

Evaluation of the interaction between nonsteroidal anti-inflammatory drugs and methotrexate using human organic anion transporter 3–transfected cells

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## ABSTRACT

Coadministration of methotrexate and nonsteroidal anti-inflammatory drugs (NSAIDs) can cause a pharmacokinetic interaction and a subsequent increase in blood methotrexate concentrations. Methotrexate and most NSAIDs are excreted into urine via organic anion transporter 3 (OAT3). The purpose of this study was to evaluate NSAIDs that compete less with methotrexate by using the renal cell line stably expressing human OAT3 (S2-hOAT3) *in vitro*. We also confirmed the pharmacokinetic interaction of methotrexate with NSAIDs *in vivo*. [<sup>3</sup>H]methotrexate uptake into S2-hOAT3 cells was inhibited by most NSAIDs in a concentration-dependent manner, but aspirin, salicylate, tiaramide, and acetaminophen did not inhibit uptake. Inhibition by sulindac and pranoprofen was weaker at therapeutic drug concentrations. Furthermore, methotrexate concentrations in rat serum were significantly increased in a NSAID concentration-dependent manner when concentrations of coadministered NSAIDs increased above the  $K_i$  values obtained in the *in vitro* study. On the other hand, drugs that were not substrates of hOAT3, such as acetaminophen, did not interact with methotrexate. The magnitude of the pharmacokinetic interaction between methotrexate and NSAIDs was significantly correlated with results of the accumulation study *in vitro* and was not significantly correlated with a reduction of urinary creatinine excretion. In conclusion, methotrexate and most NSAIDs are substrates of hOAT3, and those drugs compete via hOAT3 in tubular secretion, the major mechanism of the interaction between methotrexate and NSAIDs. The accumulation study using S2-hOAT3 cells might be useful for screening of potential interactions between methotrexate and new NSAIDs *in vivo*.

Keywords: methotrexate, tubular secretion, organic anion transporter,

pharmacokinetic interaction, NSAIDs

## 1. Introduction

Methotrexate, which inhibits dihydrofolate reductase, is widely used for cancer chemotherapy (Frei et al., 1975; Jackson, 1984), whereas nonsteroidal anti-inflammatory drugs (NSAIDs) have antipyretic and analgesic effects, achieved by inhibition of prostaglandin synthesis mediated by cyclooxygenases (Vane, 1971; Vane and Botting 1998). Although methotrexate and NSAIDs are often used concomitantly in clinical practice such as rheumatoid arthritis and cancer, the combination is reported to increase methotrexate -related adverse effects (Daly et al., 1986; Singh et al., 1986; Tuyss et al., 1986; Frenia and Long, 1992; Tracy et al., 1992). However, adequate control of pain is important for patients. Therefore, NSAIDs that do not elevate blood methotrexate levels are useful in clinical practice.

Methotrexate is excreted into urine in almost unchanged form. Both methotrexate and NSAIDs are anion compounds, and their protein binding is approximately 45–51% and >90%, respectively (Shen and Azarnoff, 1978). The following mechanisms are responsible for NSAID-induced increases in methotrexate concentrations; i) decrease in glomerular filtration of methotrexate by NSAIDs via reduction of renal blood flow with inhibition of prostaglandin synthesis (Ahern et al., 1988; Tracy et al., 1992; Murray and Brater, 1993; Brouwers and de Smet, 1994), ii) inhibition of methotrexate tubular secretion (Frenia and Long, 1992; Masada et al., 1997; Uwai et al., 2000; Uwai et al., 2004; Nozaki et al., 2007b; El-Sheikh et al., 2007), and iii) competition for protein-binding sites (Brouwers and de Smet, 1994). In general, main interaction mechanism has been known to the inhibition of prostaglandin synthesis (Brouwers and de Smet, 1994). However, recently, *in vitro* studies have revealed many renal transporters for methotrexate and NSAIDs (Van Aubel et al., 2000; Takeuchi et al., 2001; Shibayama et al., 2006). Organic anion

transporters (OAT1, OAT3, OAT4, OAT-K1) (Masada et al., 1997; Takeuchi et al., 2000; Takeda et al., 2002; Uwai et al., 2004), multidrug-resistance proteins (MRP2, MRP4) (El-Sheikh et al., 2007; Nozaki et al., 2007 b), and reduced folate carrier 1 (RFC-1) (Nozaki et al., 2004) are competitive sites between methotrexate and NSAIDs. Considering that the methotrexate main elimination route is the tubular secretion (Rubin et al., 1967; Nierenberg, 1983), it is speculated that the competition for renal transporters is important mechanism of the interaction in humans.

Human organic anion transporter 3 (hOAT3) is expressed at the second portion of the proximal tubule epithelial cells localized at the basolateral membrane (Cha et al., 2001; Takeda et al., 2001; Nozaki et al., 2007 a). In epithelial cells, methotrexate is taken from vascular fluid by hOAT3 (Takeda et al., 2002), which is significantly inhibited by NSAIDs such as salicylate and loxoprofen (Takeda et al., 2002; Uwai et al., 2004; Nozaki et al., 2007 b). Furthermore, the expression level of hOAT3 mRNA is the highest among the OAT family (Motohashi et al., 2002). It is speculated that competition for hOAT3 is the most important for the interaction between methotrexate and NSAIDs in humans (Ahern et al., 1988; Takeda et al., 2002; Nozaki et al., 2007 b). Therefore, NSAIDs that do not disturb methotrexate transport via hOAT3 are useful in clinical practice.

