of sufficient tissue.

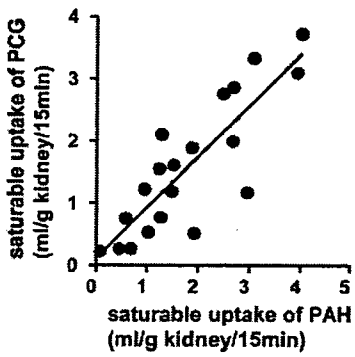

Fig. 3. Correlation of saturable uptake of PAH and PCG by human kidney slices The uptake of [^{3}H]PAH (0.1 μM) and [^{14}C]PCG (1 μM) was measured for 15 min at 37°C. The saturable uptake clearance (milliliters per gram kidney per 15 min) was obtained by subtracting the uptake clearance in the presence of 1000 μM substrate concentrations from that under tracer conditions. This plot was taken from the results of subjects 5, 22, 24 to 26, and 28 to 42 (see Fig. 2). Each point represents the results of one human kidney subject.

inhibition curves of [^{3}H]DHEAS uptake by PCG and PAH were shifted to the right in comparison with that of PCG (Fig. 6, C and D).

Discussion

Human-derived materials, such as cryopreserved hepatocytes, canalicular membrane vesicles, and microsomes, have been widely used in drug development to predict the pharmacokinetic properties of potential drug candidates (metabolism and membrane transport), and they also serve as essential tools to evaluate drug-drug interactions in addition to in vitro-in vivo scaling. Kidney slices from experimental animals have been widely used to characterize the renal uptake of organic anions through the basolateral membrane. Fleck et al. (2000) demonstrated that human kidney slices retain organic anion transport activity. The present study focused on the use of human kidney slices to characterize the uptake of hOAT1 and hOAT3 substrates. For that purpose, PAH, 2,4-D, PCG, DHEAS, and ES were selected for probing hOAT1 and hOAT3 activities. PAH and 2,4-D are common substrates of hOAT1 and hOAT3, but the transport activities by hOAT1 were markedly greater than those by hOAT3 (Tahara et al., 2005b), and PCG, DHEAS, and ES are specific substrates of hOAT3 (Tahara et al., 2005b; this study).

The mRNA expression of hOAT3 in human kidney slices was 1.7-fold higher than that of hOAT1 on average, which is

