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Interaction between methotrexate and nonsteroidal anti-inflammatory drugs in organic anion transporter

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Abstract

The antifolate drug methotrexate is mainly eliminated from the body by renal tubular secretion via organic anion transporters. In clinical situations, severe methotrexate toxicity, due to an increase in serum concentrations, was observed after coadministration with nonsteroidal anti-inflammatory drugs (NSAIDs) or probenecid. In this study, we examined the effects of NSAIDs and probenecid on methotrexate transport via the rat renal organic anion transporter rOAT1, using *Xenopus laevis* oocytes. [^{3}H]Methotrexate uptake was markedly stimulated in the rOAT1 cRNA-injected oocytes, and this uptake was inhibited by probenecid and various NSAIDs, whereas the influence of salicylate was less. The Dixon plots showed that probenecid, indomethacin and salicylate competitively inhibited rOAT1 with apparent K_i values of 15.8 μM, 4.2 μM and 1.0 mM, respectively. These findings demonstrate that rOAT1 is the major site of the transporter-mediated interaction between methotrexate and NSAIDs and/or probenecid, leading to a decrease in renal excretion of methotrexate.

Keywords: OAT1 (organic anion transporter 1); *p*-Aminohippurate; Methotrexate; Probenecid; NSAID (nonsteroidal anti-inflammatory drug); Kidney; (Rat)

1. Introduction

Methotrexate is administered for the treatment of various malignancies in high doses or for the treatment of rheumatoid arthritis and psoriasis in low doses (Frei et al., 1975; Jackson, 1984; Bannwarth et al., 1996). The agent is mainly excreted in urine, almost entirely in a nonmetabolized form, and the renal tubular secretion of methotrexate by organic anion transporters is the main route for its elimination (Huffman et al., 1973; Liegler et al., 1969). It has been reported that probenecid, a potent inhibitor of renal organic anion transporters, increases the half-life and area under the curve (AUC) of the serum concentration of methotrexate, which are associated with the adverse effects of methotrexate (McLeod, 1998).

In the management of rheumatoid arthritis, methotrexate is given concomitantly with nonsteroidal anti-inflammatory drugs (NSAIDs) (Bannwarth et al., 1996). However, there are several clinical observations that suggest that coadministration of these drugs induces severe methotrexate toxicity such as bone marrow depression, mucositis, hepatitis or renal insufficiency (Maiche, 1986; Thyss et al., 1986). Possible mechanisms for the toxic effects due to their simultaneous administration are that unbound serum levels of methotrexate are increased by displacement from protein binding sites and that the inhibition of prostaglandin synthesis by NSAIDs leads to decreased renal clearance of methotrexate (Bannwarth et al., 1996). Nierenberg (1983) showed that NSAIDs inhibit methotrexate uptake in rabbit kidney slices. Among these mechanisms, the inhibition of renal tubular secretion of methotrexate by NSAIDs has been suggested to be the most potent (Nierenberg, 1983; Statkevich et al., 1993).

Methotrexate causes the acute nephrotoxicity as a result of its intratubular precipitation (El-Badawi et al., 1996). In addition, the Rheumatoid Arthritis Clinical Trial Archive Group (1995) reported that the decline of renal function due to age or renal insufficiency is a potent risk for

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development of methotrexate nephrotoxicity. Despite methotrexate-induced nephrotoxicity being related to its renal handling, the precise mechanism for tubular secretion and/or reabsorption of methotrexate remains to be solved. Therefore, it is important to elucidate the detailed renal handling of methotrexate in order to avoid drug–drug interactions or renal failure caused by methotrexate.

To date, the transporters that principally mediate renal tubular secretion of methotrexate have not been identified. However, according to the studies by Nierenberg (1986) and Besseghir et al. (1989), methotrexate could be taken up by renal tubular cells via the transport system mediating *p*-aminohippurate uptake. In fact, the cloned rat kidney organic anion transporter OAT1 has been suggested to recognize methotrexate as a substrate (Sekine et al., 1997; Uwai et al., 1998).

In the present study, we investigated the inhibitory effects of NSAIDs and probenecid on rOAT1-mediated methotrexate transport, using the *Xenopus laevis* oocyte expression system, to obtain information on the molecular aspects of the mechanisms of the NSAID-induced increase in serum methotrexate levels. The findings of the present study suggest that rOAT1 is the site where methotrexate interacts with NSAIDs or probenecid, leading to its side effects.

