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Transport mechanism and substrate specificity of human organic anion transporter 2 (hOat2 [*SLC22A7*])

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Abstract

Human organic anion transporter 2 (hOat2 [*SLC22A7*]) is highly expressed in the human liver. Although localization, gene expression, substrate specificity and transport mechanisms of other human Oat isoforms such as human Oat1 (hOat1), human Oat3 (hOat3) and human Oat4 (hOat4) have been elucidated, information concerning human Oat2 (hOat2) is less defined. The objective of this study was to provide further information on the transport mechanism and substrate specificity of hOat2. When expressed in *Xenopus laevis* oocytes, the transport of organic compounds mediated by hOat2 was not affected by the replacement of extracellular sodium with lithium, choline and mannitol. The uptake of estrone sulfate (ES) in hOat2-expressing oocytes was significantly *trans*-stimulated by preloading the oocytes with fumarate and succinate, but not glutarate. Moreover, we observed that hOat2 mediates the transport of bumetanide, ES, glutarate, dehydroepiandrosterone sulfate, allopurinol, prostaglandin E_2, 5-fluorouracil, paclitaxel and L-ascorbic acid. These compounds are identified for the first time as hOat2 substrates. A wide range of structurally unrelated organic compounds inhibited the hOat2-mediated uptake of tetracycline, except for sulfobromophthalein. All of these findings indicate that hOat2 is a sodium-independent multi-specific organic anion/dimethyldicarboxylate exchanger. Our present findings thus provide further insights into the role of hOat2 in hepatic drug transport.

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Introduction

The liver is the central organ for drug metabolism and detoxification of structurally diverse compounds, including endo- and exogenous substrates. Generally, lipophilic compounds are catalytically converted into water-soluble substrates by cytochrome P450s and are eliminated and excreted from the liver via multi-specific organic anion transporters (Muller & Jansen 1997; Hagenbuch & Meier 2003; Koepsell & Endou 2003). Because the liver plays an important role in the maintenance of homeostasis and protects the body from toxic compounds, elucidation of the role of the transporter localized in the liver is important from a clinical and pharmacological point of view.

Several distinct multi-specific organic anion transporters, such as multi-drug resistant associated proteins (Mrps), organic anion transporting polypeptides (OATP/Oatps) and Oats, have been isolated from different species (Simonson et al 1994; Lopez-Nieto et al 1997; Sekine et al 1997; Sweet et al 1997; Kusuhara et al 1999; Kuze et al 1999; Race et al 1999; Cha et al 2000, 2001; Sun et al 2001). Among the Oat family, three members of the human Oat family, hOat1, 3 and 4, are predominantly expressed in the kidney, whereas hOat2 is abundantly expressed in the liver (Hosoyamada et al 1999; Cha et al 2000, 2001; Sun et al 2001); therefore hOat2 is considered to be a key molecule in hepatic handling of organic anions. Regarding the membrane localization of hOat2, we have reported that hOat2 is expressed at the basolateral membrane of the kidney proximal tubules (Enomoto et al 2002). In contrast, rat Oat2 (rOat2) is localized at the brush-border membrane of the tubules in the medullary thick ascending loop of Henle as well as in the cortical and medullary collecting ducts (Kojima et al 2002). Thus, the localization of Oat2 differs among species.

Regarding the substrates of hOat2, research has revealed that hOat2 mediates the uptake of methotrexate (MTX), prostaglandin E_2 (PGE_2), cAMP, α-ketoglutarate, azidodeoxythymidine (AZT), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), tetracycline and *p*-aminohippurate (PAH) (Sun et al 2001; Babu et al 2002; Kimura et al 2002; Takeda et al 2002). Although substrates and functional characterization of mouse and rat Oat2 have been reported (Sekine et al 1998; Kobayashi et al 2002), there is still limited data concerning the substrate selectivity and transport mechanism of hOat2.

In the present study, therefore, we extended our investigations of the substrate specificity and transport mechanism of hOat2. Our findings provide further insights into the role of hOat2 in the human liver.

