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Functional involvement of rat organic anion transporter 2 (*Slc22a7*) in the hepatic uptake of

the non-steroidal anti-inflammatory drug, ketoprofen

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Running title : role of Oat2 in the liver

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Abbreviations : AIC, Akaike's information criterion; cAMP, cyclic adenosine monophosphate; FBS,

fetal bovine serum; ICG, indocyanine green; NSAIDs; non-steroidal anti-inflammatory drugs; OAT,

organic anion transporter; OATP, organic anion transporting polypeptides; PAH, p-aminohippurate;

SDS, sodium dodecyl sulfate.

Abstract

Rat organic anion transporter 2 (rOat2, *Slc22a7*) is a sinusoidal multispecific organic anion transporter in the liver.   The role of rOat2 in the hepatic uptake of drugs has not been thoroughly investigated yet.   rOat2 substrates include non-steroidal anti-inflammatory drugs, such as ketoprofen, indomethacin and salicylate.   In the present study, the uptake of ketoprofen, indomethacin and salicylate by freshly isolated rat hepatocytes was characterized.   The uptake of ketoprofen, indomethacin and salicylate by hepatocytes was sodium-independent, and the rank order of their uptake activities was indomethacin > ketoprofen > salicylate.   Kinetic analysis based on Akaike's Information Criterion suggested that the uptake of ketoprofen and indomethacin by hepatocytes consists of two saturable components and one non-saturable one.   The K_m and V_{max} values for the high and low affinity components for ketoprofen uptake were 0.84 and 97 μM, and 35 and 1800 pmol/min/mg protein, respectively, while those for indomethacin were 1.1 and 140 μM, and 130 and 16000 pmol/min/mg protein, respectively.   The K_m values of the high affinity component were similar to those for rOat2 (3.3 and 0.37 μM for ketoprofen and indomethacin, respectively).   The uptake of ketoprofen by hepatocytes was significantly inhibited by probenecid and rOat2 inhibitors (indocyanine green, indomethacin, glibenclamide and salicylate).   Other inhibitors of rOatps (taurocholate and pravastatin) and rOat3 (pravastatin and *p*-aminohippurate) had a slight effect, but digoxin had no effect.   These results suggest that rOat2 accounts partly for the hepatic uptake of ketoprofen and, presumably, indomethacin as a high affinity site, and that other transporters, such as rOatps, but not rOatp2, and rOat3, are also involved.

The liver plays an important role in the detoxification/inactivation of xenobiotics including drugs and endogenous compounds in the body. The hepatic uptake mechanisms of organic anions have been extensively investigated *in vivo* and *in vitro*, and shown to consist of sodium-dependent and -independent uptake mechanisms (Faber et al., 2003; van Montfoort et al., 2003). The sodium-dependent uptake of bile acids in the liver is accounted for by sodium-taurocholate co-transporting polypeptide (Kullak-Ublick et al., 2000; Faber et al., 2003). Although the sodium-dependent uptake of cholate and bumetanide by hepatocytes has been suggested to be mediated by a transporter distinct from sodium-taurocholate co-transporting polypeptide (Honscha et al., 1993; Yamazaki et al., 1993), its molecular characteristics remains unknown. As far as the sodium-independent system is concerned, several isoforms which belong to the organic anion transporting polypeptide (Oatp/OATP, *SLC21A*/*SLCO*) family have been identified on the sinusoidal membrane of rat liver (Faber et al., 2003; Hagenbuch and Meier, 2003; van Montfoort et al., 2003). It is generally accepted that rOatps, such as rOatp1 (*Oatp1a1*), rOatp2 (*Oatp1a4*) and rOatp4 (*Oatp1b2*), play major roles in the hepatic uptake of amphipathic organic anions and certain types of cationic compounds in the rat liver.

In addition to rOatps, members of the organic anion transporter (Oat/OAT) family have been shown to be expressed in the liver. Oat/OAT is classified within the gene superfamily of solute carriers (*SLC*) as the gene family *"SLC22A"*, and four members (rOat1-3 and rOat5) have been cloned in rats (Koepsell and Endou, 2004; Youngblood and Sweet, 2004). rOat2 is the isoform expressed in the liver and female kidney (Simonson et al., 1994; Sekine et al., 1998; Kato et al., 2002), while rOat3 is expressed in the male liver, and the kidney, brain and eye (Kusuhara et al., 1999). The other members, Oat1 (*Slc22a6*) and Oat5, are predominantly expressed in the kidney

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(Sekine et al., 1997; Youngblood and Sweet, 2004).

rOat2 was initially reported as a novel liver-specific transporter without any information about its substrates which was cloned using a monoclonal antibody raised against a partially purified rat liver glucagon receptor from the liver cDNA library (Simonson et al., 1994).   It is localized in the sinusoidal membrane in the liver (Simonson et al., 1994), whereas, on the brush border membrane in the kidney, it is expressed in the tubules in the medullary ascending limb of Henle's loop and the cortical and medullary collecting ducts (Kojima et al., 2002).   Functional expression of rOat2 in *Xenopus laevis* oocytes revealed that it mediates sodium-independent uptake of salicylate, and its substrates include *p*-aminohippurate (PAH), α-ketoglutarate and methotrexate (Sekine et al., 1998).   Furthermore, we constructed stable transfectants of rOat2 using LLC-PK1 cells (rOat2-LLC) as host and demonstrated that rOat2 has the ability to transport prostaglandin E_2, non-steroidal anti-inflammatory drugs, such as salicylate and indomethacin, and nucleoside derivatives such as 3'-azido-3'-deoxythymidine and 2', 3'-dideoxycytidine (Morita et al., 2001). Sinusoidal localization of rOat2 in the liver suggests its involvement in the hepatic uptake of its substrates, however, this has not been thoroughly investigated yet.

The primary purpose of the present study is to characterize the uptake of rOat2 substrates by freshly isolated rat hepatocytes to investigate the contribution of rOat2.   The uptake of ketoprofen, indomethacin and salicylate by freshly isolated rat hepatocytes was determined, and the effect of inhibitors of rOat2 and inhibitors of other organic anion transporters (rOatps and rOat3) was examined with regard to the uptake of ketoprofen.

