Novobiocin Is a Potent Inhibitor for Human Organic Anion Transporters

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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 resistance 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 (hOAT)1, hOAT3, and hOAT4. Kinetic study revealed that novobiocin inhibited OAT-mediated uptake in a competitive manner, with K_i of $14.87 \pm 0.40 \mu\text{M}$ for hOAT1, K_i of $4.77 \pm 1.12 \mu\text{M}$ for hOAT3, and K_i of $90.50 \pm 7.50 \mu\text{M}$ for hOAT4. Furthermore, the *cis*- and *trans*-inhibition feature of novobiocin demonstrated that novobiocin was a potent inhibitor but not a substrate for hOAT1 ($\text{IC}_{50} = 34.76 \pm 0.31$

μM), hOAT3 ($\text{IC}_{50} = 4.987 \pm 0.35 \mu\text{M}$), and hOAT4 ($\text{IC}_{50} = 92.68 \pm 0.34 \mu\text{M}$). We further showed that the effects of novobiocin on OATs were not mediated through a change in transporter protein abundance on the plasma membrane. Taken together, we conclude that novobiocin seems 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. Because 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.

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, antitumor drugs, antibiotics, antihypertensive drugs, and anti-inflammatory drugs (You, 2002, 2004a,b), and is therefore critical for the survival of mammalian species.

Ten members of the 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, 1998; Sweet et al., 1997; Wolff et al., 1997; Cihlar et al., 1999; Kusuha 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_v1, urate/anion exchanger, 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 use 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, 2004a,b). 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 used 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 the following: 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

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ABBREVIATIONS: OAT, organic anion transporter; BCRP, breast cancer resistance proteins; hOAT, human OAT; PAH, [^3H]*p*-aminohippuric acid; ES, estrone sulfate; DHEA, dehydroepiandrosterone-3-sulfate; FBS, fetal bovine serum; PBS, phosphate-buffered saline; sulfo-NHS-SS-biotin, sulfosuccinimidyl 2-(biotinamido)-ethyl-1,3-dithiopropionate.

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 (Odland, 1981; Besseghir, 1985) and has been widely used as a specific inhibitor for BCRPs in both in vitro and in vivo studies (Shiozawa et al., 2004; Huss et al., 2005; Su et al., 2007). BCRP is associated with a 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 human OAT (hOAT)1, hOAT3, and hOAT4.

Materials and Methods

[³H]p-Aminohippuric acid (PAH) and [³H]estrone sulfate (ES) were obtained from PerkinElmer Life and Analytical Sciences (Waltham, MA). COS-7 cells were purchased from American Type Culture Collection (Manassas, VA). Dehydroepiandrosterone-3-sulfate (DHEA) and other reagents were purchased from Sigma-Aldrich (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 FBS, 100 U/ml penicillin/streptomycin, and 100 mg/ml glucose 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 FBS, 0.5 mg/ml geneticin (G418; Invitrogen, Carlsbad, CA), and 100 mg/ml glucose in a 5% CO₂ atmosphere at 37°C. Cells were grown to 90 to 100% confluence and transfected with the appropriate plasmids using Lipofectamine 2000 (Invitrogen). The plasmids used for transfection were as follows: 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 20 μM [³H]PAH or 100 nM [³H]estrone sulfate. The uptake experiments were performed at room temperature and at the times indicated in the figure legends, and 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 1 mM novobiocin, 1 mM PAH, or 3 or 4 μM DHEA separately for 1 h at 37°C to allow the chemical substances to diffuse into the cells, followed by rapid washing and subsequent exposure to PBS containing radio-labeled OAT substrates (20 μM [³H]PAH for hOAT1 or 100 nM [³H]estrone sulfate for hOAT3 and hOAT4). Transport measurement was performed as described above.

For trans-efflux studies, we followed the procedures described by Srimalroeng et al. (2005). Cells expressing hOAT1, hOAT3, or hOAT4 were preloaded with 20 μM [³H]PAH or 100 nM [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 the absence or presence of 1 mM PAH (for hOAT1) or 4 μM estrone sulfate (for hOAT3 and hOAT4) and 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 ice-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) and appeared in the incubation medium after 3 min of efflux.

