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Title page

Differential Role of OATPs in Estrone-3-Sulphate Uptake by Breast Epithelial Cells and Breast Cancer Cells[i]

Nilasha Banerjee, Christine Allen, Reina Bendayan

Department of Pharmaceutical Sciences, Leslie Dan Faculty of Pharmacy, University of Toronto, Toronto, Ontario, Canada, M5S 3M2 - NB, CA, RB.

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Running Title Page

Running Title: **Role of OATPs in Breast Cancers**

Corresponding author: Dr. Reina Bendayan, Department of Pharmaceutical Sciences, Leslie Dan Faculty of Pharmacy, University of Toronto, 144 College Street, Toronto, ON M5S 3M2, Tel: 416-978-6979, FAX: 416-978-8511, E-mail: r.bendayan@utoronto

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Non-standard abbreviations:

OATP; Organic Anion Transporting Polypeptide

E3S; Estrone-3-sulphate

BSP; Bromosulphophthalein

SLCO; gene for solute carrier superfamily encoding for OATPs

MCF10A; immortalized non-tumorigenic breast epithelial cells

MCF7; hormone dependent breast cancer cells

MDA435/LCC6; hormone independent, invasive and metastatic breast cancer cells

MDA-MB-468; hormone independent and epidermal growth factor receptor over expressing breast cancer cells

MDA-MB-231; triple negative breast cancer cells

T47D; hormone dependent breast cancer cells

ZR75; hormone dependent breast cancer cells

MDCK II; Madin Darby Canine Kidney II cells

MDCK II/ OATP2B1; Madin Darby Canine Kidney II cells stably over expressing OATP2B1

HEK-293; Human embryonic kidney cells

HEK-293/OATP3A1; Human embryonic kidney cells stably over expressing OATP3A1

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HEK-293/OATP4A1; Human embryonic kidney cells stably over expressing OATP4A1

DMEM; Dulbecco's modified eagle's medium

MEM α; Minimum essential medium alpha

RPMI; Cell culture medium developed at Roswell Park Memorial Institute

ATCC; American Tissue Culture Collection

STS; Steroid sulphatase

HSD-1-17β; Hydroxy steroid dehydrogenase type 1

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ABSTRACT:

The purpose of this study is to investigate the differential expression and function of Organic Anion Transporting Polypeptides (OATPs) in breast epithelial and breast cancer cells. E3S, a substrate for seven out of eleven OATPs, is a predominant source of tumor estrogen in post menopausal hormone dependent breast cancer patients. Over expression of certain OATPs (e.g. OATP1A2) reported in breast tumour tissues compared to surrounding normal tissues, could contribute towards 2-3 times higher tumoral E3S concentration. Little is known about the expression and function of other OATP family members among breast epithelial and breast cancer cells. We therefore compared gene and protein expression of seven OATPs (i.e. OATP1A2, OATP1B1, OATP1B3, OATP1C1, OATP2B1, OATP3A1 and OATP4A1) in immortalized breast epithelial cells (MCF10A), hormone dependent (MCF7) and hormone independent breast cancer cells (MDA/LCC6-435, MDA-MB-231, MDA-MB-468) by qPCR and immunoblotting. Expression of SLCO1A2, 1B1, 1B3, 2B1 and 3A1 is exclusive, similar or significantly higher in cancer cells compared to MCF10A. Protein expression of OATPs is found to be either exclusive or higher in cancer cells compared to MCF10A. Specificity of OATP mediated E3S uptake is only observed in cancer cells, with highest total uptake in MCF7 cells. Transport kinetics of E3S uptake demonstrate transport efficiency to be 10 times greater in the MCF7 cells than in the hormone independent cells. These data suggest that OATPs could be a novel therapeutic target for hormone dependent breast cancers, particularly in post menopausal patients, where the major source of tumour estrogen is E3S.

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INTRODUCTION:

Two thirds of newly diagnosed breast cancers are hormone dependent (Linden and Mankoff, 2010), where estrogen is key promoter of tumorigenesis. Surprisingly, up to 75% of hormone dependent cancers are detected in post-menopausal women (Pasqualini and Chetrite, 2005) with 90% reduced circulating plasma estradiol levels. Furthermore, breast tissue estradiol levels are comparable between pre- and post-menopausal women (Geisler, 2003;Van Landeghem, et al., 1985) indicating *in-situ* estradiol biosynthesis in breast tissues by conversion of circulating steroids through the aromatase and sulphatase pathways (Kendall, et al., 2007). Sulphatase activity is reported to be 130-200 times higher than aromatase activity and constitutes the predominant pathway (Chetrite, et al., 2000). This renders E3S, precursor for the sulphatase pathway, an important source for breast tissue estradiol. Tumour concentration of E3S is 2-20 fold higher than the corresponding plasma levels (Geisler, 2003;Pasqualini, et al., 1996;Van Landeghem, et al., 1985) and this ratio is almost double the other sources of estradiol (Geisler, 2003;Pasqualini, et al., 1997). Also, tumour concentration of sulphatase has been reported to be 3-fold higher than normal tissues (Utsumi, et al., 2000). Together this translates into 2-3 fold higher concentration of estradiol (following conversion from E3S) in the tumour compared to surrounding normal tissues (Chetrite, et al., 2000).