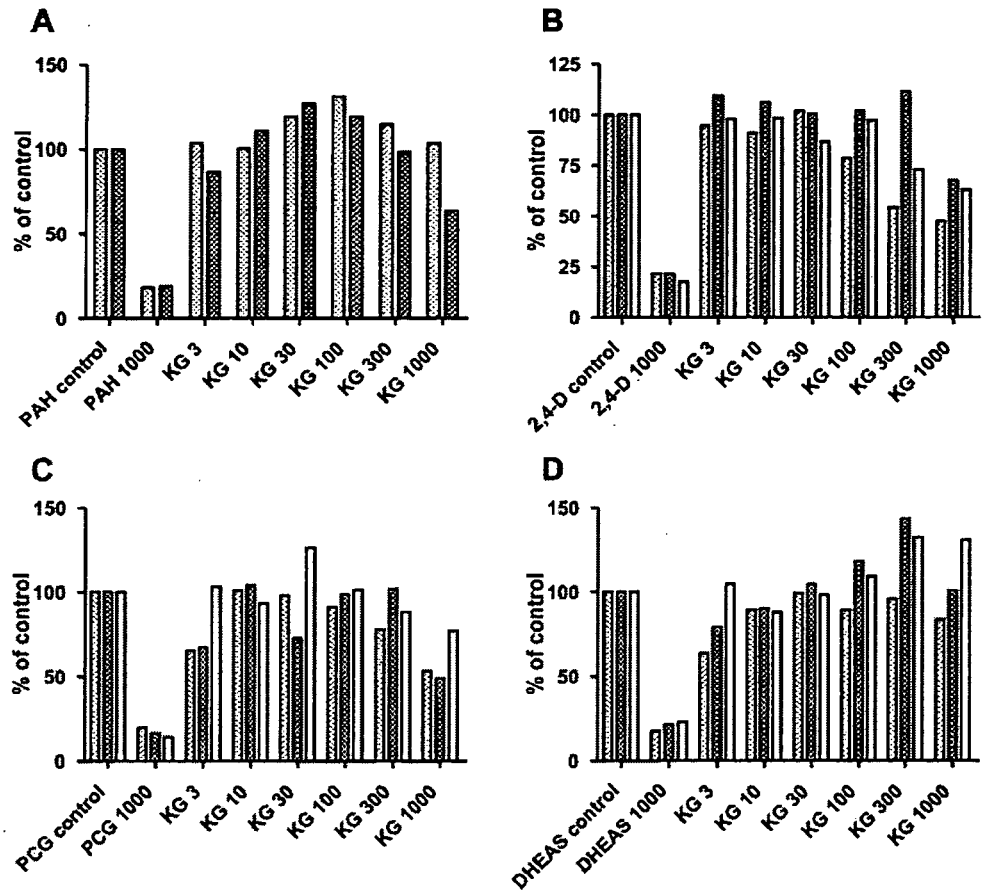

Fig. 4. Effect of α-ketoglutarate on the uptake of PAH, 2,4-D, PCG, and DHEAS by human kidney slices. The uptake of 0.1 μM [^{3}H]PAH (A), 0.1 μM [^{3}H]2,4-D (B), 1 μM [^{14}C]PCG (C), and 0.1 μM [^{3}H]DHEAS (D) was measured for 15 min at 37°C in the presence or absence of α-ketoglutarate (0–1 mM). All experiments were repeated in two or three subjects (two slices per subject), and the uptake of PAH was determined in subjects 22 and 24, that of 2,4-D in subjects 6, 11, and 12, that of PCG in subjects 6, 20, and 24, and that of DHEAS in subjects 6, 16, and 20. Each column represents an individual subject. The values are shown as a percentage of the uptake in the absence of any unlabeled compounds (n = 2 slices).

consistent with the previous determination (Motohashi et al., 2002). There was a very large interbatch difference in the mRNA expression of hOAT1 and hOAT3 (Fig. 1A) and the uptake of test compounds in human kidney slices (Fig. 2). However, the mRNA expression of hOAT1 and hOAT3 was closely correlated (Fig. 1B), and the saturable uptake of PAH and PCG by human kidney slices also correlated well (Fig. 3). The interbatch difference is probably due to the large difference in the expression of OAT1 and OAT3, but the close correlations suggests that the functional contribution of hOAT1 and hOAT3 to the net uptake is maintained irrespective of the batch of slices used (Figs. 2 and 3). Because the normal part of the kidney was obtained from surgically nephrectomized patients with renal cell carcinoma for preparation of slices, it is possible that renal cell carcinoma and chemotherapy indirectly affect the expression of OATs. Indeed, Fleck et al. (1997) reported that the uptake of PAH by slices from intact kidney was influenced by the tumor stage and size, although kidney slices were prepared from intact cortical tissues (Fleck et al., 1997). Furthermore, during the process of nephrectomy, kidney tissue is kept under warm ischemic conditions. Considering that ischemic reperfusion injury affects the mRNA and/or protein expression levels of ATP-binding cassette transporters in the kidney (Huls et al., 2006), it is also possible that the warm ischemic conditions during nephrectomy affect the expression of hOAT1 and/or hOAT3.