2. Materials and methods

2.1. Materials

p-[Glycyl-^{14}C]aminohippurate (1.9 GBq/mmol) and [3′,5′,7-^{3}H(N)]methotrexate (991.6 GBq/mmol) were purchased from Du Pont-New England Nuclear Research Product (Boston, MA, USA) and Moravek Biochemicals, (Brea, CA), respectively. Phenylbutazone, salicylate and unlabeled methotrexate (L-(+)-amethopterin) were obtained from Nacalai Tesque (Kyoto, Japan). Flufenamate, ibuprofen, indomethacin and ketoprofen were from Wako (Osaka, Japan). Other chemicals of the highest purity were purchased from Sigma (St. Louis, MO, USA).

2.2. Functional expression of rOAT1 in Xenopus laevis oocytes

The capped cRNA of rOAT1 was transcribed from *Not*I-linearized pSPORT containing rOAT1 cDNA with T7 RNA polymerase, and injected into *Xenopus* oocytes as described previously (Uwai et al., 1998). After 50 nl of water or rOAT1 cRNA (25 ng) was injected into each oocyte, they were maintained in modified Barth's medium (88 mM NaCl, 1 mM KCl, 0.33 mM $Ca(NO_3)_2$, 0.4 mM $CaCl_2$, 0.8 mM $MgSO_4$, 2.4 mM $NaHCO_3$, 10 mM HEPES) at 18°C.

2.3. p-[^{14}C]aminohippurate and [^{3}H]methotrexate uptake reaction

Three days after the injection of water or rOAT1 cRNA, the uptake reaction was initiated in a 24-well plate by incubating the oocytes in 500 μl of uptake buffer (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM HEPES; pH 7.4) containing *p*-[^{14}C]aminohippurate and [^{3}H]methotrexate at 25°C in the presence or absence of an inhibitor. Flufenamate, ibuprofen, indomethacin, ketoprofen and phenylbutazone were dissolved in uptake buffer containing 0.7% ethanol. The uptake reaction was terminated by adding 2 ml of ice-cold uptake buffer to each well, and the oocytes were washed five times with 2 ml of the buffer. After being washed, each oocyte was transferred to a scintillation counting vial and solubilized in 500 μl of 10% sodium lauryl sulfate. Five milliliters of ACSII (Amersham International, Buckinghamshire, UK) was added to each solubilized oocyte, and the radioactivity was determined in a liquid scintillation counter.

2.4. Statistical analysis

Data were analyzed statistically with analysis of variance, followed by Fisher's *t*-test for multiple comparisons.

3. Results

3.1. Interaction of methotrexate and probenecid with rOAT1

Fig. 1 shows rOAT1-mediated uptake of a typical rOAT1 substrate, *p*-[^{14}C]aminohippurate, and [^{3}H]metho-

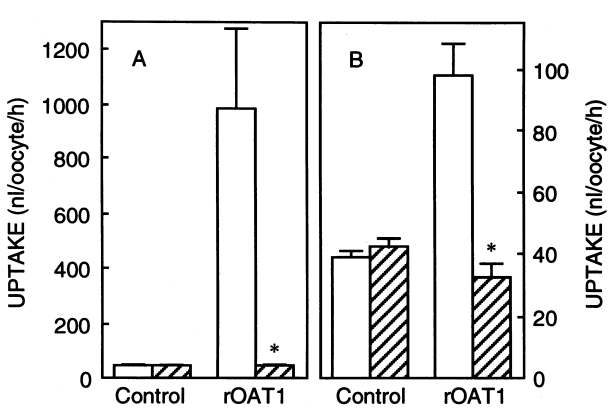

Fig. 1. Uptake of *p*-[^{14}C]aminohippurate (A) or [^{3}H]methotrexate (B) by rOAT1-expressing *Xenopus* oocytes. Water- or rOAT1 cRNA-injected oocytes were individually incubated with *p*-[^{14}C]aminohippurate (25 μM) or [^{3}H]methotrexate (1 μM) in the absence (open column) or presence of 1 mM probenecid (hatched column) for 1 h. Control means water-injected oocytes. Each column represents the mean ± S.E. of uptake clearance of *p*-[^{14}C]aminohippurate or [^{3}H]methotrexate in 10 oocytes. $^{*}P < 0.01$, significantly different from uptake without probenecid.

trexate uptake. Uptake of the two compounds was markedly stimulated in rOAT1 cRNA-injected oocytes. The rOAT1-mediated uptake clearance of *p*-aminohippurate was about 15-fold higher than that of methotrexate. The uptake of both compounds was markedly inhibited by the potent organic anion transport system inhibitor, probenecid.

