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Structure–affinity relationship in the interactions of human organic anion transporter 1 with caffeine, theophylline, theobromine and their metabolites

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Abstract

It is well known that human organic anion transporter 1 (hOAT1) transports many kinds of drugs, endogenous compounds, and toxins. However, little is known about the structure–affinity relationship. The aim of this study was to elucidate the structure–affinity relationship using a series of structurally related compounds that interact with hOAT1. Inhibitory effects of xanthine- and uric acid-related compounds on the transport of *p*-aminohippuric acid were examined using CHO-K1 cells stably expressing hOAT1. The order of potency for the inhibitory effects of xanthine-related compounds on PAH uptake was 1-methyl derivative > 7-methyl derivative > 3-methyl derivative ≒ xanthine > 1,3,7-trimethyl derivative (caffeine). The order of potency of the inhibition was 1,3,7-trimethyluric acid > 1,3-dimethyluric acid > 1,7-dimethyluric acid > 1-methyluric acid > uric acid. A significant correlation between inhibitory potency and lipophilicity of the tested uric acid-related compounds was observed. The main determinant of the affinity of xanthine-related compounds is the position of the methyl group. On the other hand, lipophilicity is the main determinant of the affinity of uric acid-related compounds.

Keywords: hOAT1; Caffeine; Theophylline; Structure–affinity relationship; Interaction

1. Introduction

Human organic anion transporters (hOATs) play an important role in the urinary excretion and reabsorption of drugs [1–9], endogenous compounds [10–12], and toxins [13]. The isoforms predominantly expressed in the kidney are hOAT1, hOAT3, and hOAT4 [14,15]. hOAT1 and hOAT3 are localized in the basolateral membrane of the proximal tubules in the cortex [14]. On the other hand, hOAT4 is localized in the apical membrane [16]. Therefore, it has been suggested that hOAT1 and hOAT3 contribute to the uptake of organic anions across the basolateral membrane of epithelial cells, which is the first step in renal excretion of many anionic drugs and endogenous anions.

In 1999, hOAT1 was cloned with two splice variants, hOAT1-1 and hOAT1-2 [17,18]. Five transcript variants have so far been identified. Although OAT1 was first characterized as a *p*-aminohippuric acid (PAH) transporter, it is now known to be a multispecific transporter that takes up a variety of organic anions with different chemical structures. Recently, it has been reported that hOAT1 transports not only organic anions such as nonsteroidal anti-inflammatory drugs [1] and diuretics [4], but also drugs without any anionic moieties such as tetracycline, acyclovir, ganciclovir, zidovudine, and cimetidine [6,8,9]. These results suggest that an anionic moiety is not essential for substrate recognition of hOAT1. Moreover, although many drugs and endogenous compounds are known as substrates of hOAT1, the structure–affinity relationship has not been clarified. The aims of this study were (1) to discover new drugs that interact with hOAT1 and (2) to elucidate a structure–affinity relationship using

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a series of structurally related compounds that interact with hOAT1 by means of cells that heterologously express hOAT1.

2. Materials and methods

2.1. Materials

p-[glycyl-2-^{3}H]-Aminohippuric acid (156 GBq/mmol) was purchased from Perkin Elmer Life Sciences (Boston, MA). *p*-Aminohippuric acid, hypoxanthine, 3-methylxanthine, caffeine, allopurinol, alloxanthine and sodium butyrate were obtained from Wako Pure Chemicals (Osaka, Japan). Purine was obtained from Nacalai Tesque (Kyoto, Japan). Xanthin sodium salt, 1-methylxanthine, 1,7-dimethylxanthine, uric acid sodium salt, 1-methyluric acid, 1,3-dimethyluric acid, 1,7-dimethyluric acid, 1,3,7-trimethyluric acid, probenecid, Nutrient Mixture F-12 Ham, penicillin (10,000 units/ml)/streptomycin (10 mg/ml) solution, and Geneticin were purchased from Sigma (St. Louis, MO). Theophylline and theobromine were obtained from Tokyo Chemical Industry (Tokyo, Japan). CHO-K1 cells (JCRB9018), a Chinese hamster ovary cell line, were obtained from Health Science Research Resources Bank (Osaka, Japan). Fetal bovine serum (FBS) was obtained from ICN Biochemicals (Aurora, OH). *Xba*I and *Xho*I were purchased from TaKaRa Bio. (Tokyo, Japan). All other chemicals were of the highest grade available.

