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Original Article

Inhibitory effects of chemotherapeutics on human organic anion transporter hOAT4

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Abstract: Human organic anion transporter 4 (hOAT4) belongs to a family of organic anion transporters which play critical roles in the body disposition of clinically important drugs. hOAT4 is expressed in the kidney and placenta. In the current study, we examined the inhibitory effects of 101 anticancer drugs from a clinical drug library on hOAT4 transport activity. The studies were carried out in hOAT4-expressing human kidney HEK-293 cells and human placenta BeWo cells. Among these drugs, only chlorambucil and cabazitaxel demonstrated more than 50% *cis*-inhibitory effect on hOAT4-mediated uptake of ^{3}H-labeled estrone sulfate, a prototypical substrate for the transporter. The IC_{50} values for chlorambucil and cabazitaxel were 44.28 and 3.5 μM respectively. Dixon plot analysis revealed that inhibition by chlorambucil was competitive with a K_i = 55.73 μM whereas inhibition by cabazitaxel was non-competitive with a K_i = 1.78 μM. Our results demonstrated that chlorambucil and cabazitaxel were inhibitors of hOAT4. Furthermore, by comparing our data with clinically relevant exposures of these drugs, we conclude that the propensity for chlorambucil and cabazitaxel to cause drug-drug interaction through inhibition of hOAT4 is low.

Keywords: Organic anion transporter, drug transporter, chemotherapeutics

Introduction

Human organic anion transporter 4 (hOAT4), a member of the organic anion transporter family, greatly affects the body disposition, clinical outcome, and toxicity risks of drugs [1-3]. hOAT4 is abundantly expressed in the kidney and placenta [1, 4, 5]. In the kidney, hOAT4 is localized at the apical membrane of the proximal tubule cells that mediates the renal elimination of anionic drugs into the tubule lumen and their reabsorption from the primary urine, consequently influencing the clinical pharmacokinetic profiles of these compounds [3, 5]. hOAT4 has been shown to interact with the inhibitors of angiotensin converting enzyme, antibiotics, antivirals, antineoplastic agents, and nonsteroidal anti-inflammatory drugs [2, 3, 6, 7]. Since hOAT4 has affinities for many compounds, drug-drug interactions (DDI) may occur when multiple drugs are co-administered, leading to altered therapeutic response. Although the implications of oncology drugs on human organic anion transporters 1 and 3, both of which are isoforms of hOAT4, have been previously reported [8-10], to our knowledge, the interrogation of anticancer drugs as inhibitors of hOAT4 and the potential implication for clinical DDIs has been relatively limited. Additionally, characterizing hOAT4-anti-cancer drugs interaction may serve as an early assessment of DDI, pharmacological outcome, and side effects.

The placenta is a highly specialized endocrine organ that functions as a structural interface between the developing embryo and the parental tissue [11]. Proper placental function is crucial for normal fetal development and a successful pregnancy [4, 11]. In addition to mediating the maternal-fetal transfer of nutrients and oxygen, the placenta also facilitates the elimination of metabolic waste products, therapeutic agents, and environmental toxins from the fetus [6]. These protective characteristics are in part attributed to the expression of transport proteins in the placenta epithelium [12-14]. Human organic anion transporter 4 (hOAT4), located in the basolateral membrane of the placenta facing the fetal circulation has been proposed to regulate the cellular uptake and disposition of steroid sulfates, xenobiotics, and clinically important drugs [4, 12, 14]. As women in

developed nations continue to delay child birth to a later age, it is expected that the coexistence of pregnancy and cancer will become a more common occurrence [15, 16]. Consequently, more and more women will be exposed to chemotherapy during gestation. Given the importance of hOAT4 in fetal development, understanding the propensity of anticancer drugs to inhibit and interact with hOAT4 is of profound clinical significance.

In the present work, a series of cell culture-based screening were conducted to identify hOAT4 inhibitors from a current FDA-approved anticancer drug library consisting of a total of 101 drugs. Two drugs chlorambucil and cabazitaxel were identified to result in a greater than 50% inhibition of hOAT4-mediated transport.

Materials and methods

Reagents

The National Institute of Health/National Cancer Institute (NIH/NCI) oncology drug set IV plate (AOD4, plate key: 4762074) was acquired from NCI Chemotherapeutic Agents Repository, Fisher BioServices. [^{3}H] estrone sulfate (ES) was obtained from PerkinElmer Life and Analytical Sciences (Waltham, MA). HEK293 cells were purchased from American Type Culture Collection (Manassas, VA). Cabazitaxel and chlorambucil used in IC_{50} and Dixon plot experiments were purchased from Selleckhem (Houston, TX) and Sigma-Aldrich (St. Louis, MO) respectively. All other reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless stated otherwise.