Next, to determine the affinity of probenecid and methotrexate for rOAT1, we induced the dose-dependent inhibition of rOAT1-mediated *p*-aminohippurate uptake using the two drugs. In the presence of 1 mM probenecid, *p*-aminohippurate uptake by rOAT1 was completely inhibited, whereas 3 mM methotrexate moderately inhibited the uptake. The estimated inhibition constants (IC_{50}) for *p*-aminohippurate uptake were determined as 18.6 μM for probenecid and 926.1 μM for methotrexate by nonlinear regression analysis of the competition curves with one component (Fig. 2).

Since we previously showed that probenecid was not transported by rOAT1 (Uwai et al., 1998), the inhibition mode of probenecid for rOAT1 was assessed by Dixon plot analysis. As shown in Fig. 3, probenecid inhibited rOAT1-mediated *p*-aminohippurate uptake in a competitive fashion with an apparent K_i value of 15.8 μM.

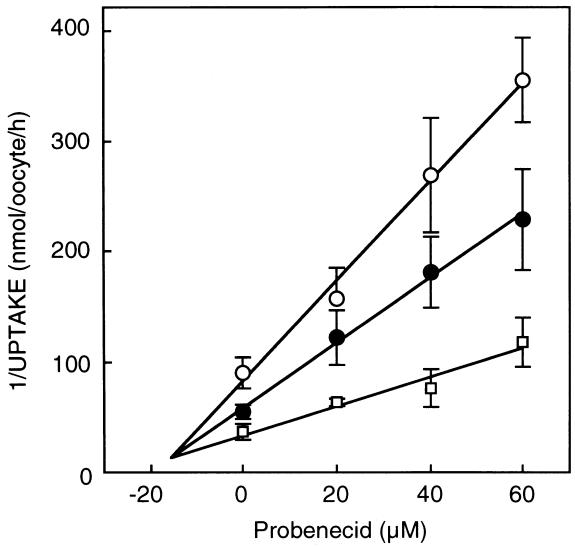

Fig. 3. Dixon plots for the inhibitory effect of probenecid on *p*-[^{14}C]aminohippurate uptake by rOAT1. rOAT1-expressing oocytes were incubated with *p*-[^{14}C]aminohippurate at 12.5 (○), 25 (●), or 50 μM (□) with 0–60 μM probenecid for 1 h. The level of *p*-[^{14}C]aminohippurate uptake was determined. Values are expressed as 1/uptake (nmol/oocyte/h). Each point represents the mean ± S.E. for 6–10 oocytes.

3.2. Inhibitory effects of NSAIDs on rOAT1-mediated [^{3}H]methotrexate uptake

Fig. 4 shows [^{3}H]methotrexate uptake by oocytes injected with water or rOAT1 cRNA in the absence or presence of indomethacin and ketoprofen. The rOAT1-mediated [^{3}H]methotrexate uptake increased linearly up to 2 h. The [^{3}H]methotrexate uptake was markedly inhibited by the presence of 1 mM indomethacin or ketoprofen. As shown in Fig. 5, flufenamate, ibuprofen and phenylbutazone as well as indomethacin and ketoprofen significantly inhibited rOAT1-mediated methotrexate uptake, whereas 1 mM salicylate had no effect.

