Novobiocin Is a Potent Inhibitor for Human Organic Anion Transporters OATs

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ABSTRACT

Organic anion transporters (OATs) mediate the body disposition of a diverse array of environmental toxins, and clinically important drugs. Previous studies have shown that novobiocin, an inhibitor for Breast Cancer Resistant Proteins (BCRP) inhibited organic anion transport. However, its interactions with specific OATs are unknown. In the current study, we characterized the inhibitory effects of novobiocin on the function of human OATs hOAT1, hOAT3 and hOAT4. Kinetic study revealed that novobiocin inhibited OAT-mediated uptake in a competitive manner, with Ki of 14.87 ± 0.40 μM for hOAT1, Ki of 4.77 ± 1.12 μM for hOAT3 and Ki of 90.50 ± 7.50 μM for hOAT4. Furthermore, the cis- and trans-inhibition feature of novobiocin demonstrated that novobiocin was a potent inhibitor but not a substrate for hOAT1 (IC50 = 34.76 ± 0.31 μM), hOAT3 (IC50 = 4.987 ± 0.35 μM) and hOAT4 (IC50 = 92.68 ± 0.34 μM). We further showed that the effects of novobiocin on OATs were not mediated through a change in transporter protein abundance on the plasma membrane. Together, we conclude that novobiocin appears to interact with the substrate-binding sites of OATs from both the intracellular and the extracellular sides and this interaction interferes with the substrate-binding site(s) on respective carriers, leading to an apparent reduction in carriers available for the substrates. Since BCRP is often expressed in the same tissue where multiple OATs are identified such as liver, kidney and placenta, when dissecting the contribution of BCRP to drug disposition using novobiocin as an inhibitor, its inhibitory effect to OATs has to be taken into consideration.
INTRODUCTION

The organic anion transporter (OAT) family mediates the body disposition of a diverse array of environmental toxins, and clinically important drugs, including anti-HIV therapeutics, anti-tumor drugs, antibiotics, anti-hypertensives, and anti-inflammatory (You, 2002; You, 2004a; You, 2004b), and is therefore critical for the survival of mammalian species.

Ten members of OAT family have been cloned, and their expressions have been identified in distinct tissues and cell membranes (Lopez-Nieto et al., 1997; Sekine et al., 1997; Sweet et al., 1997; Wolff et al., 1997; Sekine et al., 1998; Cihlar et al., 1999; Kusuhara et al., 1999; Lu et al., 1999; Cha et al., 2000; Enomoto et al., 2002a; Jutabha et al., 2003; Ekaratanawong et al., 2004; Monte et al., 2004; Youngblood and Sweet, 2004; Shin et al., 2007; Bahn et al., 2008). OAT1 and OAT3 are predominantly expressed at the basolateral membrane of kidney proximal tubule cells and at the apical membrane of brain choroid plexus. OAT2 and OAT7 are mainly expressed at the basolateral membrane of the liver. The polarized expression of OAT2 in the kidney has not been clearly defined. OAT4 is present at the basolateral membrane of the placenta and at the apical membrane of the kidney proximal tubule cells. OAT5 Oat,1, URAT1, and OAT10 are expressed at the apical membrane of the kidney proximal tubule cells. OAT6 is expressed in the olfactory mucosa.

In the kidney, OAT1 and OAT3 utilize a tertiary transport mechanism to move organic anions across the basolateral membrane into the proximal tubule cells for subsequent exit across the apical membrane into the urine for elimination (You, 2002; You, 2004a; You, 2004b). Through this tertiary transport mechanism, Na⁺/K⁺-ATPase maintains an inwardly directed (blood-to-cell) Na⁺ gradient. The Na⁺ gradient then drives a sodium dicarboxylate
cotransporter, sustaining an outwardly directed dicarboxylate gradient that is utilized by a dicarboxylate/organic anion exchanger, namely OAT, to move the organic anion substrate into the cell. This cascade of events indirectly links organic anion transport to metabolic energy and the Na\(^+\) gradient, allowing the entry of a negatively charged substrate against both its chemical concentration gradient and the electrical potential of the cell.

