¹²⁵I-Labelled 2-Iodoestrone-3-sulfate: Synthesis, characterization and OATP mediated transport studies in hormone dependent and independent breast cancer cells

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Abstract

Introduction—Organic Anion Transporting Polypeptides (OATP) are a family of membrane associated transporters that facilitate estrone-3-sulphate (E3S) uptake by hormone dependent, post-menopausal breast cancers. We have established E3S as a potential ligand for targeting hormone dependent breast cancer cells, and in this study sought to prepare and investigate radioiodinated E3S as a tool to study the OATP system.

Methods—2- and 4-Iodoestrone-3-sulfates were prepared from estrone *via* aromatic iodination followed by a rapid and high yielding sulfation procedure. The resulting isomers were separated by preparative HPLC and verified by ¹H NMR and analytical HPLC. Transport studies of 2- and 4-[¹²⁵I]-E3S were conducted in hormone dependent (i.e. MCF-7) and hormone independent (i.e. MDA-MB-231) breast cancer cells in the presence or absence of the specific transport inhibitor, bromosulphophthalein (BSP). Cellular localization of OATP1A2, OATP2B1, OATP3A1 and OATP4A1 were determined by immunofluorescence analysis using anti-Na⁺/K⁺ ATPase-α (1:100 dilution) and DAPI as plasma membrane and nuclear markers, respectively.

Results—Significantly (p<0.01) higher total accumulation of 2-[¹²⁵I]-E3S was observed in hormone dependent MCF-7 as compared to hormone independent MDA-MB-231 breast cancer cells. In contrast 4-[¹²⁵I]-E3S did not show cellular accumulation in either case. The efficiency of 2-[¹²⁵I]-E3S transport (expressed as a ratio of V_{max}/K_m) was 2.4 times greater in the MCF-7 as compared to the MDA-MB-231 breast cancer cells. OATP1A2, OATP3A1 and OATP4A1 expression was localized in plasma membranes of MCF-7 and MDA-MB-231 cells confirming the functional role of these transporters in radioiodinated E3S cellular uptake.

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Conclusion—An efficient method for the preparation of 2- and 4-[¹²⁵I]-E3S was developed and where the former demonstrated potential as an in vitro probe for the OATP system.

Keywords

Organic anion transporting polypeptide; estrone-3-sulfate; iodine-125; breast cancer

1. Introduction

Estrone-3-sulphate (E3S), a circulating inactive plasma estrogen, has been reported to serve as the predominant source for tumour estradiol in patients with post-menopausal, hormone dependent, breast cancer [1–3]. It has previously been demonstrated that the Organic Anion Transporting Polypeptides (OATPs), a family of membrane associated uptake transporters belonging to the solute carrier (SLC) superfamily, substantially contribute to specific carrier mediated E3S cellular uptake in breast cancer cells, with up to 10 times higher transport efficiency in hormone dependent (MCF-7) breast cancer cells [4]. Furthermore, the potential of E3S to serve as a novel ligand for targeting hormone dependent breast cancers was established based on tumour uptake, tumour-to-muscle and tumour-to-blood ratios of E3S, as observed in our previous pharmacokinetic and bio-distribution study conducted in mice bearing hormone dependent (MCF-7) and independent (MDA-MB-231) human breast tumour xenografts [5].

Amongst the 11 human OATP isoforms identified [6], seven (OATP1A2,1B1,1B3,1C1,2B1,3A1 and 4A1) have been reported to recognize E3S as a substrate [7, 8]. OATPs transport a wide range of structurally independent, amphipathic compounds including bile salts, hormones and their conjugates, toxins and various xenobiotics [9–12]. Based upon a comparison of known substrates, Hagenbuch et al. proposed that OATP substrates are higher molecular weight (>450) compounds and emphasized that OATPs preferentially transport compounds that have a steroid nucleus (e.g. E3S) or are small linear/cyclic peptides [10]. It was also reported that OATP substrates have three conserved hydrophobic regions, two hydrogen bonding sites and one ionic site [10, 13] (Figure 1A). Based on the nature of the known binding ligands, it was hypothesized that E3S labeled with a radionuclide at the 2 or 4-positions of the steroid could serve as an OATP substrate. Potent steroid sulfatase inhibitors based on comparable A ring modification have been developed [14–19] including 2/4 derivatives of oestrone-3-O-sulphamate [20] (Journal of Steroid Biochemistry and Molecular Biology (1998), 64(5–6), 269–275). This suggested that it should be possible to prepare potent iodinated substrates as radiolabeled compounds to support the investigation of the OATP system.