Next, we determined the dose dependence of the inhibitory effects of indomethacin, ketoprofen and salicylate on methotrexate uptake by rOAT1. The estimated inhibi-

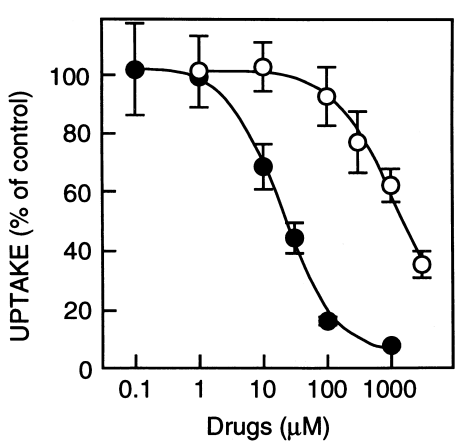

Fig. 2. Dose-dependent inhibition of *p*-[^{14}C]aminohippurate uptake by methotrexate and probenecid in rOAT1-expressing oocytes. rOAT1-expressing oocytes were incubated with *p*-[^{14}C]aminohippurate (25 μM) in the absence (control) or presence of methotrexate (○) or probenecid (●) at various concentrations for 1 h. Each point represents the mean ± S.E. of *p*-[^{14}C]aminohippurate uptake in 11–20 oocytes from two separate experiments.

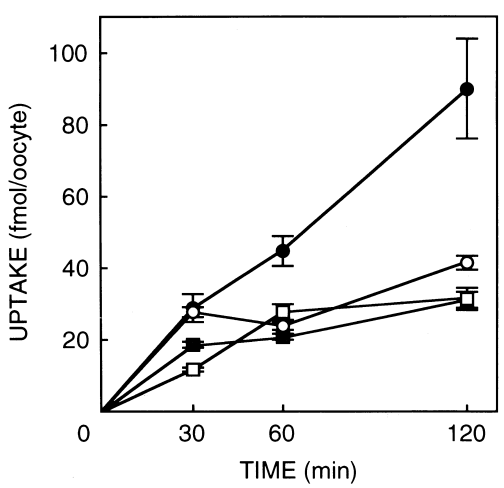

Fig. 4. Effect of indomethacin and ketoprofen on time-dependent uptake of [^{3}H]methotrexate by rOAT1-expressing oocytes. Water-injected (○) or rOAT1-expressing oocytes were incubated with 1 μM [^{3}H]methotrexate in the absence (●) or presence of 1 mM indomethacin (□) or ketoprofen (■) for the indicated periods. Each point represents the mean ± S.E. for 7–10 oocytes.

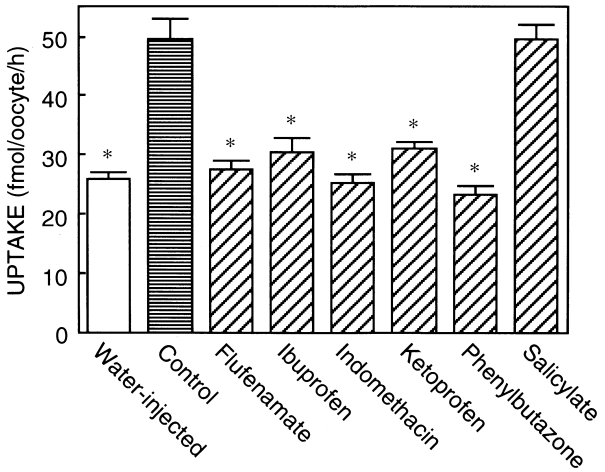

Fig. 5. Effect of various NSAIDs on [^{3}H]methotrexate uptake by rOAT1-expressing oocytes. rOAT1-mediated [^{3}H]methotrexate uptake for 1 h was determined by incubating oocytes with [^{3}H]methotrexate (1 μM) in the absence or presence of the drug at 1 mM. Control represents rOAT1 cRNA-injected oocytes incubated without an inhibitor. Water-injected means water-injected oocytes. Each column represents the mean ± S.E. of [^{3}H]methotrexate uptake in 7–10 oocytes. * $P < 0.05$, significantly different from control.

tion constants (IC_{50}) for methotrexate uptake were 2.7 μM for indomethacin, 0.5 μM for ketoprofen and 1410 μM for salicylate (Fig. 6).