Materials and Methods

Chemicals

[^{14}C]PAH (1.50 GBq mmol^{-1}), [^{3}H]bumetanide (185 GBq mmol^{-1}), [^{14}C]glutarate (2.035 GBq mmol^{-1}) and [^{3}H]paclitaxel (Taxol; 318 GBq mmol^{-1}) were purchased from American Radiolabeled Chemicals, Inc. (St Louis, MO). [^{3}H]PGE_2 (7.429 TBq mmol^{-1}), [^{3}H]estrone sulfate (ES; 1702 GBq mmol^{-1}), [^{3}H]5-fluorouracil (5-FU; 0.318 TBq mmol^{-1}), [^{14}C]salicylate (2053 MBq mmol^{-1}), [^{14}C]L-ascorbic acid (148 MBq mmol^{-1}) and [^{3}H]dehydroepiandrosterone sulfate (DHEA-S; 2738 GBq mmol^{-1}) were purchased from PerkinElmer Life Sciences (Boston, MA). [^{3}H]Allopurinol (51 GBq mmol^{-1}) and [^{3}H]tetracycline (333.6 GBq mmol^{-1}) were purchased from Moravek Biochemicals (Brea, CA). All other chemicals were of the highest grade commercially available.

Xenopus laevis oocyte preparation, cRNA synthesis and uptake experiments

Xenopus oocyte isolation was performed as previously described (Kobayashi et al 2002). Stage V and VI defolliculated oocytes obtained from adult female *Xenopus laevis* were selected throughout this study. To remove the follicular layer from the oocytes, collagenase A (Roche Applied Sciences, Mannheim, Germany) was used at a final concentration of 2.0 mg mL^{-1} in oocyte Ringer 2 (83 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.5) and slowly shaken for 1 h at room temperature. The hOat2 cDNA (GenBank/EBI/DDBJ, AY050498) was linearized with *Bam*HI, and the capped cRNA was transcribed in vitro by T7 RNA polymerase. Defolliculated oocytes were microinjected with 50 ng of in-vitro transcribed cRNA under a stereomicroscope using a microdispenser (Drummond Scientific, Broomall, PA) and incubated for 2 days in a modified Barth's solution containing gentamicin (50 μg mL^{-1}) at 18°C. Uptake experiments of radiolabelled substrates, as indicated in each experiment, were performed in an ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.4) at room temperature. Oocytes were incubated in an ND96 solution containing radiolabelled substrates for 1 h. The uptake was terminated by the addition of 2 mL of an ice-cold ND96 solution, and the oocytes were washed with the same solution at least five times. The oocytes were solubilized with 250 μL 10% (w/v) sodium dodecyl sulfate (SDS), and accumulated radioactivity was determined with a liquid scintillation counter. Each experiment was repeated more than three times to confirm the results.

trans-Stimulatory effect on transport via hOat2

In order to examine the *trans*-stimulatory effect on the influx of radiolabelled substrate, oocytes expressing hOat2 were preloaded with 5 mM dicarboxylates (glutarate, succinate, fumarate) for 1 h and then transferred into the medium containing [^{3}H]ES (50 nM). Dicarboxylates were directly dissolved in ND96 solution from stock solution. The stock solution of dicarboxylates was prepared in DMSO and diluted to a final concentration as described above. The final concentration of DMSO in the assay medium did not exceed 1.0% (v/v). We did not observe any effects of DMSO on cell integrity at this concentration.

Kinetic study

Concentration-dependent uptake experiments of [^{3}H]bumetanide, [^{3}H]5-FU and [^{3}H]Taxol via hOat2 were performed with each compound at final concentrations of 1, 2, 5, 10 and 30 μM, 1, 10, 20, 75, and 100 nM, and 1, 10, 20, 50, and 100 nM, respectively. The compounds were incubated with oocytes expressing hOat2 for 1 h at room temperature, stopped with ice-cold ND96 solution and washed five times as described above. Individual oocytes were transferred to scintillation vials and dissolved in 250 μL 10% SDS. A scintillation cocktail was added and radioactivity was counted. Counts in control (non-injected) oocytes were subtracted from the counts in cRNA-injected oocytes. Data are presented as mean $\pm$ s.e.m. except for kinetic constants, for which the error represents the error of the fit. K_m indicates the Michaelis–Menten constant (nano- or micromolar).

Inhibition study

For the inhibition study, oocytes expressing hOat2 were incubated for 1 h in ND96 solution containing [^{3}H]tetracycline in the presence or absence of various inhibitors at a final concentration of 1 mM. Tetracycline, sulfobromophthalein (BSP), indometacin, diclofenac, acetaminophen, erythromycin, chloramphenicol, ibuprofen, bumetanide and furosemide were directly dissolved in ND96 solution from stock solution. These stock solutions of the inhibitors were prepared in DMSO and diluted to a final concentration as described above. The final concentration of DMSO in the assay medium did not exceed 1.0% (v/v).

