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Species difference in the inhibitory effect of NSAIDs on the uptake of methotrexate by human kidney slices

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Running title: inhibition of renal MTX uptake by NSAIDs

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Abbreviations:

MTX, methotrexate; NSAIDs, nonsteroidal anti-inflammatory drugs; RFC-1, reduced folate carrier; Oat/OAT, organic anion transporter; PCG, benzylpenicillin; PAH, *p*-aminohippurate; DHEAS, dehydroepiandrosterone sulfate; 5-MTHF, 5-methyltetrahydrofolate; BBM, brush border membrane; MRP, multidrug resistance-associated protein; BCRP, breast cancer resistance protein

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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 ABC transporters. The uptake of methotrexate by human kidney slices was saturable with a K_m of 45 – 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 OAT3/*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*, MRP2/*ABCC2* and MRP4/*ABCC4*, which are candidates for the luminal efflux of methotrexate, ATP-dependent uptake of MTX 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, while 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.

Introduction

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 analogue of natural folate and has been widely and successfully used for the treatment of neoplastic diseases and autoimmune diseases, including rheumatoid 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 2 (MRP2), MRP4, breast cancer resistance protein (BCRP), OAT4, URAT1, and NPT1 (for review (Russel et al., 2002)). Hulot et al identified a heterozygous

mutation, which results in a loss of function of MRP2, in a patient who exhibited delayed MTX elimination (Hulot et al., 2005). 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, while 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 AUC, 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 (Imaoka et al., 2004; Hasegawa 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.

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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 Perkin-Elmer 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 Bio-Sciences (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 5 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, and 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). Since 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 ºC 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 0.2 N NaOH. The aliquots neutralized with 2 N HCl were transferred to scintillation vials containing 2 ml scintillation cocktail (Clearsol I; Nacalai Tesque Inc, Kyoto, Japan) and the radioactivities associated with the specimens were determined in a liquid scintillation counter. 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 (Imaoka et al., 2004; Hasegawa et al., 2007). Briefly, 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 48h after infection, and membrane vesicles were isolated by the hypotonic method (Imaoka et al., 2004; Hasegawa et al., 2007). Cells were diluted 40-fold with hypotonic buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH7.4, at 4 ºC), and stirred gently for 1 h on ice in the presence of 2mM 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 ºC and the resulting pellet was suspended in 10 ml isotonic TS buffer (10 mM Tris-HCl, 250 mM sucrose pH7.4 at 4 ºC) 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, pH7.4, at 4 ºC, and centrifuged in a Beckman SW41 rotor centrifuge at 280,000 g for 45 min at 4 ºC. 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 ºC. The resulting pellet was suspended in 400 ml 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. Briefly, 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 protein/5 µl), incubated at 37 ºC for designated times, and the transport reaction was stopped by the addition of 1 ml ice-cold stop solution (10 mM Tris, 250 mM sucrose and 0.1 M $NaCl_2$, pH 7.4). Then, 900 µl of the stopped reaction mixture was passed through a 0.45 µm HA filter (Millipore Corp., Bedford, MA), and the filter was washed twice with 5 ml 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. Briefly, a mixture containing 10 mg microsomal protein/ml, 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 addition of five 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 minimum volume of acetonitrile-50 mM acetic acid (10:90, v/v). The glucuronides in this solution were purified by liquid chromatography (Cosmosil 75C_{18}-PREP (Nacalai Tesque, Kyoto, Japan), 30 cm x 1.5 cm i.d.) using a stepwise gradient (acetonitrile-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 HPLC 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 :

One saturable component,

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

one saturable and one nonsaturable component,

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

where v is the uptake velocity of the substrate (nmol/g kidney/15min or pmol/mg protein/min), S is the substrate concentration of medium (μM), K_m is the Michaelis constant (μM), V_{max} is the maximum uptake velocity (nmol/g kidney/15min or pmol/mg protein/min), and P_{dif} is the nonsaturable uptake clearance (ml/g kidney/15min).

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

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

where CL represents the uptake clearance and $CL_{+inhibitor}$ represents the uptake clearance in the presence of inhibitor. I represents the concentration of inhibitor (μM). 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 $P < 0.05$.