Materials and Methods

Materials

[^{3}H]Ketoprofen (34 Ci/mmol, >99%) and [^{14}C]salicylate (55.5 mCi/mmol, >97%) were

purchased from ARC (MO, USA), and [^{14}C]indomethacin (20 mCi/mmol, >97%) was purchased

from PerkinElmer Life Science Products (Boston, MA).   Unlabeled pravastatin was kindly donated

by Sankyo (Tokyo, Japan).   The following compounds were purchased from Sigma-Aldrich (St.

Louis, MO): racemic- and (S)ketoprofen, digoxin, glibenclamide, probenecid, taurocholate,

p-aminohippurate (PAH), salicylate and NSAIDs.   Indocyanine green (ICG) was purchased from

Daiichi Pure Chemicals (Tokyo, Japan).   All other chemicals and reagents were of analytical grade

and were readily available from commercial sources, and all cell culture media and reagents were

obtained from Invitrogen Japan (Tokyo, Japan).

Uptake studies in rOat2-LLC

rOat2-LLC and its mock control (vector-LLC) were established previously (Morita et al.,

2001).   rOat2- and vector LLC were grown in M199 (BRL, Gaithersburg, MD) supplemented with

10% FBS, penicillin (100 U/ml), streptomycin (100 µg/ml) and G418 (400 µg/ml) (BRL,

Gaithersburg, MD) at 37 °C with 5% CO_2 and 95% humidity on the bottom of a dish.   Cells were

seeded in 12-well plates at a density of 1.2 x 10^5 cells/well.   They were incubated for 24h in the

presence of sodium butyrate (5mM) before starting the transport experiments to induce the

expression of rOat2 as described previously (Morita et al., 2001).   All transport assays were

performed in 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 $CaCl_2$ adjusted to pH

7.4).   rOat2-mediated uptake was obtained by subtracting the uptake values for the vector-LLC

from the net uptake by rOat2-LLC, and expressed as the accumulation of cell specimens divided by the medium concentration.

Determination of kinetic parameters

The uptake of [³H]ketoprofen (1.3 nM) by rOat2- and vector-LLC was determined at the earliest time practical (1 min) to obtain the kinetic parameters .   The uptake-concentration curve of the specific uptake by rOat2, obtained by subtracting the uptake values by vector-LLC from that by rOat2-LLC, was fitted to the following equation:

$$v = V_{max} \, S \, / \, (K_m + S) \text{ -------eq (1)}$$

where v is the uptake rate of the substrate (pmol/min/mg protein), S is the substrate concentration in the medium (μM), K_m is the Michaelis constant (μM) and V_{max} is the maximum uptake rate (pmol/min/mg protein).   Fitting was performed using a MULTI program (Yamaoka et al., 1986). The input data were weighted as the reciprocal of the observed values, and the Damping Gauss Newton Method algorithm was used for fitting.

Inhibition of ketoprofen uptake by NSAIDs, probenecid and glibenclamide

The uptake of [³H]ketoprofen (1.4 nM) by rOat2-LLC was determined at 1 min in the presence and absence of NSAIDs and probenecid which were added to the incubation buffer simultaneously with [³H]ketoprofen.   The concentration of [³H]ketoprofen was low enough compared with its own K_m value, and thus, the inhibition constants (K_i values) were obtained using the following equation regardless of inhibition type,

$$v = CL \, / \, (1 + I/K_i) \text{ -------eq (2)}$$

where CL represents the uptake clearance in the absence of inhibitors, and I is the inhibitor concentration.

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Preparation of isolated hepatocytes

Hepatocytes were isolated from Sprague-Dawley rats (male, 7 to 8 weeks old) by the

procedure described previously (Yamazaki et al., 1992).   After isolation, the hepatocytes were

suspended at 4 °C in albumin-free Krebs-Henseleit buffer to give a protein concentration of 2 mg/ml.

Cell viability was routinely checked by the trypan blue (0.4 % w/v) exclusion test.   Isolated

hepatocytes with a viability of > 85% were used for the transport studies.   In order to check that the

cell viability was sufficient to perform the uptake experiment, Na^+-dependent and independent

uptake of taurocholate by freshly isolated rat hepatocytes was also assessed using time-points at 1

and 2 min.

Uptake experiments using freshly isolated rat hepatocytes

Uptake of [3H]ketoprofen (1.4 nM), [14C]indomethacin (0.23 µM) and [14C]salicylate (2.0

µM) was initiated by adding ligand to the suspension (2 mg/ml) preincubated for 5min at 37 °C.

After the designated time, the reaction was terminated by separating the cells from the medium using

a centrifugal filtration technique (Yamazaki et al., 1992).   Briefly, 200 µl reaction mixture was

transferred to 0.4 ml centrifuge tubes containing 50 µl 2N NaOH and covered with 100 µl of a

mixture of silicone and mineral oil (density: 1.015).   Samples were then centrifuged for 10 sec in a

bench microfuge (Beckman Instruments, Fullerton, CA).   Centrifugation drove the pelleted

hepatocytes through the oil layer and into the concentrated alkaline solution (2 N NaOH).   After the

cells dissolved in alkaline solution, the tube was sliced with a razor blade and both sections (medium

portion and the bottom portion including cells) were taken to determine their radioactivity.   The

uptake value (µl/mg protein) is the cellular uptake divided by the concentration in the medium, and

obtained by subtracting the adherent water volume from the total uptake by hepatocytes.   The

adherent water volume and cell volume were determined using [^{14}C]carboxylinulin and [^{3}H]water as

marker compounds, respectively.   The volumes were determined by the same method in separate

tubes.

To determine the Na^{+}-dependent and independent uptake of ligands, the study was performed

in the absence of sodium, using Krebs-Henseleit buffer in which the NaCl and NaHCO$_3$ were

isotonically replaced with choline chloride and choline bicarbonate, respectively.

Temperature sensitivity of the uptake of [^{3}H]ketoprofen and [^{14}C]indomethacin by

hepatocytes was examined by preincubating the cells at 4 ℃ for 5 min followed by determining the

uptake for 1 min at 4 ℃.