The purpose of this study was to evaluate the degree of competition between NSAIDs and methotrexate by using proximal tubule S2 cells stably expressing hOAT3 (S2-hOAT3 cells) (Takeda et al., 2002) and then to examine the pharmacokinetic interaction of methotrexate with NSAIDs using rats to reveal the contribution of competition for tubular secretion and to evaluate the *in vitro* study.

## **2. METHODS**

### **2.1 Materials**

[3',5',7-<sup>3</sup>H]Methotrexate disodium salt (50.8, 22.5 Ci/mmol) was purchased from Moravek Biochemicals, Inc. (Brea, CA, USA), and [methoxy-<sup>14</sup>C]inulin (5–20 mCi/g) was from DuPont (Wilmington, DE, USA). Acetaminophen, mefenamic acid, diclofenac sodium, sulindac, indomethacin, etodolac, ibuprofen, naproxen, loxoprofen, piroxicam, and meloxicam were obtained from Sigma Chemical Co. (Tokyo, Japan). Aspirin and salicylate were from Wako Chemical.,Ltd. (Osaka, Japan). Pranoprofen (Mitsubishi Pharma Corp., Osaka, Japan) and tiaramide (Astellas Pharma Inc., Tokyo, Japan) were kindly provided. S2-hOAT3 cells, in which the human OAT3 gene was introduced into SV40-transfected mouse proximal tubule cells, were generous gifts of Dr. Endou (Kyorin University, Tokyo, Japan). This cell line highly and permanently expressed hOAT3 (Takeda et al., 2002).

### **2.2 *In vitro* uptake examination**

S2-hOAT3 cells were maintained as described previously, with some modifications (Takeda et al., 2002). S2-hOAT3 cells were seeded in 12-well insert plates (3  $\mu$ m, 8 $\times$ 10<sup>5</sup> pore/cm<sup>2</sup> BD Falcon; Becton Dickinson Co. NJ, USA) at a density of 1 $\times$ 10<sup>5</sup> cells/well. Incubations were done in 0.8 ml and 2 ml of medium at apical and basolateral sides, respectively. These cells were grown in an incubator for 7 days at 37°C in 5% CO<sub>2</sub> and were fed fresh medium every 2 days. The incubation medium was changed to fetal bovine serum (FBS)–free medium an hour before initiation of the uptake examination. At first, we examined a validity of incubation time and [<sup>3</sup>H]methotrexate concentrations to elucidate that not reach to the steady state using S2-hOAT3 and S2-DNA (hOAT3-mock) cells (data not shown) (Takeda et al.,

2002). The cells were incubated for 2 h in FBS-free medium containing [<sup>3</sup>H]methotrexate (26 nM), [<sup>14</sup>C]inulin, and various NSAIDs (0, 1, 10, 100 μM) at basolateral sides. When the uptake was finished, a 150-μL aliquot of the apical side medium was sampled, and its radioactivity was determined in 5 ml of ACS (Amersham Biosciences, England) by liquid scintillation counting. The uptake examination was stopped by cold phosphate-buffered saline (PBS), and the monolayer was rapidly washed 3 times adequately with cold PBS in each side. The cells in each well were dissolved with 150 μL of 1 N sodium hydroxide for 24 h at room temperature and were diluted to 10 times with distilled water. The radiation activity of each sample (150 μL) was measured using the treatment described above. Samples in which [<sup>14</sup>C]inulin transited to the apical side (>1% of samples) were excluded. Protein content of the solubilized cells was determined using the bicinchoninic acid assay (Pierce, Rockford, IL, USA).

Mefenamic acid, indomethacin, sulindac, pranoprofen, loxoprofen, piroxicam, and meloxicam were dissolved in dimethyl sulfoxide. Acetaminophen, aspirin, salicylate, ibuprofen, diclofenac sodium, etodolac, and naproxen were dissolved in methanol. Tiaramide was dissolved in distilled water. Each drug was diluted with incubation medium. Adjustments were made so that the final concentration in the incubation medium was 0.1%.

### **2.3 Kinetic analyses of [<sup>3</sup>H]methotrexate uptake**

Kinetic parameters were obtained by linear regression analysis using the following equation:

$$1/\text{accumulation} = a \times \text{concentration of NSAID} + b$$

a : slope. b : intercept.

in which the reciprocal of the percentage of accumulated methotrexate equals a multiplied by the NSAID concentration.

#### **2.4 *In vivo* pharmacokinetic examination in rats**

Male Wistar rats that weighed 250–300 g and had fasted overnight were used.

Mefenamic acid was dissolved in 1 N sodium hydroxide and was diluted to less than 10% with saline solution. Sulindac, pranoprofen, and acetaminophen were suspended with tragacanth gum. Vehicle or an NSAID—mefenamic acid (100 mg/kg) (Glazko, 1966), sulindac (5, 10, 50 mg/kg), pranoprofen (5, 10, 25 mg/kg), or acetaminophen (400, 500, 750 mg/kg) was administered perorally to each rat. After 30 min, methotrexate (40 mg/kg) (parenteral methotrexate 50mg; Wyeth K.K. Tokyo, Japan) was administered as a bolus by intraperitoneal injection (He et al., 1991), and water (3% of body weight) was given orally to obtain enough urine. Each rat was housed individually in a rat metabolic cage during the experiment.