The basolateral uptake of organic anions in renal proximal tubule cells is indirectly coupled to the Na^+ gradient through Na^+-dicarboxylate cotransport and organic anion/dicarboxylate exchange (Bakhiya et al., 2003; Sweet et al., 2003). In the kidney slices used in this study, the effect of extracellular KG was compound-dependent (Fig. 4), and KG only slightly stimulated the uptake of PAH and DHEAS (Fig. 4). There are two possibilities to account for this discrepancy. One is the difference in the incubation time. In the present study, the incubation time was shorter than in the previous study to characterize the initial uptake process (15 versus 60 min) (Sweet et al., 2003). It is also possible that the kidney slice may retain sufficient activity to allow the intracellular concentration of KG to drive OAT1 and OAT3. However, further studies are required to draw more definite conclusions.

Nonlinear regression analyses suggest that the uptake of test compounds, except DHEAS, by human kidney slices consists of one saturable and one nonsaturable component, whereas that of DHEAS consists of two saturable components, and the high-affinity component accounts for the major part (56–76%) (Fig. 5). The kinetic parameters for the uptake of organic anions by human kidney slices are summarized in Table 1. The K_m value of PAH determined in human kidney slices was similar to the K_m of hOAT1 (20 μM; Tahara et al., 2005b) and K_i of hOAT3 (100 μM; Deguchi et al., 2004), whereas the K_m values of 2,4-D and those of PCG and ES were similar to that of hOAT1 (5.8 μM; Tahara et al., 2005b) and hOAT3 (52 and 9.5 μM; Tahara et al., 2005b), respectively. For DHEAS, the K_m value for hOAT3 (13 μM, this study) is close to that of the high-affinity component rather than the low-affinity component.