In order to determine the kinetic parameters, the uptake of [^{3}H]ketoprofen and

[^{14}C]indomethacin for 45 and 60 sec, respectively, was determined at different substrate

concentrations (for ketoprofen, 0.1, 0.3, 1, 2, 5, 10, 30, 60, 100, 150, 300 µM and 1 mM; for

indomethacin, 0.088, 0.33, 1.1, 4, 11, 30, 100, 300 µM and 1 mM).   The kinetic parameters were

obtained using the following three equations:

one saturable and one non-saturable component:

$v = V_{max} S / (K_m + S) + P_{diff} S$-------eq (3)

two saturable components:

$v = V_{max1} S / (K_{m1} + S) + V_{max2} S / (K_{m2} + S)$ -------eq (4)

two saturable components and one non-saturable one:

$v = V_{max1} S / (K_{m1} + S) + V_{max2} S / (K_{m2} + S) + P_{diff} S$-------eq (5)

where P$_{diff}$ is the non-saturable uptake clearance (µl/min/mg protein).   Subscripts represent the

number of components.   The equations were fitted to the uptake by freshly isolated rat hepatocytes

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as described previously.   The rationale of the model was judged by the AIC value, a statistical

criterion generally used to judge the rationale of the numbers of independent variable.

Inhibition study of the uptake of ketoprofen by freshly isolated rat hepatocytes

The uptake of [^{3}H]ketoprofen (1.3 nM) by freshly isolated rat hepatocytes was determined for

1 min in the absence and presence of inhibitors at designated concentration.   The inhibitors were

prepared in Krebs-Henseleit buffer, and added to the cell suspension simultaneously with

[^{3}H]ketoprofen.   The inhibitors used in this study were as follows; digoxin, glibenclamide,

probenecid, taurocholate, PAH, pravastatin, indomethacin, ICG and salicylate.   ICG and

indomethacin can be used as potent inhibitors of rOat2 (Morita et al., 2001), and glibenclamide (Ki,

25 μM; this study) as a moderate inhibitor.   Probenecid (Ki, 438 μM; this study), salicylate (Ki,

399 μM; this study) and pravastatin (Ki 450 μM), taurocholate (25% of control at 1 mM), PAH (90%

of control at 1 mM) and digoxin (75% of control at 1 mM) are weak or poor inhibitors of rOat2

(Morita et al., 2001; Khamdang et al., 2004).   Taurocholate was used as an inhibitor of rOatps (K$_m$

18-50 μM), and digoxin as an inhibitor of rOatp2 (K$_m$ 0.24 μM) (Cattori et al., 2001).   Pravastatin

was used as a dual inhibitors of rOatp1 (K$_m$ 30 μM) and rOatp2 (K$_m$ 38 μM) (Hsiang et al., 1999;

Tokui et al., 1999), and rOat3 (Ki 13 μM) (Hasegawa et al., 2002).   PAH was used as an inhibitor

of rOat3 (Ki 400-1350 μM) (Hasegawa et al., 2002; Nagata et al., 2002).   The concentrations of

inhibitors were chosen based their K$_m$ or K$_i$ values for their target transporters.   Statistical

differences were examined by one-way ANOVA followed by Fisher's LSD.

Results

The uptake of ketoprofen by rOat2-LLC

The time-profiles of the uptake of ketoprofen in vector- and rOat2-LLC are shown in Figure 1. The uptake of racemic [^{3}H]ketoprofen by rOat2-LLC was significantly greater than that by vector-LLC (Figure 1a). The rOat2-mediated uptake was saturated at high substrate concentrations, and kinetic analysis using racemate and (S)ketoprofen revealed that their K_m and V_{max} values were 3.31 ± 0.18 and 5.88 ± 0.15 µM, and 490 ± 20 and 821 ± 17 pmol/min/mg protein, respectively (Figure 1b).

Effect of NSAIDs, probenecid and glibenclamide on rOat2-mediated uptake of ketoprofen was examined (Figure 2). Their Ki values are summarized in Table 1. The inhibition studies revealed that NSAIDs, other than ketoprofen and indomethacin, showed moderate and weak inhibition potency for rOat2 (Figure 2). Naproxen had a minimal effect at the concentration examined ($K_i > 1$ mM) (data not shown). Probenecid was a weak inhibitor of rOat2 with a K_i value of 438 ± 137 µM, while glibenclamide was a moderate inhibitor with a Ki value of 25.1 ± 9.9 µM (Figure 2).

The uptake study using freshly isolated rat hepatocytes

Triplicate determinations (mean $\pm$ S.E.) of the uptake of [^{3}H]water and [^{14}C]carboxylinulin, marker compounds for cell volume and adherent water space, were 4.9 ± 0.34 µl/mg protein and 2.0 ± 0.1 µl/mg protein, respectively. Triplicate determinations of the uptake of taurocholate were 101 ± 11 and 176 ± 12 µl/mg protein at 1 and 2 min, respectively, in the presence of sodium, while removal of sodium from the uptake buffer significantly reduced the uptake of taurocholate to 42.5 ± 6.2 and 63.4 ± 6.33 µl/mg protein at 1 and 2 min, respectively. These values were comparable with

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previously reported values (Yamazaki et al., 1992).

The time-profiles for the uptake of ketoprofen, indomethacin and salicylate by freshly isolated rat hepatocytes are shown in Figure 3. Indomethacin showed approximately 5-fold greater uptake by freshly isolated rat hepatocytes than ketoprofen (Figure 3), while the uptake of salicylate was small compared with that of ketoprofen and indomethacin, but still greater than the adherent water space (Figure 3). Isotonic replacement of sodium by choline in the uptake buffer, which markedly reduced the uptake of taurocholate, had no effect on the uptake of ketoprofen, indomethacin and salicylate (Figure 3), suggesting that their uptake mechanism is sodium-independent. Incubating cells at 4 °C caused a significant reduction in the cellular accumulation of ketoprofen and indomethacin for 1 min (Figure 4). The uptake of ketoprofen and indomethacin for 1 min was 104 ± 2 and 21.1± 2.3 µl/mg protein, and 352 ± 30 and 43.6 ± 25.3 µl/mg protein, at 37 and 4 °C, respectively.

The uptake of ketoprofen and indomethacin by hepatocytes was saturable (Figure 5). Eadie-Hofstee plots indicated an involvement of multiple components in their uptake. Kinetic parameters were obtained for the uptake of ketoprofen and indomethacin by freshly isolated rat hepatocytes at the earliest time practical (45 and 60 sec, respectively) assuming three models. Statistical analysis based on AIC values suggested that the model assuming two saturable components and one non-saturable one is the most appropriate to account for the saturation kinetics of the uptake of ketoprofen and indomethacin by freshly isolated rat hepatocytes (Table 2).