In this study, 2-[¹²⁵I]-iodo-estrone-3-sulphate (2-[¹²⁵I]-E3S) and 4-[¹²⁵I]-iodo-estrone-3-sulphate (4-[¹²⁵I]-E3S) were synthesized, along with the corresponding non-radioactive analogues, and OATP mediated transport characterized in hormone dependent (MCF-7) and hormone independent (MDA-MB-231) human breast cancer cells. The cellular uptake and affinity kinetics (K_m and V_{max}) of the iodinated E3S compounds were compared between hormone dependent and independent breast cancer cells to determine if greater transport efficiency (V_{max}/K_m) is observed in hormone dependent breast cancers cells, as was

previously observed for E3S. In order to further confirm the role of OATPs in the transport of iodinated-E3S, localization of OATP1A2, OATP2B1, OATP3A1 and OATP4A1, four predominant OATP isoforms reported to facilitate E3S cellular uptake in breast cancers [15, 21–23], was assessed in both MCF-7 and MDA-MB-231 cells. The results provide key insight into the feasibility of using radioiodinated E3S as a probe for the OATP and to assess the potential use of analogous compounds labelled with iodine-123/131 or iodine-124 as radiopharmaceuticals for SPECT or PET imaging [24–26].

2. Materials and methods

2.1 Chemicals, general procedures and equipment

Chemicals were purchased from Sigma-Aldrich Canada unless otherwise stated. All reactions were carried out under a nitrogen atmosphere with dry solvents under anhydrous conditions, unless otherwise specified. [^{125}I]NaI was obtained from McMaster Nuclear Reactor as a 1.48 GBq/mL solution in aqueous NaOH (pH 8–11). Reactions were monitored by thin-layer chromatography (TLC) carried out on 0.25 mm E. Merck silica gel plates (60F-254) using UV light and an ethanolic solution of phosphomolybdic acid and cerium sulfate followed by heating as visualizing strategies. Merck silica gel (60, particle size 0.040–0.063 mm) was used for flash column chromatography. ^1H and ^{13}C NMR spectra were recorded on Bruker AVIII 700 MHz NMR spectrometer. Chemical shifts were calibrated using residual non-deuterated solvent as an internal reference. The following abbreviations were used to explain the multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, br = broad.

Analytical HPLC-MS of non-radioactive materials was performed using a Waters Acquity HPLC-MS system including a Binary Solvent Manager, Sample Manager (samples cooled to 10°C), Column Manager (column temperature 30°C), Photodiode Array Detector (monitoring at 254 nm and 214 nm), TQD with electrospray ionization and a Waters Acquity BEH C18, 2.1×100 mm or 2.1×50 (1.7 μm) column. Preparative HPLC of non-radioactive and radioactive materials and analytical HPLC of radioactive materials were performed using a Waters 1525 Binary HPLC pump, a Waters 2489 UV/Visible Detector (monitoring at 254 nm and 214 nm), Bioscan Flow Count radiodetector (FC-3300) and a Waters XBridge Prep C18 19×100 mm (5 μm) column (non-radioactive) or a Waters XBridge Prep C18 10×100 mm (5 μm) column (radioactive-preparative) or a Waters XBridge C18 4.6×100 mm (5 μm) column (radioactive-analytical). HPLC elution method 1: Waters XBridge Prep C18 19×100 mm (5 μm) column; mobile phase A: H_2O (0.1% v/v TFA); mobile phase B: acetonitrile (0.1% v/v TFA); flow rate: 10 mL/min; 0→10 minutes, 50%→0% A. HPLC elution method 2: Waters XBridge Prep C18 19×100 mm (5 μm) column; mobile phase A: H_2O (0.4% w/v ammonium formate); mobile phase B: methanol; flow rate: 10 mL/min; 0→10 minutes, 80%→30% A. HPLC elution method 3: Waters XBridge Prep C18 10×100 mm (5 μm) column; mobile phase A: H_2O (0.1% v/v TFA); mobile phase B: acetonitrile (0.1% v/v TFA); flow rate: 5 mL/min; 50% A isocratic. HPLC elution method 4: Waters XBridge Prep C18 10×100 mm (5 μm) column; mobile phase A: H_2O (0.4% w/v ammonium formate); mobile phase B: methanol; flow rate: 5 mL/min; 45% A isocratic. HPLC elution method 5: Waters Acquity BEH C18 2.1×50 (1.7 μm) column; mobile phase A: H_2O (0.1%

v/v TFA); mobile phase B: acetonitrile (0.1% v/v TFA); flow rate = 0.3 mL/min; 0→8 min, 90→0% A; HPLC elution method 6: Waters XBridge C18 4.6×100 mm (5 μm) column; mobile phase A: H₂O (0.1% v/v TFA); mobile phase B: acetonitrile (0.1% v/v TFA); flow rate: 1 mL/min; 0→10 minutes, 90%→0% A.