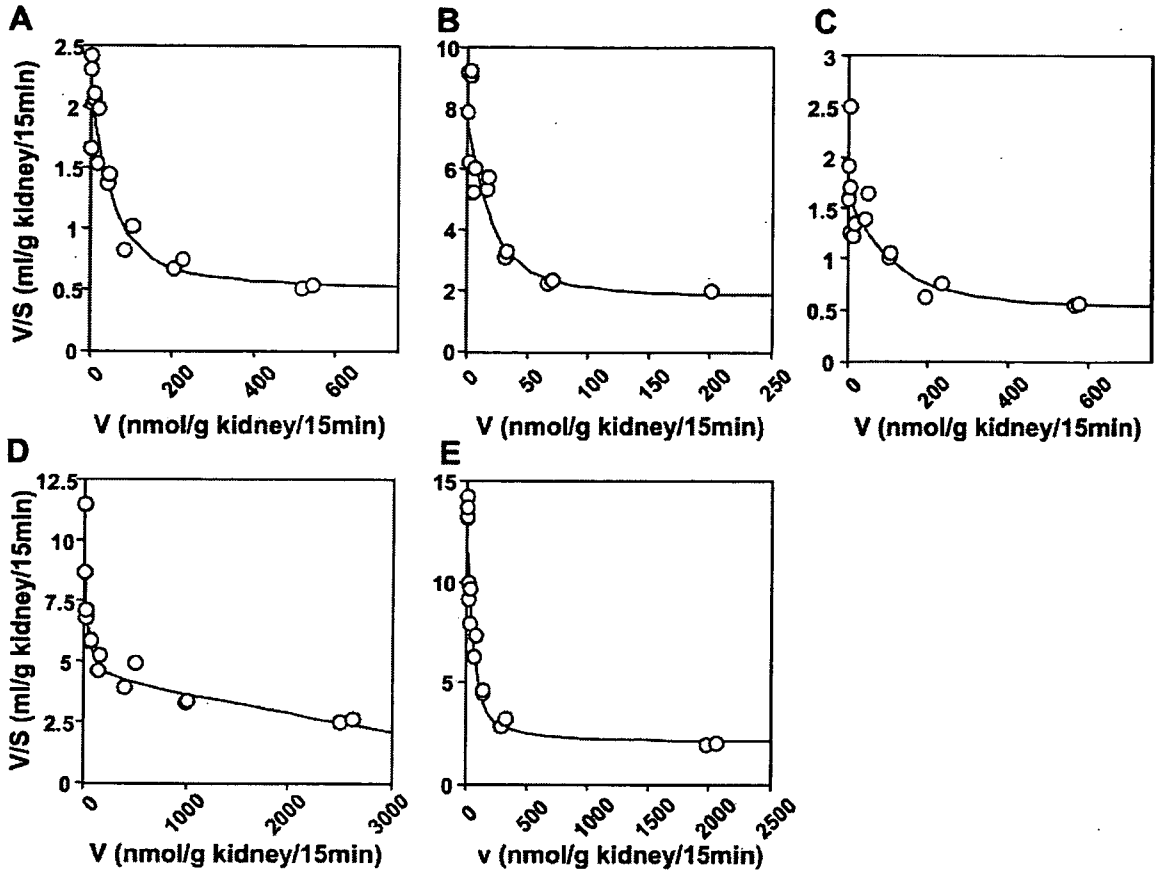

Fig. 5. Concentration dependence of the uptake of PAH, 2,4-D, PCG, DHEAS, and ES by human kidney slices. The concentration dependence of the uptake of [^{3}H]PAH (A), [^{3}H]2,4-D (B), [^{14}C]PCG (C), [^{3}H]DHEAS (D), and [^{3}H]ES (E) is shown as an Eadie-Hofstee plot. The uptake was measured for 15 min at 37°C. The concentration dependence of PAH, 2,4-D, PCG, and DHEAS was determined using three different human kidney batches and that of ES was determined using two different batches. A to E, data for human kidney slices prepared from subjects 30, 20, 20, 3, and 37, respectively. Each point represents the result from one slice. Solid lines represent the fitted lines obtained by nonlinear regression analysis.

TABLE 1

Kinetic parameters of the uptake of organic anions by human kidney slices

Kinetic parameters were determined by nonlinear regression analysis using data shown in Fig. 5. The parameters within parentheses represent that of low-affinity components. Each value represents the mean ± S.D.

Substrate	Human Kidney Slices			
	Subject Nos.	K_m	V_{max}	P_{dif}
		μM	*nmol/g kidney/15 min*	
PAH	30	39.5 ± 1.0	62.9 ± 15.4	0.438 ± 0.048
	31	31.1 ± 18.9	25.4 ± 14.9	0.412 ± 0.060
	36	47.8 ± 26.4	50.1 ± 27.5	0.436 ± 0.079
2,4-D	20	3.94 ± 1.04	23.0 ± 5.5	1.65 ± 0.13
	24	0.727 ± 0.449	7.03 ± 2.91	1.41 ± 0.23
	30	4.85 ± 1.39	25.6 ± 6.2	1.09 ± 0.11
PCG	20	89.8 ± 44.0	103 ± 53	0.461 ± 0.096
	24	42.9 ± 10.0	126 ± 27	0.439 ± 0.064
	30	13.9 ± 4.6	22.9 ± 6.4	0.455 ± 0.041
DHEAS	3	3.57 ± 1.67 (1340 ± 260	19.2 ± 8.9 (5750 ± 760)	
	4	2.19 ± 0.91 (1270 ± 580)	20.9 ± 8.2 (3810 ± 1240)	
	5	3.91 ± 1.38 (1260 ± 490)	26.6 ± 10.3 (4800) ± 1340)	
ES	37	9.18 ± 2.01	91.4 ± 17.4	2.01 ± 0.17
	39	10.7 ± 2.2	148 ± 26	2.31 ± 0.21

To estimate the contribution of OAT1 and OAT3, an inhibition study was carried out. Hasegawa et al. (2002) previously demonstrated different inhibition potencies of PAH and PCG for rOat1- and rOat3-mediated uptake. According to the published data, the K_m of PAH for hOAT1 is only 5-fold different from the K_i value for hOAT3 (20 versus 100 μM), whereas PCG exhibits a 30-fold difference (52 versus 1700 μM) (Deguchi et al., 2004; Tahara et al., 2005b). As shown in Fig. 6, PAH and PCG showed different potencies for the uptake of PAH and 2,4-D and that of PCG and DHEAS in