Cell culture

Parental HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) in 5% CO_2 atmosphere at 37°C. Cells were seeded at 5×10^5 per well of 6-well cluster plate 24 h before transfection. The plasmid used for stable transfection was hOAT4-pcDNA. Myc epitope was tagged to the carboxyl termini of hOAT4 to facilitate the detection of the transporter protein. For transfection of hOAT4 cDNA plasmid, a Lipofectamine 2000 reagent was used following the manufacturer's instruction. After 7-8 days of selection in medium containing 0.5 mg/mL geneticin (G418; Invitrogen, Carlsbad, CA), resistant colonies were replated to 96-wells for cloning, expansion, and analyzing positive clones. Previously established stable hOAT4-expressing BeWo b30-10 cells were cultured as described [17].

Transport measurements

Cells were plated at a density of 140,000 cells/well in 48-well collagen coated plate. Uptake solution consisted of phosphate-buffered saline (PBS) (1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.3) and 100 nM [^{3}H]ES. The uptake experiments were conducted at room temperature with time points indicated in the figure legends. Uptake was terminated with rapid washing of the cells with 500 µL ice-cold PBS solution twice. Cells were lysed in 0.2 M NaOH, neutralized in 0.2N HCl and placed in individual scintillation vials. Radioactivity was measured using Beckman LC6500 scintillation counter.

Concentration-dependent inhibition studies

Inhibition studies were performed at varying concentrations of chlorambucil and cabazitaxel. hOAT4 specific uptake was obtained by subtracting [^{3}H]ES uptake into parental cells from the uptake into hOAT4-expressing cells. The IC_{50} (concentration of the drugs required to inhibit 50% of ES uptake) was determined by nonlinear regression using GraphPad Prism.

Dixon plot

The mechanism of inhibition was determined by linear regression analysis of reciprocal saturable uptake (1/v) for different substrate concentrations (1.2 µM or 2.4 µM ES) as a function of inhibitor concentration. hOAT4 uptake was determined at 3 min in both the absence and presence of varying concentrations of chlorambucil and cabazitaxel. The specific uptake was obtained by subtracting [^{3}H]ES uptake into parental cells from the uptake into hOAT4-expressing cells. The data were analyzed by linear regression with GraphPad Prism. K_i values were calculated from the intersection of lines representing [ES] = 1.2 µM and [ES] = 2.4 µM.

Statistical analysis

Each experiment was repeated a minimum of two times. Statistical analysis was performed

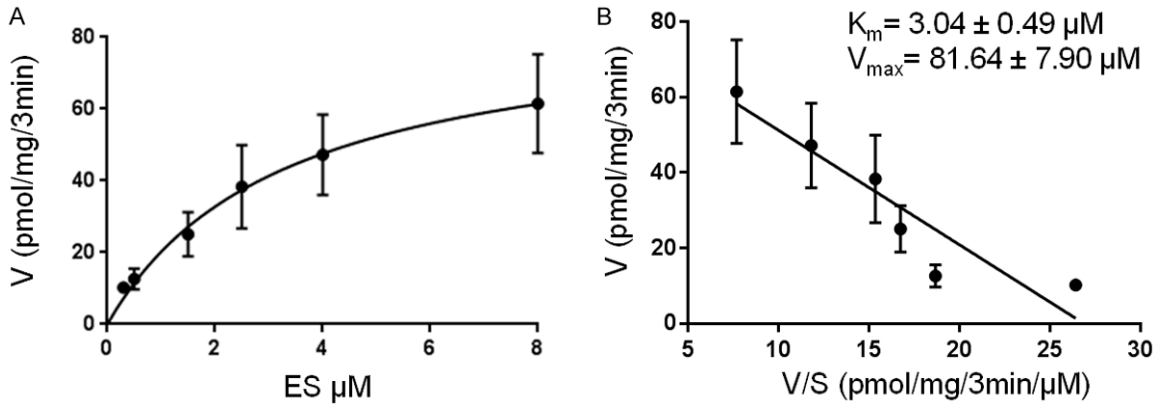


Figure 1. Kinetic analysis of hOAT4-mediated estrone sulfate (ES) transport in HEK293 cells. A. Kinetic characteristics were determined at substrate concentration ranging from 0.05 to 8 μM (3-min uptake). The data represent uptake into pcDNA-hOAT4 transfected cells minus uptake into pcDNA vector-transfected cells. Values are mean ± S.E. (n = 3). B. Transport kinetic values were calculated using the Eadie-Hofstee transformation.

using GraphPad Prism software (GraphPad Software Inc., San Diego, CA), one-way ANOVA, multiple comparisons Tukey's test. A p value of < 0.05 was considered significant.