2.2 Synthesis of 2- and 4- iodoestrones (2a and 2b)

To a solution of estrone (135 mg, 0.5 mmol) in acetonitrile (10 mL) were added *N*-iodosuccinimide (115 mg, 0.5 mmol) and *p*-TsOH (95 mg, 0.5 mmol) at room temperature. The reaction mixture was stirred for 12 hours before quenching with sodium thiosulfate (10 mL, sat. aq.) and products extracted with ethyl acetate (3 × 10 mL). The organic layer was concentrated and purified by flash column chromatography (silica gel, 3:1→1:1 v/v hexanes/ethyl acetate) to afford the desired mono-iodo compounds as a mixture of 1.5:1 positional isomers, which were separated by prep-HPLC (Method 1, retention time: 6.44 and 6.9 minutes for **2a** and **2b**, respectively; 69 mg of **2a**, 34% yield; 46 mg of **2b**, 22% yield). *2-Iodoestrone (2a)* [27]: LC-MS elution method 5; retention time: 5.07 min; MS (negative ESI): found *m/z* 509.1 [M+CF₃CO₂]⁻; C₂₀H₂₁F₃IO₄ (509.04). ¹H NMR (700 MHz, CDCl₃/CD₃OD 1:1 v/v): δ 7.52 (1 H, s), 6.57 (1 H, s), 2.80-1.34 (15 H, m), 0.90 (3 H, s). *4-Iodoestrone (2b)* [28]: LC-MS elution method 5; retention time: 5.26 min; MS (negative ESI): found *m/z* 509.2 [M+CF₃CO₂]⁻; C₂₀H₂₁F₃IO₄ (509.04). ¹H NMR (700 MHz, CDCl₃): δ 7.21 (1 H, d, *J* = 8.5 Hz), 6.85 (1 H, d, *J* = 8.5 Hz), 5.42 (1 H, s, br), 2.93-1.37 (15 H, m), 0.90 (3 H, s).

2.3 Synthesis of 2- and 4-iodoestrone 3-sulfates (3a and 3b)

Sulfur trioxide pyridine complex (15 mg, 94 μmol) was added to a solution of 2-iodoestrone **2a** (5 mg, 12.6 μmol) in pyridine (70 μL), at room temperature. The reaction mixture was stirred for 12 hours at 50°C, then quenched with NaOH (0.1 mL, 1.0 M aq.). The final product 2-iodoestrone-3-sulfate (**3a**) was obtained after prep-HPLC purification (Method 2, retention time: 7.9 minute; 3.0 mg, 48% yield). 4-Iodoestrone-3-sulfate (**3b**) was prepared using the same procedure in 48% yield. *2-Iodoestrone-3-sulfate*: LC-MS elution method 5; retention time: 3.44 min; MS (negative ESI): found *m/z* 474.9 [M]⁻; C₁₈H₂₀IO₅S (475.01). ¹H NMR (700 MHz, CD₃OD): δ 7.67 (1 H, s), 7.32 (1 H, s), 2.91-1.41 (15 H, m), 0.93 (3 H, s); ¹³C NMR (175 MHz, CD₃OD): δ 152.16, 139.72, 139.53, 137.40, 122.55, 87.43, 51.76, 45.20, 39.54, 36.92, 32.91, 30.38, 27.57, 27.12, 22.69, 14.44. *4-Iodoestrone-3-sulfate*: LC-MS elution method 5; retention time: 3.47 min; MS (negative ESI): found *m/z* 475.0 [M]⁻; C₁₈H₂₀IO₅S (475.01). ¹H NMR (700 MHz, CD₃OD): δ 7.38 (1 H, d, *J* = 8.6 Hz), 7.31 (1 H, d, *J* = 8.6 Hz), 3.01-1.35 (15 H, m), 0.92 (3 H, s); ¹³C NMR (175 MHz, CD₃OD): δ 152.38, 141.70, 139.21, 127.23, 119.33, 100.08, 51.76, 45.95, 39.15, 38.76, 36.96, 32.96, 28.68, 27.43, 22.66, 14.41.

2.4 Preparation of [¹²⁵I]-3a and [¹²⁵I]-3b

To a clear max recovery HPLC vial containing estrone (0.1 mg, 0.4 μmol in 100 μL methanol) and acetic acid (5 μL) was added carrier-free Na¹²⁵I (77.3 MBq) in 0.1 M aqueous sodium hydroxide. To this mixture was added a solution of iodogen (5.0 μg, 11.5 nmol) in 20 μL methanol. The reaction vial was capped and allowed to sit for 15 minutes at

