

Structural dissection of 13-epiestrones based on the interaction with human Organic anion-transporting polypeptide, OATP2B1

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ABSTRACT

Human OATP2B1 encoded by the *SLCO2B1* gene is a multispecific transporter mediating the cellular uptake of large, organic molecules, including hormones, prostaglandins and bile acids. OATP2B1 is ubiquitously expressed in the human body, with highest expression levels in pharmacologically relevant barriers, like enterocytes, hepatocytes and endothelial cells of the blood-brain-barrier. In addition to its endogenous substrates, OATP2B1 also recognizes clinically applied drugs, such as statins, antivirals, antihistamines and chemotherapeutic agents and influences their pharmacokinetics. On the other hand, OATP2B1 is also overexpressed in various tumors. Considering that elevated hormone uptake by OATP2B1 results in increased cell proliferation of hormone dependent tumors (e.g. breast or prostate), inhibition of OATP2B1 can be a good strategy to inhibit the growth of these tumors.

13-epiestrones represent a potential novel strategy in the treatment of hormone dependent cancers by the suppression of local estrogen production due to the inhibition of the key enzyme of estrone metabolism, 17 β -hydroxysteroid-dehydrogenase type 1 (HSD17 β 1). Recently, we have demonstrated that various phosphonated 13-epiestrones are dual inhibitors also suppressing OATP2B1 function. In order to gain better insights into the molecular determinants of OATP2B1 13-epiestrone interaction we investigated the effect of C-2 and C-4 halogen or phenylalkynyl modified epiestrones on OATP2B1 transport function. Potent inhibitors (with EC₅₀ values in the low micromolar range) as well as non-inhibitors of OATP2B1 function were identified. Based on the structure-activity relationship (SAR) of the various 13-epiestrone derivatives we could define structural elements important for OATP2B1 inhibition. Our results may help to understand the drug/inhibitor interaction profile of OATP2B1, and also may be a useful strategy to block steroid hormone entry into tumors.

1. Introduction

Organic anion-transporting polypeptides (OATPs) encoded by the *SLCO* (solute carrier for organic anions) genes are membrane proteins that mediate the cellular uptake of large (> 300 Da) organic molecules in a Na⁺- and ATP-independent manner [1]. 11 human OATPs are known, that are highly variable in their tissue distribution and substrate recognition. Some OATPs are ubiquitously expressed in the human body (OATP4A1, OATP3A1), while the expression of others is restricted to a given organ, like OATP1B1 and OATP1B3 expression which is restricted to hepatocytes [2,3]. Also, based on the substrate interaction

profile, multispecific OATPs (1A2, 1B1, 1B3, and 2B1) recognizing a plethora of organic compounds (including clinically applied drugs), and OATPs with a more limited substrate recognition (e.g., the thyroid transporter OATP1C1) can be distinguished. Although not all of the 11 OATPs are properly characterized with regard to their expression and function, steroids (e.g. bile acids, estrone-3-sulfate (E1S) and dehydroepiandrosterone sulfate (DHEAS)) can be considered as general OATP substrates [4]. Consequently, the OATPs 1A2, 1B1, 2B1 and 4A1 are supposed to be key participants in the cellular uptake of the steroid hormone conjugates E1S and DHEAS [3,5]. Besides their role in the maintenance of steroid hormone homeostasis, multispecific OATPs,

Abbreviations: OATP, Organic anion-transporting polypeptide; E1S, estrone-3-sulfate; HSD17 β 1, 17 β -hydroxysteroid-dehydrogenase type 1; SAR, structure activity relationship; STS, steroid sulfatase

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1A2, 1B1, 1B3 and 2B1 are important determinants of pharmacokinetics [6]. In addition, OATPs are often up-regulated in tumors [7–9]. Hence, they are promising targets for anti-tumor therapy.

OATP2B1 is a ubiquitously expressed transporter, with highest protein levels in pharmacologically relevant barrier tissues, like the intestine, liver and blood-brain barrier [1]. Besides, it is also expressed in the placenta, mammary gland and in skeletal muscle cells [1]. OATP2B1 is a multispecific transporter that recognizes molecules with largely variable size and structure. The most relevant endogenous substances transported by OATP2B1 are taurocholate, leukotriene C₄, E1S, DHEAS and prostaglandin E₂ [10]. OATP2B1 also promotes cellular uptake of clinically applied drugs, like statins, antibiotics, anti-hypertensives, anti-inflammatory drugs and chemotherapeutics [10]. Considering its tissue distribution and substrate recognition pattern, OATP2B1 may be a key player in intestinal drug absorption and also drug transport across the blood–brain barrier [11,12]. On the other hand, OATP2B1 overexpression has been detected in tumors of the colon, bone, breast, prostate and also in gliomas [13–15]. Considering its transport of anti-cancer agents, OATP2B1 is one of the main candidates of tumor-targeted drug delivery. On the other hand, it has been shown that increased steroid hormone (E1S, DHEAS) uptake by OATP2B1 promotes growth of steroid-dependent tumors. Matsumoto and colleagues demonstrated that overexpression of OATP2B1 results in increased survival of breast cancer cells *in vitro* [14]. Moreover, *in vivo* data revealed that DHEAS uptake by OATP2B1 has crucial role in prostate cancer progression [16]. Also, the SLCO2B1 rs12422149 GG (Arg312Gln) genotype resulting in increased OATP2B1 function correlates with shorter time to progression in prostate cancer patients who received androgen deprivation therapy [17,18]. Therefore, inhibition of OATP2B1 function presents a possible strategy to suppress steroid hormone uptake and hence the proliferation of hormone dependent cancers.

13-epiestrones are stereoisomers of natural estrone, lacking hormonal activity [19,20]. Previous work has demonstrated that certain 13-epiestrones are potent inhibitors of the 17 β -hydroxysteroid-dehydrogenase type 1 (HSD17 β 1) and steroid sulfatase (STS) enzymes crucial in estrone metabolism [21]. These enzymes are responsible for local estrogen formation and generation of the transcriptionally active estradiol therefore promoting proliferation of hormone dependent cancers [22,23]. Hence their inhibition e.g. by 13-epiestrones can be a potential anti-tumor strategy.

Recently we found that phosphonated 13-epiestrones inhibit the function of OATP2B1 [24]. In the current work, in order to get a better insight into the molecular determinants involved in this inhibition we analyzed the interaction between OATP2B1 and a large set of 13-epiestrones containing modifications on C-3 and C-2 or C-4. In addition, we systematically investigated the influence of certain substituents on inhibitory activity by correlation analysis.