All of the cloned OATs share several common structural features including 12 transmembrane domains flanked by intracellular amino- and carboxyl termini; multiple glycosylation sites localized in the first extracellular loop between transmembrane domains 1 and 2, and multiple potential phosphorylation sites present in the intracellular loop between transmembrane domains 6 and 7, and in the carboxyl terminus. The investigation from our laboratory on the structure-function relationship of OATs revealed that glycosylation was necessary for the targeting of these transporters to the plasma membrane (Tanaka et al., 2004; Zhou et al., 2005).

Novobiocin, an aminocoumarin antibiotic, has been shown to inhibit organic anion transport in several systems (Odllind, 1981; Besseghir, 1985) and has been widely used as a specific inhibitor for Breast Cancer Resistant Proteins (BCRP) both in vitro and in vivo studies (Shiozawa et al., 2004; Huss et al., 2005; Su et al., 2007). BCRP is associated with high level of cellular resistance to a variety of anticancer agents (Shiozawa et al., 2004; Huss et al., 2005; Su et al., 2007; Mao, 2008). However, the effect of novobiocin on organic anion transport mediated by OATs has not been explored. In the current study, we examined the effects of novobiocin on organic anion transport mediated by hOAT1, hOAT3 and hOAT4.
MATERIALS AND METHODS

[^3H] ρ-aminohippuric acid (PAH), [^3H] estrone sulfate was from NEN Life Science Products (Hercules, CA). COS-7 cells were purchased from American Type Culture Collection (Manssas, VA). Dehydroepiandrosterone-3-sulfate (DHEAs) and other reagents were purchased from Sigma (St. Louis, MO).

Cell Culture and Transfections – COS-7 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (FBS) and antibiotics. Parental human placental BeWo b30-10 cells were grown in Dulbecco's modified Eagle's/F-12 medium (phenol red free) supplemented with 5% charcoal/dextran-stripped fetal bovine serum, penicillin/streptomycin (100 U/ml), and glucose (100 mg/ml) in a 5% CO2 atmosphere at 37°C. BeWo b30-10 cells stably expressing hOAT4 (Zhou et al., 2007) were maintained in Dulbecco's modified Eagle's/F-12 medium supplemented with 10% charcoal/dextran-stripped fetal bovine serum, 0.5 mg/ml geneticin (G418; Invitrogen, Carlsbad, CA), and glucose (100 mg/ml) in a 5% CO₂ atmosphere at 37°C. Cells were grown to 90–100% confluence and transfected with the appropriate plasmids using Lipofectamine 2000 (Invitrogen, CA). The plasmids used for transfection were: hOAT1-myc-pcDNA, hOAT3-myc-pcDNA, and hOAT4-pcDNA. Myc epitope was tagged to the carboxyl termini of hOAT1 and hOAT3 to facilitate the detection of the transporter proteins (Hong et al., 2004; Tanaka et al., 2004).

Transport Measurements – For each well, uptake solution was added. The uptake solution consisted of phosphate-buffered saline (PBS)/CM (137 mM NaCl, 2.7 mM KCl, 4.3 mM
Na₂HPO₄, 1.4 mM KH₂PO₄, 0.1 mM CaCl₂, and 1 mM MgCl₂, pH 7.3) and [³H] PAH (20 μM) or [³H] estrone sulfate (100 nM). The uptake experiments were proceeded at room temperature and at the times indicated in the figure legends, the uptake was stopped by aspirating the uptake solution off and rapidly washing the cells with ice-cold PBS solution. The cells were then solubilized in 0.2 N NaOH, neutralized in 0.2 N HCl, and aliquoted for liquid scintillation counting. The uptake count was standardized by the amount of protein in each well.

For trans-influx studies, cells expressing hOAT1, hOAT3 or hOAT4 were preloaded with novobiocin (1 mM), PAH (1mM) or DHEA (3 or 4 µM) separately for 1 hour at 37 ºC to allow the chemical substances to diffuse into the cells, followed by rapid washing and subsequent exposure to PBS containing radio-labeled OAT substrates ([³H] PAH (20 µM) for hOAT1, or [³H] estrone sulfate (100 nM) for hOAT3 and hOAT4). Uptake experiment was preceded as described above.