Statistical analysis

Statistical differences were evaluated using ANOVA. The values represent the mean ± s.e.m. (*$P < 0.05$).

Results and Discussion

Functional characterization of hOat2

We and other investigators have revealed that r- and m-Oat2 are sodium-independent multispecific organic anion transporters (Sekine et al 1998; Kobayashi et al 2002). We firstly examined, based on these findings, whether the uptake of organic anions mediated via hOat2 is also sodium independent. Since the uptake of [^{14}C]PAH by uninjected oocytes was equal to the oocytes injected with 50 nL of water (data not shown), uninjected oocytes were used as the control instead of water-injected oocytes throughout this study. As shown in Table 1, the uptake of [^{14}C]PAH via hOat2 was not affected by the replacement of extracellular sodium with lithium, choline and mannitol. The result indicates that hOat2, as well as m- and r-Oat2, is a sodium-independent transporter.

Previous reports on Oat1 and Oat3 function in the *Xenopus* oocyte expression system found evidence for dicarboxylate gradients (Sekine et al 1997; Sweet et al 2003). Sweet et al (2003) suggested that Oat2 may be an organic anion/dicarboxylate exchanger therefore we examined whether or not Oat2 has a similar function. As shown in Table 2, the preloaded fumarate and succinate *trans*-stimulates influx of [^{3}H]ES via hOat2. However, intracellularly accumulated unlabelled glutarate did not stimulate the uptake of [^{3}H]ES, indicating that hOat2 is an organic anion/dimethyldicarboxylate exchanger.

Substrate selectivity of hOat2

Regarding the substrate of Oat2 orthologs, we and other investigators have reported that hOat2 mediates the uptake of MTX, cAMP, α-ketoglutarate, AZT, PGE_2, $PGF_{2\alpha}$, tetracycline and PAH (Sun et al 2001; Babu et al 2002; Kimura et al 2002; Takeda et al 2002). Morita et al (2001) and Sekine et al (1998) revealed that rOat2 mediates the uptake of salicylate, PGE_2, MTX, acetylsalicylate, PAH, α-ketoglutarate, indometacin and AZT. Recently, we reported that mOat2 mediates the transport of glutarate and ochratoxin A (Kobayashi et al 2002). Thus, evidence concerning the substrates of Oat2 orthologs is rapidly accumulating; information on the substrate of hOat2 is still limited. To expand further the known substrate range of hOat2, transport experiments were performed using a *Xenopus* oocyte expression system. Since the cell-associated count of [^{3}H]tetracycline increased linearly up to 2 h (data not shown), we performed all uptake experiments at 1 h. As shown in Table 3, [^{3}H]bumetanide, [^{3}H]ES, [^{14}C]glutarate,

Table 1 Effect of extracellular cation on [^{14}C]PAH uptake in *X. laevis* oocytes expressing hOat2

	Na^+	Li^+	Choline$^+$	Mannitol
Control	0.11 ± 0.02	0.09 ± 0.01	0.08 ± 0.02	0.11 ± 0.02
hOat2	0.23 ± 0.09*	0.19 ± 0.02*	0.18 ± 0.02*	0.21 ± 0.02*

The tracer concentration used was 10 μM. The uptake rate of [^{14}C]PAH by control oocytes or hOat2-expressing oocytes for 1 h was measured at room temperature in the presence or absence of extracellular Na^+. Extracellular Na^+ was replaced with equimolar concentration of lithium, choline or mannitol. Values are mean ± s.e.m. of 9–18 oocyte determinations (nmol oocyte^{-1} h^{-1}). The significance between control (non-injected) and hOat2-cRNA-injected oocytes was determined using ANOVA (*$P < 0.05$). Other experimental conditions and details are described in the Materials and Methods section.

Table 2 *trans*-Stimulatory effect of fumarate, succinate and glutarate on the transport of [^{3}H]ES via hOat2

Non-injected	ES	Glutarate	Succinate	Fumarate
7.41 ± 0.92	25.37 ± 1.11*	9.25 ± 1.11	12.41 ± 0.56*	15.93 ± 2.96*

Oocyte expressing hOat2 was preloaded with 5 mM fumarate, succinate or glutarate for 1 h before starting the uptake experiment. Transport of [^{3}H]ES mediated via hOat2 was *trans*-stimulated by fumarate and succinate. No *trans*-stimulatory effect was observed in the presence of glutarate. A quantity of 50 nM [^{3}H]ES was used for the uptake experiments. Data were expressed as fmol oocyte^{-1} h^{-1}. Values are mean ± s.e.m. of 18–27 oocyte determinations. The significance between control (non-injected) and hOat2-cRNA-injected oocytes was determined using ANOVA (*$P < 0.05$). Other experimental conditions and methods are described in the Materials and Methods section.