room temperature with gentle swirling every 3 minutes. Aqueous sodium thiosulfate (100 μ L, 0.1 M) was added to quench the reaction and radiolabeled **2a** and **2b** were isolated by prep-HPLC (Method 3, ^{125}I -**2a** R_t = 7.02 min; ^{125}I -**2b** R_t = 8.10 min). The HPLC fraction containing ^{125}I -**2a** was diluted with water (7 mL) and loaded onto a C-18 Sep-Pak light cartridge, and then washed with water (10 mL). The product was then eluted with ethanol (3 mL) into a microwave vial. Ethanol was evaporated to dryness under nitrogen flow at 50 °C and the co-evaporation/drying process was repeated twice with anhydrous acetonitrile (1.0 mL). To the microwave vial containing ^{125}I -**2a** was added sulfur trioxide pyridine complex (220 mg, 1.38 mmol), anhydrous pyridine (0.1 mL), and anhydrous acetonitrile (1.0 mL). The mixture was capped and stirred at 50 °C for 20 hours, after which the crude reaction was diluted with water (8 mL) and passed through a C-18 Sep-Pak plus cartridge. The cartridge was washed with water (20 mL) and then ethanol (10 mL), which eluted the desired product and residual pyridine. The desired product was isolated by semi-preparative HPLC (Method 4) and trapped onto a C18 Sep-Pak light cartridge as above, washed with water (20 mL) and eluted with ethanol (3 mL) and concentrated to dryness. The final product, ^{125}I -**3a**, was formulated in assay buffer in 19% radiochemical yield (14.7 MBq) and in >99% radiochemical purity (determined by analytical HPLC, method 6). The above sulfonation reaction procedure was performed on ^{125}I -**2b** to afford [^{125}I]-4-iodoestrone-3-sulfate (^{125}I -**3b**) in 28% radiochemical purity (determined by analytical HPLC, method 6).

2.5 Cell culture

MCF-7 and MDA-MB-231 cells were purchased from American Tissue Culture Collection (ATCC, Manassas, VA). Tissue culture reagents were obtained from Invitrogen (Carlsbad, CA), unless indicated otherwise. The MCF-7 and MDA-MB-231 cells were grown in Dulbecco's modified Eagle's medium (DMEM). Culture media were supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum. Cells were maintained at 37°C in humidified 5% CO₂ with fresh medium replaced every 2 to 3 days. Cells were sub-cultured with 0.05% trypsin-EDTA upon reaching 95% confluence. For transport experiments, cells were seeded into 24-well plates with a cell density of 25×10^3 cells/cm².

2.6 Transport experiments

Transport experiments were conducted as previously described [4, 29] with minor modifications. Briefly, for the time course studies, confluent cell monolayers were incubated with 20 nM 2- or 4-iodoestrone-3-sulfates spiked with 11.1 kBq/mL 2- or 4- [^{125}I]-E3S, in the presence or absence of 100 μ M of the OATP transport inhibitor BSP. The iodo-E3S and BSP concentrations were chosen based on previous studies [4, 30]. Cell aliquots were removed at various time points, lysed, and the soluble contents analysed for total ^{125}I and total protein content to normalize values of ^{125}I -E3S uptake. For the concentration dependent studies, the cells were incubated with **3a** or **3b** ranging from 5 nM to 1 mM concentration (buffer was spiked with 11.1 kBq/mL of the corresponding isomer of [^{125}I]-E3S).

2.7 Immunofluorescence

To identify the cellular localization of OATP transporters OATP1A2, OATP2B1, OATP3A1 and OATP4A1 immunofluorescence analysis was performed as previously described with

minor modifications [31]. Samples were prepared by culturing MCF-7 and MDA-MB-231 cells on glass coverslips at a cell density of approximately 5000 cells/cm². The samples were fixed in 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. Fixed cell samples were then incubated with primary rabbit polyclonal anti-OATP1A2 (Sigma-Aldrich, Canada), rabbit polyclonal anti-OATP2B1 (Sigma-Aldrich, Canada), goat polyclonal anti-OATP3A1 (Santa Cruz Biotechnology, Inc.) or goat polyclonal anti-OATP4A1 (Santa Cruz Biotechnology, Inc.) antibodies at 1:500, 1:100, 1:200 and 1:200 dilutions respectively. Cells were also incubated with rabbit or goat anti-Na⁺/K⁺ ATPase- α (Santa Cruz Biotechnology, Inc.) (1:100 dilution) for goat polyclonal OATP3A1 and OATP4A1, and for the rabbit polyclonal OATP1A2 and OATP2B1, respectively. This enzyme was used as a marker of plasma membrane localization. Fixed cells were then incubated with the respective secondary (anti-goat or anti-rabbit IgG) antibodies conjugated to Alexa Fluor 488 and Alexa Fluor 568 (Sigma-Aldrich, Canada). Samples were mounted on glass slides with Vectashield hardset mounting medium containing DAPI (excitation 358 nm/emission 386 nm) as a marker of DNA. For negative controls, samples were incubated only with secondary antibodies. Cells were then visualized using a 40X oil differential interference contrast objective and Zeiss LSM 510 META NLO two-photon confocal laser-scanning microscope (Carl Zeiss AG, Oberkochen, Germany) equipped with argon (458, 476, 488, and 513 nm wavelengths), a helium-neon (533 nm wavelength), and a tuneable Chameleon (720–930 nm wavelengths) laser lines.