Concentration-Dependent Inhibition Studies. Inhibition studies were performed at varying concentrations of novobiocin (10–1000 μM). hOAT-specific uptake was obtained by subtracting uptake ([³H]PAH for hOAT1 or [³H]estrone sulfate for hOAT3 and hOAT4) into the 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 eq. 1 (Akarawut and Smith, 1998) using GraphPad Prism software (GraphPad Software Inc., San Diego, CA). In eq. 1, V_{max} is the maximal rate of saturable uptake (in the 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.

$$V = \frac{V_{\max}}{1 + 10^{X - \text{LogIC}_{50}}} \quad (1)$$

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 (20, 50, and 100 μM) was determined at 3 min in both the absence and presence of varying concentrations of novobiocin (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 were analyzed by linear regression with GraphPad Prism. For hOAT3, [³H]estrone sulfate (100, 200, and 500 nM) uptake was determined at 3 min in the absence and presence of varying concentrations of novobiocin (2, 5, 15, and 50 μM). Uptake data analysis was the same as that described for hOAT1. For hOAT4, [³H]estrone sulfate (100, 200, and 500 nM) uptake was determined at 3 min in both the absence and presence of varying concentrations of novobiocin (50–600 μM). Uptake data analysis was the same as that described for both hOAT1 and hOAT3.

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

$$K_i = \frac{\text{IC}_{50}}{1 + C/K_m} \quad (2)$$

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 complete 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-Aldrich)]. The cell lysates were cleared by centrifugation at 16,000g at 4°C. Fifty microliters of streptavidin-agarose beads (Pierce) was then added to the supernatant to isolate cell membrane protein. OATs were detected in the pool of surface proteins by SDS-polyacrylamide gel electrophoresis and immunoblotting.

Electrophoresis and Immunoblotting. Immunoblotting was performed as described previously (Hong et al., 2004; Zhou et al., 2006). In brief, protein samples were resolved on 7.5% SDS-polyacrylamide gel electrophoresis minigels and electroblotted onto 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 incubation with horseradish peroxidase-conjugated secondary antibodies. Because 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). Antibody against hOAT4 (Zhou et al., 2006) (1:1000) was

FIG. 1. *cis*-effect of novobiocin (Nov) on OAT-mediated transport in COS-7 cells. a, three-minute uptake of [³H]PAH (20 μ M) in COS-7 cells transiently expressing hOAT1. b, three-minute uptake of [³H]ES (100 nM) in COS-7 cells transiently expressing hOAT3. c, three-minute uptake of [³H]ES (100 nM) COS-7 cells transiently expressing hOAT4. All the uptakes were measured in the presence (hatched bars) and absence (solid bars) of Nov (100 μ M). All data represent 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 novobiocin. The results shown are means $\pm$ S.E. ($n = 3$).

acquired from Alpha Diagnostic International Inc. (San Antonio, TX). The signals were detected by SuperSignal West Dura extended duration substrate kit (Pierce). Nonsaturating, immunoreactive protein bands were quantitated by scanning densitometry with the FluorChem 8000 imaging system (Alpha Innotech, 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 analysis of variance 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 μ M, 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 that 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

FIG. 2. *cis*-effect of novobiocin (Nov) on hOAT4-mediated transport in BeWo Cells. Three-minute uptake of [³H]ES (100 nM) in BeWo cells stably expressing hOAT4 was measured in the presence (hatched bar) and absence (solid bar) of Nov (100 μ M). All data represent 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 novobiocin. The results shown are means $\pm$ S.E. ($n = 3$).

concentrations of novobiocin (10–1000 μ M). 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 , 4.987 ± 0.35 , and

FIG. 3. Dose-response effect of novobiocin (Nov) on OAT-mediated transport in COS-7 cells. a, three-minute uptake of [3 H]PAH (20 μ M) in hOAT1-expressing cells was measured in the presence of increasing concentrations of Nov (10 μ M–1 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 vector-transfected cells. The line represents a best fit of the data using nonlinear regression analysis. b, three-minute uptake of [3 H]ES (100 nM) in hOAT3-expressing cells was measured in the presence of increasing concentrations of Nov (10 μ M–1 mM). The unit of x-axis is the log value of the concentration (molar) of novobiocin. Data were calculated as described above. c, three-minute uptake of [3 H]ES (100 nM) in hOAT4-expressing cells was measured in the presence of increasing concentrations of Nov (10 μ M–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.

$92.68 \pm 0.34 \mu$ M, respectively (Table 1). 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 K_i 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 were listed in Table 2 (established in our laboratory), and with the use of these K_m , the inhibition constant (K_i) of novobiocin was then calculated (Table 1). For all analyses, the coefficient of determination (r^2) was ≥ 0.9 .

Trans-Effect of Novobiocin on OAT-Mediated Transport. hOAT1, hOAT3, and hOAT4 are known to function as an exchanger (You, 2002, 2004a,b) 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 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 radiolabeled 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 radiolabeled OAT substrates. Novobiocin showed the trans-inhibition of uptake of radiolabeled 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 radiolabeled OAT substrates ([3 H]PAH for hOAT1 and [3 H]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

radiolabeled OAT substrates ([3 H]PAH for hOAT1 and [3 H]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 radiolabeled 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-SS-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 (data 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 BCRP, multidrug resistance protein, and P-glycoprotein. Therefore, identification of specific inhibitors 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 radiolabeled substrates. We

TABLE 1
Inhibitory constants of novobiocin on OAT-mediated organic anion transport