Table 3 Uptake of various ^{14}C- and ^{3}H-labelled compounds by hOat2-expressing oocytes

Tracer	Concentration	Non-injected	hOat2
[^{3}H]Bumetanide	2 μM	0.13 ± 0.01	2.23 ± 0.10*
[^{3}H]ES	50 nM	6.77 ± 0.09	26.67 ± 1.35*
[^{14}C]Glutarate	5 μM	0.15 ± 0.02	0.43 ± 0.05*
[^{3}H]DHEA-S	50 nM	5.26 ± 0.01	14.22 ± 0.32*
[^{3}H]Allopurinol	10 μM	0.05 ± 0.01	13.13 ± 0.42*
[^{3}H]PGE_2	10 nM	1.39 ± 0.02	18.68 ± 0.63*
[^{3}H]5-FU	20 nM	9.83 ± 0.21	41.52 ± 1.69*
[^{3}H]Taxol	50 nM	20.68 ± 2.58	106.55 ± 18.1*
[^{14}C]L-Ascorbic acid	25 μM	0.41 ± 0.08	1.41 ± 0.10*
[^{14}C]Salicylate	10 μM	4.42 ± 0.02	3.85 ± 0.63

After 2 days of incubation, uptake experiments were performed in a solution containing Na^+ for 1 h. Values are mean ± s.e.m. of 8–18 oocyte determinations. The significance between control and hOat2-cRNA-injected oocytes was determined using ANOVA (*$P < 0.05$). The units of measure were femtomoles per oocyte per hour for ^{3}H tracers and picomoles per oocyte per hour for ^{14}C tracers. Other experimental conditions and methods are described in the Materials and Methods section.

[^{3}H]DHEA-S, [^{3}H]allopurinol, [^{3}H]PGE_2, [^{3}H]5-FU, [^{3}H]Taxol and [^{14}C]ascorbic acid were significantly transported via hOat2. Although Sun et al (2001) revealed that EcR293 cells expressing hOat2 do not transport DHEA-S, our results clearly indicate that these compounds are identified for the first time as novel hOat2 substrates. Despite functional transporter expression, hOat2-expressing oocytes did not take up [^{14}C]salicylic acid compared with non-injected control oocytes despite the fact that salicylate is transported by rOat2 (Sekine et al 1998). We therefore concluded that salicylate is not a substrate of m- and h-Oat2.

The loop diuretic bumetanide has been used for many years as therapy for oedema associated with congestive heart failure and hepatic diseases. Bumetanide has been indicated to act as a substrate of hepatic organic anion transport system (Davies et al 1973). In this respect, Honscha et al demonstrated that the bile acid-sensitive rat liver MTX carrier 1/2 (RL-Mtx1/2) mediates the transport of bumetanide (Honscha et al 2000). Their results indicate that RL-Mtx1/2 is a transporter for the uptake of bumetanide in rat liver. In the present study, we demonstrate that bumetanide is transported via hOat2 (Table 3). Nonlinear regression analysis yielded a K_m value of $7.52 \pm 2.3\ \mu$M for bumetanide uptake (Figure 1A). However, it has been reported that bumetanide is not transported by hOat2 when expressed in the second segment of the kidney proximal tubular (S_2) cells (Hasannejad et al 2004). At present, we have no explanation for this discrepancy.

The antineoplastic agent has been used for the treatment of several different types of malignancies (Jeung et al 2001). Over 80% of an intravenous administration of 5-FU is known to be inactivated by hepatic dihydropyrimidine dehydrogenase (Diasio & Harris 1989). Regarding the uptake of 5-FU into hepatocytes, Wohlhueter et al (1980) have suggested that 5-FU enters the hepatocytes via a carrier-mediated transport process. However, the molecular mechanism is still unknown. As shown in Figure 1B, oocytes expressing hOat2 mediated the transport of 5-FU with a K_m value of 53.8 ± 7.9 nM. Our results indicate that hOat2 may, at least partly, be responsible for the hepatic uptake of 5-FU.