2.8 Data analysis

All experiments were performed in triplicate using cells obtained from three different passages where for each experiment, all data points represent triplicate trials. Results are presented as mean $\pm$ SD or mean $\pm$ S.E.M as appropriate. Statistical analysis was performed with Graphpad InStat version 3.0 software (GraphPad Software, Inc., San Diego, CA). Statistical significance was assessed by two-tailed Student's *t* test for unpaired experimental values or one-way analysis of variance (ANOVA) for analysis of repeated measures, as appropriate. $p < 0.05$ was considered statistically significant.

In the time dependent uptake experiments, the non-specific uptake was estimated by measuring E3S accumulation in the presence of a high concentration of the OATP transport inhibitor BSP (100 μ M). Specific uptake was determined by calculating the difference between the total E3S uptake and the non-specific E3S uptake. In the concentration dependent experiments, kinetic analysis for estimation of the Michaelis-Menten affinity constant (K_M) and the maximal velocity (V_{max}) were performed using the following equation, which consists of one saturable Michaelis-Menten component combined with a nonsaturable component:

$$V = \frac{V_{max}[S] + k_{ns} \times [S]}{K_M + [S]} \quad (1)$$

where V is the total rate of uptake of the studied substrate, V_{max} is the maximum uptake rate, K_M is the dissociation constant, $[S]$ is the substrate concentration, and k_{ns} is the

coefficient for nonspecific uptake or diffusion. In the concentration dependent uptake experiments performed in the different cells, the total uptake was corrected for the nonspecific binding (i.e., background radioactivity at zero time) and the nonsaturable or diffusion component. The fitting of data into each model was performed by nonlinear least-squares analysis for “one site-fit total and nonspecific binding” using Prism 5 software (GraphPad Software Inc., San Diego, CA).

3.0 Results

3.1 Synthesis and Characterization of 2- or 4-iodoestrone-3-sulfates

Initial attempts to label estrone sulfate directly using conventional iodination methods (Na^{125}I , iodogen) did not produce the desired sulfated radioiodinated species. Iodination in the presence of the sulfate group was expected to be difficult as its electron withdrawing nature would reduce the reactivity of the aryl ring toward electrophilic substitution reactions. However, “desulfated” 2- and 4-iodoestrone (^{125}I -**2a** and ^{125}I -**2b**) were formed exclusively (Supplementary Information- Fig. S1). It is likely that loss of the sulfate group occurred first, generating the reactive phenol, which was sequentially radioiodinated. This indicated that iodination of estrone should precede sulfation.

An alternative approach to produce the non-radioactive standards was to first iodinate estrone, followed by in situ preparation of the sulfate. The 2- and 4-iodoestrone-3-sulfates (Figure 1: compounds **3a** and **3b** respectively) were prepared from estrone (**1**) *via* aromatic iodination followed by sulfation. Estrone was treated with one equivalent of *N*-iodosuccinimide in the presence of *p*-toluenesulfonic acid to produce a mixture of 2- and 4-iodoestrone (**2a** and **2b**) in a ~1.5:1 ratio. The two structurally similar isomers were then separated by preparative HPLC (34% and 22% yield, respectively). The two isolated products (2- and 4-iodoestrone) were identical by mass spectra and could be readily identified by ^1H NMR spectroscopy (Supplementary Information- Fig. S2). 2-Iodoestrone (**2a**) exhibits two singlets in the aromatic region (6.5~7.5 ppm), whereas 4-iodoestrone (**2b**) shows two sets of doublets due to the coupling of the adjacent aromatic protons.

Sulfation of each isomer was performed successfully using sulfur trioxide in the presence of pyridine. As seen in Figure 2, 2- and 4-iodoestrone sulfates (**3a** and **3b**) were obtained after preparative HPLC purification in 48% yield (for both isomers). HPLC-MS analysis of the final products showed the negatively charged sulfates, which, as expected, are significantly more hydrophilic than the parent phenols. The chemical shifts of aromatic protons for the estrone-3-sulfates (**3a** and **3b**) were downfield compared to that for the free phenols (**2a** and **2b**) due to the presence of the electronic withdrawing sulfate group (Figure 3). For instance, the aromatic protons for **2a** exhibit two singlets at 7.52 and 6.57 ppm in ^1H NMR spectrum, whereas the corresponding sulfate **3a** displays two singlets at 7.67 and 7.32 ppm. In addition, the existence of the sulfate group is evident by the detection of the desired mass in ESI-MS (negative ion mode) at $m/z = 475$ (M^-).