2.2. Establishment of the cells stably expressing hOAT1

A full-length clone of hOAT1 was generated by RT-PCR from human kidney total RNA using TaKaRa RNA LA PCR Kit (TaKaRa, Tokyo, Japan) according to the manufacture's instructions. Forward primer (5′-ATATATCGACTCGAGCCATGGCCTTTAATGACCTCCT-3′) contains *Xho*I site and reverse primer (5′-ATATATCGATCTAGATGTGGTTCTGGTGGGGTTTAT-3′) contains *Xba*I site. Amplification was performed for 40 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 90 s. The PCR fragments obtained were purified and subcloned into pGEM-T Easy vector (Promega, Leiden, The Netherlands). Sequence was analyzed using an ABI310 sequencer (Applied Biosystems, Foster City, CA). The coding sequence was identical to the published sequence of hOAT1-2 (SLC22A6, transcript variant 2). The hOAT1 cDNA fragment was obtained by digestion of a pGEM-T Easy-hOAT1 cDNA construct with *Xho*I and *Xba*I. The fragment was subcloned into pcDNA3.1(+) (Invitrogen, Carlsbad, CA). The expression construct was transfected into CHO-K1 cells using Lipofectamine transfection reagent (Invitrogen), and stable transformants were isolated by selection with 600 mg/ml Geneticin. A stable transformant transfected with pcDNA3.1(+) (no insert) was used as a control. Cells were grown in Nutrient Mixture F-12 Ham supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin.

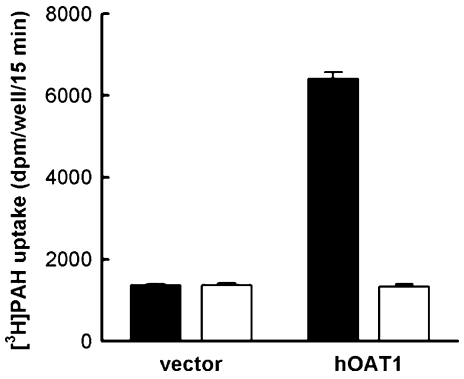

Fig. 1. Uptake of [^{3}H]PAH into vector-transfected and hOAT1 cDNA-transfected cells in the absence (■) or presence (□) of 1 mM probenecid. The cells were incubated with 0.24 μM [^{3}H]PAH for 15 min at room temperature. Each column represents the mean with S.E. of three measurements.

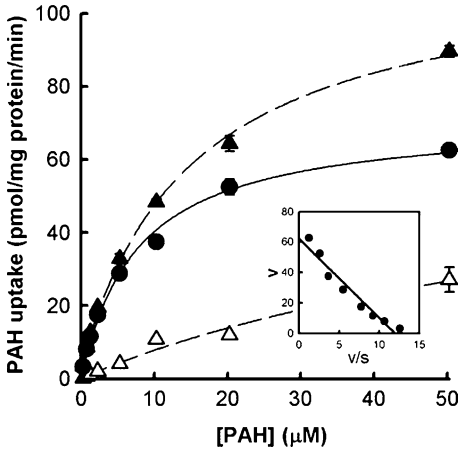

Fig. 2. Saturation kinetics of PAH transport. Transport of PAH (0.26–50 μM) was measured in vector-transfected cells (△) and hOAT1 cDNA-transfected cells (▲) at room temperature for 1 min. hOAT1-specific transport (●) was calculated by subtracting the value of vector-transfected cells from the value of hOAT1 cDNA-transfected cells. Each value represents the mean ± S.E. of four measurements. Inset, Eadie–Hofstee plot.

2.3. Uptake of PAH by hOAT1-expressing cells

hOAT1- and vector-transfected cells were seeded in 24-well tissue culture plates at a density of 5×10^4 cells/well. The culture medium was replaced with a medium supplemented with 5 mM sodium butyrate 24 h before transport studies to induce the expression of hOAT1 according to the method of Hasegawa et al. [19], who studied about rOat1 and rOat3. In this study, CHO-K1 cells between the 6th and 10th passages were used. Uptake experiments were carried out as described previously [20] with some modifications. The transport medium consisted of 137 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.8 mM $MgSO_4$, 0.4 mM H_2PO_4, 0.3 mM Na_2HPO_4, 25 mM D-glucose, and 10 mM HEPES/Tris