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Results

The uptake of typical hOAT1 and hOAT3 substrates by human kidney slices.

The saturable uptake clearance (ml/g kidney/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 (Figs. 1A and 1B). The uptake of MTX by two batches of human kidney slices consists of one saturable and one non-saturable 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/15min, respectively (mean ± S.D.).

Fig. 2 describes the inhibitory effect of PAH, PCG and 5-methyltetrahydrofolate (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), while the inhibitory effect of 5-MTHF was weak (65.0 ± 4.0 % of control at 1 mM) (Fig. 2).

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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-, 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 (Figs. 4A, 4B and 4C, respectively). The concentration-dependence of BCRP-, MRP2- and MRP4-mediated transport of MTX was examined (Figs. 4D, 4E and 4F, respectively), and their K_m values were 5,210 ± 500, 1,540 ± 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/5 min, respectively.

Inhibitory effect of NSAIDs and other drugs on ATP-dependent transport of MTX via BCRP, MRP2 and MRP4.

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We examined the inhibitory effect of NSAIDs and other drugs on the ATP-dependent transport of MTX via BCRP, MRP2 and MRP4 (Figs. 5A, 5B and 5C, 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 (Figs. 5D, 5E and 5F, respectively). Diclofenac glucuronide significantly inhibited MRP2-mediated transport of MTX in a concentration-dependent manner, while 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 ABC 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 non-saturable component, while the uptake in rat kidney slices consisted of three components (two saturable components and one non-saturable 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 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, while almost fifty percent 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 non-inhibitable fraction for ketoprofen and probenecid, while 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. Since 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 kidney slices, respectively) (Nozaki et al. 2004 and 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 values of salicylate and diclofenac determined in human kidney slices were smaller than those 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, 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). Since 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

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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 (Figs. 4A, B and 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 effect of NSAIDs, probenecid and PCG on the BCRP-, MRP2- and MRP4-mediated transport of MTX was examined (Figs. 5A, 5B and 5C, 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, 1997b; Davies and Anderson, 1997a), 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 (figure 5). Addition of experimental points at higher concentrations gave a Ki 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. Since 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 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 ABC transporters (MRP2 and MRP4) by glucuronide conjugates of NSAIDs.

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Footnotes

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Legends for Figures

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 – 10000 μM for 15 min at 37 ºC. Figs. 1A and 1B represent the 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.

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 ºC. 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).

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 ºC. 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).

Fig. 4. The uptake of MTX by BCRP-, MRP2- and MRP4-expressing vesicles. Time-profiles of ATP-dependent uptake of MTX (A, B and 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 ºC 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, E and 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 ºC. 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).

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 ºC in the presence or absence of inhibitors (A, B and 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, E and F, respectively). Each value represents the mean ± S.E. (n = 3). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ statistically different from control.

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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.

	clinical concentration		K_i (μM)	R value
inhibitors	total (μM)	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[a]	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)

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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. N.D., not determined.

	K_i (μM)	
inhibitors	hOAT1	hOAT3
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

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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.

	clinical concentration		K_i (μM)	R value
inhibitors	total (μM)	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	48.5[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)