2. Materials and methods

2.1. Materials

Materials if not stated otherwise were purchased for Sigma Aldrich (Budapest, Hungary). 13-epiestrones investigated in this study were synthesized as described elsewhere [21,25].

2.2. Generation and maintenance of the cell lines

A431 (human epidermoid carcinoma) cells overexpressing human OATP2B1 or mock transfected controls used in the current study were generated earlier as described in [26]. Briefly, OATP2B1 expressing cells were generated by transposase mediated genomic insertion of the OATP2B1 cDNA (BC041095.1, HsCD00378878). As a negative control mock transfected (pSB-CMV) cells were used. After 2 weeks of puromycin (1 μ g/ml) selection, cells were sorted based on Live/Dead Green

uptake. After recovery, cell were grown in DMEM (Gibco, Thermo Fischer Scientific (Waltham, MA, US)) without puromycin supplemented with 10 % fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37 °C with 5% CO₂ and 95 % humidity.

2.3. Western blot detection of OATP2B1 expression

OATP2B1 expression was confirmed by Western blot as described earlier [26]. Briefly, whole cell lysates of A431 cells were separated on 7.5 % SDS-PAGE gels and transferred onto a PVDF membrane. OATP2B1 was detected by using an anti-OATP2B1 antibody (a courtesy of Dr. Bruno Stieger, Department of Clinical Pharmacology and Toxicology, University Hospital, 8091 Zurich, Switzerland) [27]. As a secondary antibody HRP-conjugated anti-rabbit antibody (Jackson ImmunoResearch, Suffolk, UK) was used in a dilution of 20,000 \times . An anti- β -actin antibody (A1978, Sigma) and HRP-conjugated anti-mouse antibody (Jackson ImmunoResearch, Suffolk, UK, 20,000 \times dilution) were used to detect β -actin. Luminescence was detected using the Luminer Enhancer Solution kit by Thermo Fisher Scientific (Waltham, MA, US).

2.4. Fluorescent dye uptake determined by flow cytometry

The transport function of OATP2B1 was determined by flow cytometry. A431 cells (mock and OATP2B1 overexpressing) were collected after 0.1 % trypsin treatment. The cells were washed in Uptake buffer (125 mM NaCl, 4.8 mM KCl, 1.2 mM CaCl₂, 1.2 mM KH₂PO₄, 12 mM MgSO₄, 25 mM MES, and 5.6 mM glucose, with the pH adjusted to 5.5 using 1 M HEPES and 1 N NaOH). After washing 5 \times 10⁵ cells were incubated for 15 min at 37 °C with Zombie Violet (BioLegend®, San Diego, CA, US) (0.4 μ l ZV/5 \times 10⁵ cell) in a final volume of 100 μ l. The reaction was stopped by the addition of 1 ml ice-cold PBS (phosphate buffered saline) and the cells were kept on ice until the flow cytometry analysis. The fluorescence of 10,000 living cells was determined using Attune NxT Flow Cytometer (Invitrogen, Carlsbad, CA). Dead cells were excluded by propidium iodide (1 μ g/ml) labeling.

2.5. 96 well plate-based transport assay

Effect of 13-epiestrones on OATP2B1 function was determined by measuring Zombie Violet (BioLegend®, San Diego, CA, US) fluorescent dye uptake on microplates [26]. Briefly, A431 cells were seeded on 96 well-plates (8 \times 10⁴ cells in 200 μ l final volume/well) and cultured for 16–24 h at 37 °C, 5% CO₂ prior to the transport measurements. Next day after repeated washing with 200 μ l PBS, cells were pre-incubated with 50 μ l Uptake buffer (125 mM NaCl, 4.8 mM KCl, 1.2 mM CaCl₂, 1.2 mM KH₂PO₄, 12 mM MgSO₄, 25 mM MES, and 5.6 mM glucose, with the pH adjusted to 5.5 using 1 M HEPES and 1 N NaOH) containing the appropriate concentrations of the 13-epiestrones (0–100 μ M) for 5 min at 37 °C. Reaction was started by the addition of 770 \times diluted Zombie Violet dye in 50 μ l/well Uptake buffer followed by a 30 min incubation at 37 °C. The reaction was stopped by the addition of 200 μ l ice-cold PBS. After repeated washing, 200 μ l ice-cold PBS was added to each well and fluorescence in the wells was determined in an Enspire fluorescent plate reader (Perkin Elmer, Waltham, MA) at Ex/Em: 405/423 nm. Experiments were repeated at least three times.

2.6. Measurement of ³H-E1S uptake

A431 control and A431-OATP2B1 cells (10⁶ cells/sample) were incubated in the absence or presence of 2-bromo-13-epiestrone (final concentration 50 μ M) for 5 min at 37 °C in uptake buffer pH 5.5. Transport reaction was started by the addition of ³H-E1S (250 mCi/ml, final concentration 9.65 nM (Perkin Elmer, Waltham, MA)). After incubation for further 10 min at 37 °C, the reaction was stopped by the addition of 1 ml ice-cold PBS and the cells were centrifuged at 300 g.

The cell pellet was collected in 100 μ l PBS and pipetted into 1 ml Opti-Fluor (Perkin Elmer, Waltham, MA). Radioactivity was measured in a Wallac Liquid Scintillator Counter. Experiments were repeated three times.

2.7. Data calculation

Transport data were obtained by subtracting the fluorescence in mock transfected cells from that measured in OATP2B1 cells. Kinetic parameters of dye uptake and half inhibitory concentrations (EC_{50}) obtained from at least three independent experiments were determined by Hill fit using the GraphPad prism software (GraphPad, La Jolla, CA, USA).

2.8. Structure-activity relationship (SAR) analysis

Compounds were drawn using the structure editor integrated into the ChemSpider chemical structure database webservice (freely available at <https://www.chemspider.com/About.aspx>). Daylight SMILES structural format was generated for every unique compound of the dataset. KNIME Analytics Platform (version 3.4) [28] was used to create an automated workflow for Structure-Activity Relationship (SAR) analysis of 13-epiestrones. First, Daylight SMILES format for input compounds was converted into the canonical form ('RDKit Canon SMILES' node). Murcko scaffolds were generated and the maximum common substructure (MCS) was derived from the retrieved Murcko scaffolds ('RDKit MCS' node). MCS was used as a structural query for substructure mining in order to perform R-group decomposition ('RDKit R Group Decomposition' node).