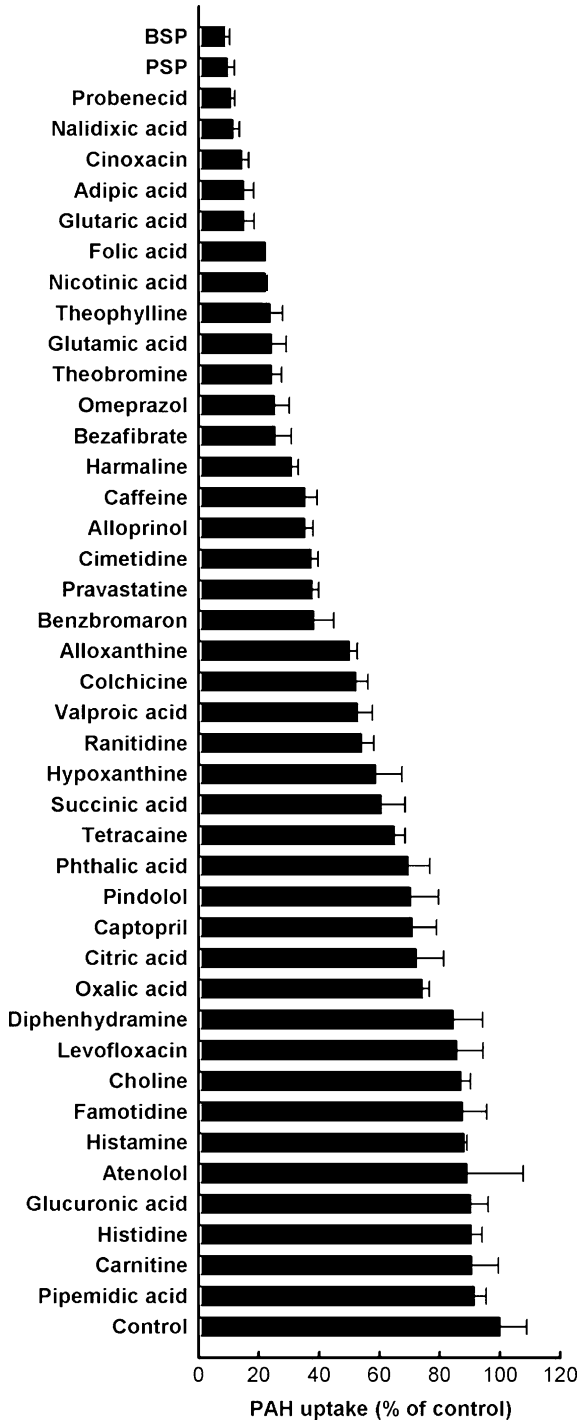

Fig. 3. Inhibitory effects of various drugs on the uptake of PAH by hOAT1-expressing cells. The cells were incubated with 0.24 μM [^{3}H]PAH for 1 min at room temperature in the presence or absence of 1 mM of a drug. Because of their low solubility, benzbromarone, bezafibrate, and omeprazole were used as saturated solutions. Each column represents the mean with S.E. of three or four measurements.

(pH 7.5). After washing the cells with 1 ml of the transport buffer, uptake was started by adding 0.25 ml of the substrate solution. The temperature for the transport was room temperature. Transport was terminated by aspiration of the substrate solution followed by washing twice with 1 ml of ice-cold transport medium. The cells were lysed with 0.25 ml of 1% SDS in 0.2 M NaOH, and the radioactivity was measured. A small portion of the cell lysate was used for the determination of protein concentration. Uptake values are expressed as pmol/mg protein.

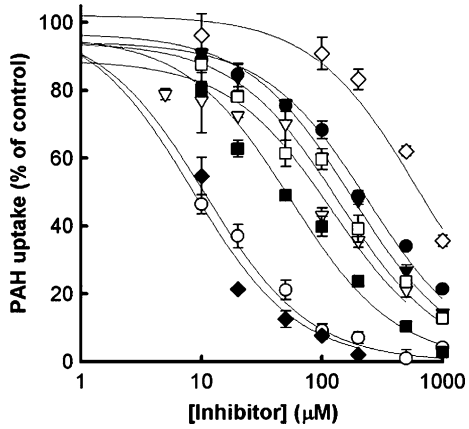

Fig. 4. Dose–response relationships of the inhibition of hOAT1-mediated transport of PAH by xanthine-related compounds (●, xanthine; ○, 1-methylxanthine; ▼, 3-methylxanthine; ▽, 7-methylxanthine; ■, theophylline; □, theobromine; ◆, 1,7-dimethylxanthine; ◇, caffeine). Transport of 0.24 μM labeled PAH into vector- or hOAT1 cDNA-transfected cells was measured for 1 min at room temperature. hOAT1-specific transport was calculated by subtracting the transport in vector-transfected cells from the transport in cDNA-transfected cells. Control transport in the absence of an inhibitor was taken as 100%. Each point represents the mean ± S.E. of three measurements.