3.2 Synthesis and Characterization of [¹²⁵I]-2- or 4-iodoestrone-3-sulfates

Radioiodinated estrone-3-sulfates were synthesized from estrone in a similar manner to the cold reference standards, except that the iodination step was performed using Na¹²⁵I and iodogen (Fig. 1B). The labeling conditions produced ¹²⁵I-2a and ¹²⁵I-2b in reasonable radiochemical yield (19% and 28% respectively) and in high purity (>98%). The isolated yields were reduced as a result of binding of the product to glassware and tubing during purification, rather than the formation of multiple impurities. The identities of the final ¹²⁵I-labeled iodoestrone 3-sulfates were confirmed by analytical HPLC analysis and co-injection with the non-radioactive standards (Supplementary Information- Fig. S4). It should be noted that this method was suitable for the preparation of ample quantities of the ¹²⁵I labeled compounds for in vitro assays but would need to be further optimized before the ¹²³I analogue for imaging applications could be considered.

3.3 Characterization of [¹²⁵I]2- or 4-iodoestrone-3-sulfates uptake by the breast epithelial cells

3.3.1 Specificity of OATP mediated uptake and total accumulation of [¹²⁵I]-2- or 4-iodoestrone-3-sulfates—

In order to determine the specific contribution of the OATP transporters towards E3S uptake by the hormone-dependent MCF-7 and hormone-independent MDA-MB-231 cells, time course experiments were performed using 20 nM 2- or 4-[¹²⁵I]-E3S, in the presence or absence of an excess (100 μM) of the specific transport inhibitor BSP [29]. Significant reduction of uptake in the presence of BSP would indicate that the OATP transporters contribute towards E3S intracellular accumulation. Specific OATP mediated uptake of 2-[¹²⁵I]-E3S was observed in MCF-7 cells (Figure 4A) at all time points while in MDA-MB-231 cells the effect of BSP was only observed at the longest time point (Figure 4B). Total accumulation of 2-[¹²⁵I]-E3S was significantly ($p < 0.01$) higher in MCF-7 cells as compared to MDA-MB-231 cells (Figure 4C). No specific OATP mediated uptake by either cell was observed for 4-[¹²⁵I]-E3S (data not shown).

3.3.2 Transport kinetics of [¹²⁵I]-2-iodoestrone-3-sulfate in hormone dependent and hormone independent breast cancer cells—

Transport kinetics (V_{max} and K_m) and transport efficiency (expressed as a ratio of V_{max}/K_m) of 2-[¹²⁵I]-E3S were determined in both cell lines, as previously described [32]. The affinity (determined by K_m) and capacity (determined by V_{max}) of 2-[¹²⁵I]-E3S transport were $51.5 \pm 3.6 \mu M$ and 229.2 ± 33.1 pmol/mg protein/min, respectively, in hormone dependent (MCF-7) (Figure 5A) and $96 \pm 1.7 \mu M$ and 183.9 ± 49.4 pmol/mg protein/min, respectively, in hormone independent (MDA-MB-231) breast cancer cells (Figure 5B). Both cell lines show one site saturation binding kinetics. The efficiency of transport was 2.4 times greater in the hormone dependent (MCF-7) as compared to the hormone independent (MDA-MB-231) breast cancer cells (Figure 5C).

3.3.3 Cellular localization of OATP transporters in breast cancer cells—

Cellular localization of OATP1A2, OATP2B1, OATP3A1 and OATP4A1 was investigated in MCF-7 and MDA-MB-231 cells to further verify the presence of the transporter. Both cells were also incubated with a Na⁺/K⁺ ATPase antibody as a plasma membrane marker [33] and a mounting media containing DAPI, to serve as a nuclear marker. OATP1A2 (Figure 6A, 7A),

OATP3A1 (Figure 6B, 7B) and OATP4A1 (Figure 6C, 7C) all showed cell plasma membrane localization in MCF-7 and MDA-MB-231 cells. However, we could not detect plasma membrane localization of OATP2B1 in MCF-7 or MDA-MB-231 cells (data not shown). Localization of OATPs with Na⁺/K⁺ ATPase suggests the plasma membrane localization of these transporters. Negative controls were conducted for all markers in both cell systems. Absence of primary antibody resulted in lack of fluorescence signal with only DNA staining for DAPI present in the mounting media.

4.0 Discussion

The objective of this study was to prepare 2- and 4-[¹²⁵I]-E3S and assess the isomers as radioligands for studying OATP in hormone dependent breast cancer cells. 2- and 4-[¹²⁵I]-E3S were synthesized using a new two-step labelling approach. The first goal was to prepare the cold reference standards, which involved direct aromatic iodination of estrone, which produced two isomers (Figure 2). Prior to sulfation, 2-iodo- and 4-iodo-estrone were separated using preparative HPLC and characterized using ¹H NMR. Following addition of the sulfate group the final products were again isolated using preparative HPLC and characterized by ¹H and ¹³C NMR, analytical HPLC and LC-MS (Figures 2 and 3). HPLC-MS analysis of the final products were consistent with the formation of the negatively charged sulfates, which as expected were more hydrophilic than the parent phenols. Furthermore, the addition of the sulfate group was confirmed by ESI-MS, which showed the expected mass-to-charge ratio [C₁₈H₂₀IO₅S (475.01)]. The radioactive [¹²⁵I]-2- or 4-iodoestrone-3-sulfates were similarly synthesized using a two step approach and were identified by analytical HPLC analysis of co-injected samples with the fully characterized non-radioactive standard compounds (Supplementary Information- Fig. S4).