Effect of inhibitors on the uptake of ketoprofen by freshly isolated rat hepatocytes

The inhibitory effect of several typical inhibitors of rOatps (taurocholate, digoxin and pravastatin), rOat2 (ICG, indomethacin, glibenclamide, salicylate) and rOat3 (PAH and pravastatin),

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was examined for the uptake of ketoprofen by freshly isolated rat hepatocytes (Figure 6).   The

concentrations of inhibitors were chosen considering their K_m or K_i values for the target transport to

minimize any non-specific effects.   Except digoxin, all inhibitors had a significant effect on

ketoprofen uptake.   Probenecid, salicylate, glibenclamide, indomethacin and ICG significantly

inhibited the uptake of ketoprofen by freshly isolated rat hepatocytes in a concentration-dependent

manner although the degree of inhibition by ICG was smaller than that achieved by other inhibitors.

Pravastatin, taurocholate and PAH had a slight inhibitory effect.

Discussion

rOat2 is an Oat/OAT family isoform abundantly expressed in the liver and female kidney of rats. It remains unknown if it is involved in the tissue uptake of its substrates in the liver. The purpose of the present study was to characterize the uptake of an rOat2 substrate, ketoprofen, by freshly isolated rat hepatocytes.

In addition to previously reported substrates, ketoprofen was newly identified as rOat2 substrate (Figure 1). Comparison of K_m values determined using racemate and (S)ketoprofen suggests that the K_m value of ketoprofen shows stereoselectivity to rOat2 (Figure 1b), and whether this is associated with stereoselectivity of the intrinsic transport activity should be examined in future analysis. The K_i values of other NSAIDs were determined for comparison (Table 1). Unlike ketoprofen and indomethacin, the other NSAIDs are weak inhibitors of rOat2 (Table 1). In particular, although ketoprofen, ibuprofen and naproxen contain a phenylisopropionic acid group, the K_i values of ibuprofen and naproxen are markedly greater than the K_m value of ketoprofen. NSAIDs, other than ketoprofen and indomethacin, might be low affinity substrates of rOat2. In contrast to rOat2, NSAIDs are potent inhibitors of rOat3 except salicylate, and most NSAIDs are potent inhibitors of rOat1 (Table 2).

The liver plays an important role in the pharmacokinetics of NSAIDs. The NSAIDs are eliminated from the body mainly by the liver by biliary excretion and metabolism including glucuronidation in rats (Yesair et al., 1970; Sumner et al., 1975; Stierlin and Faigle, 1979; Cayen et al., 1981; Foster and Jamali, 1988; Dietzel et al., 1990; Iwakawa et al., 1991) except salicylate and sulindac. For salicylate, the renal and hepatic elimination occurs to a similar degree (Yue and Varma, 1982). Sulindac, a sulfide prodrug, is converted to its active form both in the liver and

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kidney, but its hepatic distribution is markedly greater than its renal distribution (Duggan et al.,

1980). Previous transport studies using primary cultured rat hepatocytes suggest that the

transporter(s)-mediated fraction accounts for the major part of the uptake of indomethacin, and that

the major hepatic transporter responsible for the uptake of indomethacin is distinct from rOatp1 and

sodium-taurocholate co-transporting polypeptide (Kouzuki et al., 2000). It is possible that rOat2

accounts for the hepatic uptake of ketoprofen, indomethacin and salicylate. This possibility was

examined using freshly isolated rat hepatocytes.

A sodium-independent uptake of ketoprofen, indomethacin and salicylate was observed in

freshly isolated rat hepatocytes (Figure 3). Unlike ketoprofen and indomethacin, the uptake of

salicylate was too small for further characterization, but greater than the adherent water volume.

The uptake of ketoprofen and indomethacin by hepatocytes was temperature-sensitive, and markedly

reduced at 4 °C (Figure 4). Non-linear regression analyses revealed that the uptake-substrate

concentration curves could be fitted to the equations assuming multiple components (Figure 5).

The AIC values suggested that the model assuming two saturable components (high- and

low-affinity component) and one non-saturable one best fitted the curve for the uptake of ketoprofen

and indomethacin by hepatocytes (Table 2). The uptake of ketoprofen and indomethacin remaining

at 4 °C was comparable with their corresponding P_{dif} values, supporting the presence of a

non-saturable component. Based on the intrinsic transport activity (V_{max}/K_m), the clearance

corresponding to the high and low affinity components account for 60 and 25% of the total uptake of

ketoprofen, while they contribute equally to the uptake of indomethacin (40 % of the total uptake).

The K_m values of the high affinity component for the uptake of ketoprofen and indomethacin by

hepatocytes were similar to those determined in rOat2-LLC, suggesting an involvement of rOat2 in

hepatic uptake of ketoprofen and indomethacin as high affinity site.

Inhibition studies were carried out to characterize the uptake of ketoprofen by freshly isolated

rat hepatocytes using inhibitors of rOat2 and other candidate transporters, such as rOatps and rOat3

(Figure 6).   Since ketoprofen and indomethacin are high affinity substrates of rOat2, the substrate

concentration in the incubating buffer has to be lower than their K_m values for rOat2.   The specific

activity of [^{14}C]indomethacin was too low for this purpose.   Indomethacin and ICG can be used as

potent inhibitors of rOat2, and glibenclamide is a moderate inhibitor of rOat2.   Salicylate and

probenecid are weak inhibitors of rOat2.   These rOat2 inhibitors showed a significant inhibitory

effect on the uptake of ketoprofen by hepatocytes (Figure 6).   ICG did not fully inhibit the uptake

of ketoprofen by hepatocytes at the concentration examined (approximately 40% inhibition), and

indomethacin had a partial effect at a concentration sufficient to inhibit rOat2-mediated uptake (55%

inhibition).   Furthermore, probenecid and glibenclamide showed a significant effect even at

concentrations lower than their Ki values for rOat2.   These results suggest that the hepatic uptake

of ketoprofen cannot be accounted for only by rOat2.   Inhibitors of other organic anion transporters

(rOatps and rOat3) had a slight effect on the uptake of ketoprofen by hepatocytes, but digoxin had no

effect.   It is possible that other transporters, such as rOatps and rOat3, but not rOatp2, play a role in

the hepatic uptake of ketoprofen.