Results

Functional characterization of hOAT4

To investigate the regulation of hOAT4 in renal cells, we established human embryonic kidney (HEK)-293 cells stably expressing hOAT4. The functional properties of hOAT4 in these cells were then characterized. The time-dependent uptake of [^{3}H]-estrone sulfate (ES), a prototypical substrate, in hOAT4-expressing cells was markedly faster than that in control cells. As the uptake increased linearly within 5 min, an uptake period of 3 min (initial rate) was chosen for future studies. The kinetics of ES transport was analyzed. The initial rate of ES uptake over a wide range of ES concentrations was determined (**Figure 1**). The transport of ES across the cell membrane was saturable (**Figure 1A**). Based on Eadie–Hofstee plot analysis (**Figure 1B**), the K_m value for ES was 3.04 ± 0.49 μM and V_{max} was 81.64 ± 7.90 pmol/mg/3 min.

Cis-inhibition of hOAT4-mediated ES uptake by anti-cancers drugs in HEK293 cells

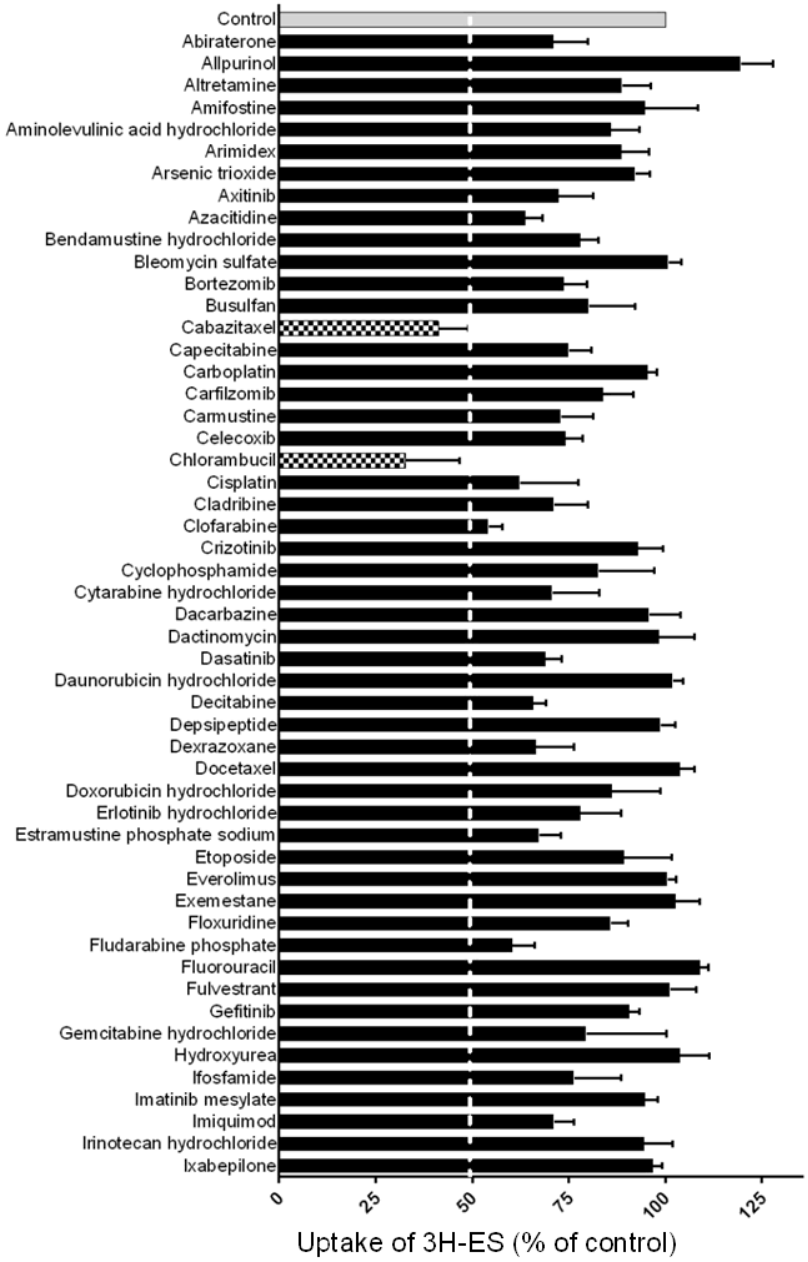
To identify hOAT4 inhibitors from the NIH/NCI drug library oncology drugs set IV plate (plate key: 4762074), *cis*-inhibition studies were performed in hOAT4-expressing HEK293 cells. Although many of the drugs tested demonstrated some level of inhibition, only chlorambucil and cabazitaxel demonstrated greater than 50% suppression of hOAT4-mediated [^{3}H]ES uptake at the indicated concentrations (**Figure 2**). Compounds without appreciable inhibitory activity suggest a lack of hOAT4 interaction. Thus, their probability to cause drug interactions via hOAT4 inhibition can be excluded. Probenecid, a known inhibitor for OAT family members [18], was used as an inhibitor control for this study. We therefore, focus on chlorambucil and cabazitaxel in the following studies.