For trans-efflux studies, we followed the procedures described by Srimaroeng et al (Srimaroeng et al. 2005). Cells expressing hOAT1, hOAT3 or hOAT4 were preloaded with 20 µM of [³H] PAH, or 100 nM of [³H] ES respectively for 45 min to build up intracellular accumulation of labeled substrates. After incubation, the cells were quickly washed with PBS/CM (room temperature) twice. Then cells were incubated in absence or presence of 1 mM PAH (for hOAT1) or 4µM of estrone sulfate (for hOAT3 and hOAT4), 0.1 mM novobiocin (Nov) respectively for 3 min. At the end of incubation, [³H] PAH or [³H] estrone sulfate in the incubation medium (efflux) would be collected and counted by liquid
scintillation counter. The cells were then quickly washed twice with cold PBS/CM and lysed as described above. The intracellular accumulation of labeled substrates was counted by liquid scintillation. The efflux study results were interpreted as a mean percentage of the total efflux (count in the efflux medium/count in the efflux medium+ count in the intracellular medium) appeared in the incubation medium after 3min of efflux.

Concentration-dependent inhibition studies – Inhibition studies were performed at varying concentrations of novobiocin (10-1000 µM). Specific uptake was obtained by subtracting uptake ([3H] PAH for hOAT1) or ([3H] estrone sulfate for hOAT3 and hOAT4) into parental cells from the uptake into hOAT-expressing cells. The IC50, the concentration of novobiocin required to inhibit 50% of specific substrate uptake, was obtained by fitting data with Equation 1 (Akarawut and Smith, 1998) using GraphPad Prism software (GraphPad Software Inc. San Diego, CA 92130 USA). In Equation 1, Vmax is the maximal rate of saturable uptake (in absence of novobiocin). IC50 is the concentration causing 50% inhibition of the maximal novobiocin effect. X is the log of concentration (unit is molar). LogIC50 has the same unit as X. Each experiment had triplicate measurements.

Equation 1:  
\[ V = \frac{V_{\text{max}}}{1 + 10^{(x-\log IC_{50})}} \]  
(Akarawut and Smith, 1998)

Dixon plot -To determine the mechanism underlying the inhibition of hOAT-mediated uptake by novobiocin, Dixon plot analysis was used. For hOAT1, uptake with [3H] PAH (20µM, 50µM and 100 µM) was determined at 3 min both in the absence and presence of varying concentration of novobiocin (20, 40 and 100 µM). The specific uptake was obtained.
by subtracting $[^3]H$ PAH uptake into parental cells from the uptake into hOAT1-expressing cells. The data was analyzed by linear-regression with GraphPad Prism®. For hOAT3, $[^3]H$ estrone sulfate (100nM, 200nM and 500nM) uptake was determined at 3 min both in the absence and presence of varying concentration of novobiocin (2, 5, 15 and 50 µM). Uptake data analysis was the same as described for hOAT1. For hOAT4, $[^3]H$ estrone sulfate (100nM, 200nM and 500nM) uptake was determined at 3 min both in the absence and presence of varying concentration of novobiocin (50µM to 600µM). Uptake data analysis was the same as described for both hOAT1 and hOAT3.

Inhibition constant $K_i$ was obtained by fitting the data with Equation 2 (Akrawut and Smith, 1998), where $C$ is the concentration of substrates (µM), $K_m$ is the Michaelis constant (µM).

Equation 2:

$$K_i = \frac{IC_{50}}{1 + C/K_m}$$

(Akrawut and Smith, 1998)

*Cell surface biotinylation* – Cell surface expression levels of hOATs were examined using the membrane-impermeable biotinylation reagent, NHS-SS-biotin (Pierce, Rockford, IL). Individual hOAT was transfected in cells grown in 6-well plates using Lipofectamine 2000 as described above. After 24 h, the medium was removed and the cells were washed twice with 3 ml of ice-cold PBS/CM (pH 8.0). The plates were kept on ice, and all solutions were ice-cold for the rest of the procedure. Each well of cells was incubated with 1 ml of freshly prepared NHS-SS-biotin (0.5 mg/ml in PBS/CM) in two successive 20-min incubations on ice with very gentle shaking. After biotinylation, each well was briefly rinsed with 3 ml of
PBS/CM containing 100 mM glycine and then incubated with the same solution for 20 min on ice to ensure completely quenching of the unreacted NHS-SS-biotin. The cells were then dissolved on ice for 1 h in 400 µl of lysis buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100 with 1:100 protease inhibitor mixture (Sigma, USA). The cell lysates were cleared by centrifugation at 16,000 x g at 4 °C. 50 µl of streptavidin-agarose beads (Pierce, USA) was then added to the supernatant to isolate cell membrane protein. OATs were detected in the pool of surface proteins by SDS-PAGE and immunoblotting.