Physicochemical descriptors (RDKit) for the substituents (R groups) at position C-2 and C-4 were calculated. Descriptor values were normalized ('Normalizer' node using Z-score normalization). The Pearson correlation coefficient was calculated to identify positive or negative correlations between pEC_{50} values and respective physicochemical descriptors at a given R-group position ('Linear Correlation' node).

3. Results

3.1. Effect of C-2 or C-4 halogenated 13-epiestrones on the transport activity of OATP2B1

Recently, we have reported that various phosphonated 13-epiestrones are potent inhibitors of OATP2B1 function [24]. In order to gain better insights into the molecular determinants of this inhibition, we investigated the inhibitory effect of various 2- or 4-halogenated 3-hydroxy- (3-OH) or 3-methoxy (3-OMe) 13-epiestrones (Fig. 1).

Interaction was measured in A431 mock transfected (control) and OATP2B1 overexpressing cell lines using the Zombie Violet (ZV) assay. The A431 cell line overexpressing OATP2B1 was generated earlier [26]. Prior to the interaction tests OATP2B1 overexpression was confirmed by Western blot (Fig. 2A). ZV is one of the newly identified fluorescent dyes applicable for testing drug interactions and function of OATP2B1, and a good alternative to the generally used radioactive functional assays [26]. Fig. 2B shows that OATP2B1 overexpression results in increased uptake of the viability dye, ZV.

When testing the inhibitory effect of the epiestrone derivatives, ZV uptake was measured in A431-OATP2B1 (and control) cells seeded in 96-well plates in the presence of increasing amounts of the tested compounds. As shown in Fig. 3, the compounds showed various degrees of transport inhibition. The first striking difference could be observed between the C-3 OH and OMe compounds. 13-epiestrone (C-3 OH, termed as **compound 1**) had practically no effect on ZV transport (EC_{50} around 50 μ M), while its methylether counterpart (3-OMe derivative, **compound 2**) resulted in a quite effective inhibition (EC_{50} 2.98 μ M) (see Fig. 3 and Table 1).

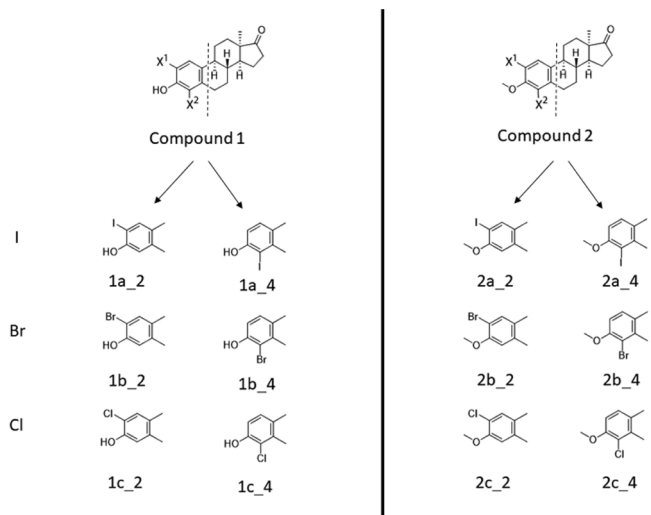


Fig. 1. Structure of the halogenated 13-epiestrones investigated in the current study. In the case of **compounds 1 and 2** $X^1 = H$ and $X^2 = H$.

Interestingly, introduction of a second modification, halogenation of either at C-2 or C-4 resulted in opposite changes in the inhibitory potential of 13-epiestrones. In the case of **compound 1**, halogenation at C-2 (compounds **1a_2**, **1b_2** and **1c_2**) resulted in a striking increase in inhibitory potential, with EC_{50} values between 0.5 and 2.1 μ M. However, the C-4 chlorinated derivative (compound **1c_4**) showed practically no effect on OATP2B1 transport function, while 4-iodo and 4-bromo 13-epiestrones (**1a_4** and **1b_4**) proved to be weak inhibitors. This reveals a strong dependence of the regioisomerism of the halogenated 3-OH compounds, the most potent inhibition detected with the 2-halogenated derivatives. In the case of 3-OMe derivatives, halogenation caused lower alteration in the inhibitory potential, although all of the compounds showed increased EC_{50} values compared to the initial compound. Iodinated derivatives (**2a_2** and **2a_4**) were more effective than the brominated or chlorinated compounds (**2b_2**, **2b_4** or **2c_2**, **2c_4**). Regioisomer specific inhibitory effect in the case of the 3-OMe variants could only be observed for the chlorinated compounds, with an approximately 5-fold increase in the EC_{50} of the C-4 vs. C-2 chlorinated epiestrones (**2c_2** and **2c_4**, Table 1).

3.2. Effect of C-2 or C-4 phenylalkynylated 13-epiestrones on the transport activity of OATP2B1

Next, we investigated the effect of the introduction of a phenylalkynyl group in position C-2 or C-4 on OATP2B1 function (Fig. 4).

In the case of 3-OH 13-epiestrones, introduction of a large ring resulted in various effects. In general, although some phenylalkynylated 13-epiestrones showed detectable interaction with OATP2B1, these compounds were less effective inhibitors than the C-2 halogenated epiestrones (see Table 2 and Fig. 5). The only exception is **1gS_4** containing a (4-methoxyphenyl)ethynyl substituent, that was almost as effective inhibitor as the C-2 halogenated 13-epiestrones. Interestingly, in the case of phenylalkynylated 3-OH 13-epiestrones no clear rule in preference in interaction with C-2 over C-4 modified compounds could be observed. Compounds **1eS_4** and **1gS_4** were more potent inhibitors than their C-2 counterparts (**1eS_2** and **1gS_2**). However, in the case of 13-epiestrones bearing fluorinated substituents (**1hS** and **1fS**), this tendency changed, C-2 compounds being slight inhibitors (EC_{50} around 10 μ M) compared to C-4 modified derivatives lacking any inhibitory potential (at least in the concentrations tested).