Cis-inhibition of hOAT4-mediated ES uptake by chlorambucil and cabazitaxel in BeWo cells

hOAT4 is expressed in both the kidney and placenta. The inhibition effects of chlorambucil and cabazitaxel were next characterized in human placenta BeWo cells stably expressing hOAT4. This cell line was previously established in our lab [6]. At the concentration of 100 μM, significant inhibition of hOAT4-mediated ES uptake by both drugs was observed in these cells (**Figure 3**). Probenecid was again used as an inhibitor control for this study.

Dose dependent effects of chlorambucil and cabazitaxel on hOAT4-mediated transport

Dose response curves were constructed to evaluate the effectiveness of chlorambucil and cabazitaxel as inhibitors of hOAT4 uptake in HEK293 cells. 25-500 μM chlorambucil (**Figure 4A**) and 1-100 μM cabazitaxel (**Figure 4B**) significantly inhibited hOAT4-mediated ES uptake


Control
Abiraterone
Allpurinol
Altretamine
Amifostine
Aminolevulinic acid hydrochloride
Arimidex
Arsenic trioxide
Axitinib
Azacitidine
Bendamustine hydrochloride
Bleomycin sulfate
Bortezomib
Busulfan
Cabazitaxel
Capecitabine
Carboplatin
Carfilzomib
Carmustine
Celecoxib
Chlorambucil
Cisplatin
Cladribine
Clofarabine
Crizotinib
Cyclophosphamide
Cytarabine hydrochloride
Dacarbazine
Dactinomycin
Dasatinib
Daunorubicin hydrochloride
Decitabine
Depsipeptide
Dexrazoxane
Docetaxel
Doxorubicin hydrochloride
Erlotinib hydrochloride
Estramustine phosphate sodium
Etoposide
Everolimus
Exemestane
Floxuridine
Fludarabine phosphate
Fluorouracil
Fulvestrant
Gefitinib
Gemcitabine hydrochloride
Hydroxyurea
Ifosfamide
Imatinib mesylate
Imiquimod
Irinotecan hydrochloride
Ixabepilone
0
25
50
75
100
125
Uptake of 3H-ES (% of control)