The OATP mediated 2- or 4-[¹²⁵I]-E3S transport was characterized in hormone dependent (MCF-7) and hormone independent (MDA-MB-231) breast cancer cells. Specific transporter mediated uptake was only observed for one isomer i.e. 2-[¹²⁵I]-E3S. Although specific OATP mediated 2-[¹²⁵I]-E3S uptake was seen in both hormone dependent and independent breast cancer cells, the extent of transport inhibition was significantly higher (p<0.001) in the hormone dependent MCF-7 cells (Figure 4). Furthermore, it was observed that total accumulation of 2-[¹²⁵I]-E3S was significantly higher (p<0.01) in the hormone dependent MCF-7 cells as compared to the hormone independent MDA-MB-231 cells (Figure 4C). Similar to 2-[¹²⁵I]-E3S uptake, we previously demonstrated higher uptake of E3S by MCF-7 cells [4]. The total uptake of 2-[¹²⁵I]-E3S (0.16 ± 0.006 pmol/mg protein) is also comparable to that of ³H-E3S (0.1 ± 0.002 pmol/mg protein) shown previously for hormone dependent MCF-7 cells [4]. These observations suggest that similar to ³H-E3S, 2-[¹²⁵I]-E3S also has potential as a novel ligand for targeting OATP in hormone dependent breast cancers.

Upon establishing the specific functional role of OATP in 2-[¹²⁵I]-E3S cellular uptake, the transport kinetics i.e. K_m, V_{max} and efficiency of 2-[¹²⁵I]-E3S transport were determined in MCF-7 and MDA-MB-231 cells. Although the total uptake of 2-[¹²⁵I]-E3S was comparable to E3S (Figure 4), the overall affinity (51.5 ± 3.6 μM) of 2-[¹²⁵I]-E3S for MCF7 cells was lower than the one we previously observed for ³H-E3S (6.5 ± 1.9 μM) (Figure 5A) [4, 30]. Interestingly the transport efficiency is 2.4 times higher in MCF-7 cells as compared to

MDA-MB-231 cells for 2-[¹²⁵I]-E3S (Figure 5C). This suggests that 2-[¹²⁵I]-E3S is a more effective targeting ligand for hormone dependent breast cancers. However, we previously showed that ³H-E3S transport efficiency was ten times greater for MCF-7 than MDA-MB-231 cells. This reduction in transport efficiency could potentially result from the lower affinity of MCF-7 cells for 2-[¹²⁵I]-E3S, as compared to ³H-E3S. Although affinity and transport efficiency are lower for 2-[¹²⁵I]-E3S in the MCF-7 cells, there is comparable total uptake observed, suggesting its potential as a specific targeting ligand.

It has been previously documented that seven OATP isoforms recognize E3S as a substrate. Additionally, we have previously analysed gene and protein expression of six OATP isoforms i.e. OATP1A2, OATP1B1, OATP1B3, OATP2B1, OATP3A1 and OATP4A1 in both MCF-7 and MDA-MB-231 cells uptake [4]. Both cell lines express OATP1A2, and OATP3A1, suggesting the functional role of these transporters in E3S cellular. Miki *et al.* and Meyer Zu Schwabedissen *et al.* also reported 10 fold higher OATP1A2 expression in breast cancer tissues as compared to surrounding normal tissues [21, 22]. Pizzagalli *et al.* detected the localization of the OATP 2B1 in the luminal epithelium and myoepithelial cells in invasive ductal carcinoma tissues and in the surrounding normal tissues of the breast, respectively [23]. Wlcek *et al.* and others reported SLCO3A1 and SLCO4A1 gene expression in various breast cancer cells [21–23, 34]. To verify the presence of the transporter in the cells used in the present study we characterized the expression and sub-cellular localization of OATP1A2, OATP2B1, OATP3A1 and OATP4A1. It was observed that OATP1A2, OATP3A1 and OATP4A1 isoforms were predominantly located on the plasma membrane as was confirmed with their overlapping signals along with the plasma membrane marker, Na⁺/K⁺ ATPase [33]. These observations further confirmed the functional role of OATP in cellular uptake of 2-[¹²⁵I]-E3S. In this study, the localization of OATP1A2, OATP3A1 and OATP4A1 were predominant, but not limited to the plasma membrane in both MCF-7 and MDA-MB-231 cells. Our observations were similar to previously reported localization data in the MCF-7 cells [35]. These data also support gene and/or protein expression of the transporters that we had previously quantified in the MCF-7 and MDA-MB-231 cells [4].