Approximately 500  $\mu$ L of blood was collected via the subclavian vein under light ether anesthesia 0.5, 2, 5, 8, 12, and 24 h after intraperitoneal injection of methotrexate. Serum samples were obtained after centrifugation at  $5200 \times g$  for 10 min. Serum and urinary concentrations of methotrexate were determined by fluorescence polarization immunoassay using TDx (Abbott Japan co., Ltd, Tokyo, Japan). Concentrations of NSAIDs (mefenamic acid, sulindac, and pranoprofen) and acetaminophen were determined by high-performance liquid chromatography (HPLC).

Urine samples were collected 0–2, 2–5, 5–12, and 12–24 h after

intraperitoneal injection of methotrexate. Supernatant fluid was obtained after centrifugation at  $2400 \times g$  for 10 min. Methotrexate concentrations in urine were determined as in serum. The protocol(s) were approved by the institutional ethics committee

#### 2.4.1 HPLC condition

Serum concentrations of NSAIDs (mefenamic acid, sulindac, and pranoprofen) and acetaminophen were determined by the following HPLC method:

The chromatographic system (Jasco co., Tokyo, Japan) consisted of an 880-PU intelligent pump equipped with an 880-51 two-line degasser, 851-AS intelligent autosampler, 875-UV intelligent UV detector, and CO-965 column oven. The Capcell Pak MF Ph-1 column (5- $\mu\text{m}$  particle size, 150 $\times$ 4.6 mm ID; Shiseido co.,Ltd, Tokyo, Japan) was fitted with the C18 guard column (5  $\mu\text{m}$ , 3.9 $\times$ 20 mm; Waters association Inc., Milford, MA,USA) under 40°C. The mobile-phase flow rate was maintained at 0.5 ml/min for all drugs.

#### 2.4.2 Measurement of NSAIDs (Hirai et al., 1997)

The mobile phase was a mixture of acetonitrile and acetic acid (0.12%), 6:4 (v/v) for mefenamic acid and 4:6 (v/v) for sulindac and pranoprofen. The UV wavelength that determined mefenamic acid, sulindac, and pranoprofen was set at 340, 327, and 307 nm, respectively. A serum volume of 50  $\mu\text{L}$  was deproteinized by adding 150  $\mu\text{L}$  of acetonitrile and vortexing for 10 seconds. After centrifugation at  $12,000 \times g$  for 10 min in 4 °C, 20  $\mu\text{L}$  of supernatant was injected onto HPLC.

#### 2.4.3 Measurement of Acetaminophen (Dawson et al., 1988)

The mobile phase was a mixture of methanol and acetic acid (0.12%), 25:75 (v/v). The UV wavelength was set at 249 nm. The sample for injection was prepared with methanol in the same manner as that described for NSAIDs.

## **2.5 Statistical analysis**

Data were analyzed statistically by one-way analysis of variance followed by Scheffe's post hoc test. Levels of statistical significance were assessed using the *t*-test (vs. vehicle).

### **3. RESULTS**

#### **3.1 Inhibition of methotrexate accumulation by NSAIDs and acetaminophen in S2-hOAT3 cells**

We examined the inhibitory effects of various NSAIDs and acetaminophen on methotrexate accumulation using S2-hOAT3 cells. As shown in Fig. 1, most NSAIDs used— mefenamic acid, diclofenac sodium, sulindac, indomethacin, etodolac, ibuprofen, naproxen, pranoprofen, loxoprofen, piroxicam, and meloxicam—inhibited methotrexate accumulation mediated by hOAT3 in a concentration-dependent manner, but aspirin, salicylate, tiaramide, and acetaminophen did not inhibit methotrexate uptake into S2-hOAT3 cells. The  $K_i$  value for each drug was calculated by linear regression analysis with reciprocal of accumulation percent plot (Table 1). In addition, using  $K_i$  values, the degree of inhibition between methotrexate and NSAIDs was calculated in order to predict an inhibitory effect for each drug at therapeutic concentrations from the literature (Table 1).

Furthermore, to reveal the validity of  $K_i$  values which were calculated from the linear regression analysis, we performed kinetic analysis using Dixon plots (Dixon 1953). As shown in Fig. 2, mefenamic acid, sulindac, and pranoprofen inhibited [ $^3$ H]methotrexate accumulation in S2-hOAT3 cells in a competitive manner.  $K_i$  values for these three drugs were 29  $\mu$ M, 82  $\mu$ M, and 84  $\mu$ M, respectively.

#### **3.2 Interaction between methotrexate and NSAIDs in rats**

As shown in Fig. 3, serum methotrexate concentrations were significantly increased in proportion to the dose of NSAIDs. In mefenamic acid, the increase was significant by therapeutic concentration (100 mg/kg;  $P = 0.0091$ ). While it was significant only by supra-therapeutic concentrations in sulindac (5mg/kg;  $P = 0.9983$ ,

50 mg/kg;  $P = 0.0002$ ) and pranoprofen (5mg/kg;  $P = 0.9664$ , 25 mg/kg;  $P = 0.0207$ ) (Figs. 3 and 4). In all NSAIDs, the statistical differences became obvious when concentrations of NSAIDs were above the  $K_i$  values. On the other hand, acetaminophen which was not substrates of hOAT3 had little effect with more than 100 times of the therapeutic concentrations (750 mg/kg;  $P = 0.1238$ ) (Fig. 3).