In the case of the 3-OMe compounds with a second, subst. phenylalkynyl modification, similarly to that observed for the halogenated

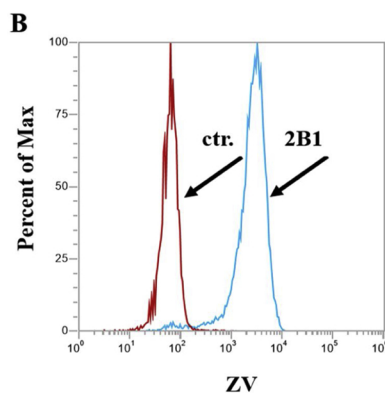
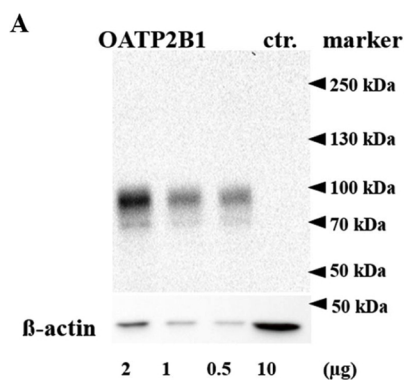


Fig. 2. A) Western blot detection of OATP2B1 expressed in A431 cells. OATP2B1 was detected by an anti-OATP2B1 antibody [27], and β-actin was used as a loading control. Control (ctr.) stands for mock transfected A431 cells. Multiple migratory bands may represent differentially glycosylated forms of OATP2B1. B) Zombie Violet uptake in A431-OATP2B1 and control cells. Histograms show the uptake of ZV (250x dilution) in the cells incubated with the fluorescent dye for 15 min at 37 °C in uptake buffer (pH 5.5). Living (propidium-iodide negative) cells are shown. Mock transfected cells are indicated with a red line and OATP2B1 transfected are with blue.

compounds, a decrease or even a complete loss of inhibition compared to the parental compound 3-OMe 13-epiandrosterone could be observed. The only exceptions were compounds **2gS_2** and **2gS_4** that proved to be effective inhibitors. A clear rule of regioselectivity could not be observed for the 3-OMe phenylalkynyl modified compounds.

3.3. Effect of 2-bromo 13-epiandrosterone on OATP2B1-mediated estrone-3-sulfate uptake

Inhibition of hormone uptake by OATP2B1 in tumor cells could be the major goal of the newly identified inhibitors. Therefore, in order to

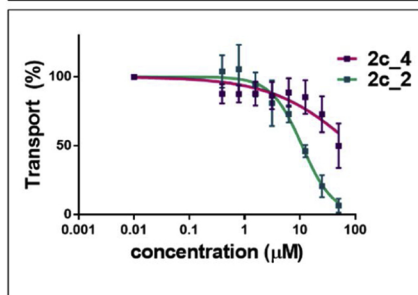
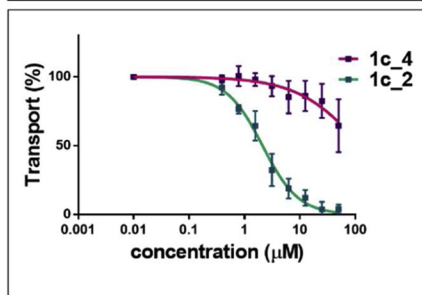
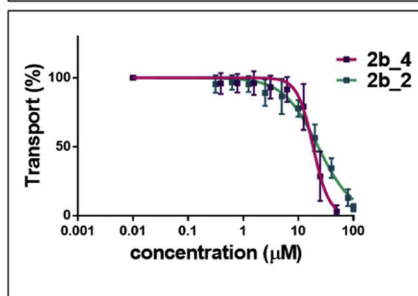
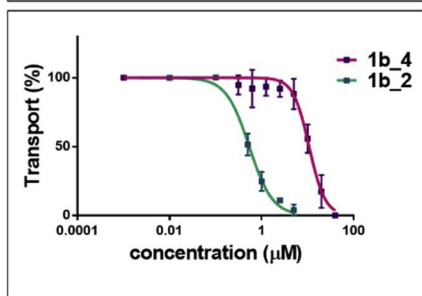
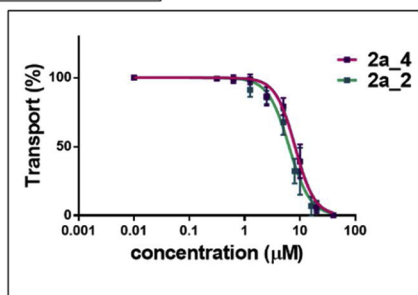
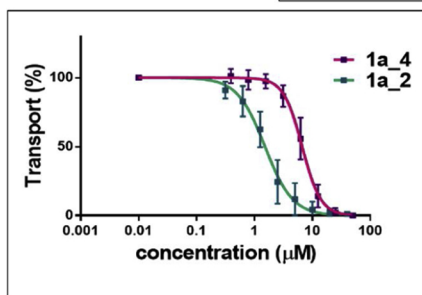
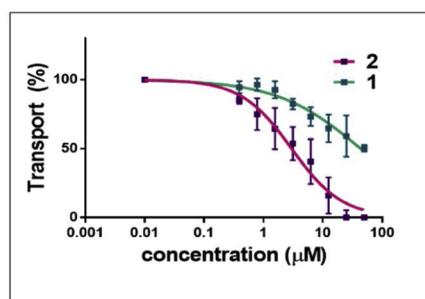


Fig. 3. Inhibition of dye uptake in A431-OATP2B1 cells by halogenated 13-epiandrosterones. A431-OATP2B1 cells and their mock transfected counterparts were incubated in the presence or absence of increasing amounts of the 13-epiandrosterones (1-100 μM, as indicated on the x axis) with the Zombie Violet dye for 30 min at 37 °C. Fluorescence was measured in an Enspire plate reader. Fluorescence obtained in mock transfected A431 cells was subtracted from that measured in A431-OATP2B1 cells. Transport was calculated based on the fluorescence measured in the absence of 13-epiandrosterones (100 %). Data points show the average +/- SD values obtained in at least three independent biological replicates.