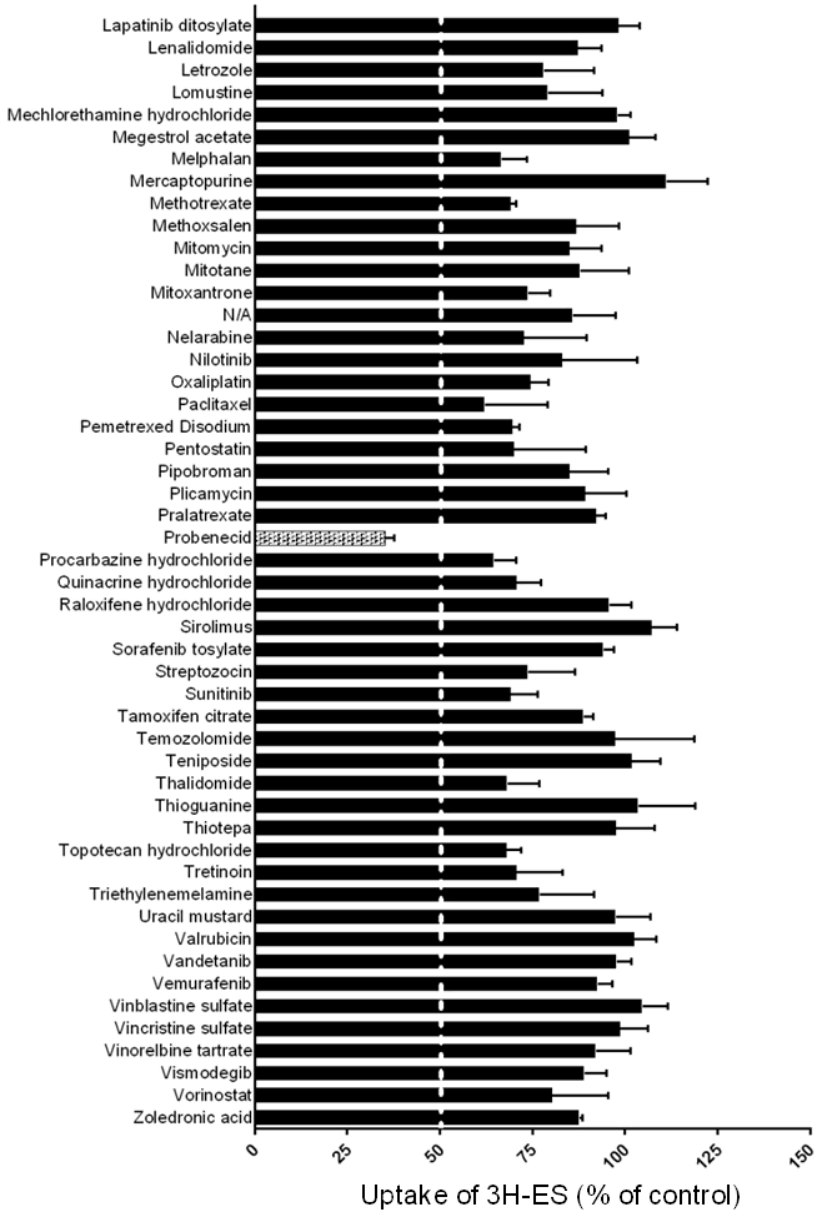

Lapatinib ditosylate
Lenalidomide
Letrozole
Lomustine
Mechlorethamine hydrochloride
Megestrol acetate
Melphalan
Mercaptopurine
Methotrexate
Methoxsalen
Mitomycin
Mitotane
Mitoxantrone
N/A
Nelarabine
Nilotinib
Oxaliplatin
Paclitaxel
Pemetrexed Disodium
Pentostatin
Pipobroman
Plicamycin
Pralatrexate
Probenecid
Procarbazine hydrochloride
Quinacrine hydrochloride
Raloxifene hydrochloride
Sirolimus
Sorafenib tosylate
Streptozocin
Sunitinib
Tamoxifen citrate
Temozolomide
Teniposide
Thalidomide
Thioguanine
Thiotepa
Topotecan hydrochloride
Tretinoin
Triethylenemelamine
Uracil mustard
Valrubicin
Vandetanib
Vemurafenib
Vinblastine sulfate
Vincristine sulfate
Vinorelbine tartrate
Vismodegib
Vorinostat
Zoledronic acid
0
25
50
75
100
125
150
Uptake of 3H-ES (% of control)

Figure 2. Interaction of hOAT4 with 101 anticancer drugs from the NIH/NCI oncology drug set IV, plate key: 4762074. hOAT4 mediated [^{3}H]ES uptake was measured in HEK293 cells stably expressing hOAT4. The uptake of 100 nM [^{3}H]ES in the absence (control) or presence of test compounds (10 μM) for 3 mins were measured. Each data point represent only carrier mediated transport after subtraction of values from parental cells. Uptake activity was expressed as percentage of uptake measured in control cells from three independent experiments. Results shown are means ± S.E.

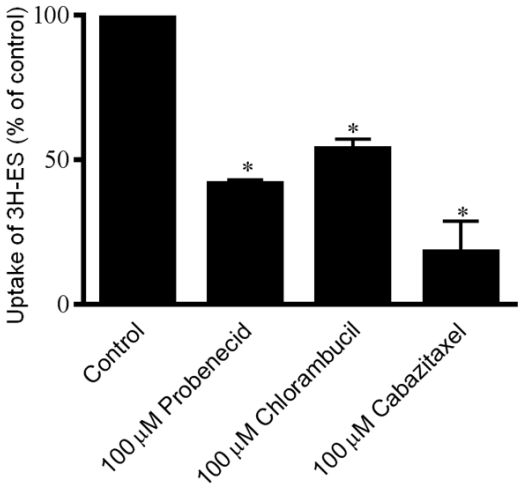


Figure 3. *Cis*-effect of cabazitaxel and chlorambucil on hOAT4 uptake in BeWo cells. hOAT4-mediated [^{3}H]ES uptake was measured in BeWo cells stably expressing hOAT4. The uptake of 100 nM [^{3}H]ES in the absence (control) or presence of test compounds (100 μM) for 3 mins were measured. Each data point represent only carrier mediated transport after subtraction of values from parental cells. Uptake activity was expressed as percentage of uptake measured in control cells from three independent experiments. Results shown are means ± S.E. of three separate experiments. Data were analyzed statistically with ANOVA, followed by Tukey's post-hoc test. *P < 0.05.