Transporters	Uptake of Substrates	K_i	IC_{50}
hOAT1	PAH	14.87 ± 0.40	34.76 ± 0.31
hOAT3	Estrone sulfate	4.77 ± 1.12	4.99 ± 0.35
hOAT4	Estrone sulfate	90.50 ± 7.50	92.68 ± 0.34

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, [3 H]PAH (20, 50, and 100 μ M) uptake was determined at 3 min in the absence and presence of varying concentrations of Nov (20, 40, and 100 μ M). The specific uptake was obtained by subtracting [3 H]PAH uptake into parental cells from the uptake into hOAT1-expressing cells. The data were fitted by linear regression. b, for the effect of novobiocin to hOAT3, [3 H]ES (100, 200, and 500 nM) uptake was determined at 3 min in both the absence and presence of varying concentrations of Nov (2, 5, 15, and 50 μ M). Uptake data analysis was the same as that described for hOAT1. c, for hOAT4, [3 H]ES (100, 200, and 500 nM) uptake was determined at 3 min in the absence and presence of varying concentrations of Nov (50–600 μ M). Uptake data analysis was the same as that described for hOAT1 and hOAT3.

showed that uptake of radiolabeled substrates mediated by hOAT1, hOAT3, and hOAT4 in the presence of novobiocin was significantly reduced (Fig. 1). The inhibition potency of novobiocin for OATs is 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 with or even greater than that caused by 200 μ M 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 radiolabeled

TABLE 2

K_m values of OAT-mediated organic anion transport

Transporters	Uptake of Substrates	<i>K_m</i>
		μM
hOAT1	PAH	14.8 ± 0.10
hOAT3	Estrone sulfate	2.18 ± 0.14
hOAT4	Estrone sulfate	4.20 ± 0.60

substrates 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, 2004a,b). 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 radiolabeled 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 occurs, novobiocin masks the substrate binding sites on respective carriers and thus reduces the number of free carriers available for the substrates (Fig. 7). The *cis*- and trans-inhibition nature of novobiocin was similar to that of probenecid, a classic 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. Coadministration of novobiocin with various cancer drugs can significantly increase their therapeutic efficiency (Murren et al., 2000).

FIG. 5. Trans-effect of novobiocin (Nov) on OAT-mediated transport in COS-7 cells. Top panels, influx studies: cells expressing hOAT1, hOAT3, or hOAT4 were preloaded (PL) with novobiocin (Nov, 1 mM) or respective unlabeled OAT substrates (1 mM PAH for hOAT1, 3 μM 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 radiolabeled OAT substrates (20 μM [³H]PAH for hOAT1, 100 nM [³H]ES for hOAT3 and hOAT4). Three minutes later, the uptake was stopped by rapidly washing the cells with ice-cold PBS. Intracellular accumulation of radiolabeled 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 $\pm$ S.E. ($n = 3$). Bottom panels, efflux studies: cells expressing hOAT1, hOAT3, or hOAT4 were preloaded (PL) with respective radiolabeled OAT substrates (20 μM [³H]PAH for hOAT1 and 100 nM [³H]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 (1 mM PAH for hOAT1 and 4 μM ES for hOAT3 and hOAT4). After 3-min incubation, the incubation media were collected and counted as described under *Materials and Methods*. The intracellular accumulation of labeled substrates was also counted after cell lysis. Efflux was expressed as the percentage of total PAH or ES (intracellular + efflux medium) and appeared in the efflux medium after 3 min of incubation. The results shown are means $\pm$ 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 30 min. The cell surface expression levels of these OATs were then examined using the membrane-impermeant biotinylation reagent, NHS-SS-biotin, followed by Western blotting as described under *Materials and Methods*. The primary antibodies used for Western blotting were as follows: anti-myc (9E10, 1:500) for detecting hOAT1 or hOAT3 (myc epitope was tagged to the carboxyl termini of hOAT1 and hOAT3; see *Materials and Methods*); and anti-hOAT4 (1:1000) for detecting hOAT4. Top panels, Western blot results. Bottom panels, densitometry analysis of the results from the top panels. 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 the treated group and the control group.

FIG. 7. Model of novobiocin (Nov) interaction with OATs. Top panel, OATs are known to be an exchanger. Under normal conditions, 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.

Novobiocin was normally used in *in vitro* studies with a concentration range from 30 to 300 μM and with a plasma concentration approximately 150 μM *in vivo* (Kennedy et al., 1995; Su et al., 2007). Such *in vivo* concentration is significantly higher than its IC_{50} for hOATs demonstrated in our current studies and is expected to be sufficient to affect the function of OATs and can subsequently affect the elimination of OATs substrates. Because the OAT family mediates the body disposition of a diverse array of environmental toxins and clinically important drugs, the potential drug-drug interaction that 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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