Assuming a partial contribution of rOat2 (40% based on the degree of inhibition by ICG), the

rOat2-mediated uptake of other rOat2 substrates by hepatocytes can be estimated based on the

relative activity factor method using ketoprofen as a reference compound (Hasegawa et al., 2003;

Hirano et al., 2004).   Since the intrinsic transport activity ratio of indomethacin and ketoprofen was

2.5 in rOat2-LLC, the rOat2-mediated uptake of indomethacin in hepatocytes was estimated to be

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120 µl/min/mg protein, which is almost identical to the clearance corresponding to the high affinity component (Table 2). rOat2-mediated uptake of salicylate and prostaglandin E_2, the intrinsic activities of which were 20% of ketoprofen uptake in rOat2-LLC (Morita et al., 2001) will be 10 µl/min/mg protein in hepatocytes, and that of nucleoside analogs, the transport activities of which were 2~3% of ketoprofen (Morita et al., 2001) will be 1 µl/min/mg protein in hepatocytes. This estimation is consistent with the low uptake of salicylate by hepatocytes (Figure 3), and the previous results in which saturation of the uptake of 3'-azido-3'-deoxythymidine by hepatocytes was hardly observed (Bezek et al., 1994).

The therapeutic concentrations of ketoprofen and indomethacin are 1.2 and 0.84-84 µM, respectively (Insel, 1995). According to report by Jamali and Brocks (1990), the C_{max} of ketoprofen after oral administration ranged from 3 to 90 µM. Both ketoprofen and indomethacin are highly protein-bound in plasma. Taking into consideration their unbound fraction in humans (0.007 and 0.1 for ketoprofen and indomethacin, respectively: Insel, 1995), the unbound plasma concentration of ketoprofen is lower or comparable with the K_m value of the high affinity component determined in rats, while that of indomethacin is within the range of unbound concentrations. It is possible that the high affinity component of indomethacin is saturated to some degree in clinical situations. It should be noted that drawing conclusions from the rat data could be misleading, as far as human significance is concerned, due to a species difference in the substrate recognition by rat and human OAT2. Khamdang et al (2002) demonstrated a significant uptake of salicylate by S_2 cells expressing hOAT2, whereas the specific uptake of ketoprofen and indomethacin by hOAT2 was below the limit of detection (Khamdang et al., 2002). Furthermore, the K_i values of ketoprofen and indomethacin for hOAT2 are greater than those for rOat2 (Table 1). These reports suggest that the

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transport characteristics involved in the hepatic uptake of ketoprofen and indomethacin by human

hepatocytes are different from those in rat hepatocytes.   Further studies are necessary to elucidate

the importance of hOAT2 in the hepatic uptake of ketoprofen and indomethacin, and other drugs

using human hepatocytes and cDNA transfectants.

In conclusion, we have demonstrated that ketoprofen is a substrate of rOat2.   Hepatic uptake

of ketoprofen and indomethacin consists of high and low affinity components.   The results of the

present study suggest that rOat2 accounts partly for the high affinity component of ketoprofen

uptake and, presumably, indomethacin, and that other transporters, such as rOatps and rOat3, but not

rOatp2, are also involved in the uptake of ketoprofen by hepatocytes.

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Footnotes

Figure legends

Figure 1.   The uptake of ketoprofen by rOat2-LLC.

a. The uptake of ketoprofen (1.3 nM) by rOat2-LLC (closed circles) and vector-LLC (open circles) was examined in Krebs-Henseleit buffer.   b Concentration-dependence of the uptake of [3H]ketoprofen by rOat2 was examined using racemic and (S)ketoprofen (closed and open squares, respectively).   The results were expressed as an Eadie-Hofstee plot.   Each data point represents the net uptake obtained by subtracting the uptake by vector-LLC from that by rOat2-LLC.   Solid line represents the fitted line.   The details of the fitting were described in Materials and Methods. Each data point represents the mean ± S.E.   Where vertical bars are not shown, the S.E. is contained within the limits of the symbol.

Figure 2.   Effect of NSAIDs, probenecid on the rOat2-mediated uptake by rOat2-LLC.

The uptake of ketoprofen (1.3 nM) by rOat2-LLC and vector-LLC for 1 min was determined in the presence and absence of NSAIDs (panel a: etodolac, panel b: ibuprofen, panel c: indomethacine, panel d: phenybutazone, panel e: salicylate panel f: sulindac, panel g: tolmetin), probenecid (panel h) and glibenclamide (panel i).   The concentration range of inhibitors ranged from 1 to 1000 µM (1, 10, 30, 100, 300 and 1000 µM) except for indomethacin (0.03, 0.1, 0.3, 1, 3, 10 and 100 µM) and glibenclamide (0.3, 1, 3, 10, 30 and 100 µM).   Each data point was obtained by subtracting the uptake by vector-LLC from that by rOat2-LLC, and represents the mean ± S.E. of triplicate determinants.   Where vertical bars are not shown, the S.E. is contained within the limits of the symbol.   Solid line represents the fitted line.   The details of the fitting are described in Materials and Methods.

Figure 3.   Time-profiles of the uptake of ketoprofen and indomethacin by freshly isolated rat hepatocytes.

The uptake of ketoprofen (1.3 nM, panel a), and indomethacin (0.23 µM, panel b) and salicylate (2 µM, panel c) by freshly isolated rat hepatocytes was measured at 37 °C in the presence and absence of sodium (closed and open symbols, respectively) at designated time.   Sodium ion in the incubating buffer was isotonically replaced with choline.   Each data point represents the mean ± S.E. of triplicate determinations.   Where vertical bars are not shown, the S.E. is contained within the limits of the symbol.

Figure 4.   Temperature sensitivity of the uptake of ketoprofen and indomethacin by freshly isolated rat hepatocytes.

The uptake of [3H] ketoprofen (1.3 nM) and [14C]indomethacin (0.23 µM) by freshly isolated rat hepatocytes for 60 sec was determined at 4 or 37 °C.   For the uptake at 4 °C, the cells were incubated for 5 min at 4 °C prior to the uptake experiment.   Each bar represents the mean ± S.E. of triplicate determinations.

Figure 5.   Eadie-Hofstee plot of the uptake of ketoprofen and indomethacin by freshly isolated rat hepatocytes.

The uptake of ketoprofen and indomethacin by freshly isolated rat hepatocytes was examined at different substrate concentrations (panel a and b).   The uptake of ketoprofen (panel a) and indomethacin (panel b) was determined at 45 and 60 sec, respectively, at 37 °C.   The substrate

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concentrations ranged from 0.1 to 300 µM (0.1, 0.3, 1, 2, 5, 10, 30, 60, 100, 150, 300 µM) for

ketoprofen, and from 0.088 to 1000 µM (0.088, 0.33, 1.1, 4, 11, 30, 100, 300 µM and 1 mM). Each

point represents the mean ± S.E. of triplicate determinants. Where vertical bars are not shown, the

S.E. is contained within the limits of the symbol. Solid lines represent the least-squares fit of data

to the Michaelis-Menten equation. The details of the fitting are described in Material and Methods.