in a concentration-dependent manner with IC_{50} values (the concentrations at which 50% inhibition was achieved) of 44.28 μM and 3.49 μM respectively.

Dixon plot analysis

To further characterize the mechanism of inhibition and to determine the K_i values (inhibition constants), uptake in the absence and presence of chlorambucil and cabazitaxel were analyzed via Dixon plot (**Figure 5**). Chlorambucil demonstrated a competitive mechanism of inhibition of ES uptake by hOAT4 (as the lines for substrate concentrations converge above the x axis) with K_i values of 55.73 μM (**Figure 5A**), whereas cabazitaxel demonstrated a non-competitive mechanism of inhibition of ES uptake by hOAT4 (as the lines for substrate concentrations converge at the x axis) with K_i values of 1.78 μM, (**Figure 5B**).

Discussion

hOAT4 plays an important role in body disposition of numerous drugs and environmental toxins, and therefore is a critical determinant for drug efficacy and toxicity. In the current study, we conducted a comprehensive culture-based screening of hOAT4 inhibitors from a drug library consisting of a total of 101 drugs provided by the National Cancer Institute.

hOAT4 is mainly expressed in kidney and placenta. As a platform to study the interactions of hOAT4 with anticancer compounds, we established a human kidney HEK293 cell line stably expressing hOAT4 (**Figure 1**). The transport kinetics of hOAT4 in these cells exhibited a characteristic comparable to that of hOAT4 in other systems [6], suggesting that HEK293 cells are good model for the characterization of hOAT4. In these cells, we examined the inhibitory effects of the 101 anticancer drugs for hOAT4 (**Figure 2**). Although many of the drugs tested demonstrated some level of inhibition, only chlorambucil and cabazitaxel demonstrated greater than 50% suppression of hOAT4-mediated [^{3}H]-ES uptake. On the contrary, allpurinol showed a stimulatory effect on hOAT4-mediated transport. One possible explanation for such a stimulatory effect is that it may result from a conformational change of hOAT4 induced upon binding of allopurinol to the transporter, a similar situation observed with other transporters including [9, 17, 19-21]. This idea remains to be fully explored.

The inhibitory effects of chlorambucil and cabazitaxel, which showed the highest inhibitory potency during our initial screening, were further tested in human placenta BeWo cells. Similar to what was observed in HEK293 cells, both chlorambucil and cabazitaxel exhibited significant inhibition of hOAT4-mediated transport, suggesting that the inhibition characteristics of these drugs are not cell type-specific but are general properties as inhibitors for hOAT4.