The percentage of methotrexate excreted in urine over 24 h was not affected by any drugs, but the urinary excretion of methotrexate was significantly increased during the period of 5–24 h ( $P < 0.05$ ) when serum methotrexate concentrations were increased by combination with NSAIDs (mefenamic acid, sulindac, and pranoprofen) (Fig. 5). Most rats who received acetaminophen 750 mg/kg had anuria for 0–2 h, which might have caused the increase in urinary methotrexate over 2–5 h ( $P < 0.05$ ).

We examined urinary excretion of creatinine as indicator of glomerular filtration. As shown in Table 2, the urinary excretion of creatinine was not decreased with the addition of sulindac (50 mg/kg) although serum methotrexate concentrations were significant increased. Whereas, pranoprofen (5, 10 mg/kg) and acetaminophen (400, 500, 750 mg/kg), which had no effect on serum methotrexate concentrations, decreased the creatinine excretion in urine ( $P < 0.05$ ).

#### 4. DISCUSSION

Recently, many transporters that compete with methotrexate and NSAIDs have been identified (Masada et al., 1997; Takeuchi et al., 2000; Takeda et al., 2002; Nozaki et al., 2004; Uwai et al., 2004; El-Sheikh et al., 2007; Nozaki et al., 2007 b). In particular, inhibition of tubular methotrexate secretion mediated by hOAT3 is a proposed mechanism (Takeda et al., 2002; Uwai et al., 2004; Nozaki et al., 2007 b). To evaluate the safety of NSAIDs coadministered with methotrexate, we established an *in vitro* screening system using S2-hOAT3 cells in this study.

We examined the accumulation of [<sup>3</sup>H]methotrexate from the basolateral side into S2-hOAT3 cells. Aspirin, salicylate, tiaramide, and acetaminophen did not inhibit [<sup>3</sup>H]methotrexate uptake mediated by hOAT3, which indicates that these drugs are not substrates of hOAT3 (Fig. 1). Antipyrine, salicylate, and basicity drug groups did not compete with methotrexate, and the inhibitory effects of other NSAIDs were similar within the groups. Therefore, it is suggested that the interaction with methotrexate depends on a structural analogue.

Ki values were calculated for the NSAIDs that inhibited in a concentration-dependent manner. Moreover, the degrees of inhibition were predicted from maximum concentrations given in the literature (Table 1). Ki values calculated from linear regression analysis were changed by substrate concentrations, and these values were calculated with 26nM [<sup>3</sup>H]methotrexate (Table 1). On the other hand, the values can be calculated by Dixon plots (Dixon, 1953), which was not influenced by the substrate concentrations, and they were 29 μM for mefenamic acid, 82 μM for sulindac, and 84 μM for pranoprofen, respectively (Fig. 2). The Ki values obtained from the Dixon plots were closeness values to compare with linear regression analysis in this study. Therefore, the Ki and the degree of inhibition values calculated

through linear regression analysis with reciprocal of accumulation plot equation were satisfactorily appreciated.

The degree of inhibition for mefenamic acid, sulindac, and pranoprofen were 53%, 22%, and 17%, respectively. We presume that a drug at a concentration that is more than 50% of the degree of inhibition competes strongly with methotrexate and a drug at a concentration less than 50% of the degree of inhibition competes weakly. In addition, drugs that are not substrates of hOAT3, such as acetaminophen, do not interact with methotrexate.

We also determined pharmacokinetic interactions between methotrexate and NSAIDs, including acetaminophen, in rats at the MTX concentration during a high-dose therapy (He et al., 1991). Serum methotrexate concentrations were increased in proportion to NSAID concentrations. Furthermore, the statistical differences became obvious when concentrations of NSAIDs were more than the  $K_i$  values obtained by *in vitro* studies (Figs. 3 and 4). Thus, our *in vitro* data with hOAT3-expressing cells can be used in evaluating renal pharmacokinetic interactions in rats. This finding is compatible with a previous report that the character of OAT3 in humans is comparable to that in rats (Matsumoto et al., 2007). Our *in vitro* study may provide useful information when we consider the pharmacokinetic drug interaction in humans. Although aspirin and salicylate were not substrates of hOAT3 in the present study, it has been reported that aspirin interacts with methotrexate in humans (Furst et al., 1990). Further study is needed to address this apparent discrepancy.

Urinary excretion of methotrexate over 24 h was not statistically different when methotrexate was administered alone versus when it was combined with NSAIDs, including acetaminophen. These results are similar to those of a previous study

(Hwang et al., 1995). But excretion of methotrexate after 5 h was significantly increased with coadministration of NSAIDs (mefenamic acid; 100mg/kg, sulindac; 50mg/kg, and pranoprofen; 25mg/kg) (vs. vehicle;  $P < 0.05$ ) in our study (Fig. 5). Thus, the inhibitory effect was weakened when the serum NSAID concentrations were decreased. We think this is because that excretion of methotrexate in urine was strongly inhibited by high NSAID concentrations and the inhibition was weakened when NSAID concentrations were decreased.

In the case of humans, the percentage of methotrexate recovery in urine was reported to be almost 100% (Shen and Azarnoff, 1978). On the other hand, urinary recovery of methotrexate in rats was relatively low in our study ( $28.0 \pm 4.4\%$ ), which indicates that the majority of methotrexate accumulates in rat renal cells (Murray and Brater, 1993). Therefore, other transporter on apical side may also affect the urinary excretion of methotrexate in rats.