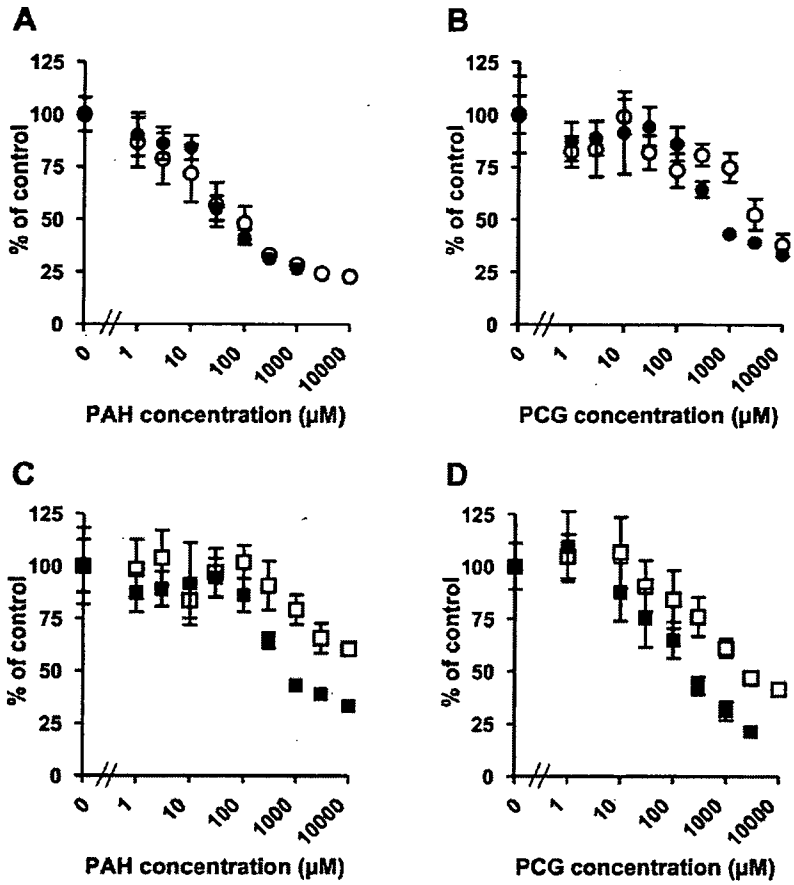

Fig. 6. Inhibitory effect of PAH and PCG on the uptake of [^{3}H]PAH, [^{3}H]2,4-D, [^{14}C]PCG, and [^{3}H]DHEAS by human kidney slices. The uptake of [^{3}H]PAH (0.1 μM, closed circles), [^{3}H]2,4-D (0.1 μM, open circles), [^{14}C]PCG (1 μM, closed squares), and [^{3}H]DHEAS (0.1 μM, open squares) was determined in the presence and absence of unlabeled PAH (A and C) and PCG (B and D) for 15 min at 37°C. The values are shown as a percentage of the uptake in the absence of any unlabeled compounds. All experiments were repeated in three subjects (two slices per subject), and each point represents the mean ± S.E. (n = 6).

human kidney slices. PAH inhibited 2,4-D uptake with an IC_{50} value similar to its own K_m value, whereas it inhibited the uptake of PCG and DHEAS, but with lower potencies. On the contrary, PCG had only a weak effect on the uptake of PAH and 2,4-D by the kidney slices, and, particularly, at a concentration similar to its K_m value, it had no effect. These mutual inhibition studies kinetically suggest that PAH shares the same transporter (hOAT1) with 2,4-D, but not with PCG. PAH and 2,4-D are also substrates of hOAT3 with low transport activities (Tahara et al., 2005b), and its contribution to PAH and 2,4-D uptake by human kidney slices is probably smaller than hOAT1. As in the case of PCG uptake, PCG more potently inhibited the uptake of DHEAS than PAH (Fig. 6, C and D). However, the inhibition curves shifted to the right in comparison with that of PCG uptake (Fig. 6D). Because the uptake DHEAS consists of two saturable components, PCG and PAH may have different inhibition potencies to these two components.