2.4. Partition coefficient

The partition coefficient at pH 7.5 (D_{oct}) was determined by shaking a buffered aqueous solution of the tested com-

Table 1
IC_{50} values, types of inhibition induced by xanthine- and uric acid-related compounds, and log(D_{oct}) values

Compounds	IC_{50} (μM)	Type of inhibition	log(D_{oct})*
Xanthine-related compounds			
Xanthine	243.9	Competitive	−0.993 ± 0.082
1-Methylxanthine	10.3	Competitive	−0.352 ± 0.020
3-Methylxanthine	178.6	Competitive	−0.545 ± 0.000
7-Methylxanthine	122.0	Mix	−0.930 ± 0.006
1,3-Dimethylxanthine (Theophylline)	54.6	Competitive	0.015 ± 0.007
3,7-Dimethylxanthine (Theobromine)	138.9	Competitive	−0.599 ± 0.011
1,7-Dimethylxanthine	8.4	Competitive	−0.300 ± 0.005
1,3,7-Trimethylxanthine (Caffeine)	625.0	Mix	−0.060 ± 0.004
Uric acid-related compounds			
Uric acid	312.5	Competitive	−2.930 ± 0.253
1-Methyluric acid	79.4	Competitive	−2.280 ± 0.273
1,3-Dimethyluric acid	9.2	Competitive	−1.711 ± 0.017
1,7-Dimethyluric acid	15.0	Competitive	−1.890 ± 0.043
1,3,7-Trimethyluric acid	3.9	Competitive	−0.457 ± 0.011

* Each value represents the mean ± S.E. of three measurements.

pound with *n*-octanol (saturated with distilled water before use) for 30 min at room temperature. The buffer used in this study was the transport buffer described above. After centrifugation at 1500×*g* for 5 min, the concentration of the tested drug in the aqueous phase was determined. The difference between the drug concentrations in the aqueous phase before and after *n*-octanol partitioning was taken as the concentration of the drug that partitioned into the organic phase.

2.5. Analytical methods

The determination of [^{3}H]PAH was carried out by liquid scintillation counting. Protein concentration was determined according to the method of Lowry et al. using bovine serum albumin as a standard [21]. The concentrations of tested compounds were determined using an HPLC system, Hitachi L-6000 (Hitachi, Tokyo, Japan), equipped with a Hitachi L-4200H UV-VIS detector. Separation of the compounds was