Until now, the mechanism of this interaction has been reported to be the competition for protein-binding sites and/or the reduction of the glomerular filtration rate of MTX by NSAIDs via inhibition of prostaglandin synthesis (Ahern et al., 1988; Tracy et al., 1992; Murray and Brater, 1993; Brouwers and de Smet, 1994; Brouwers and de Smet, 1994). It has been reported that protein binding of mefenamic acid is low (48.0%) (Glazko, 1966) whereas that of most other NSAIDs is high (>90%) (Lin et al., 1987). In addition, sulindac exerts a little inhibition on cyclooxygenase in the kidney (Sedor et al., 1984). However, in our study, serum methotrexate concentrations were similarly elevated by mefenamic acid, sulindac and pranoprofen when their concentrations were more than their  $K_i$  values. Thus, the effects on competition for protein-binding sites and inhibition of cyclooxygenase in the kidney

were small with regard to the mechanism of the pharmacokinetic interaction between methotrexate and NSAIDs. Furthermore, it was elucidated that the elevation of serum methotrexate concentrations was not correlated with the reduction of the glomerular filtration (Table 2). The present data suggest that the interaction of methotrexate with NSAIDs involves inhibition of the transporter in proximal renal tubules. In particular, competition at hOAT3 from the basolateral side into renal cells might be an important mechanism. In this study, NSAIDs dose levels were relatively higher than clinical dose in humans, because they were required dose in rats to reproduce serum NSAIDs concentrations in clinical practice owing to the distinction of formulation and bioactivity between rats and humans.

In conclusion, methotrexate and most NSAIDs are substrates of hOAT3 in S2-hOAT3 cells. The magnitude of the pharmacokinetic interaction between methotrexate and NSAIDs in rats can be speculated on, given the results of the accumulation study *in vitro*. Thus, when a therapeutic drug concentration is below the drug's  $K_i$  value, obtained by *in vitro* study, the interaction with methotrexate will be relatively small *in vivo*. Therefore, our accumulation study using S2-hOAT3 cells might be useful for screening potential interactions between methotrexate and new NSAIDs *in vivo*.

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Table 1. Ki value and degree of inhibition for each drug that inhibits [<sup>3</sup>H]methotrexate accumulation in S2-hOAT3 cells

S2-hOAT3 cells were incubated with solution containing [<sup>3</sup>H]methotrexate at a concentration 26 μM for 2hr at 37°C in the absence or presence of various drugs. Ki values represents a half of the accumulation compared with the absence of NSAIDs and the degree of inhibition(%) represents the inhibitory effect for [<sup>3</sup>H]methotrexate accumulation in the therapeutic drug concentrations of NSAIDs in comparison with the absence of NSAIDs (inhibitory effect 0).The values were determined by linear regression analysis from the [<sup>3</sup>H]methotrexate accumulation experiment at each NSAID concentration.

| Drugs          | Ki (μM) | Clinical Practice |           |                | Degree of Inhibition (%) |
|----------------|---------|-------------------|-----------|----------------|--------------------------|
|                |         | Dose (mg)         | Cmax (μM) | References     |                          |
| Acetaminophen  | 720     | 1000              | 28.58     | Sanaka, 1999   | N.D.                     |
| Aspirin        | 5000    | 650               | 52.8      | Siebert, 1983  | N.D.                     |
| Salicylate     | 2200    |                   | 328.3     | Siebert, 1983  | N.D.                     |
| Mefenamic acid | 36      | 250               | 41.44     | Glazko, 1966   | 53                       |
| Diclofenac Na  | 68      | 50                | 3.77      | Terhaag, 1991  | 13                       |
| Sulindac       | 53      | 200               | 11.36     | Kanfer, 1996   | 22                       |
| Indomethacin   | 56      | 50                | 11.74     | Caille, 1987   | 29                       |
| Etodolac       | 360     | 200               | 55.33     | Brater, 1989   | 16                       |
| Ibuprofen      | 230     | 600               | 274.38    | Pugh, 1984     | 51                       |
| Naproxen       | 110     | 500               | 415.18    | Caille, 1987   | 79                       |
| Pranoprofen    | 88      | 75                | 19.16     | Kajiyama, 1991 | 17                       |
| Loxoprofen     | 170     | 60                | 11.73     | Jhee, 2007     | 21                       |
| Piroxicam      | 74      | 20                | 9.05      | Olkkola, 1994  | 14                       |
| Meloxicam      | 81      | 30                | 5.09      | Busch, 1996    | 13                       |
| Tiaromide      | 450     | 200               | 4.33      | Klunk, 1981    | N.D.                     |

Table 2. Effects of NSAIDs and acetaminophen on creatinine excretion in urine.