Taken together, it is likely that the uptake of PAH and 2,4-D in human kidney slices is due to hOAT1 and that of PCG and ES, and part of DHEAS uptake is due to hOAT3. There was a 6.3-fold difference in the intrinsic transport activities (V_{max}/K_m) of PAH and 2,4-D in hOAT1-expressing HEK293 cells (Tahara et al., 2005b), which was close to the difference observed in the human kidney (6-fold) (Table 1). This holds true for PCG, ES, and DHEAS. Assuming that the high-affinity component of DHEAS uptake is solely explained by hOAT3, the intrinsic transport activities of ES and DHEAS were 5.2- and 7.5-fold greater than that of PCG in human kidney, and the corresponding values were 4.7 and 7.7 in hOAT3-expressing HEK293 cells, respectively. The transport activities relative to PAH and PCG transport are probably preserved between human kidney slices and the cDNA transfectants, which allows the application of relative activity factor method in which the transport activities of test compounds in the kidney/liver were predicted from the intrinsic parameter of test compound in cDNA transfectants multiplied by the scaling factor obtained using reference compounds (Hasegawa et al., 2003; Hirano et al., 2004).

The present study shows that human kidney slices maintain the transport activities due to hOAT1 and hOAT3 and thus enable us to determine the contribution of hOAT1 and hOAT3 ex vivo. This experimental system will help in the characterization of the renal uptake of novel drugs and the quantitative evaluation of likelihood of drug-drug interactions caused by inhibition of the renal uptake transporters. However, the possible impact of disease state and patient drug treatments on OAT function in the available source tissues is unknown, and caution must be used when extrapolating such data to quantitative evaluation of the normal human response.

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Address correspondence to: Dr. Hiroyuki Kusuhara, Department of Molecular Pharmacokinetics, Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyoku-Tokyo 13-0033, Japan. E-mail: kusuhara@mol.f.u-tokyo.ac.jp

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

Yoshitane Nozaki, Hiroyuki Kusuhara, Tsunenori Kondo, Masahiro Iwaki, Yoshiyuki Shiroyanagi, Hideki Nakayama, Shigeru Horita, Hayakazu Nakazawa, Teruo Okano, and Yuichi Sugiyama

Department of Molecular Pharmacokinetics, Graduate School of Pharmaceutical Sciences, University of Tokyo, Tokyo, Japan (Y.N., H.K., Y.Su.); Department of Urology, Kidney Center, Tokyo Women's Medical University, Tokyo, Japan (T.K., Y.Sh., H.N., S.H.); Division of Biopharmaceutical Sciences, Faculty of Pharmaceutical Sciences, Kinki University, Osaka, Japan (M.I.); Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, Tokyo, Japan (Y.Sh., T.O.); and Department of Urology, Tokyo Women's Medical University, Medical Center, East, Tokyo, Japan (H.N.)

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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.