Taxol, a diterpenoid plant product, has been widely used to treat solid tumours such as breast and ovarian cancer (Rowinsky & Donehower 1993; Kroger et al 1999). The major factors that affect the efficacy of Taxol appear to be the intracellular concentration of Taxol. Sparreboom et al (1997) reported that Taxol appears to be a substrate of the multi-drug resistance protein P-glycoprotein (P-gp), an energy-dependent drug-efflux pump. Parekh & Simpkins (1996) have proposed the presence of an alternative Taxol transport system that is distinct from the P-gp and MRP2/cMOAT system. However, as far as we know, there is no report concerning the hepatic uptake of Taxol. Our uptake experiments demonstrated that Taxol is transported via hOat2 with a K_m value of 142.8 ± 22.7 nM (Figure 1C). Although Taxol is a hydrophobic compound without anionic moiety at physiological pH, hOat2 may be a transporter for the uptake of Taxol at the sinusoidal membrane of the human liver. Further detailed studies will be needed.

L-Ascorbic acid has been known to be an effective antioxidant, free radical scavenger and essential cofactor

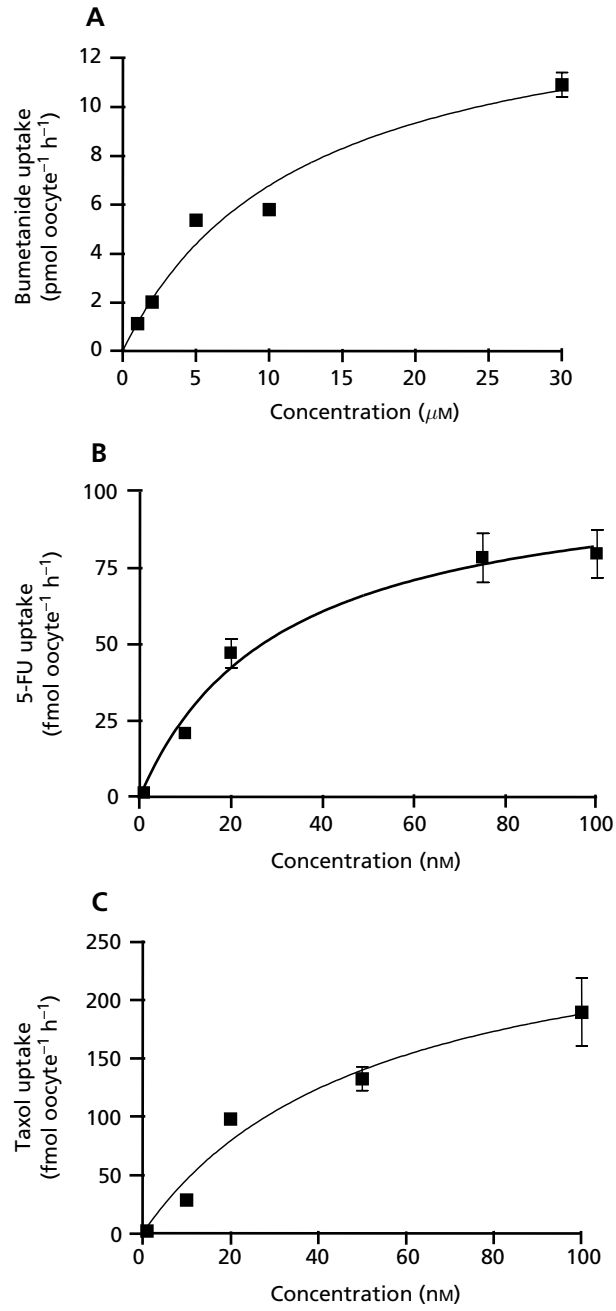

Figure 1 Concentration-dependent uptake of [^{3}H]bumetanide (A), [^{3}H]5-FU (B) and [^{3}H]Taxol (C) mediated by hOat2. The uptake experiments of bumetanide, 5-FU and taxol via hOat2 were performed with these compounds at final concentrations of 1, 2, 5, 10 and 30 μM; 1, 10, 20, 75 and 100 nM; and 1, 10, 20, 50, and 100 nM, respectively. The uptake rates of bumetanide, 5-FU and Taxol by the control (non-injected) or hOat2-expressing oocytes for 1 h were measured at variable concentrations. The uptake was saturable with K_m values of $7.52 \pm 2.3\ \mu$M, 53.8 ± 7.9 nM and 142.8 ± 22.7 nM for bumetanide, 5-FU and Taxol, respectively, and fitted to the Michaelis–Menten curve. Values are mean ± s.e.m. of 9–21 oocyte determinations. The hOat2-mediated transport was determined by subtracting the transport velocity in control (non-injected) oocytes from that in hOat2-expressing oocytes. Other experimental conditions and details are described in the Materials and Methods section.