| Drugs          | Dose (mg/kg) | Urinary creatinine (mg/h) |                        |                        |                        |
|----------------|--------------|---------------------------|------------------------|------------------------|------------------------|
|                |              | 0-2                       | 2-5                    | 5-12                   | 12-24 (h)              |
| mefenamic acid | vehicle      | 0.34±0.24                 | 0.33±0.09              | 0.32±0.06              | 0.40±0.04              |
|                | 100          | 0.29±0.08                 | 0.19±0.05 <sup>a</sup> | 0.22±0.07 <sup>a</sup> | 0.32±0.09              |
| sulindac       | vehicle      | 0.35±0.15                 | 0.27±0.09              | 0.24±0.07              | 0.37±0.06              |
|                | 50           | 0.31±0.10                 | 0.31±0.06              | 0.27±0.11              | 0.36±0.05              |
|                | 10           | 0.40±0.13                 | 0.14±0.02 <sup>a</sup> | 0.31±0.04              | 0.38±0.07              |
|                | 5            | 0.51±0.21                 | 0.20±0.10              | 0.31±0.10              | 0.41±0.05              |
| pranoprofen    | vehicle      | 0.34±0.10                 | 0.39±0.11              | 0.28±0.14              | 0.30±0.19              |
|                | 25           | 0.34±0.14                 | 0.26±0.04 <sup>a</sup> | 0.28±0.10              | 0.38±0.04              |
|                | 10           | 0.36±0.16                 | 0.25±0.05 <sup>a</sup> | 0.31±0.12              | 0.39±0.10              |
|                | 5            | 0.53±0.14 <sup>b</sup>    | 0.24±0.09 <sup>a</sup> | 0.29±0.08              | 0.39±0.09              |
| acetaminophen  | vehicle      | 0.43±0.17                 | 0.28±0.12              | 0.37±0.08              | 0.35±0.05              |
|                | 750          | 0.17±0.07 <sup>a</sup>    | 0.15±0.03 <sup>a</sup> | 0.12±0.08 <sup>a</sup> | 0.27±0.05 <sup>a</sup> |
|                | 500          | 0.40±0.18                 | 0.19±0.05              | 0.17±0.08 <sup>a</sup> | 0.29±0.08              |
|                | 400          | 0.39±0.14                 | 0.18±0.04              | 0.17±0.06 <sup>a</sup> | 0.32±0.03              |

Shown are means ± S.D. (n = 6). <sup>a</sup>P < 0.05 : significant low values compared with vehicle.

<sup>b</sup>P < 0.05 : significant high values compared with vehicle.

## FIGURE LEGENDS

Fig. 1. Effects of NSAIDs and acetaminophen on [<sup>3</sup>H]methotrexate accumulation in S2-hOAT3 cells. Shown are means ± S.D. (*n* = 3).

Fig. 2. Dixon plots for the effect of NSAIDs on [<sup>3</sup>H]methotrexate accumulation in S2-hOAT3 cells. Each plot shows means ± S.D. (*n* = 3). Data are expressed 1/accumulation (pmol/mg protein). [<sup>3</sup>H]methotrexate accumulation is shown for each drug concentration: 10 nM (▲), 50 nM (△), and 100 nM (●).

Fig. 3. Serum methotrexate concentration after intraperitoneal injection of methotrexate. Each plot shows means ± S.D. (*n* = 6). \**P* < 0.05, \*\**P* < 0.01 vs. vehicle.

Fig. 4. NSAID and acetaminophen concentrations in serum after intraperitoneal injection of methotrexate. Each plot shows means ± S.D. (*n* = 6).

Fig. 5. Percentage of methotrexate excreted in urine. Shown are means ± S.D. (*n* = 6). \**P* < 0.05, \*\**P* < 0.01: significant high values compared with vehicle. #*P* < 0.05: significant low values compared with vehicle.