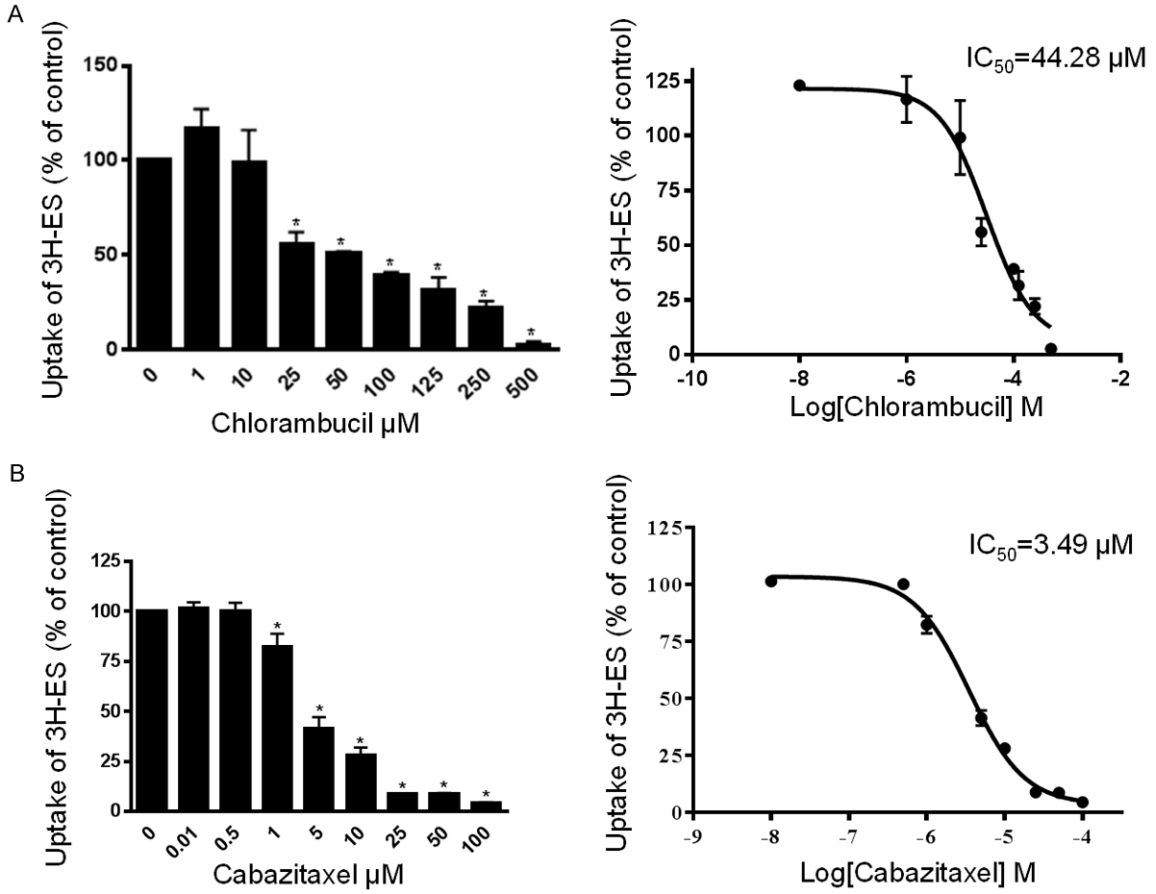


Figure 4. Concentration dependence of chlorambucil (A) and cabazitaxel (B) inhibition on hOAT4 mediated uptake. HEK293 cells stably expressing hOAT4 were incubated for 3 min with PBS containing 100 nM [^{3}H]ES in the presence or absence of various concentrations of test compounds. Each data point represent only carrier mediated transport after subtraction of values from parental cells. Uptake activity was expressed as percentage of uptake measured in control cells from three independent experiments. Results shown are means ± S.E. of three separate experiments. Data were analyzed statistically with ANOVA, followed by Tukey's post-hoc test. *P < 0.05. The line represents a best fit of data using nonlinear regression analysis.

Our characterization of the inhibition mechanism by Dixon plot revealed that the modes of action of chlorambucil and cabazitaxel are distinct. Chlorambucil showed a competitive mechanism of inhibition of ES uptake by hOAT4. Competitive inhibition is a form of inhibition where binding of the inhibitor to the active site on the transporter prevents binding of the substrate and *vice versa.* In contrast, cabazitaxel showed a non-competitive mechanism of inhibition of ES uptake by hOAT4. Non-competitive inhibition is a type of inhibition where the inhibitor reduces the activity of the transporter by binding an area other than the substrate binding site. Structurally, chlorambucil carries an anionic charge and possesses an aromatic center, which is more similar to estrone sulfate (ES), the prototypical substrate for hOAT4 (**Figure 6**). Cabazitaxel, on the other hand, is a neutral molecule at the physiological pH. Such structural similarity between chlorambucil and estrone sulfate likely makes chlorambucil easier to access to the substrate binding site of hOAT4, which may explain its competitive mode of inhibition.

The IC_{50} value of chlorambucil for hOAT4 determined in the present study is 44.28 μM. The therapeutic dose of chlorambucil suggested by Oppitz *et. al* [22] are 0.1-0.2 mg/kg body weight and would yield a maximum plasma concentration (C_{max}) of approximately 1.6 μM. Corrected by unbound fraction value of 0.01, the unbound maximum plasma concentration ($C_{u,max}$) of chlorambucil is ~0.02 μM. The IC_{50} value of cabazitaxel obtained in our study is 3.49 μM. According to Paller et al., a peak plasma cabazitaxel concentration of 535 μg/L or 0.64 μM