Electrophoresis and immunoblotting – immunoblotting was preceded similarly as we described before (Hong et al., 2004; Zhou et al., 2006). Briefly, protein samples were resolved on 7.5% SDS-PAGE minigels and electroblotted on to polyvinylidene difluoride membranes. The blots were blocked for 1 h with 5% nonfat dry milk in PBS containing 0.05% Tween 20, and then washed, and incubated for 1 h at room temperature with appropriate primary antibodies followed by the incubation with horseradish peroxidase-conjugated secondary antibodies. Since Myc epitope was tagged to the carboxyl termini of hOAT1 and hOAT3, the detection of hOAT1 and hOAT3 was facilitated by an antibody against Myc (9E10; 1:500) (Mount Sinai Medical Center, USA). Antibody against hOAT4 (Zhou et al., 2006) (1:1000) was acquired from Alpha Diagnostic Intl. Inc. (Texas, USA). The signals were detected by SuperSignal West Dura extended duration substrate kit (Pierce, Rockford, IL). Nonsaturating, immunoreactive protein bands were quantitated by scanning densitometry with the FluorChem 8000® imaging system (Alpha Innotech Corp., San Leandro, CA).
Statistical Analysis – Each experiment was repeated a minimum of three times. The statistical analysis given was from multiple experiments. Statistical analysis was performed using Student's paired t tests or Anova analysis as appropriate. A $p$ value of <0.05 was considered significant.
RESULTS

Cis Effect of Novobiocin on OAT-Mediated Transport

We first investigated the cis-effect of novobiocin on organic anion transport mediated by hOAT1, hOAT3, and hOAT4. Cis refers to the inhibitor being present on the same side of the membrane as radiolabeled substrates. The studies were performed in kidney COS-7 cells. As shown in Fig. 1, novobiocin, at a concentration of 100 μM, markedly reduced hOAT1-mediated uptake of [3H] PAH to 30%, reduced hOAT3-mediated uptake of [3H] estrone sulfate to 10%, and reduced hOAT4-mediated uptake of [3H] estrone sulfate to 50%.

hOAT4 is the only member of OAT family, which is expressed in the placenta. We previously showed (Zhou et al., 2008) that regulation of hOAT4 by scaffolding proteins are different between kidney cells and placental BeWo cells. Therefore, the inhibition effect of novobiocin on hOAT4 activity was also examined in human placental BeWo cells. As shown in Fig. 2, novobiocin reduced hOAT4-mediated uptake of [3H] estrone sulfate to 50%, similar to that observed in COS-7 cells.

Dose-Response Effect of Novobiocin on OAT-Mediated Transport

The effectiveness of novobiocin as an inhibitor of OAT uptake was confirmed by constructing a dose-response curve. OAT-mediated uptake of their substrates was measured in the presence of increasing concentrations of novobiocin (10-1000μM). As shown in Fig. 3, novobiocin inhibited OAT-mediated uptake of their substrates in a concentration-dependent manner. The IC50 values of novobiocin for hOAT1, hOAT3 and hOAT4 are 34.76 ± 0.31 μM, 4.987 ±0.35 μM and 92.68 ±0.34 μM respectively (Table 2). To further characterize the
mechanism of novobiocin inhibition of OAT-mediated uptake, uptake in the absence and presence of novobiocin was determined and analyzed by Dixon plot as depicted in Fig. 4. Our results showed that novobiocin inhibition of OAT-mediated transport was competitive in nature. We then determined the inhibition constant Ki by fitting the data to a competitive inhibition model, as described by Eq. 2 (Cheng and Prusoff, 1973; Akarawut and Smith, 1998). The $K_m$ values of OATs for their respective substrate was listed in Table 1 (established in our laboratory), and with the use of these $K_m$, the inhibition constant (Ki) of novobiocin was then calculated (Table 2). For all analysis, the coefficient of determination ($r^2$) was $\geq 0.9$.