Table 1
Inhibition of OATP2B1 activity by C-2 or C-4 halogenated 13-epiestrones.

compound "name"	EC ₅₀ ± SD (μM)	compound "name"	EC ₅₀ ± SD (μM)	compound "name"	EC ₅₀ ± SD (μM)	compound "name"	EC ₅₀ ± SD (μM)
1	50			2	2.98 ± 0.05		
1a_2	1.52 ± 0.01	1a_4	6.63 ± 0.01	2a_2	6.34 ± 0.02	2a_4	8.14 ± 0.02
1b_2	0.54 ± 0.02	1b_4	10.8 ± 0.02	2b_2	22.88 ± 0.02	2b_4	18.6 ± 0.02
1c_2	2.11 ± 0.02	1c_4	> 50	2c_2	10.89 ± 0.03	2c_4	> 50

The inhibitory effect of 13-epiestrones was measured using *Zombie Violet* as a test substrate [26]. The kinetic parameters of inhibition shown on Fig. 3 were determined by the Graphpad Prism software.

determine whether the best performing newly identified inhibitor, 2-bromo-13-epiestrone (compound **1b_2**) can be applied to inhibit hormone uptake, we determined its effect on ³H-E1S uptake in A431 control and A431-OATP2B1 cells. Fig. 6 shows that compound **1b_2** can attenuate E1S uptake mediated by OATP2B1, therefore it is a good candidate to block hormone uptake in OATP2B1 expressing cells.

3.4. SAR analysis

Since the compounds under study do all possess a common core structure (scaffold) with largest variance in terms of different substituents in positions C-2 and C-4, it appears interesting to perform Structure-Activity Relationship (SAR) analysis for positions C-2 and C-4 separately. The rationale behind is that the increase or decrease of bioactivities within a congeneric SAR series of compounds can possibly be explained by the variations in physicochemical properties at a specific substitution site (R or X-group site). However, since the initial (parent) compounds 3-OH and 3-OMe are possessing very different inhibitory potential (EC₅₀ value of compound **1** (3-OH) is 50 μM while for compound **2** (3-OMe) is 2.98 μM), we have performed the SAR analysis for 3-OH and 3-OMe derivatives separately.

In the case of the 3-OH derivatives, the SAR analysis (Table 3) for substituents at position C-2 shows that the number of atoms and heavy atoms in the substituent is negatively correlated with activity (R = -0.89 and -0.84). Of equal effect is the number of aromatic carbocycles in that side chain: possessing no rings is more favorable (R = -0.90). Further, molar refractivity ('SMR') which reflects the charge distribution and hence corresponds to the polarizability of a given functional group is inversely correlated to bioactivity: less polarizable is more favourable (R = -0.88). Also Labute's Approximative Surface Area ('LabuteASA') (R = -0.82) [29] - a measure of the size of a molecules' surface area - as well as the partition coefficient ('SlogP') are negatively

correlated (R = -0.73) to activity, corresponding to a favorable lower size and lipophilicity of substituents at position C-2. Concrete values of Pearson correlation coefficients for given descriptors are listed in Table 3 (a heat map representation of correlation values is given in Supplementary Figure S1).

The only significant positive correlation at position C-2 was identified for the HallKier Alpha index ('HallKier α', R = 0.88), as introduced by Hall and Kier (equation nr. 58 in provided reference) [30]. HallKier α belongs to the class of topological descriptors which can quantify molecular shape similarity within a set of molecules. HallKier α relates to the size contribution of a query fragment to C(sp³)-hybridized atoms, which are taken as a reference (HallKier α for sp³ carbon equals to 0). HallKier α thus encodes the effect of both covalent radius and hybridization state of a given group of atoms. From Supplementary Table 1 it becomes clear that the halogenated derivatives are generally having positive HallKier α values and the phenylalkynyl compounds are showing negative values. Interestingly, in the case of 3-OMe derivatives none of the investigated physicochemical properties of the C-2 substituents showed a meaningful correlation with the bioactivity values of these compounds.

In contrast to substituents at position C-2, the SAR analysis for substituents at position C-4 did not allow to prioritize physicochemical features influencing the overall bioactivity on OATP2B1 (Table 3 and Supplementary Figure S1). In general, correlations of physicochemical features and bioactivity for substituents in position C-4 are comparable for both 3-OH and 3-OMe derivatives. Most strikingly, the number of heteroatoms of substituents at position C-4 is slightly negatively correlated with bioactivity for both 3-OH and 3-OMe derivatives (R = -0.47 and -0.45). Due to the chemical composition of our compounds, this effect points to an unfavorable effect of electronegative atoms at this position (Cl, I, Br, F) which is inverse to the trends observed for position C-2 (where halogens seem to be favorable).

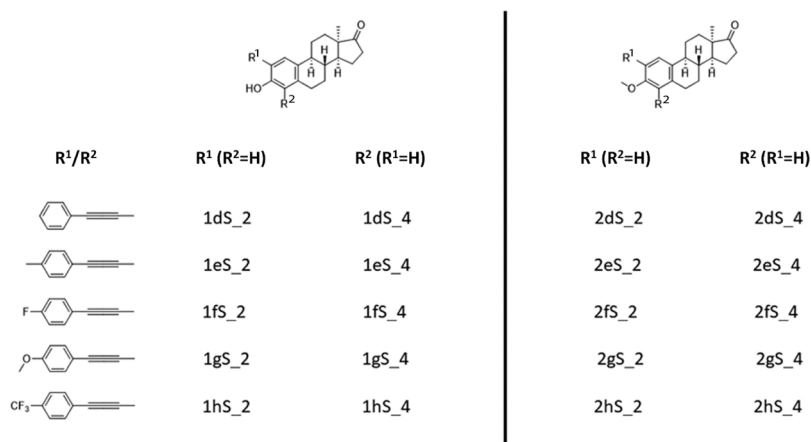


Fig. 4. Structure of the phenylalkynyl modified 13-epiestrones investigated in the current study.

Table 2
Inhibition of OATP2B1 activity by C-2 or C-4 phenylalkynylated 13-epiestrones.

compound "name"	EC ₅₀ ± SD (μM)	compound "name"	EC ₅₀ ± SD (μM)	compound "name"	EC ₅₀ ± SD (μM)	compound "name"	EC ₅₀ ± SD (μM)
1dS_2	15.53 ± 0.03	1dS_4	11.55 ± 0.02	2dS_2	> 50	2dS_4	12.83 ± 0.04
1eS_2	> 50	1eS_4	11.36 ± 0.02	2eS_2	20.77 ± 0.03	2eS_4	11.86 ± 0.02
1fS_2	12.95 ± 0.01	1fS_4	> 50	2fS_2	> 50	2fS_4	8.73 ± 0.02
1gS_2	11.08 ± 0.04	1gS_4	4.57 ± 0.01	2gS_2	3.79 ± 0.05	2gS_4	3.4 ± 0.02
1hS_2	9.27 ± 0.04	1hS_4	> 50	2hS_2	10.5 ± 0.03	2hS_4	46.09 ± 0.04