Finally, we performed a Dixon plot analysis to determine the inhibitory mode of indomethacin and salicylate on rOAT1-mediated methotrexate uptake. Both drugs inhibited rOAT1 competitively, and the apparent K_i values

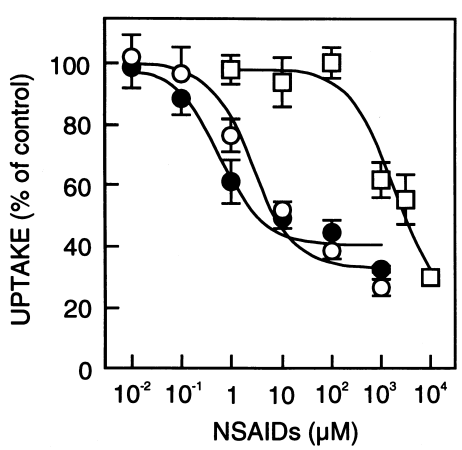

Fig. 6. Dose-dependent inhibition of [^{3}H]methotrexate uptake by indomethacin, ketoprofen and salicylate with rOAT1-expressing oocytes. rOAT1-expressing oocytes were incubated with [^{3}H]methotrexate (1 μM) in the absence (control) or presence of indomethacin (○), ketoprofen (●) and salicylate (□) at various concentrations for 2 h. Each point represents the mean ± S.E. of [^{3}H]methotrexate uptake in 9–20 oocytes from two separate experiments.

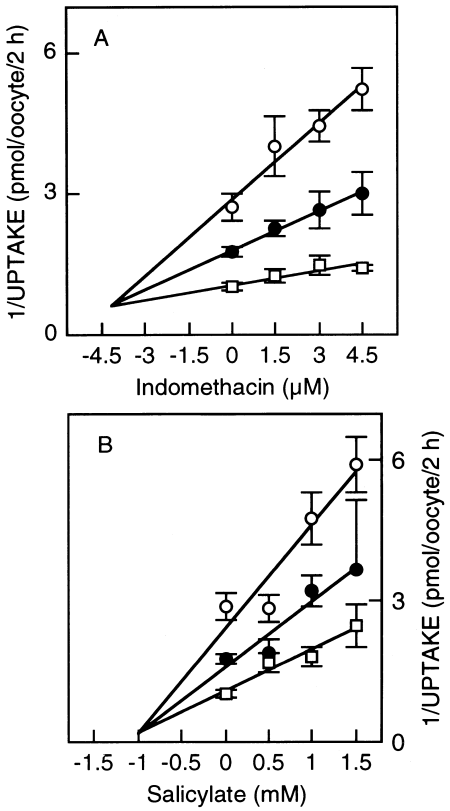

Fig. 7. Dixon plots for the inhibitory effects of indomethacin (A) and salicylate (B) on [^{3}H]methotrexate uptake by rOAT1. rOAT1-expressing oocytes were incubated with [^{3}H]methotrexate at 1 (○), 2 (●), or 4 μM (□) with the two NSAIDs at the indicated concentrations for 2 h. The level of [^{3}H]methotrexate uptake was determined and the figures were drawn after subtraction of the uptake in water-injected oocytes from that in rOAT1 cRNA-injected oocytes. Values are expressed as 1/uptake (pmol/oocyte/2 h). Each point represents the mean ± S.E. for 4–10 oocytes.

were estimated to be 4.2 μM for indomethacin and 1.0 mM for salicylate (Fig. 7).

4. Discussion

The main elimination route of methotrexate is considered to be renal tubular secretion by organic anion transporters in the proximal tubules (Liegler et al., 1969). Previous studies suggested that rOAT1 is the major transporter for *p*-aminohippurate uptake from blood to renal epithelial cells at the basolateral membranes of proximal tubular cells (Sekine et al., 1997; Sweet et al., 1997; Uwai et al., 1998). Nierenberg (1986) suggested that the *p*-aminohippurate transport pathway in proximal tubule is identical to the pathway for methotrexate by comparing K_i values of phenylbutazone, probenecid, chlorothiazide, indomethacin and salicylate for uptake of *p*-aminohippurate and methotrexate. Moreover, using rabbit isolated proximal tubules, it was reported that the accumulation of methotrexate in the proximal S2 segment was higher than that in the S1 segment and the S3 segment (Besseghir et

al., 1989). The accumulation pattern of methotrexate in proximal tubules corresponds to the distribution of rOAT1 expression in proximal tubules (Tojo et al., 1999). Taken together with these data, rOAT1 has been suggested to play a main role in the elimination of methotrexate from blood.