in various enzymatic reactions (Padh 1991; Rose & Bode 1993). To date, two distinct Na^+-dependent vitamin C transporters (SVCT1 [*SLC23A1*] and SVCT2 [*SLC23A2*]) and two orphan isoforms have been isolated (Daruwala et al 1999; Tsukaguchi et al 1999). The gene coding for SVCT1 is expressed in the liver, whereas no SVCT2 mRNA is localized in human hepatocytes (Daruwala et al 1999; Tsukaguchi et al 1999). SVCT1 is therefore considered to be the molecule responsible for the Na^+-dependent uptake of L-ascorbic acid in the human liver. Our results suggest that there exists an Na^+-independent transport mechanism for the uptake of L-ascorbic acid into human hepatocytes.

We subsequently studied the inhibitory effect of the hOat2-mediated [^{3}H]tetracycline uptake by various compounds to elucidate further the substrate specificity of hOat2. As shown in Figure 2, *cis*-inhibitory effects were observed for various structurally dissimilar drugs. The inhibition of the hOat2-mediated transport of [^{3}H]tetracycline exhibited a rank order of ibuprofen = bumetanide = furosemide > chloramphenicol > erythromycin = acetaminophen. We have already reported that BSP is a strong inhibitor for mOat2-mediated uptake (Kobayashi et al 2002). This compound stimulated hOat2-mediated uptake of [^{3}H]tetracycline to about 1.5-fold that of the control oocytes. This result indicates that the substrate selectivity of hOat2 is different from that of mOat2.

The present study describes the transport mechanism and substrate specificity of hOat2 (*SLC22A7*). hOat2 mediated the transport of various kinds of organic compounds in a sodium-independent manner. With the exception of BSP, a wide range of structurally unrelated organic compounds inhibited the hOat2-mediated uptake. Moreover, we found that fumarate and succinate, but not glutarate, *trans*-stimulates the transport of organic compounds. All of these findings lead us to conclude that hOat2 is a sodium-independent multispecific organic anion/dimethyldicarboxylate exchanger.

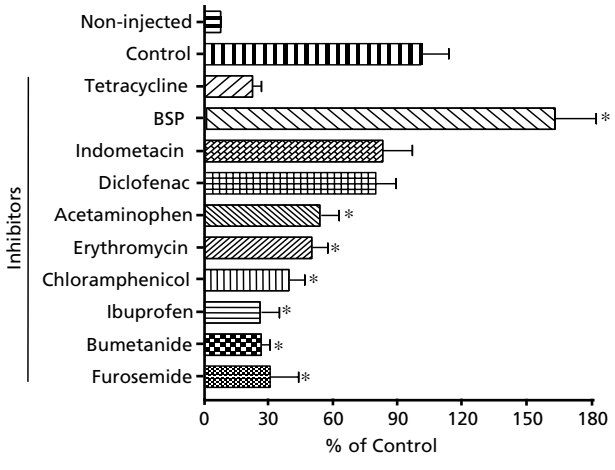

Figure 2 *cis*-Inhibitory effect of hOat2-mediated [^{3}H]tetracycline uptake by various organic compounds. The uptake rate of [^{3}H]tetracycline by hOat2-expressing oocytes or control (non-injected) oocytes was determined in the presence or absence of 1 mM inhibitors. The concentration of [^{3}H]tetracycline was 3 μM. The values were expressed as a percentage of [^{3}H]tetracycline uptake in hOat2-expressing oocytes in the absence of the inhibitors. Values are mean ± s.e.m. of 12–18 oocyte determinations. The significance between control (non-injected) and hOat2-cRNA-injected oocytes was determined by using ANOVA (*$P < 0.05$). Other experimental conditions and methods are described in the Materials and Methods section.

Conclusions

In the present study, we demonstrate for the first time the substrate selectivity and the mechanism of organic anion transport by hOat2. hOat2 is a sodium-independent multispecific organic anion/dimethyldicarboxylate exchanger. Thus, in light of the present findings, hOat2 plays a pivotal role in the hepatic handling of organic drugs and chemicals. Although other members of liver-predominant organic anion transporters such as OATP-B (*SLCO2B1*), OATP-C (*SLCO1B1*) and OATP8 (*SLCO1B3*) are known to be localized at the sinusoidal membrane of the human liver (Hagenbuch & Meier 2003), it would be interesting to elucidate which transporter(s) plays a major role in the hepatic uptake of these organic drugs and chemicals.

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