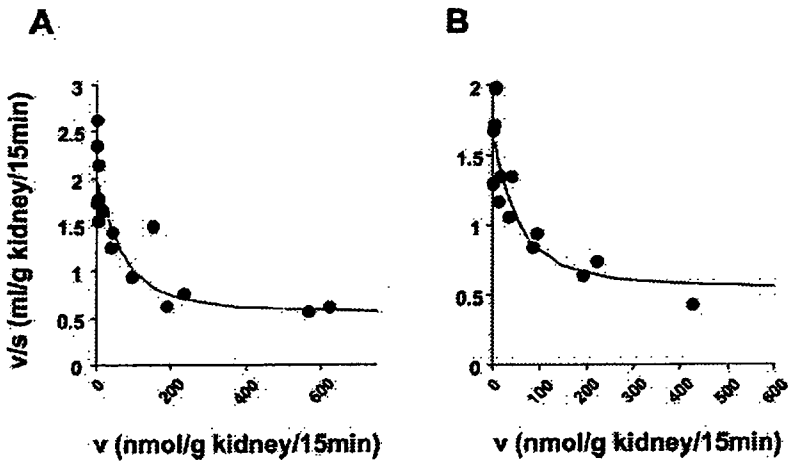

Fig. 1. Concentration dependence of the uptake of MTX by human kidney slices. The concentration dependence of the uptake of MTX is shown as an Eadie-Hofstee plot. The uptake of MTX was measured at concentrations between 0.1 and 10,000 μM for 15 min at 37°C. A and B, data for human kidney slices prepared 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

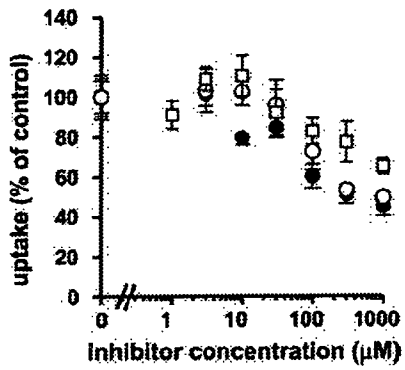

Fig. 2. Inhibitory effect of PAH, PCG, and 5-MTHF on the uptake of MTX by human kidney slices. The uptake of [^{3}H]MTX (0.1 μM) was determined in the presence and absence of unlabeled PAH (open circles), PCG (closed circles), and 5-MTHF (open squares) for 15 min at 37°C. The values are shown as a percentage 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).

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.