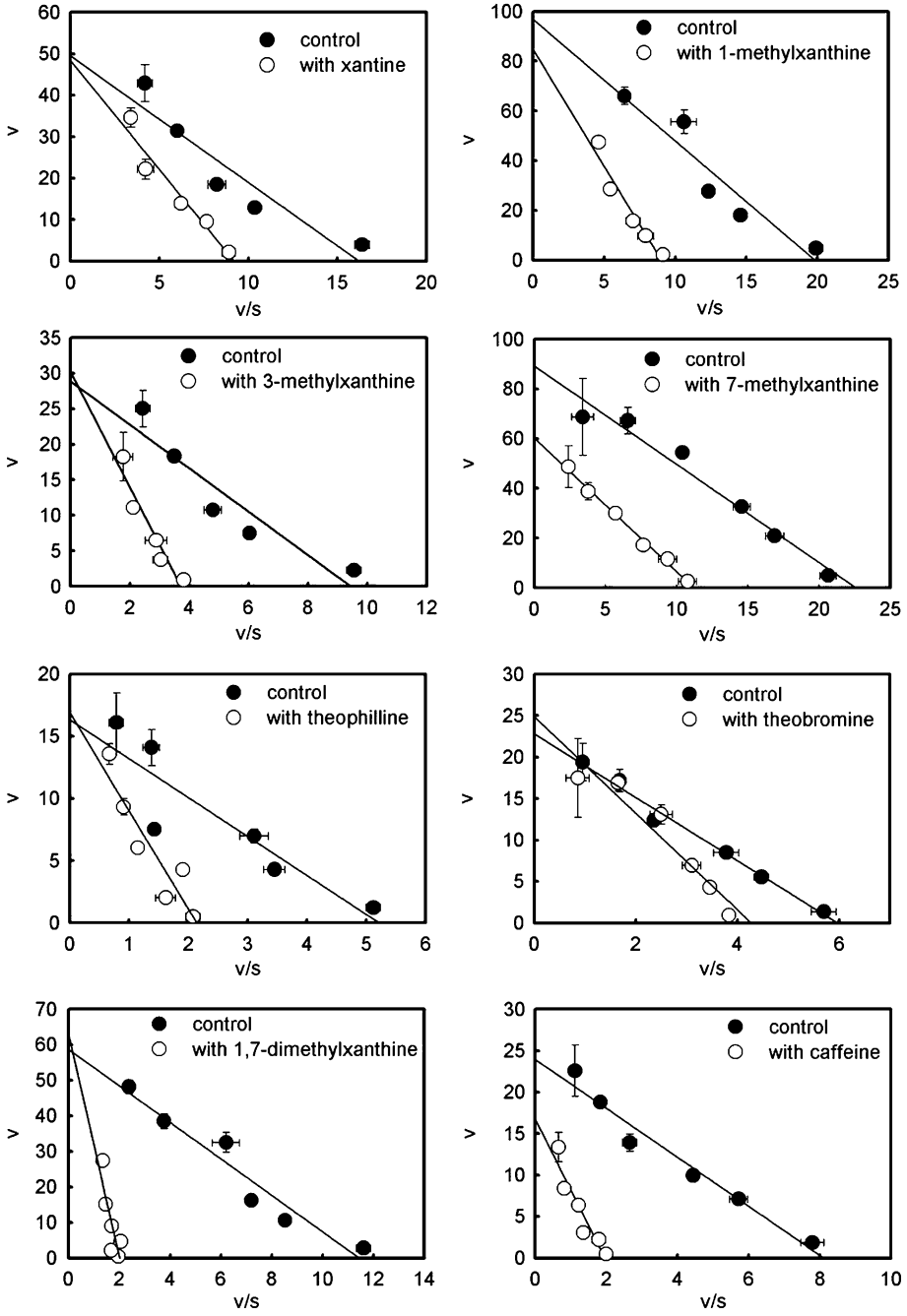

Fig. 5. Eadie–Hofstee plots of the uptake of PAH in the presence or absence of various xanthine-related compounds. Each value represents the mean±S.E. of 3–4 measurements. Since OAT1 activity of the stably-expressing cells decreased gradually by every subcultibation, maximum velocity of each experiment varied. v, uptake rate (pmol/mg protein/30 s); s, substrate concentration (μM).

done on a reversed-phase column Hitachi Gel #3053 (4 mm in i.d., 250 mm in length) at 55 °C. The composition of the mobile phase was 50 mM KH_2PO_4 either in 5% acetonitrile (for purine, hypoxanthine, xanthine, 1-methylxanthine, 3-methylxanthine, 7-methylxanthine, uric acid, 1-methyluric acid, 1,3-demethyluric acid, 1,7-dimethyluric acid, and 1,3,7-trimethyluric acid) or in 11.5% acetonitrile (for theophylline, theobromine, 1,7-dimethylxanthine, and caffeine). The wavelength for detection was 273 nm.

3. Results

3.1. Functional characteristics of hOAT1 in CHO-K1 cells

The uptake of PAH into hOAT1 cDNA-transfected (CHO-hOAT1) cells was remarkably greater than the uptake into vector-transfected (CHO-pcDNA) cells. In the presence of 1 mM probenecid, a typical inhibitor of organic anion transporters, hOAT1-specific PAH uptake was almost completely inhibited (Fig. 1). Since a linear uptake was observed for at least 2 min (data not shown), the uptake over a period of 1 or 2 min was measured in this study. Fig. 2 shows saturation kinetics of the uptake of PAH. The hOAT1-specific transport of PAH was saturable with values of Michaelis–Menten constant (K_t) and maximal velocity (V_{max}) for the transport process of 7.7±0.8 μM and 71±2.5 pmol/mg protein/min, respectively.

3.2. Inhibitory effects of various drugs on the uptake of PAH by CHO-hOAT1 cells

The effects of various drugs on the uptake of PAH by CHO-hOAT1 cells were examined. In this experiment, data were not compensated by subtracting the uptake into CHO-pcDNA cells. As shown in Fig. 3, many drugs inhibited the uptake of PAH. Not only anionic compounds but also compounds that do not have any anionic moiety (even organic cations) such as benzbromaron, colchicine, omeprazole, caffeine, theophylline, theobromine, harmaline, and tetracaine remarkably inhibited the uptake of PAH.

3.3. Inhibitory effects of xanthine, uric acid and their structurally related compounds on the transport of PAH

As can be seen in Fig. 3, xanthine-related compounds (theophylline and theobromine) showed relatively strong inhibitory effects. The structure of xanthine is similar to the structures of uric acid and antiviral drugs that are transported by hOAT1. Moreover, many compounds structurally related to xanthine and uric acid are commercially available. We therefore studied the structure–affinity relationship using these compounds.