Figure 6. Effects of nine compounds on the uptake of ketoprofen by freshly isolated rat hepatocytes.

The uptake of ketoprofen (3 nM) was examined in the presence of inhibitors at the concentrations

indicated. The values are expressed as remaining ketoprofen uptake by freshly isolated rat

hepatocytes for 1 min. Each column represents the mean ± S.E. (n = 3). * ; $P < 0.05$, ** ; $P < 0.01$

Table 1 K_m and K_i values of NSAIDs among organic anion transporters.

The effect of NSAIDs was examined for the uptake of ketoprofen by rOat2-LLC, and their Ki values were determined by nonlinear regression analysis using the data shown in Figure 2.   The values in each column represent the means ± computer-calculated S.D.   An asterisk represents the Km value.   Superscripts, a and b represent the previously reported values by Nozaki et al (2002) and Khamdang et al (2002), respectively.
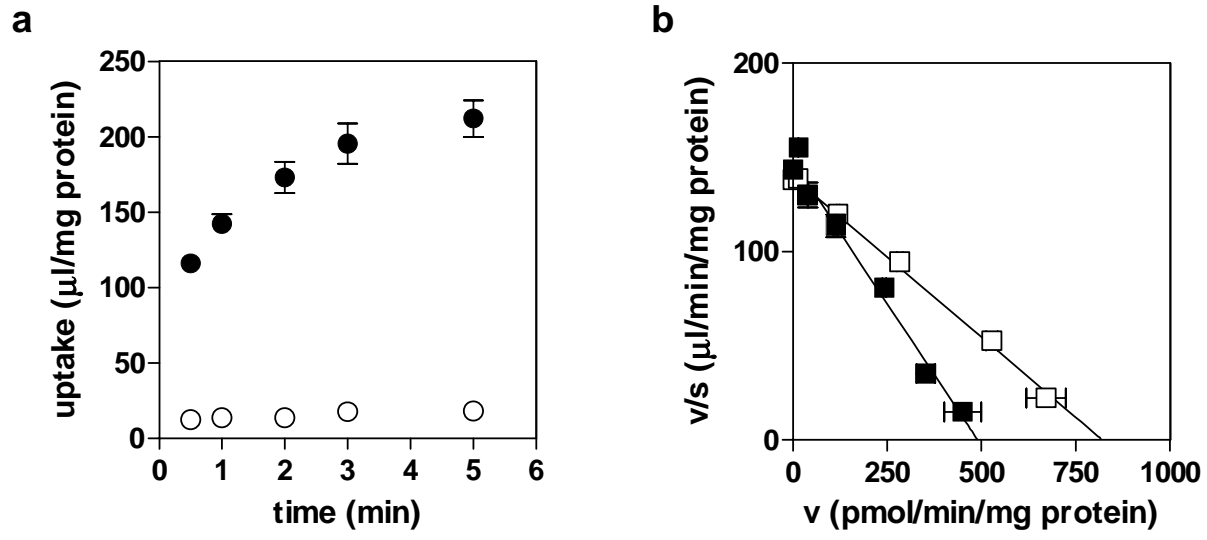
NSAIDs	rOat1[a] (µM)	rOat2 (µM)	rOat3[a] (µM)	hOAT2[b] (µM)
salicylate	2110 ± 190	399 ± 133	519 ± 132	N.D.
diclofenac	4.56 ± 1.24	107 ± 29	3.17 ± 0.43	14.3
sulindac	99.9 ± 18.6	537 ± 282	7.72 ± 1.32	440
indomethacin	7.44 ± 3.88	1.25 ± 0.40	1.29 ± 0.46	64.1
etodolac	> 100	344 ± 99	9.98 ± 1.48	N.D.
tolmetin	15.4 ± 3.2	288 ± 118	8.78 ± 2.32	N.D.
ibuprofen	4.33 ± 0.40	346 ± 100	3.57 ± 1.09	692
ketoprofen	6.11 ± 1.66	3.31 ± 0.18[*]	4.31 ± 1.28	400
naproxen	5.54 ± 0.89	> 1000	19.1 ± 6.9	486
phenylbutazone	47.9 ± 9.2	387 ± 129	8.48 ± 0.78	N.D.

N.D., not determined.