Methotrexate and NSAIDs have often been coadministered to patients affected by rheumatoid arthritis (Bannwarth et al., 1996). There are, however, several clinical reports showing that simultaneous administration is associated with striking enhancement of serum methotrexate levels and resultant severe toxicity (Maiche, 1986; Thyss et al., 1986). Nierenberg (1983) reported that methotrexate uptake in rabbit kidney slices was inhibited by various NSAIDs as well as by the typical organic anions, *p*-aminohippurate and probenecid. Statkevich et al. (1993) demonstrated that tubular clearance of methotrexate was depressed by the concomitant administration of indomethacin or flurbiprofen in the isolated perfused rat kidney. Based on these findings, it is suggested that NSAIDs have potent inhibitory effects on rOAT1-mediated methotrexate uptake, leading to the side effects of methotrexate. To clarify the rOAT1-mediated interaction between methotrexate and NSAIDs, we examined the interactions of these drugs at the molecular level using rOAT1-expressing oocytes. Methotrexate transport via rOAT1 was strongly inhibited by flufenamate, ibuprofen, indomethacin, ketoprofen and phenylbutazone (Figs. 4 and 5). As shown in Fig. 6, the inhibition constants (IC_{50}) of methotrexate uptake were estimated to be 2.7 μM for indomethacin and 0.5 μM for ketoprofen. It is noteworthy that pharmacologically effective blood concentrations of indomethacin and ketoprofen are in the range of several micromolars, suggesting that the two NSAIDs could be potent inhibitors of rOAT1 in vivo. Taylor and Halprin (1977) reported that indomethacin did not influence the protein binding of methotrexate at clinically used concentrations. According to these findings, it is suggested that the dominant mechanism for the marked increase in serum methotrexate concentration caused by indomethacin could be the inhibition of renal tubular uptake of methotrexate via rOAT1.

The clinical serum concentration of salicylate ranges from several hundred micromolars to several millimolars, which is reported to influence not only the renal clearance of methotrexate but also its protein binding (Liegler et al., 1969; Taylor and Halprin, 1977). The inhibition constant of salicylate for rOAT1-mediated methotrexate uptake was 1410 μM, indicating the much lower affinity of salicylate for rOAT1 compared with the affinity of indomethacin or ketoprofen (Fig. 6). Accordingly, it is reasonable to assume that the toxic effects of methotrexate, when used concomitantly with salicylate, may be caused by mixed phenomena with both an enhancement of unbound methotrexate due to displacement of protein binding and a decrease in its renal uptake via rOAT1. The mechanisms of the toxicity of methotrexate when given together with salicylate or other NSAIDs may be different, and the difference in their affinity for rOAT1 could be one of the mechanisms (Fig. 6). Consequently, rOAT1 is thought to be a potential site for the inhibitory interaction of methotrexate with NSAIDs in the kidney.

Masuda et al. (1997) showed that methotrexate accumulates extensively in the rat kidney after intravenous administration. The accumulation in the kidney cortex was approximately two-fold higher than that in the kidney medulla, which is considered to be associated with rOAT1 distribution along the nephron (Tojo et al., 1999). In the kidney cortex, [^{3}H]methotrexate accumulation was strongly inhibited by co-injection of unlabeled methotrexate, indomethacin or ketoprofen (Masuda et al., 1997); however, in the kidney medulla, their inhibitory effects were much less potent. Therefore, it can be assumed that other types of organic anion transporters are involved in methotrexate uptake in the kidney medulla. The rOAT2 has been shown to transport methotrexate (Sekine et al., 1998). However, the renal distribution and a detailed kinetic analysis of methotrexate transport via rOAT2 remain to be resolved.

Probenecid is a typical inhibitor of renal organic anion transporters. The elimination of various agents, which are secreted via renal organic anion transporters, is delayed by the simultaneous administration of probenecid. Although probenecid has clinically important characteristics, the interaction of probenecid with rOAT1 has not been examined in detail. Previously, we demonstrated that probenecid was not transported by rOAT1 (Uwai et al., 1998). In this study, probenecid inhibited rOAT1 in a competitive manner and its K_i value was 15.8 μM (Fig. 3). Taking these findings into consideration, probenecid has potent binding affinity for rOAT1, but it is not translocated by the transporter. The high lipophilicity of probenecid might prevent the translocation step due to slow dissociation from the binding site.

In conclusion, we showed the interaction of methotrexate, NSAIDs and probenecid via rOAT1. These findings suggest that rOAT1 is the main site in the kidney, where methotrexate interacts with NSAIDs or probenecid, leading to its severe toxicity.

Acknowledgements

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