**Trans Effect of Novobiocin on OAT-Mediated Transport**

hOAT1, hOAT3 and hOAT4 are known to function as an exchanger (You, 2002; You, 2004a; You, 2004b) with one organic anion transported into the cells to be exchanged for another organic anion effluxed from the cells. From previous experiments, novobiocin was shown to be a competitive inhibitor. However it is uncertain whether novobiocin could be transported by these hOATs. If novobiocin was a substrate of OATs, it would be expected that the presence of novobiocin in the opposite side of the membrane to the labeled substrates would increase the flux of labeled substrates. Instead, if novobiocin is not transported by the OATs but rather than binds to the carrier and prevents it from being available again for other substrates, then trans-inhibition would take place. Therefore, trans-effect on hOAT1-, hOAT3- and hOAT4-mediated transport by novobiocin was investigated via two different approaches (Fig. 5). In the first approach (Fig 5, top panel), cells expressing hOAT1, hOAT3, or hOAT4 were preloaded with or without novobiocin followed
by exposure to the medium containing respective radio-labeled OAT substrates. Parallel experiments were performed as positive controls with cells preloaded with unlabeled OAT substrates (PAH, for hOAT1 or DHEA for hOAT3 and hOAT4) followed by exposure to the medium containing respective radio-labeled OAT substrates. Novobiocin showed the trans-inhibition of uptake of radio-labeled OAT substrates rather than trans-stimulation shown by positive control. In the second approach (Fig. 5, bottom panel), cells expressing hOAT1, hOAT3, or hOAT4 were preloaded with respective radio-labeled OAT substrates ([3H] PAH for hOAT1 and [3H] estrone sulfate for hOAT3 and hOAT4), followed by exposure to medium containing novobiocin. Parallel experiments were performed as positive controls with cells preloaded with respective radio-labeled OAT substrates ([3H] PAH for hOAT1 and [3H] estrone sulfate for hOAT3 and hOAT4), followed by exposure to medium containing respective unlabeled OAT substrates. Again, novobiocin showed the trans-inhibition of efflux of radio-labeled OAT substrates rather than trans-stimulation shown by positive control.

Cell Surface and Total Cell Expression of OATs

It is known that the expression of certain transporters could be affected by their own substrates or inhibitors (Kilberg et al., 1980; Hyatt et al., 1997). To investigate whether the decrease in transport activity of OATs after novobiocin treatment is due to a decrease in the amount of transporter protein at the plasma membrane, we performed cell surface biotinylation experiments using the membrane-impermanent reagent Sulfo-NHS-LC-Biotin. Our results showed that cells treated with novobiocin did not have any effect on OAT protein
expression at the cell surface (Fig. 6). The total expression of OATs was also not affected by novobiocin treatment (not shown).
DISCUSSION

Drug transport across key epithelia such as kidney, liver, brain and placenta depends on the concerted action of transporters differentially expressed at the apical membrane and the basolateral membrane. For example, in renal proximal tubule cells, transporters at the basolateral membrane such as OAT1 and OAT3 are responsible for the movement of anionic drugs from blood across the basolateral membrane into the proximal tubule cells. Once inside the cells, these drugs are then extruded from the cells across apical membrane into the urine for the subsequent elimination. The apical step is accomplished by transporters at the apical membrane, such as Breast Cancer Resistant Proteins (BCRP), Multidrug Resistant Protein (MRP1) and P-glycoprotein (P-gp). Therefore, identification of specific inhibitor for each transporter will be important for dissecting the contribution of these transporters in the overall drug elimination process.

Novobiocin, a commonly used inhibitor for BCRP (Shiozawa et al., 2004; Huss et al., 2005; Su et al., 2007) has been indicated to inhibit organic anion transport in several systems. However, the interaction of novobiocin with specific OATs has never been explored. The main finding of the current study is that novobiocin is a potent inhibitor for hOAT1, hOAT3, and hOAT4. The first evidence that novobiocin interacts with OATs came from our cis-inhibition studies. Cis refers to the inhibitor being present on the same side of the membrane as radio-labeled substrates. We showed that uptake of radio-labeled substrates mediated by hOAT1, hOAT3 and hOAT4 in the presence of novobiocin was significantly reduced (Fig. 1). The inhibition potency of novobiocin for OATs are hOAT3 > hOAT1 > hOAT4 (Fig. 3). Dixon plot demonstrated that novobiocin is a competitive inhibitor for OATs (Fig. 4). The inhibitory effect of novobiocin to these hOATs was comparable to or...
even greater than that caused by 200µM of probenecid, a specific inhibitor for OATs (data not shown).