4. Discussion

Recognizing numerous clinically applied drugs and promoting their intestinal, hepatic and central nervous system (through the blood-brain-barrier) uptake, OATP2B1 is a key determinant of drug pharmacokinetics [31]. Hence understanding the mechanism of its substrate/inhibitor recognition can promote drug development and may also help in predicting/avoiding adverse effects caused by OATP2B1 mediated drug-drug interactions. In addition, OATP2B1 is a dedicated conjugated steroid hormone transporter. Its steroid hormone substrates are E1S, pregnenolone-sulfate and DHEAS [32,33]. OATP2B1 has been identified as a key uptake transporter of DHEAS and E1S in the placenta and mammary gland that are largely dependent on these hormone precursors [15,34]. In addition, OATP2B1 expressed in endothelial cells of the blood-brain barrier is considered as an important mediator of the uptake of the neuroactive steroids DHEAS and pregnenolone-sulfate into the brain [35]. On the other hand, increased steroid hormone uptake by OATP2B1 may also be favorable for tumor progression, as was demonstrated in breast and prostate cancer [36,37]. Therefore, inhibition of OATP2B1 function may be an alternative/successful strategy to inhibit the growth of various tumors.

During the last two decades, since OATP2B1 was cloned [27], numerous inhibitors of OATP2B1 have been described. Most of these are clinically applied drugs, like cyclosporin A, rifampicin and statins [38,39]. However these compounds are also inhibiting other drug transporters, like P-glycoprotein [40] and additional OATPs or even CYP enzymes [6,41], therefore they are lacking OATP2B1 specificity. In addition, various steroids, e.g. estrone or testosterone that are themselves not transported by OATP2B1 have been documented as OATP2B1 inhibitors [32]. However, besides again the lack of specificity, these steroids are not effective inhibitors, since only low levels of inhibition could be observed even at concentrations well above their physiological occurrence. Grube et al. documented only 20–30 % decrease in OATP2B1-mediated E1S uptake by the application of 10 or 100 μM testosterone or estrone, respectively [32].

13-epiestrones represent a new class of OATP2B1 inhibitors. They have no steroidogenic effect, hence their application may be void of side effects. In our preliminary work we found that phosphonated 13-epiestrones are potent inhibitors of OATP2B1 function, with EC₅₀ values in the micromolar range [24]. In order to map the molecular determinants of this inhibition, here we analyzed the inhibitory effect of a series of 3-hydroxy or 3-methylether 13-epiestrones containing a second, C-2 or C-4 halogen or phenylalkynyl modification. **Compound 1**, (3-OH 13-epiestrone) showed no interaction with OATP2B1 (EC₅₀ around 50 μM), but the 3-methoxy counterpart **compound 2** (3-OMe 13-epiestrone) performed a strong interaction (EC₅₀ 2.98 μM). Introduction of a second modification on C-2 or C-4 resulted in various effects. In the case of 3-OMe 13-epiestrones, the second modification issued in a decrease or loss of inhibitory activity with the exception of 2-iodo or 4-iodo (**2a_2** and **2a_4**), (4-fluorophenyl)ethynyl (**2fS_4**), (2-methoxyphenyl)ethynyl (**2gS_2**) and (4-methoxyphenyl)ethynyl

(**2gS_4**) derivatives having similar EC₅₀ values as the initial compound 3-OMe 13-epiestrone. In contrast, in the case of 13-epiestrone, the second modification had various effects depending on the site (C-2 or C-4) or nature (halogen or phenylalkynyl) of the substitution. In general, introduction of a phenylalkynyl substituent did not result in potent inhibitors. The only exception was the C-4 modified compound **1gS_4** having an EC₅₀ value well below 10 μM. However, the most striking change in the inhibitory effect was observed in the case of the 2-halogenated 13-epiestrones (**1a_2**, **1b_2** and **1c_2**). These compounds potently inhibited OATP2B1 function with EC₅₀ values between 0.5 and 2.1 μM. In addition, halogenated 13-epiestrones revealed a strong regioselectivity, 4-halogenated compounds showing no or very weak inhibition of OATP2B1 activity. This C-2 halogen preference has already been observed in the case of 2-iodo-estrone-3-sulfate [42]. Banerjee and colleagues have demonstrated that 2-[¹²⁵I]-estrone-3-sulfate is transported by OATP2B1 while transport in the case of its 4-iodo counterpart could not be observed.

SAR analysis by R-group decomposition was performed in order to elucidate molecular determinants potentially being responsible for bioactivity of 13-epiestrones. Different potency of respective compounds was correlated to the subtle changes in physico-chemical properties at a specific R or X -group position. It has to be emphasized that the observed trends are showing correlations of side chain features and bioactivity, but these correlations are not necessarily pointing to a causal relationship of activity and chemical descriptor value. In other words, some of the correlations we see might be artefacts caused by high intercorrelation of related features (e.g. molecular weight and lipophilicity are often intercorrelated). The presence of halogens in position C-2 was identified to drive the activity against OATP2B1. The importance of fluor and 4-fluorophenyl functional groups for OATP2B1 substrate activity was already demonstrated by the substructural fragment analysis performed by Shaikh et al. [43]. Our findings are suggesting the likelihood of halogen bond formation in OATP2B1-ligand binding complexes. As an outlook, we suggest to prove such a hypothesis by e.g. molecular docking combined with quantum mechanics techniques. SAR analysis of functional groups at position C-4 delivered negative correlation with the number of heteroatoms. These trends, however, appear to be less pronounced for position C-4 substituents and therefore it is required to repeat the analyses with a bigger data set showing a larger range of structural variations in this position for further investigations. SAR analysis revealed that 3-OH derivatives are showing more pronounced positive and negative correlation with different physicochemical properties at position C-2. In general, 3-OH derivatives are more sensitive to the substitutions at position C-2 when compared to 3-OMe derivatives.