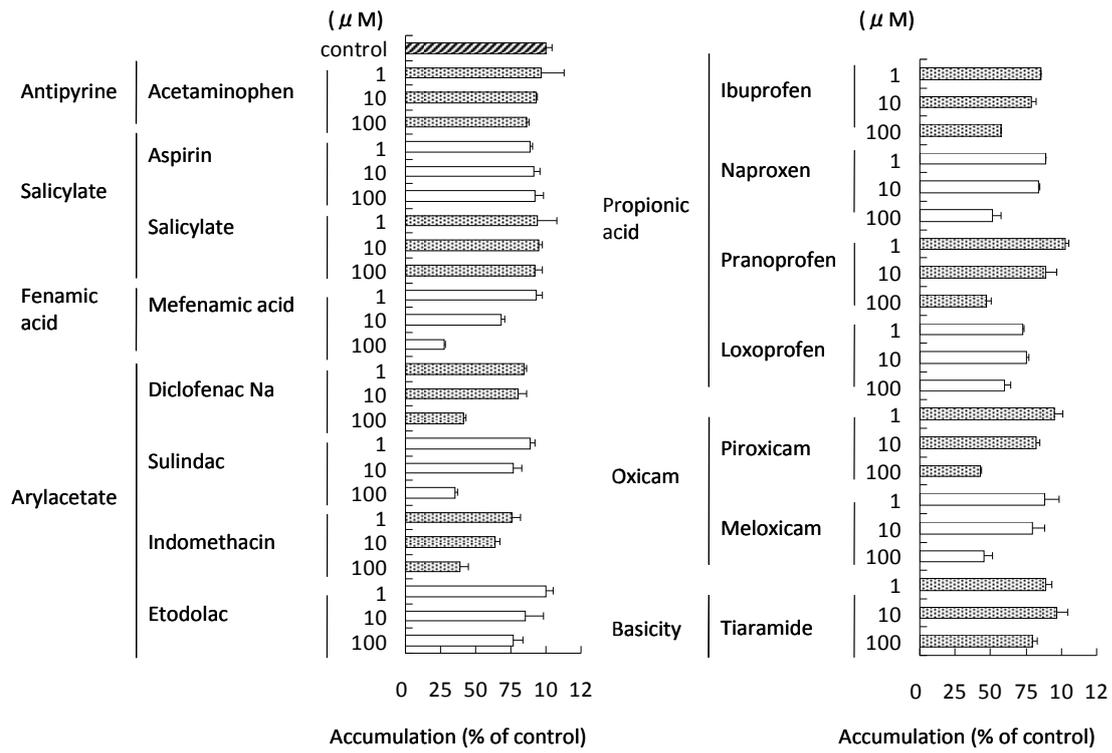


Fig.1

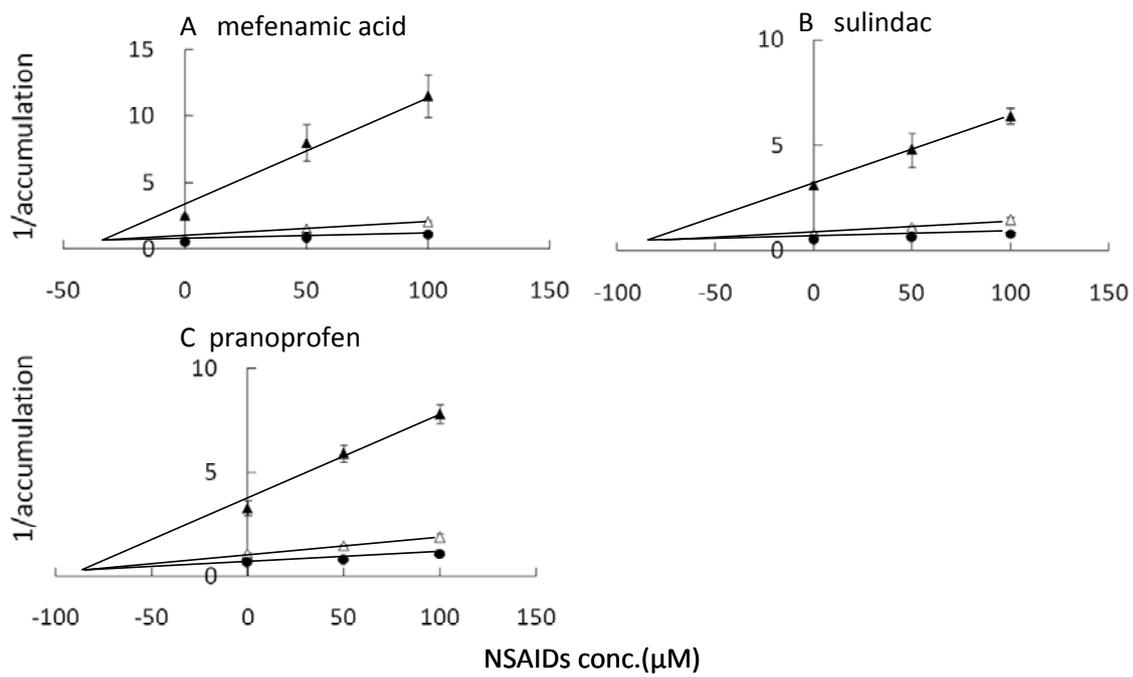


Fig.2

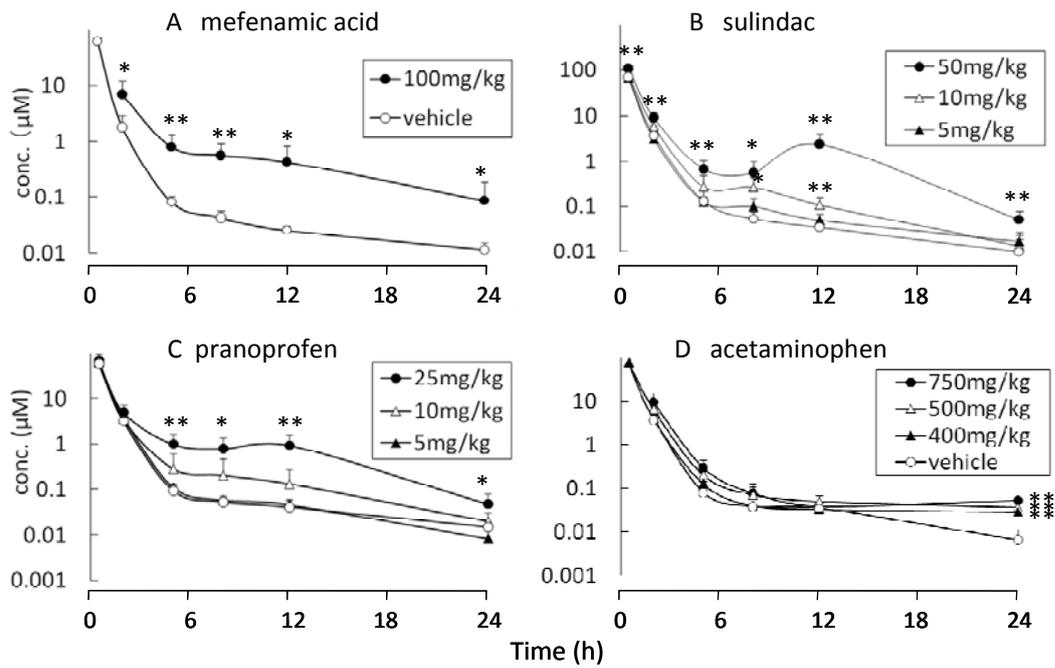