The cis inhibition feature of novobiocin (Figs.1-4) indicates that it might be a substrate of OATs, which competes with other radio-labeled substrate to be transported. The definitive criteria for showing a compound as a substrate for a specific transporter/exchanger are the ability to exhibit both cis-inhibition and trans-stimulation (Holohan and Ross, 1980). hOAT1, hOAT3 and hOAT4 are known to function as an exchanger (You, 2002; You, 2004a; You, 2004b). Therefore, if novobiocin is a substrate of OATs, we would expect that novobiocin present on one side of the membrane of the cells would result in an enhanced exchange of OAT substrate on the other side of the membrane of the cells. Our experiment with novobiocin present on the side opposite to that of radio-labeled OAT substrates (Fig. 5) showed a trans-inhibition instead of trans-stimulation of OAT-mediated transport, suggesting that novobiocin is not a substrate but an inhibitor for these transporters. The substrate-binding site of OATs is accessible to novobiocin from both the intracellular and the extracellular sides and once binding, novobiocin masks the substrate-binding sites on respective carriers, and thus reduces the number of free carriers available for the substrates. The cis- and trans-inhibition nature of novobiocin was similar to that of probenecid, a classical competitive inhibitor for OATs (Takeda et al., 2001; Enomoto et al., 2002b; Srimaroeng et al., 2005).

It is known that the expression of certain transporters is affected by their own substrates or inhibitors (Kilberg et al., 1980; Hyatt et al., 1997). To investigate whether inhibition of OAT activity by novobiocin resulted from a reduced cell surface expression of the transporter, we measured the expression of OATs at the cell surface and in total cell
lysate in cells treated with novobiocin. Our results (Fig. 6) showed that novobiocin treatment had no significant effect on cell surface and total cell expression of OATs, suggesting that the inhibition by novobiocin was not mediated through a change in the abundance of the transporters at the cell surface.

BCRP inhibitors or inhibitors for transporters related with drug resistance have been widely studied in the clinical cancer therapy. Co-administration of novobiocin with various cancer drugs can significantly increase their therapeutic efficiency (Murren et al., 2000). Novobiocin was normally used in vitro studies with a concentration range from 30 μM to 300 μM and with a plasma concentration around 150 μM in vivo (Kennedy et al., 1995; Su et al., 2007). Such in vivo concentration is significantly higher than its IC50 for hOATs demonstrated in our current studies, and is expected to be sufficient to affect the function of OATs and subsequently can affect the elimination of OATs substrates. Since the organic anion transporter (OAT) family mediates the body disposition of a diverse array of environmental toxins, and clinically important drugs, therefore, potential drug-drug interaction resulted from the inhibitory effect of novobiocin to OATs should be taken into consideration while using novobiocin to argument cancer drug resistance.
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Footnotes

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FIGURE LEGENDS

Fig. 1. Cis Effect of Novobiocin (Nov) on OAT-Mediated Transport in COS-7 cells. a. 3-min uptake of [³H] PAH (20 μM) in COS-7 cells transiently expressing hOAT1. b. 3-min uptake of [³H] estrone sulfate (ES, 100 nM) in COS-7 cells transiently expressing hOAT3. c. 3-min uptake of [³H] estrone sulfate (ES, 100 nM) COS-7 cells transiently expressing hOAT4. All the uptakes were measured in the presence (hatched bars) and the absence (solid bars) of novobiocin (Nov, 100 μM). Each data represents only the carrier-mediated transport after subtraction of values from vector-transfected cells. Uptake activity was expressed as a percentage of the uptake measured in cells without treatment of Nov. The results shown are means ± S.E. (n = 3).

Fig. 2. Cis Effect of Novobiocin (Nov) on hOAT4-Mediated Transport in BeWo Cells. 3-min-uptake of [³H] estrone sulfate (ES, 100 nM) in BeWo cells stably expressing hOAT4 was measured in the presence (hatched bar) and the absence (solid bar) of novobiocin (Nov, 100 μM). Each data represents only the carrier-mediated transport after subtraction of values from parental cells. Uptake activity was expressed as a percentage of the uptake measured in cells without treatment of Nov. The results shown are means ± S.E. (n = 3).