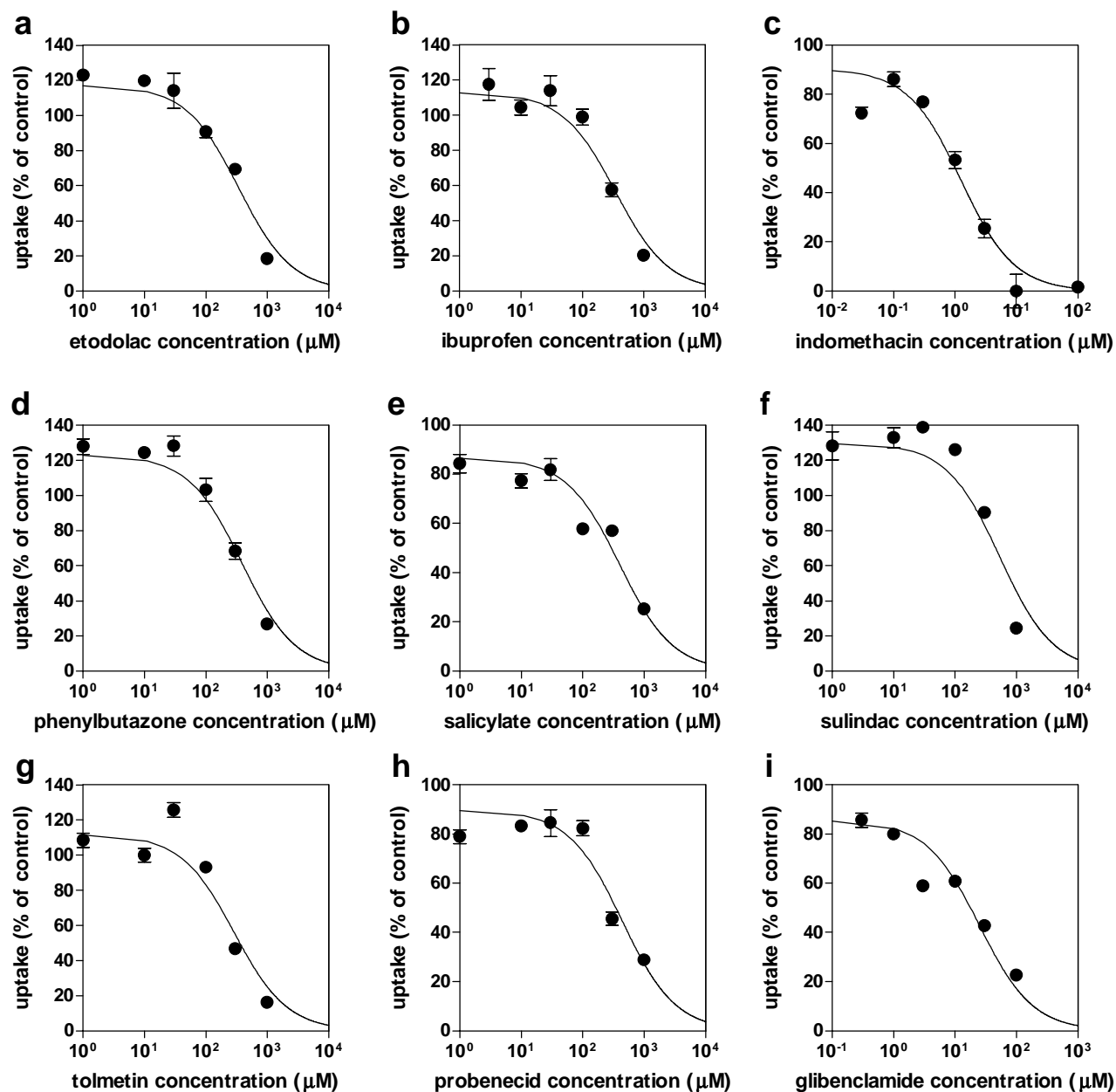**Table 2 Kinetic parameters of the uptake of ketoprofen and indomethacin by freshly isolated rat hepatocytes.**

The kinetic parameters for the uptake of ketoprofen and indomethacin by freshly isolated rat hepatocytes (data were taken from Figure 4) were obtained by fitting the uptake-concentration curve to the three equations assuming 1) one saturable and one non-saturable components (K_{m1}, V_{max1} and P_{dif}), 2) two saturable components (K_{m1}, V_{max1}, K_{m2} and V_{max2}), and 3) two saturable and one non-saturable components (K_{m1}, V_{max1}, K_{m2}, V_{max2} and P_{dif}).   The details of fitting are described in Materials and Methods. Values are means $\pm$ computer-calculated S.D.   AIC represents the Akaike's information criterion.
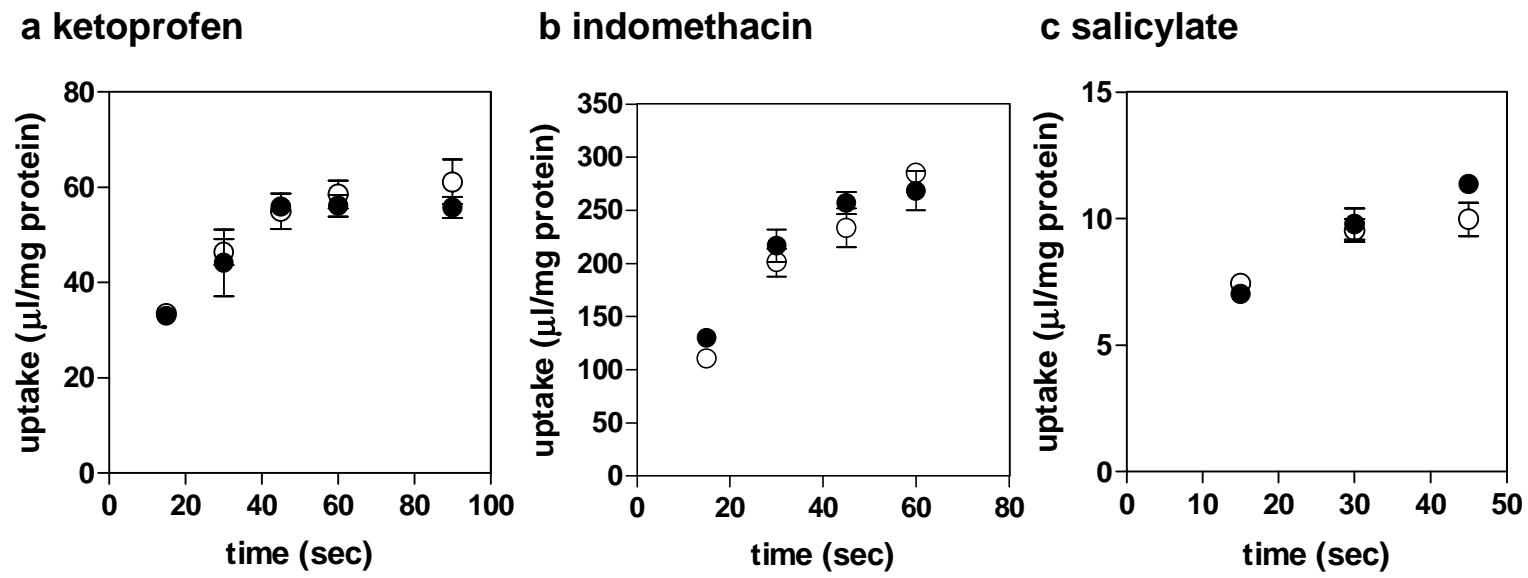
substrate	K_{m1} (µM)	V_{max1} (pmol/min/mg)	K_{m2} (µM)	V_{max2} (pmol/min/mg)	P_{dif} (µl/min/mg)	AIC
ketoprofe	0.84 ± 0.20	34.8 ± 7.8	97.4 ± 24	1750 ± 360	11.2 ± 0.6	-48
	2.03 ± 0.61	88.6 ± 22.5	1270 ± 261	27100 ± 4720	-	-28
	7.46 ± 3.31	297 ± 112	-	-	15.1 ± 1.3	-14
indometh	1.14 ± 0.80	133 ± 93.3	139 ± 49	16300 ± 4810	82.8 ± 6.4	-33
	3.21 ± 1.68	333 ± 163	1220 ± 166	14800 ± 10500	-	-25
	28 ± 12.5	3540 ± 1520	-	-	74.8 ± 7.4	-18