Fig. 1

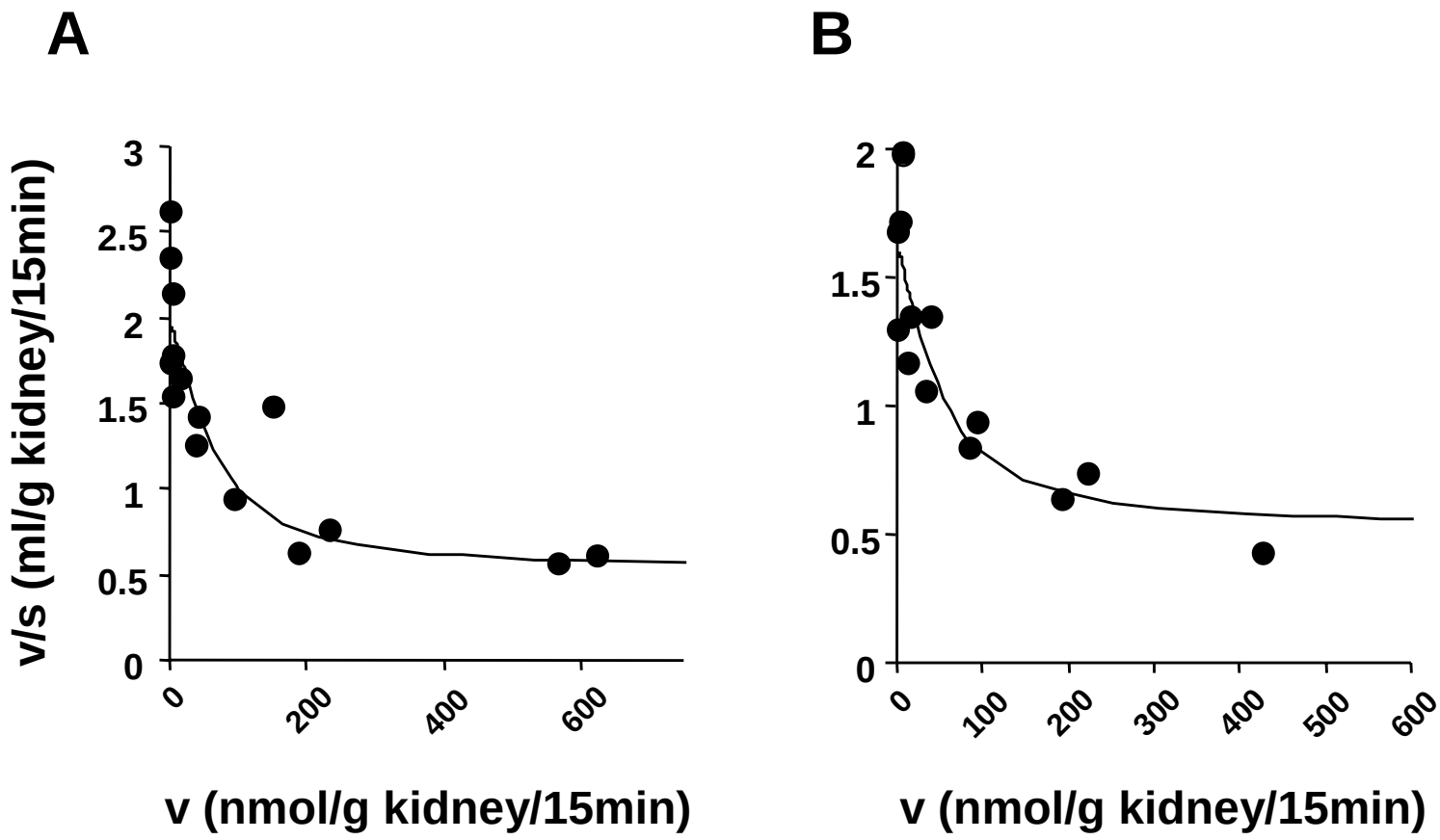

Fig. 2