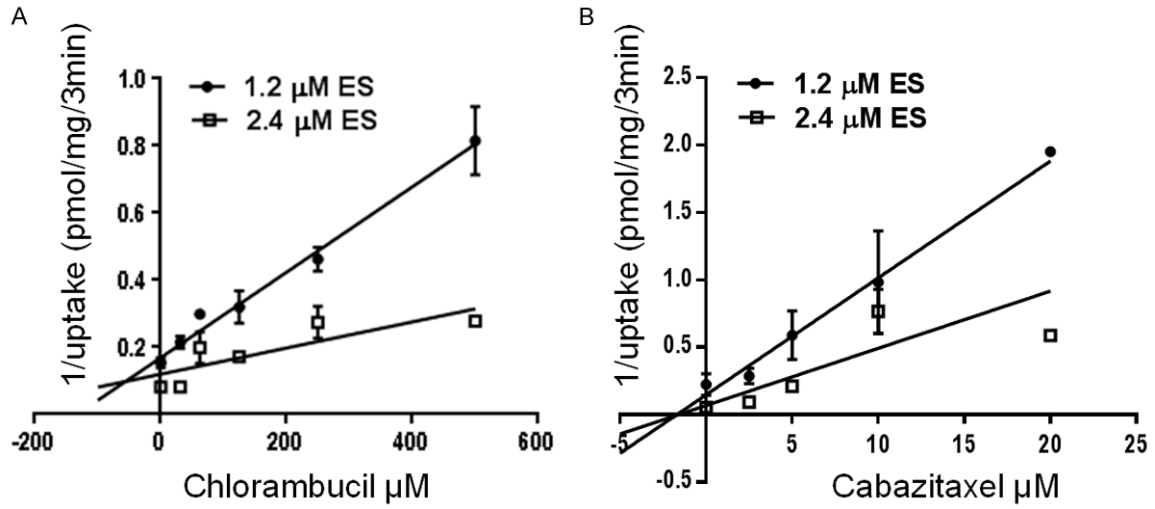


Figure 5. Dixon plot analysis of the inhibitory effects of (A) chlorambucil and (B) cabazitaxel on hOAT4-mediated transport in HEK293 cells. 1.2 μM and 2.4 μM [^{3}H]ES uptake was determined at 3 min in the absence or presence of varying concentrations of test compounds. Each data point represent only carrier mediated transport after subtraction of values from parental cells. Results shown are means ± SE percentage of uptake measured in control cells from two independent experiments. The data were fitted by linear regression and K_i was calculated.

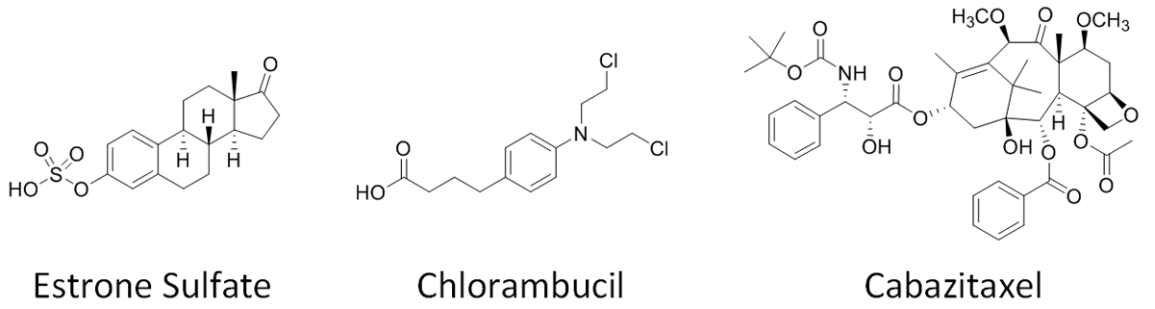


Figure 6. Chemical structures of estrone sulfate, chlorambucil and cabazitaxel.

was achieved in prostate cancer patients [23]. Corrected by unbound fraction value of 0.11, the unbound maximum plasma concentration ($C_{u,max}$) of chlorambucil is ~0.07 μM. A $C_{u,max}$/IC_{50} value greater than 0.1 would indicate a potential for drug-drug interaction [24]. The $C_{u,max}/IC_{50}$ value of chlorambucil and cabazitaxel for hOAT4 were < 0.1. Therefore, the propensity for chlorambucil and cabazitaxel to cause drug-drug interaction through inhibition of hOAT4 is low.

In conclusion, our results demonstrated that although chlorambucil and cabazitaxel significantly inhibit hOAT4-mediated transport, the likelihood for these drugs to cause drug-drug interaction through inhibition of hOAT4 is limited. Therefore, dosage adjustment appears to be an efficient, safe way to manipulate its systemic exposure.

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Disclosure of conflict of interest

None.

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