Figure 1

Figure 2

a ketoprofen   **b indomethacin**   **c salicylate**
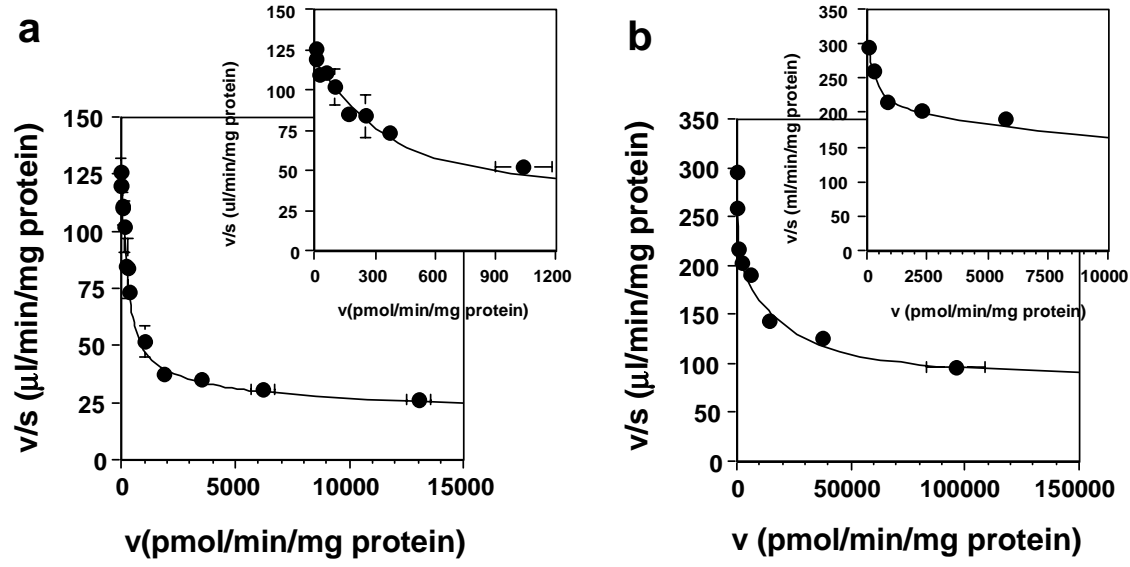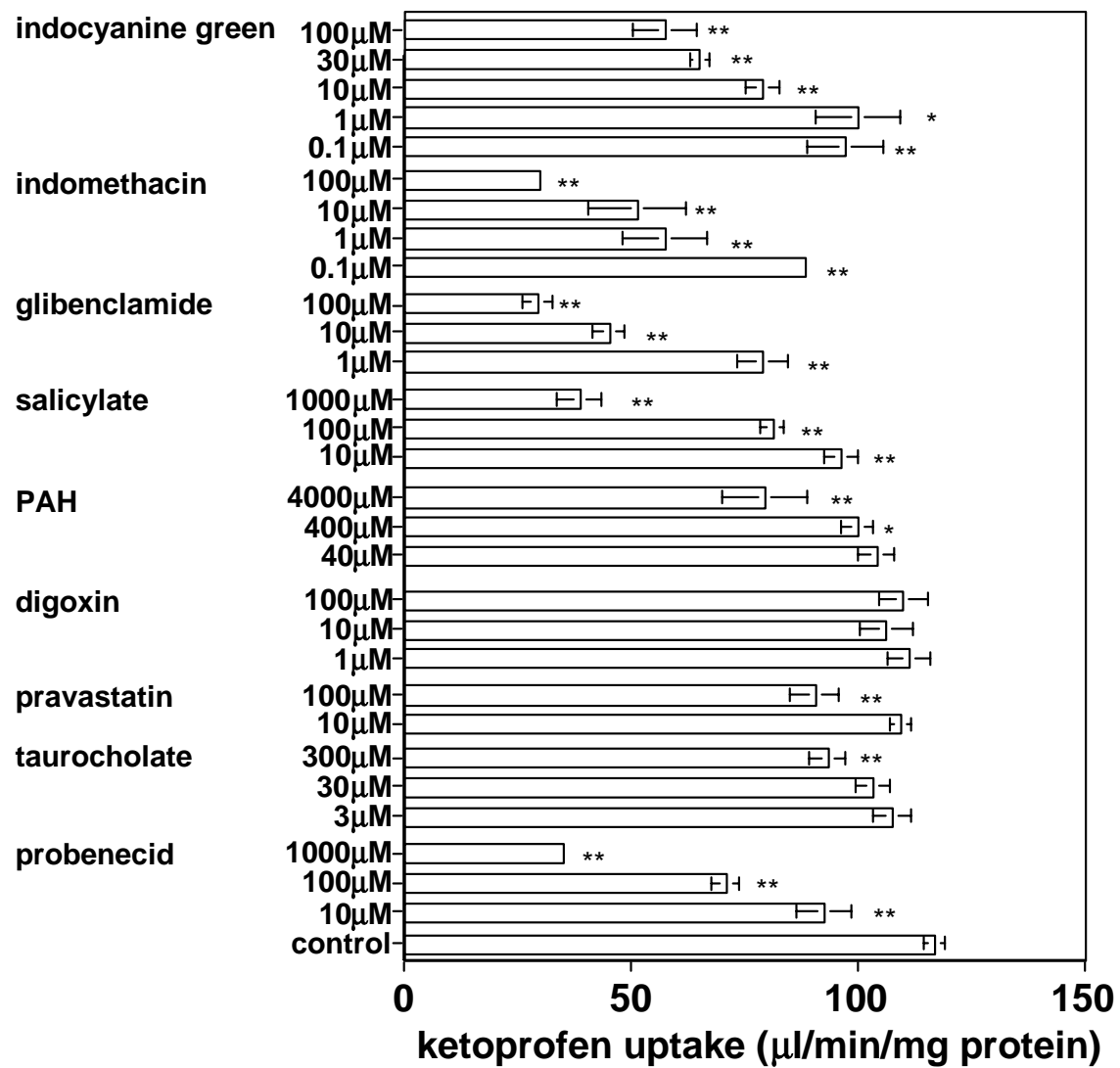

Figure 3

Figure 4

Figure 5

ketoprofen uptake (µl/min/mg protein)

Figure 6