The dose–response relationships and calculated 50% inhibitory concentrations (IC_{50}) for the inhibition of PAH uptake by xanthine-related compounds are shown in Fig. 4 and Table 1, respectively. Inhibition of the uptake showed the following order of potency: 1,7-dimethylxanthine ≧ 1-methylxanthine > 1,3-dimethylxanthine (theophylline) > 7-methylxanthine ≧ 3,7-dimethylxanthine (theobromine) > 3-methylxanthine ≒ xanthine > 1,3,7-trimethylxanthine (caffeine). Determination of the kinetics of the inhibitory effects of xanthine-related compounds on hOAT1-mediated PAH uptake showed that xanthine, 3-methylxanthine, 1-methylxanthine, 1,3-methylxanthine (theophylline), 1,7-dimethylxanthine and 3,7-dimethylxanthine (theobromine) inhibited hOAT1-mediated PAH uptake in a competitive manner, whereas 7-methylxanthine and 1,3,7-trimethylxanthine (caffeine) inhibited the uptake in a mix-type manner (Fig. 5, Table 1).

The dose–response relationships and calculated IC_{50} values for the inhibition of PAH uptake by uric acid-related compounds are shown in Fig. 6 and Table 1, respectively. The order of potency of inhibition was 1,3,7-trimethyluric acid > 1,3-dimethyluric acid > 1,7-dimethyluric acid > 1-methyluric acid > uric acid. Determination of the kinetics of inhibition of hOAT1-mediated PAH uptake showed that all of the tested uric acid-related compounds inhibited the uptake in a competitive manner (Fig. 7, Table 1).

3.4. Correlations between lipophilicity and inhibitory effects of xanthine-related and uric acid-related compounds

A plot of inhibitory effects versus lipophilicity determined using D_{oct} is shown in Fig. 8. Values of log(D_{oct}) of tested compounds are shown in Table 1. As shown in Fig. 8A, no significant relationship was found when xanthine-related compounds were used. On the other hand, as shown in Fig. 8B, a significant correlation between inhibitory

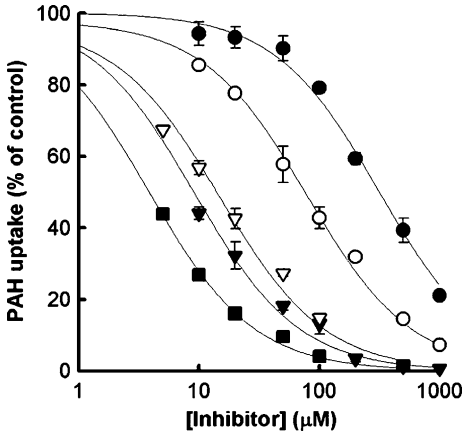

Fig. 6. Dose–response relationships of the inhibition of hOAT1-mediated transport of PAH by uric acid-related compounds (●, uric acid; ○, 1-methyluric acid; ▼, 1,3-dimethyluric acid; ▽, 1,7-dimethyluric acid; ■, 1,3,7-trimethyluric acid). Transport of 0.24 μM labeled PAH into vector- or hOAT1 cDNA-transfected cells was measured for 1 min at room temperature. hOAT1-specific transport was calculated by subtracting the transport in vector-transfected cells from the transport in cDNA-transfected cells. Control transport in the absence of an inhibitor was taken as 100%. Each point represents the mean±S.E. of three measurements.