Fig.3

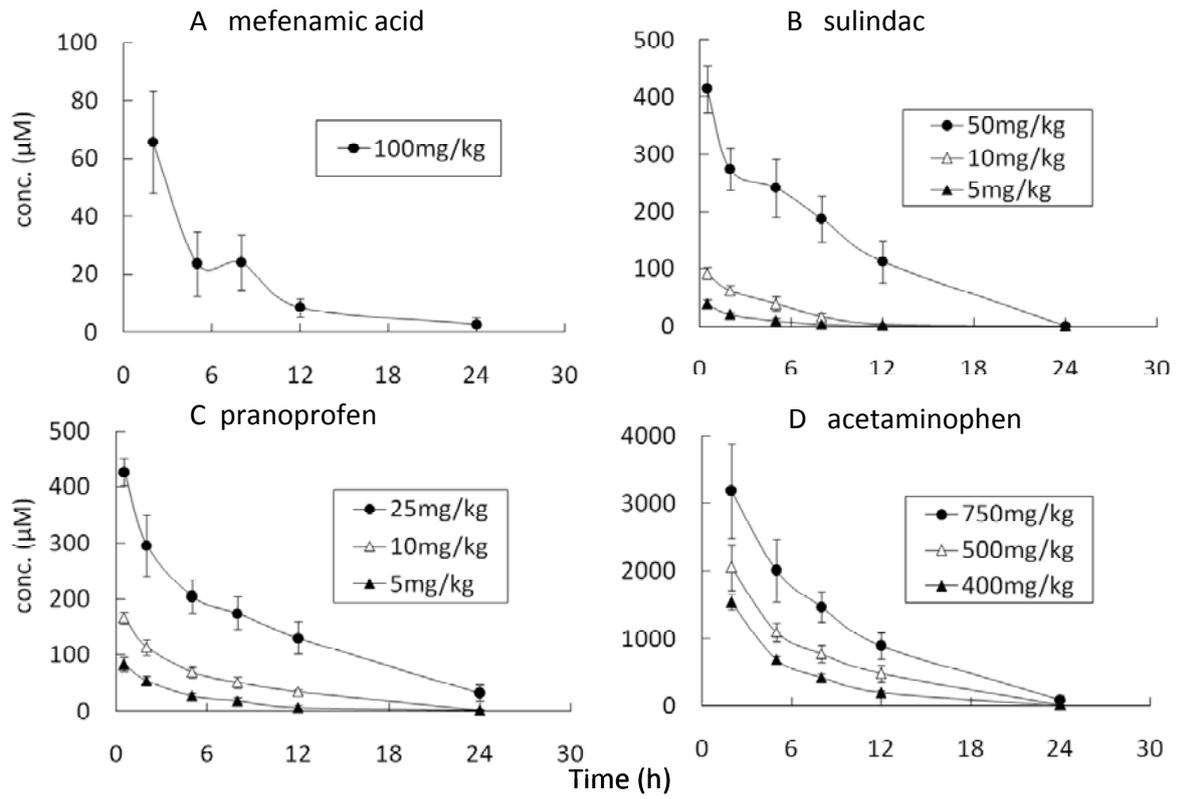


Fig.4

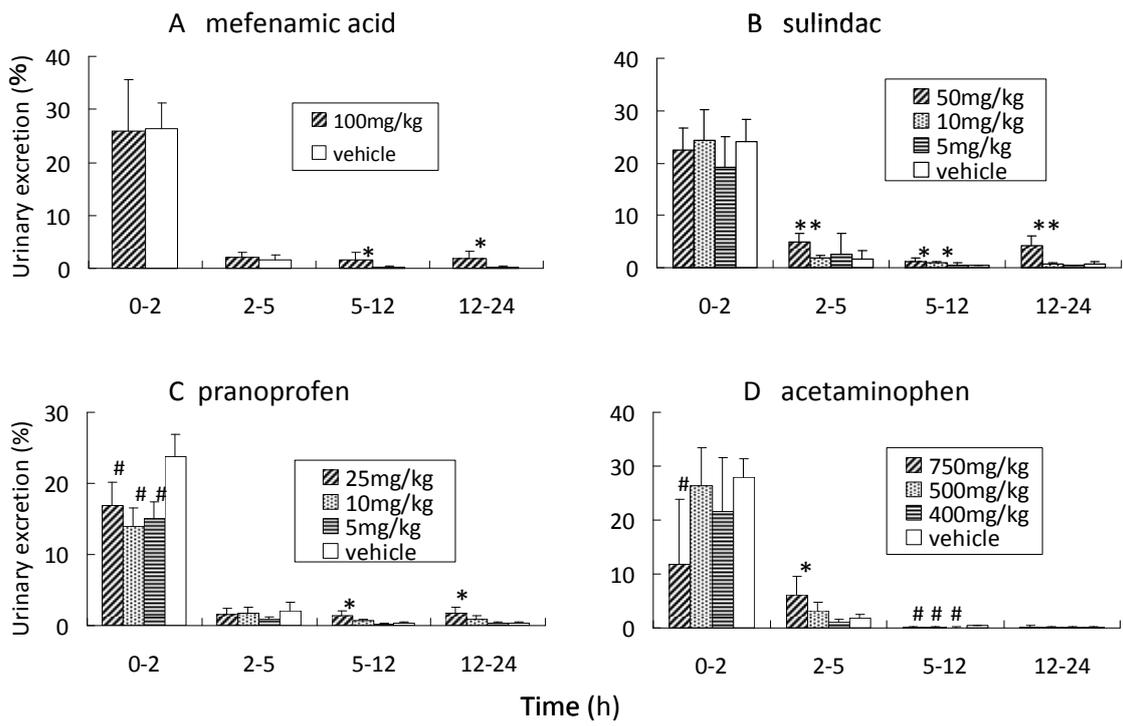


Fig.5