In our previous study we investigated 3-hydroxy, 3-methylether and 3-benzylether (3-OBn) 13-epiestrones with a C-2 or C-4 diethyl phosphono or diphenylphosphine oxide substitution [24]. In that study, in accordance with our current findings, we found that the second (C-2 or C-4) modification results in dramatic increase in the inhibitory potential of 13-epiestrone (3-OH). Also in harmony with our current results,

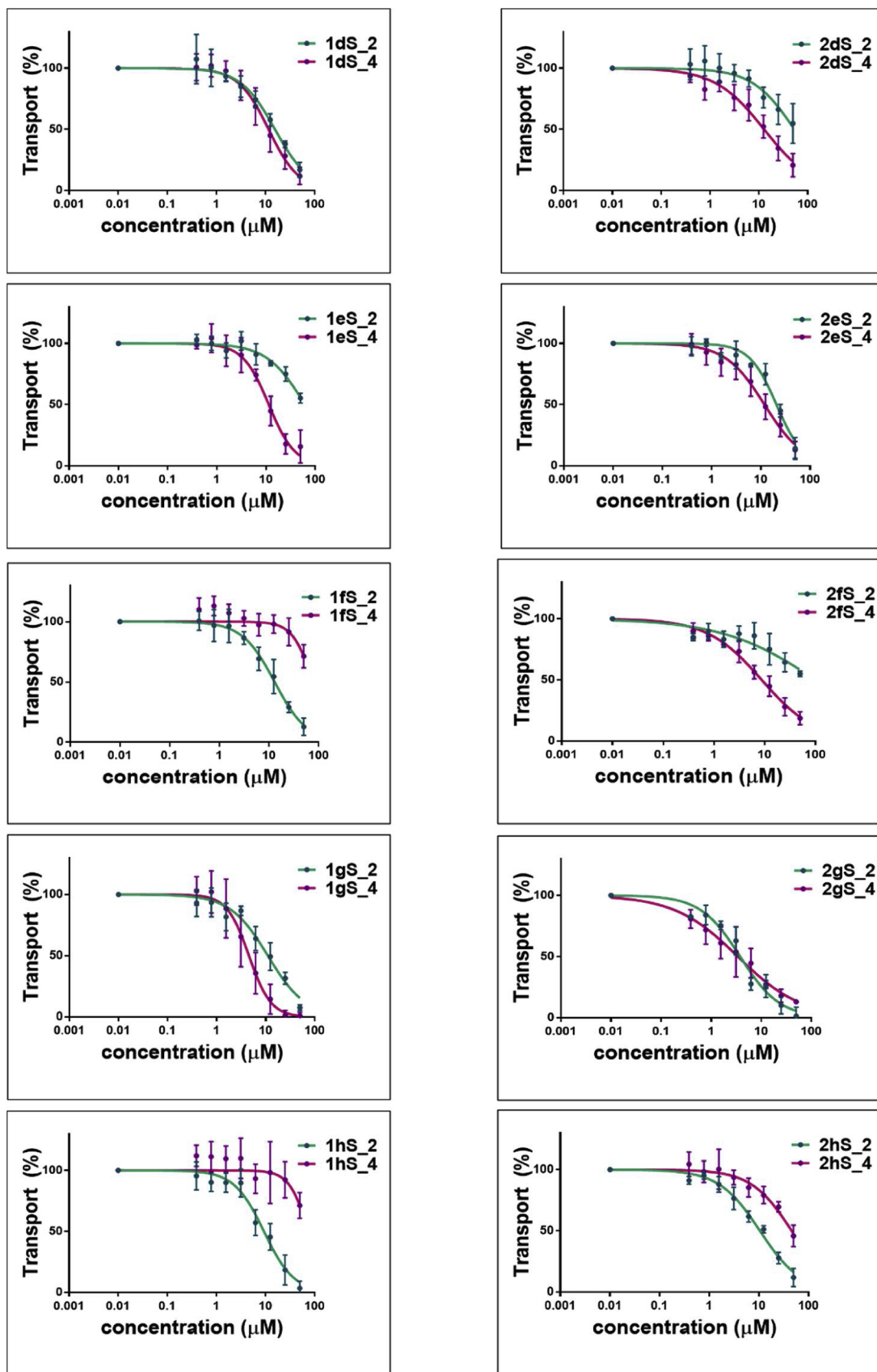


Fig. 5. Inhibition of dye uptake in A431-OATP2B1 cells by phenylalkynylated 13-epiandrosterones. Inhibition of Zombie Violet uptake in A431-OATP2B1 and mock transfected cells was measured as described at Fig. 3. Average \pm SD values obtained in at least three independent biological replicates are shown.

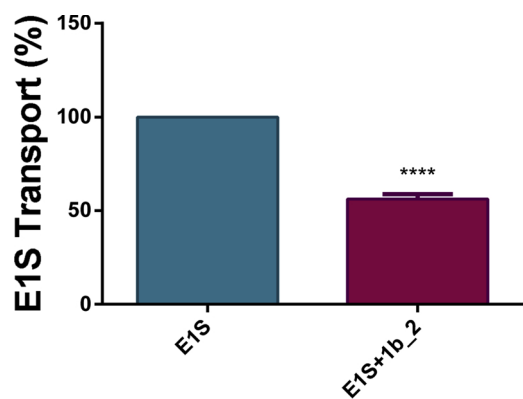


Fig. 6. Inhibition of ³H-E1S uptake in A431-OATP2B1 cells by 2-bromo-13-epiestrone (1b₂). A431-OATP2B1 cells and their mock transfected counterparts were incubated in the presence or absence of 50 μM 1b₂ with 9.65 nM ³H-E1S for 10 min at 37 °C. The radioactivity was measured by Wallac Liquid Scintillator Counter. Radioactivity obtained in mock transfected A431 cells was subtracted from that measured in A431-OATP2B1 cells. Transport was calculated based on the measured radioactivity of ³H-E1S in the absence of 2-bromo-13-epiestrone (100 %). Data points show the average ± SD values obtained in three independent biological replicates. Significance was calculated with GraphPad Prism, using unpaired t-test, ****P < 0.0001.

Table 3

Pearson correlation coefficients for physico-chemical descriptors at position C-2 and C-4.

A) 3-OH derivatives		
Descriptor name	Pearson correlation coefficient	
	Position C-2	Position C-4
NumAtoms	-0.89	0.01
NumHeavyAtoms	-0.84	-0.13
NumHeteroAtoms	0.23	-0.47
NumAromaticCarbocycles	-0.90	-0.06
SMR	-0.88	0.08
LabuteASA	-0.82	-0.03
SlogP	-0.73	-0.26
HallKierAlpha	0.88	0.13
B) 3-Ome derivatives		
Descriptor name	Pearson correlation coefficient	
	Position C-2	Position C-4
NumAtoms	-0.05	0.31
NumHeavyAtoms	-0.06	0.20
NumHeteroAtoms	0.39	-0.45
NumAromaticCarbocycles	-0.21	0.30
SMR	-0.05	0.39
LabuteASA	-0.01	0.28
SlogP	-0.08	-0.04
HallKierAlpha	0.16	-0.23

the second modification could not further improve the potent inhibition by 3-Ome 13-epiestrone. However, in the case of phosphonated 13-epiestrones, the OATP2B1 inhibitory action did not substantially depend on the regioisomerism (C-2 vs. C-4).