5. Conclusion

An approach for synthesizing 2- and 4-[¹²⁵I]-E3S was developed where for the former specific uptake was observed in both hormone dependent and independent breast cancer cells. Even with the large iodine atom added to E3S, OATPs continue to recognise 2-[¹²⁵I]-E3S as a substrate. Although the transport efficiency of OATP mediated uptake of 2-[¹²⁵I]-E3S was less than E3S, there were significant differences between the hormone dependent and independent cells. This data aligns with the known physiological role of E3S which is to serve as the predominant source for tumour tissue estrogen in post-menopausal hormone dependent patients. Specific carrier mediated 2-[¹²⁵I]-E3S uptake was detected in both hormone dependent and independent breast cancer cells, suggesting that cellular uptake is independent of estrogen receptor (ER) expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Advances in Knowledge and Implications for Patient Care

The new E3S probe can be used to study the OATP system and as a platform to create radiopharmaceuticals for imaging breast cancer.

Figure 1. Chemical structure and schematic OATP binding of E3S (A), and the synthesis scheme for radioiodinated E3S and 2- and 4-[¹²⁵I]-E3S (B).

Figure 2. Analytical HPLC traces monitored at 214 and 254 nm of 2- and 4-iodoestrones (A) and the corresponding sulfates (B).

Figure 3.
¹H NMR of 2- (A) and 4-iodoestrone-3-sulfates (B).

Figure 4.

Time course of 2- $[^{125}\text{I}]$ -E3S uptake in MCF-7 (A) and MDA-MB-231 (B) breast cancer cells. Total uptake (solid line) of 2- $[^{125}\text{I}]$ -E3S in MCF-7 and MDA-MB-231 cells was evaluated over 30 min at pH 7.4 and 37°C. The non-specific uptake (dashed line) was determined by measuring 2- $[^{125}\text{I}]$ -E3S uptake in the presence of a transport inhibitor (BSP 100 µM). (C) Total E3S accumulation at 30 min was determined in both cell line systems and significant differences are observed among the hormone dependent and independent cells. Note the slight difference in y-axis scales between A. and B. Statistical significance was assessed by two-tailed Student's *t* test for unpaired experimental values. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$ are considered to be statistically significant.

Figure 5.

Kinetics of 2-[¹²⁵I]-E3S uptake by (A) MCF7 and (B) MDA-MB-231 cells at concentrations ranging from 5 nM to 200 μM and 5 nM to 1000 μM, respectively, measured at 2 min, pH 7.4, and 37°C. The saturable (solid triangle) and nonsaturable (solid square, dashed line) components of the total (solid circle) uptake rates were determined by fitting the data into the Michaelis-Menten equation. Saturable E3S rate of uptake by the MCF-7 and MDA-MB-231 cells were fitted into a “one site-Fit total and nonspecific binding” kinetics model. The kinetic parameters K_M and V_{max} were estimated by least-square nonlinear regression analysis. Data represent the mean $\pm$ S.E.M. for $n = 3$ independent experiments. (C) Transport efficiency of 2- [¹²⁵I]-E3S uptake MCF7 and MDA-MB-231 cells. Note the difference in y-axis scales between A. and B. ** $p < 0.01$ was considered to be statistically significant.

Figure 6.

Immunocytochemical localization of OATP1A2 (A), OATP3A1 (B) and OATP4A1 (C) in MCF-7 cells. Cells were stained with DNA dye, DAPI (blue), and examined by immunofluorescence using anti-OATP1A2, anti-OATP3A1 and anti-OATP4A1 antibodies. Anti-Na⁺/K⁺ ATPase- α antibody (polyclonal goat for cells stained with anti-OATP1A2 and polyclonal rabbit for cells stained with anti-OATP3A1 and anti-OATP4A1) was used as a plasma membrane marker. Cells were stained with Alexa Fluor-conjugated secondary antibodies 488/568. Cells were visualized using a 40X oil differential interference contrast objective.

Figure 7. Immunocytochemical localization of OATP1A2 (A), OATP3A1 (B) and OATP4A1 (C) in MDA-MB-231 cells. Cells were stained with DNA dye, DAPI (blue), and examined by immunofluorescence using anti-OATP1A2, anti-OATP3A1 and anti-OATP4A1 antibodies. Anti-Na⁺/K⁺ ATPase- α antibody (polyclonal goat for cells stained with anti-OATP1A2 and polyclonal rabbit for cells stained with anti-OATP3A1 and anti-OATP4A1) was used as a plasma membrane marker. Cells were stained with Alexa Fluor-conjugated secondary antibodies 488/568.