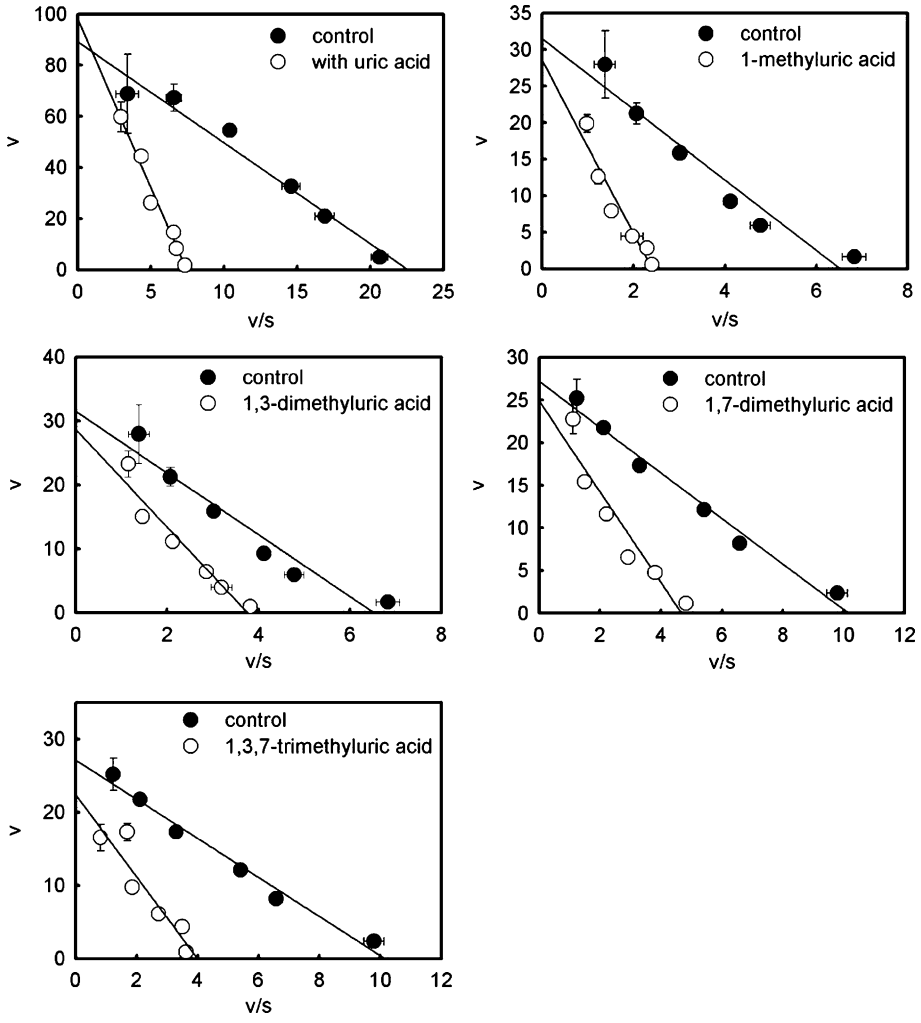

Fig. 7. Eadie–Hofstee plots of the uptake of PAH in the presence or absence of various uric acid-related compounds. Each value represents the mean ± S.E. of 3–4 measurements. Since OAT1 activity of the stably-expressing cells decreased gradually by every subcultibation, maximum velocity of each experiment varied. v, uptake rate (pmol/mg protein/30 s); s, substrate concentration (μM).

potency and lipophilicity of the tested uric acid-related compounds was found.

4. Discussion

In the present study, we established CHO-K1 cells that stably express hOAT1. Using these cells, we examined the effects of various compounds to discover new drugs that interact with hOAT1 (Table 1). As a result, we found that benzbromaron, colchicine, omeprazole, caffeine, theophylline, theobromine, hypoxanthine, tetracaine, and harmaline interact with hOAT1, though they are not anionic compounds (tetracaine and harmaline in fact being organic cations). Among these compounds, caffeine, theophylline, and theobromine (xanthine derivatives) showed relatively strong inhibitory effects. It has been demonstrated that hOAT1 mediates the transport of relatively small hydrophilic organic anions such as PAH. In contrast, hOAT3 prefers larger or more hydrophobic compounds [22]. The xanthine derivatives tested in this study are relatively small compounds. Therefore, it is possible that they are good substrates or inhibitors for hOAT1. Since the structure of xanthine is similar to that of uric acid, a substrate of hOAT1, we chose these xanthine derivatives and uric acid derivatives for the structure–affinity relationship study.

It is well known that hOAT1 transports many kinds of drugs, endogenous compounds, and toxins [1–13]. However, there has been little study on the structure–affinity relationship using a series of structurally related compounds. In the present study, we compared the inhibitory effects of xanthine-related compounds, namely, the effects of position and number of methyl groups on the affinity. As shown in Fig. 4 and Table 1, the potency of inhibitory effects of monomethyl derivatives was in the order of 1-methylxanthine > 7-methylxanthine > 3-methylxanthine ≒ xanthine and the potency of inhibitory effects of dimethyl derivatives was in the order of 1,7-dimethylxanthine > 1,3-dimethylxanthine > 3,7-dimethylxanthine > xanthine. There-

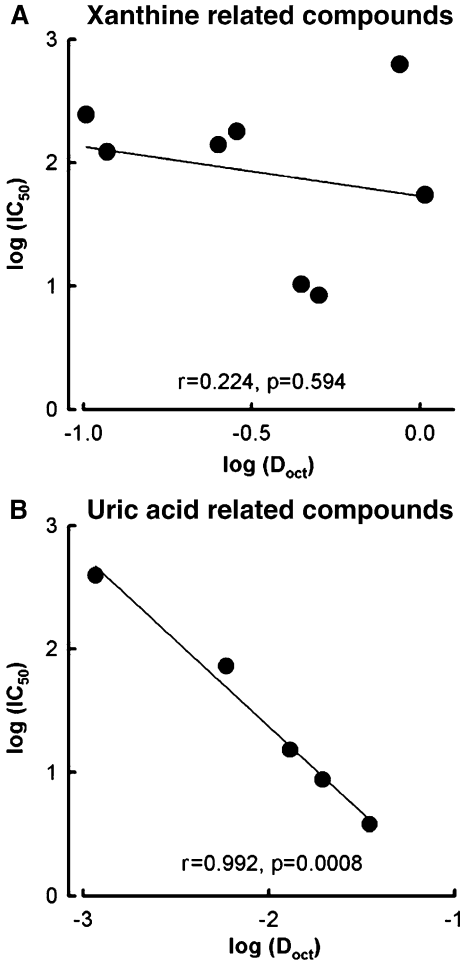

Fig. 8. Relationships between potency of the inhibitory effect and lipophilicity of the tested xanthine-related compounds (A) and uric acid-related compounds (B).