HSD17β1 and STS are key enzymes in local estrogen production. Inhibition of their activity by specific or more desirably by dual inhibitors could be a good strategy to prevent tumor progression [44]. The most promising STS inhibitor STX-64, Irosustat performed well in phase I trial, however the phase II trial was not that satisfactory [44], suggesting the need for combined treatment with HSD17β1 inhibitors [45]. Moreover, it has been demonstrated that blocking the aromatase pathway resulted in the upregulation of the STS enzyme and OATPs [46]. Therefore, simultaneous blockage of the different estrogen metabolic pathways and the function of the steroid uptake transporter

Table 4

Comparison of the inhibitory effect of 13-epiestrones on OATP2B1, HSD17β1 and STS activity.

C-3	C-2	C-4	Inhibition of OATP2B1 (determined in the current study)	Inhibition of HSD17β1 (as described earlier in [21,25])	STS (as described earlier in [21])
OH	H	H	non	+	non
	halogen	H	+ / + +	+ +	non
	phenylalkynyl	H	non	+ +	+ / non
	H	halogen	+ / non	+	+ / non
Ome	H	phenylalkynyl	+ / non	non	non
	halogen	H	+	+	n.d.
	phenylalkynyl	H	+ / non	non	n.d.
	H	halogen	+ / non	+ +	n.d.
	H	phenylalkynyl	+ / non	non	n.d.

Columns labeled with C-2, C-3 or C-4 indicate modifications on the 2nd, 3rd or 4th carbon of 13-epiestrone. n.d.: no data available.

+ : EC50 below 10 μM.

+ + : EC50 below 1 μM.

OATPs could be the only successful strategy to inhibit hormone dependent cancers. Our experiments show that the newly identified best performing inhibitor, 2-bromo-13-epiestrone (compound 1b₂) can be used to inhibit OATP2B1-mediated E1S uptake (Fig. 6). Some of the inhibitors identified in our current study, including compound 1b₂, are also potent inhibitors of the HSD17β1 enzyme (see Table 4), therefore they can be good candidate dual inhibitors to be tested in hormone dependent cell lines.

Although OATP2B1 is not related evolutionally to the STS and HSD17β enzyme families, considering their overlapping inhibitor specificities, one may speculate that knowledge gathered from the inhibitor recognition profile of these enzymes can be used to design effective inhibitors of OATP2B1. This is especially important since a protein structure of OATPs is not yet available. Therefore, we have compared the inhibition data obtained in the current study for OATP2B1 with that previously measured for HSD17β1 and STS enzymes [21,25]. Table 4 shows, that inhibition of HSD17β1 reveals few similar features to that of the inhibition of OATP2B1.

Namely, HSD17β1 also has a C-2 preference and both 2-halogenated and phenylalkynyl conjugates potently inhibit HSD17β1 function. Also, similarly to OATP2B1, 3-Ome 13-epiestrones are less effective inhibitors of HSD17β1. However, C-4 halogenation of 3-Ome 13-epiestrones also results in effective inhibitors that is in contrast to that observed for OATP2B1. Unfortunately, data about the effect of the 13-epiestrones investigated in the current study on STS activity are incomplete. Nevertheless, although estrone-3-sulfate is a common substrate of OATP2B1 and STS, based on the interaction with halogenated 13-epiestrones, opposite inhibitor preference could be observed for OATP2B1 and STS. STS was only inhibited by some of the C-4 halogenated compounds that were not the most effective inhibitors of OATP2B1. We suggest that a larger data set of HSD17β1, STS, OATP and 13-epiestrone interactions should be generated in order to determine whether common trends in structural elements important for their inhibition can be observed. Also it would be interesting to investigate the inhibitory effect of 13-epiestrones on the function of other OATPs up-regulated in hormone dependent cancers, OATP1A2, OATP1B3, OATP3A1 and OATP4A1 [47]. On the other hand, hepatic OATPs, 1B1, 1B3 and 2B1 have overlapping substrate specificities, hence, although desirable, specific inhibitors amenable to distinguish between their function are scarce. The OATP2B1 inhibitor 13-epiestrones (1a₂, 1b₂ and 1c₂) identified in the current study can be good candidates to be tested for OATP1B interaction.

In summary, we identify potent inhibitors of OATP2B1. The EC₅₀ of the most potent inhibitor 2-bromo-13-epiestrone falls within the range

of previously documented OATP2B1 inhibitors (BSP 1.26 μM [26], antivirals: 0.5–1 μM , erlotinib: 0.03 μM [48] and EIS: 0.56 μM [26]. However, although potent inhibitors were identified, our assay cannot distinguish between an inhibition caused by transported substrates or non-competitive inhibitors. Further experiments, e.g. measurement of direct uptake of the best performing 13-epiestrones (showing the highest inhibition) are needed to clarify this issue. Still, one may speculate that if OATP2B1 can mediate the uptake of these HSD17 β 1 inhibitors, a more potent anti-tumor effect can be achieved in tumors expressing both HSD17 β 1 and OATP2B1. Since certain 2-halogenated-13-epiestrones, and the previously investigated phosphonated 13-epiestrones [24] are dual inhibitors of HSD17 β 1 and OATP2B1, their effect on the survival of hormone dependent cell lines with OATP2B1 overexpression, and/or HSD17 β 1 expression is reasonable to be investigated.

CRedit authorship contribution statement

Réka Laczkó-Rigó: Methodology, Visualization, Writing - original draft. **Rebeka Jójárt:** Resources. **Erzsébet Mernyák:** Resources, Writing - review & editing. **Éva Bakos:** Methodology, Supervision, Conceptualization. **Alzbeta Tuerkova:** Methodology, Formal analysis. **Barbara Zdrzil:** Methodology, Formal analysis, Writing - review & editing. **Csilla Özvegy-Laczka:** Conceptualization, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.jsbmb.2020.105652>.

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