Fig. 3. Dose-Response Effect of Novobiocin (Nov) on OAT-Mediated Transport in COS-7 Cells. a. The 3-min uptake of [³H] PAH (20 μM) in hOAT1-expressing cells was measured in the presence of increasing concentrations of novobiocin (Nov, 10 μM- 1mM). The unit of X-axis is the log value of the concentration (molar) of novobiocin. Each data
point is the mean of triplicate values from a typical experiment and represents only the carrier-mediated transport after subtraction of values from vector-transfected cells. The line represents a best fit of the data using nonlinear regression analysis. b. The 3-min uptake of $[^3H]$ estrone sulfate (ES, 100 nM) in hOAT3-expressing cells was measured in the presence of increasing concentrations of novobiocin (Nov, 10 μM- 1mM). The unit of x-axis is the log value of the concentration (molar) of novobiocin. Data was calculated as described above. c. The 3-min uptake of $[^3H]$ estrone sulfate (ES, 100 nM) in hOAT4-expressing cells was measured in the presence of increasing concentrations of novobiocin (Nov, 10 μM- 0.8 mM). The unit of x-axis is the log value of the concentration (molar) of novobiocin. Each data point is the mean of triplicate values from a typical experiment and represents only the carrier-mediated transport after subtraction of values from cells transfected with vector. The line represents a best fit of the data using nonlinear regression analysis.

**Fig 4. Dixon Plot Analysis of Inhibitory Effect of Novobiocin (Nov) on OAT-mediated Transport in COS-7 Cells.** a. For the effect of novobiocin to hOAT1 - mediated uptake, $[^3H]$ PAH (20, 50 and 100 μM) uptake was determined at 3 min both in the absence and presence of varying concentration of novobiocin (Nov, 20, 40 and 100 μM). The specific uptake was obtained by subtracting $[^3H]$ PAH uptake into parental cells from the uptake into hOAT1-expressing cells. The data was fitted by linear-regression. b. For the effect of novobiocin to hOAT3, $[^3H]$ estrone sulfate (ES, 100, 200 and 500 nM) uptake was determined at 3 min both in the absence and presence of varying concentration of novobiocin (Nov, 2, 5, 15 and 50 μM). Uptake data analysis was the same as described for hOAT1. c. For hOAT4, $[^3H]$ estrone sulfate (ES, 100, 200 and 500 nM) uptake was
determined at 3 min both in the absence and presence of varying concentration of novobiocin (Nov, 50 to 600 μM). Uptake data analysis was the same as described for hOAT1 and hOAT3.

Fig.5 **Trans Effect of Novobiocin (Nov) on OAT-mediated Transport in COS-7 cells.** Top panel: Influx studies: Cells expressing hOAT1, hOAT3 or hOAT4 were preloaded (PL) with novobiocin (Nov, 1 mM) or respective unlabeled OAT substrates (1mM PAH for hOAT1, 3μM DHEA for hOAT3, 4 μM DHEA for hOAT4) (experimental groups) or with PBS (control) for 1 h, followed by washing with PBS and a subsequent exposure (EXP) to medium containing radio-labeled OAT substrates (20 μM [³H] PAH for hOAT1, 100nM [³H] estrone sulfate (ES) for hOAT3 and hOAT4). 3 min later, the uptake was stopped by rapidly washing the cells with ice-cold PBS. Intracellular accumulation of radio-labeled substrates was then counted. Each value represents the difference between experimental group and control group and was expressed as a percentage of the uptake measured in cells without preloading with novobiocin or positive control. The results shown are means ± S.E. (n = 3).

Bottom panel: Efflux studies: Cells expressing hOAT1, hOAT3 or hOAT4 were preloaded (PL) with respective radio-labeled OAT substrates (20 μM [³H] PAH for hOAT1, 100 nM [³H] estrone sulfate (ES) for hOAT3 and hOAT4) for 45 min, followed by washing with PBS and a subsequent exposure (EXP) to medium containing 0.1 mM Nov, or unlabeled OAT substrates (1mM PAH for hOAT1, 4μM estrone sulfate (ES) for hOAT3 and hOAT4). After 3 min incubation, the incubation medium were collected and counted as described in the **MATERIALS and METHODS.** The intracellular accumulation of labeled substrates was also counted after lysis the cells. Efflux was expressed as the percentage of total PAH or ES.
(intracellular+efflux medium) appeared in the efflux medium after 3 min of incubation. The results shown are means ± S.E. (n = 3).