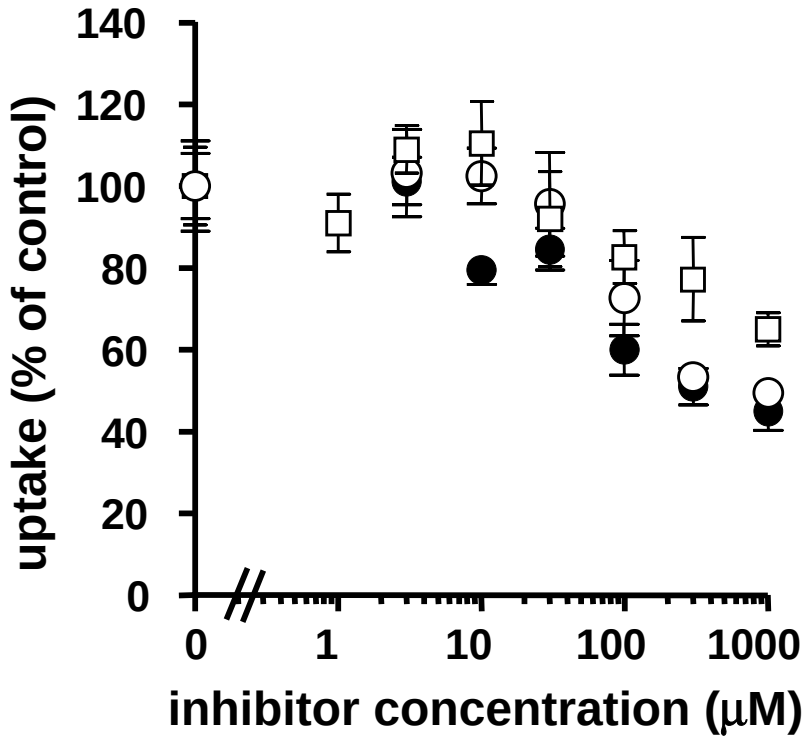

Fig. 3

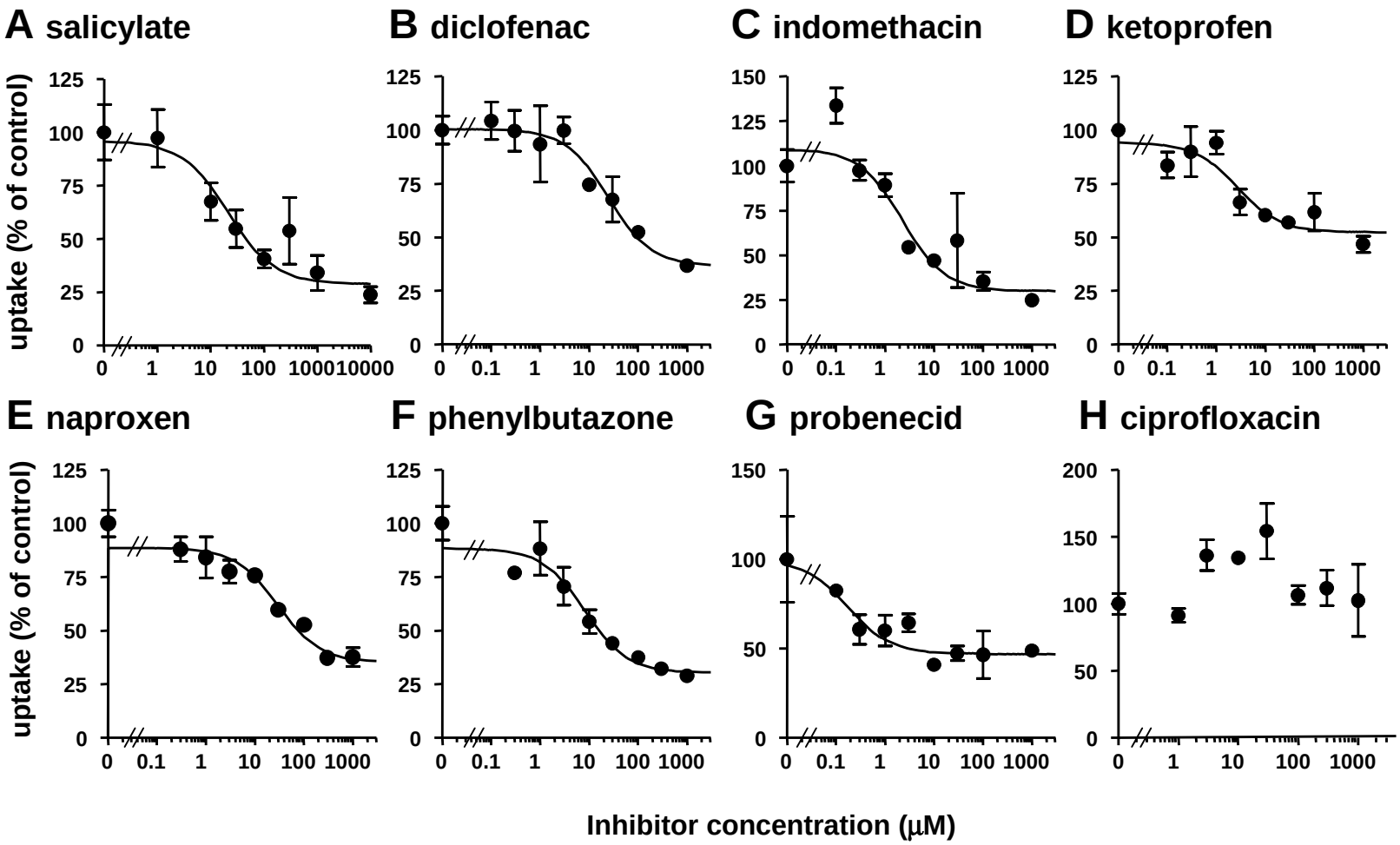