fore, the order of contribution of methyl group position that increases the potency of the inhibitory effect is 1>7>3. These data suggest that the position of the methyl group plays an important role in the affinity of xanthine-related compounds to hOAT1. It is possible that steric hindrance is the reason for 1,3,7-trimethylxanthine (caffeine) showing the lowest potency. The effect of the methyl group on the affinity of uric acid-related compounds was also examined (Fig. 5, Table 1). If the increasing effect of the methyl group on affinity is the same as that in the case of xanthine (1>7>3), the order of potency of inhibitory effects of uric acid-related compounds should be 1,7-methyluric acid ≧ 1-methyluric acid ≧ 1,3-dimethyluric acid > uric acid ≧ 1,3,7-trimethyluric acid. However, the actual order was 1,3,7-trimethyl derivative > 1,3-dimethyl derivative > 1,7-dimethyl derivative > 1-methyl derivative > uric acid. These results suggest that determinant of affinity of uric acid-related compounds is different from that of xanthine-related compounds. To elucidate the determinant of affinity of uric acid-related compounds, we studied the correlations between lipophilicity and inhibitory effects of tested compounds. Lipophilicity has been reported to be an important determinant of the inhibitory effects of a variety of organic compounds on transporters, enzymes and ion channels [23–26]. Fig. 8B shows a significant correlation between the potency of inhibitory effect and lipophilicity for uric acid-related compounds. On the other hand, as shown in Fig. 8A, lipophilicity was not a determinant of the inhibitory effect of xanthine-related compounds. Our results suggest that there are at least two processes involved in the affinity of substrates/inhibitors to hOAT1. One is interaction with the lipophilic environment of the transporter protein and the other is direct interaction of some amino acid residues in the substrate-binding pocket with substrates or inhibitors, like report of Feng et al. that described about substrate recognition of rOAT3 [27]. Even though the chemical structures of xanthine and uric acid are quite similar, their main determinants of affinity were different. The major structural difference is carbonyl moiety on the 8-position of purine ring. It causes the difference of pK_a values of these compounds (7.7 for xanthine; 5.7 for uric acid). Therefore, at the pH of 7.5 in this study, percentage of ionized form of uric acid is approximately 98%, whereas that of xanthine is less than 40%. The difference of pK_a and the existence of carbonyl moiety itself might be one of the reasons for the difference of their determinants of affinity.

Although caffeine itself has low affinity (IC_{50}=625 μM), its main metabolites, 1-methyluric acid, 1,7-dimethyluric acid, 1-methylxanthine and 1,7-methylxanthine [28], have strong inhibitory effects. In the same way, although theophylline and its metabolite, 1-methyluric acid, have intermediate affinity, its main metabolite, 1,3-dimethyluric acid [29], has high affinity. Therefore, it is possible that these compounds cause drug interaction on hOAT1-mediated transport in the urinary excretion. Moreover, since hOAT1-mediated transport of uric acid has been reported [11], it is likely that uric acid-related compounds (metabolites) are transported as substrates of hOAT1. However, when [^{3}H]caffeine and [^{3}H]theophylline were tested, their hOAT1-mediated transport was not observed (data not shown). Therefore, these compounds might work only as inhibitors. There is a report regarding the drug interaction between caffeine (or its metabolites) and the drugs that are substrates of OAT. Vrtic et al. reported that ibuprofen and probenecid inhibited the renal excretion of some of the caffeine metabolites [30]. They concluded that the inhibitory effect was due to the interaction on the transport via OAT and/or other transport system. Also, possible interaction on the transport via OAT3, another major subtype of OAT family, should be further investigated.

In conclusion, our results indicate that drugs related to xanthine (caffeine, theophylline, and theobromine) were found to inhibit the transport activity of hOAT1. Their metabolites, including xanthine and uric acid-related com-

pounds, had stronger inhibitory effects. In the case of xanthine-related compounds, the main determinant of affinity was the position of the methyl group. On the other hand, lipophilicity was the main determinant of affinity of uric acid-related compounds.

Acknowledgement

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