**Fig. 6. Cell Surface Expression of OATs.** Cells expressing hOAT1, hOAT3 or hOAT4 were treated with or without 0.1 mM of novobiocin (Nov) for 30min. The cell surface expression levels of these OATs were then examined using the membrane-impermanent biotinylation reagent, NHS-SS-biotin, followed by Western blotting as described in the section of *MATERIALS and METHODS*. The primary antibodies used for Western blotting were: anti-myc (9E10, 1:500) for detecting hOAT1 or hOAT3 (myc epitope was tagged to the carboxyl termini of hOAT1 and hOAT3; see the section of *MATERIALS AND METHODS*). anti-hOAT4 (1:1000) for detecting hOAT4. **Top panel:** Western blot results. **Bottom panel:** Densitometry analysis of the results from the top panel. a. Cell surface expression of hOAT1-myc. b. Cell surface expression of hOAT3-myc. c. Cell surface expression of hOAT4. There is no statistical difference between treated group and control group.

**Fig 7. Model of Novobiocin (Nov) Interaction with OATs.** Top panel: OATs are known to be an exchanger. Under normal condition, one organic anion was transported into the cells with exchange of another organic anion efflux from the cells.

**Bottom panel:** When Nov binds to the substrate binding site, it blocks the access of other substrates (either extracellular or intracellular) to this binding site, and therefore reduces the number of transporters available to carry substrates and subsequently inhibits the function of OATs.
Table 1. Km values of OAT-mediated Organic Anion Transport.

<table>
<thead>
<tr>
<th>Transporters</th>
<th>Uptake of substrates</th>
<th>Km (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hOAT1</td>
<td>PAH</td>
<td>14.8 ± 0.10</td>
</tr>
<tr>
<td>hOAT3</td>
<td>estrone sulfate</td>
<td>2.18 ± 0.14</td>
</tr>
<tr>
<td>hOAT4</td>
<td>estrone sulfate</td>
<td>4.20 ± 0.60</td>
</tr>
</tbody>
</table>
Table 2. Inhibitory Constants of Novobiocin on OAT-mediated Organic Anion Transport.

<table>
<thead>
<tr>
<th>Transporters</th>
<th>Uptake of substrates</th>
<th>Ki (μM)</th>
<th>IC₅₀ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hOAT1</td>
<td>PAH</td>
<td>14.87± 0.40</td>
<td>34.76 ± 0.31</td>
</tr>
<tr>
<td>hOAT3</td>
<td>estrone sulfate</td>
<td>4.77 ± 1.12</td>
<td>4.99 ± 0.35</td>
</tr>
<tr>
<td>hOAT4</td>
<td>estrone sulfate</td>
<td>90.50 ± 7.50</td>
<td>92.68 ± 0.34</td>
</tr>
</tbody>
</table>
Fig. 1

(a) Uptake of $^3$H-PAH (% of control)

(b) Uptake of $^3$H-ES (% of control)

(c) Uptake of $^3$H-ES (% of control)

hOAT1

hOAT3

hOAT4
Fig. 2

Uptake of $^3$H-ES (% of control)

- Control
- Nov-treated
Fig. 3

a. hOAT1
IC$_{50}$ = 34.76 µM

b. hOAT3
IC$_{50}$ = 4.987 µM

c. hOAT4
IC$_{50}$ = 92.68 µM
Fig. 5

- **hOAT1**
  - PL: PBS, Nov, PAH
  - Uptake of $^3$H-PAH (% of control)
  - Uptake of $^3$H-ES (% of control)
  - Efflux of $^3$H-PAH (% of control)
  - Efflux of $^3$H-ES (% of control)

- **hOAT3**
  - PL: PBS, Nov, DHEA
  - Uptake of $^3$H-PAH (% of control)
  - Uptake of $^3$H-ES (% of control)
  - Efflux of $^3$H-PAH (% of control)
  - Efflux of $^3$H-ES (% of control)

- **hOAT4**
  - PL: PBS, Nov, DHEA
  - Uptake of $^3$H-PAH (% of control)
  - Uptake of $^3$H-ES (% of control)
  - Efflux of $^3$H-PAH (% of control)
  - Efflux of $^3$H-ES (% of control)
Fig. 6

a) hOAT1

b) hOAT3

c) hOAT4

Arbitrary unit

control  Nov-treated

control  Nov-treated

control  Nov-treated
Fig 7

Symbol Legend

- OATs
- Substrate 1
- Substrate 2
- Substrate binding sites of OATs
- Novobiocin