Fig. 4

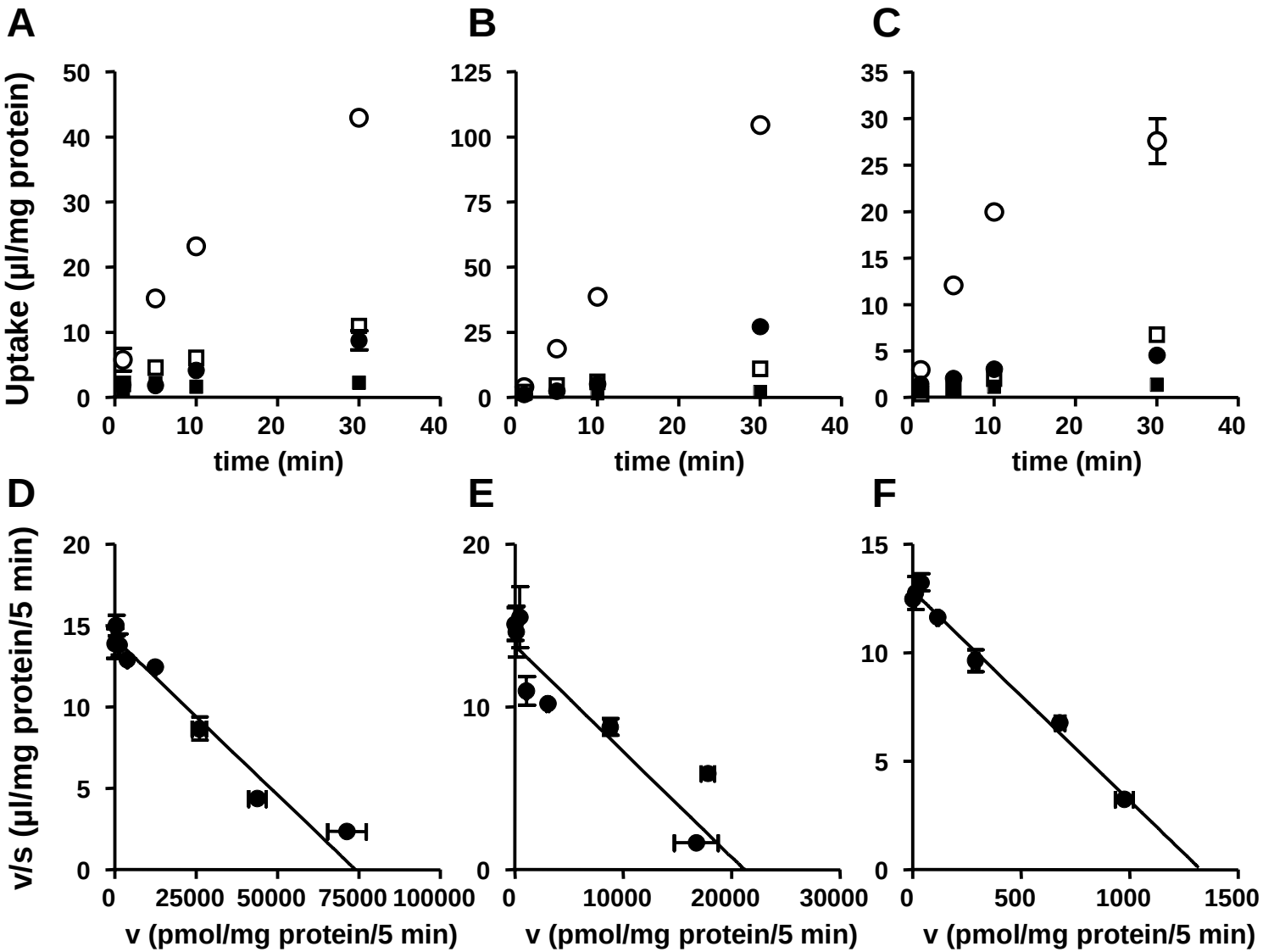