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

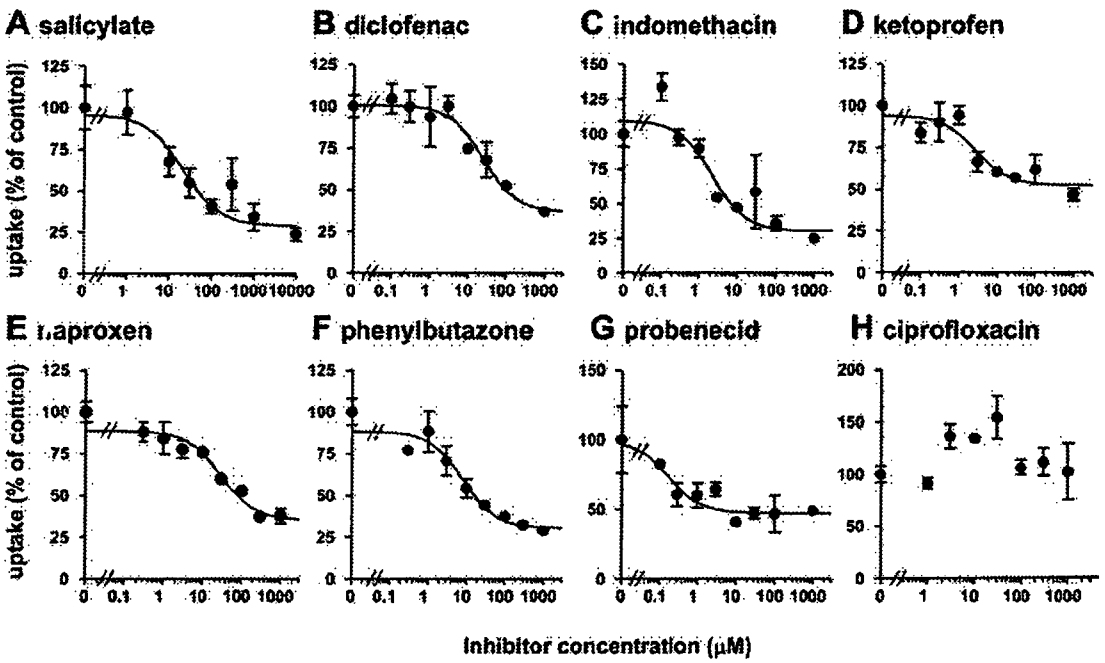

Fig. 3. Inhibitory effect of NSAIDs and other drugs on the uptake of MTX by human kidney slices. The uptake of MTX (0.1 μM) was determined in the presence and absence of unlabeled salicylate (A), diclofenac (B), indomethacin (C), ketoprofen (D), naproxen (E), phenylbutazone (F), probenecid (G), and ciprofloxacin (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[c]	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

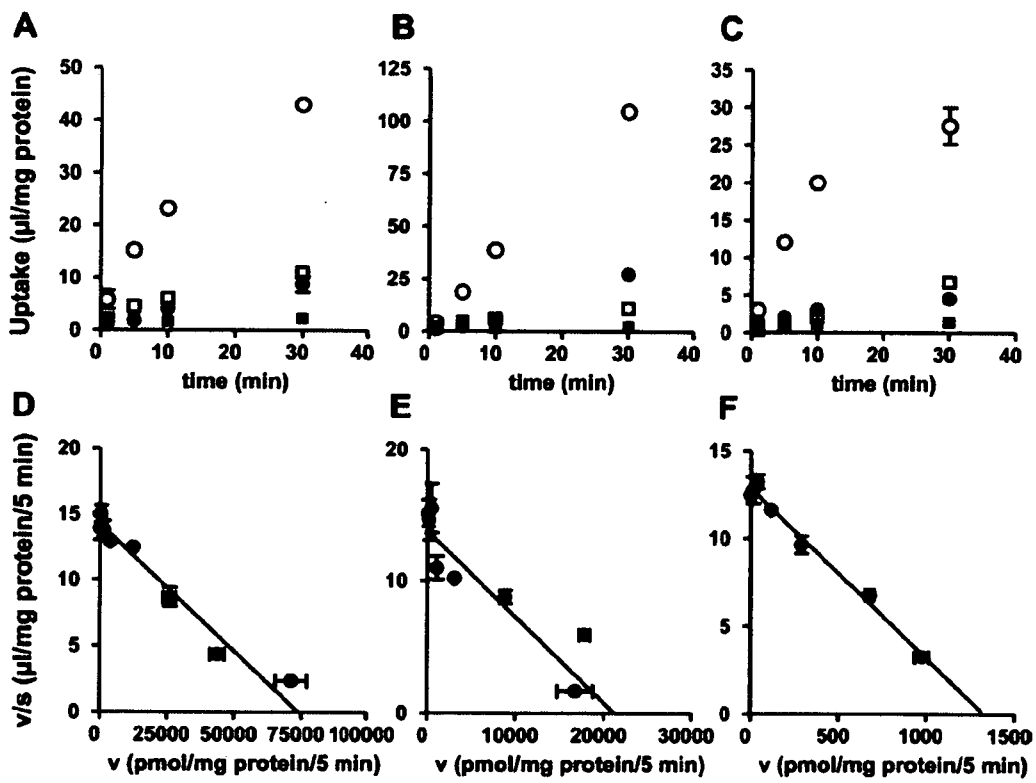

Fig. 4. The uptake of MTX by BCRP-, MRP2-, and MRP4-expressing vesicles. Time profiles of ATP-dependent uptake of MTX (A–C). Membrane vesicles (5 μg) prepared from HEK293 cells infected with BCRP (A), MRP2 (B), and MRP4 (C) adenoviruses (circles) or GFP adenoviruses (squares) were incubated at 37°C in 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).