Fig. 5

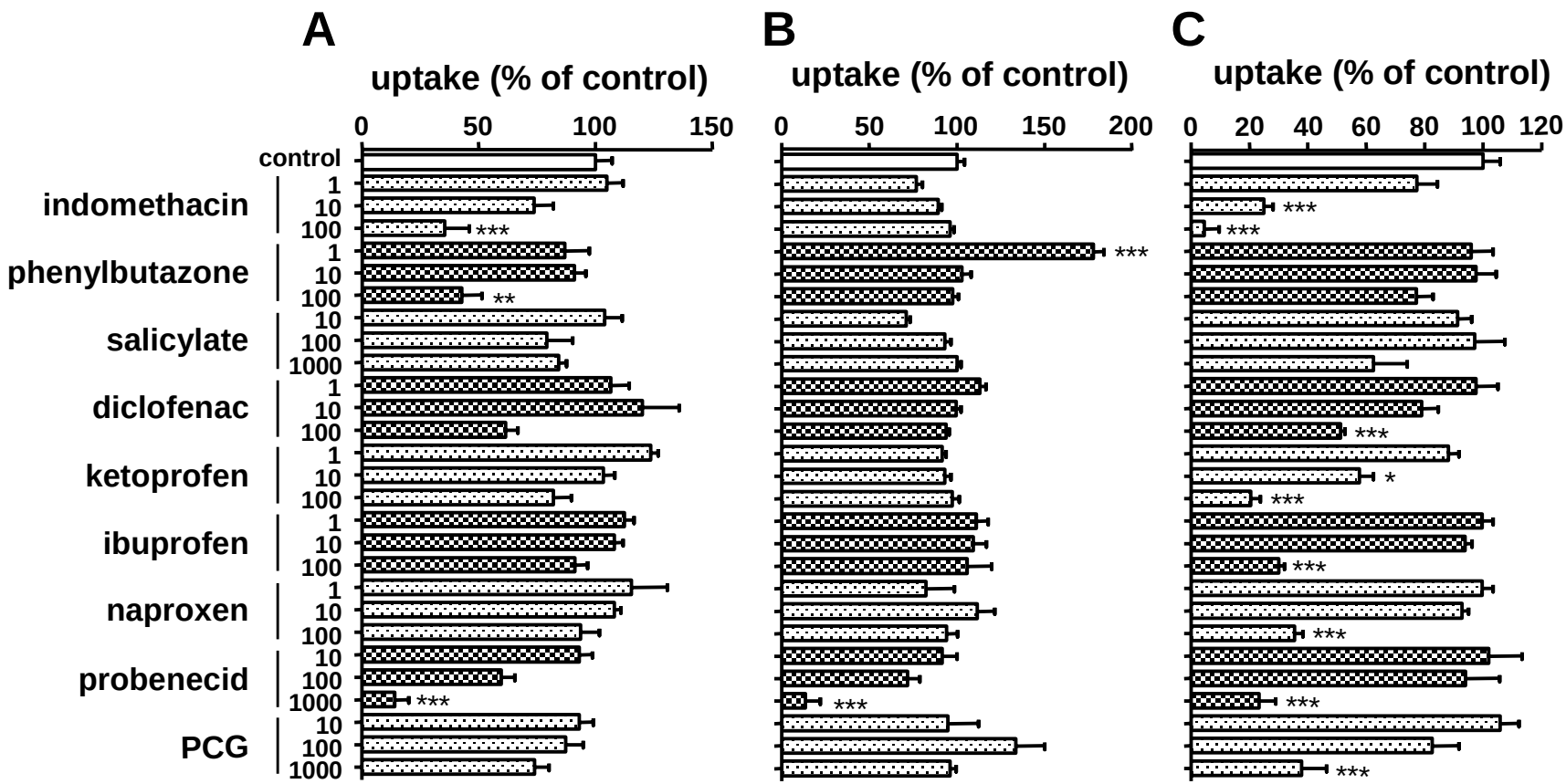

Fig. 5 (continued)