Unlike lipohilic estrone and estradiol, which readily diffuse through plasma membrane, E3S, due to it's hydrophilicity and net negative charge can not readily cross the plasma membrane by diffusion (Tan, et al., 1999;Purohit, et al., 2011;Verheugen, et al., 1984). Hence, an active transporter mediated mechanism is proposed to be involved in its cellular uptake. The OATPs facilitate E3S uptake in the liver, intestine, kidney, brain and various other sites (Konig, 2011;Kis, et al., 2010a). This suggests that OATPs are involved in E3S uptake by breast cancer

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cells. Furthermore, Nozawa *et al.* showed that inhibition of an E3S transporter results in the suppression of hormone dependent breast cancer cells proliferation (MCF7 and T47D) (Nozawa, et al., 2004;Nozawa, et al., 2005). These data suggest a potential role of OATPs in hormone dependent breast cancers proliferation by facilitating E3S cellular uptake. Recently Maeda, *et al.* showed the role of OATP1B3 in E3S uptake in two subclones of MCF7 cells (Maeda, et al., 2010). Amongst the 11 human OATP isoforms identified (Hagenbuch and Meier, 2004), seven (OATP1A2,1B1,1B3,1C1,2B1,3A1 and 4A1) were reported to recognize E3S as a substrate (Obaidat, et al., 2011). Among these, 10 fold higher expression of OATP1A2 was observed in breast cancer tissues as compared to their non-malignant counterparts (Miki, et al., 2006;Meyer Zu Schwabedissen, et al., 2008). Pizzagalli et al. reported OATP2B1 expression in luminal epithelium in invasive ductal carcinoma tissues and myoepithelial cells in the surrounding normal tissues (Pizzagalli, et al., 2003) and also demonstrated the functional role of OATP2B1 in the transport of E3S in these tissues. Recently, Kindla et al. compared the expression and localization of OATP2B1, OATP3A1 and OATP5A1 in paired samples of normal breast tissue and breast cancer tissue, and reported that while OATP3A1 and OATP5A1 are localized to the plasma membrane of epithelial cells of lactiferous ducts in normal breast tissue, these transporters are highly expressed in the plasma membrane and cytoplasm of breast cancer tissues (Kindla, et al., 2011). Despite all these data, very little is known about the difference in expression and function of the OATPs between hormone dependent and hormone independent breast cancers.

The purpose of this study is to investigate transcript and protein expression of seven OATP isoforms that recognize E3S as a substrate, and further evaluate their differential functional role in E3S uptake, among immortalized breast epithelial cells, hormone dependent breast cancer

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cells and hormone independent breast cancer cells. Although previous literature reports OATP expression in hormone dependent breast cancer cells (MCF7, T47D, ZR75)(Maeda, et al., 2010;Nozawa, et al., 2004;Nozawa, et al., 2005), for the first time, we compare OATP expression and function in immortalized breast epithelial cells (MCF10A), hormone dependent breast cancer cells (MCF7), hormone independent, invasive and metastatic breast cancer cells (MDA435/LCC6, derived from MDA-MB-435 cells)(Leonessa, et al., 1996), hormone independent and epidermal growth factor receptor over expressing breast cancer cells (MDA-MB-468)(Armstrong, et al., 1994) as well as triple negative breast cancer cells (MDA-MB-231)(Grundker, et al., 2010). Confirmed differences in OATP expression between these cell lines may aid in elucidating the basis for the high tumor concentrations of E3S reported in post-menopausal patients with hormone dependent breast cancers. In addition we examined the functional role of the OATPs and characterized E3S transport kinetics and efficiency in the breast cancer cell lines that showed specific OATP mediated E3S uptake.

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MATERIALS AND METHODS

A. Cell culture: MCF7, MDA-MB-468 and MDA-MB-231 cells are purchased from American Tissue Culture Collection (ATCC, Manassas, VA). MDA435/LCC6 and MCF10A cells are kindly donated by Dr. Robert Clarke, Georgetown University School of Medicine (Washington, U.S.A.) and Dr. Fei-Fei Liu, University Health Network (UHN) (Toronto, Canada), respectively. Madin-Darby canine kidney II (MDCKII) cells stably expressing OATP2B1 (MDCKII/OATP2B1) are kindly donated by Dr. M. Grube, Ernst-Moritz-Arndt-University (Greifswald, Germany). Human embryonic kidney (HEK-293) cells stably expressing OATP3A1 (HEK/OATP3A1) and OATP4A1 (HEK/OATP4A1) are kindly donated by Dr. Martin F. Fromm, Institute of Experimental and Clinical Pharmacology and Toxicology, Friedrich-Alexander-Universität Erlangen-Nürnberg, (Erlangen, Germany). Tissue culture reagents were obtained from Invitrogen (Carlsbad, CA), unless indicated otherwise.

The MCF7, MDA-MB-231, HEK-293, HEK/OATP3A1, HEK/OATP4A1 and MDCKII/OATP2B1 cells are grown in Dulbecco's modified Eagle's medium (DMEM). The MDCKII/OATP2B1 medium is additionally supplemented with 1% L-glutamine, and 400 µg/ml hygromycin B (Sigma-Aldrich) to provide selection pressure for stably transfected cells (Kis, et al., 2010b). HEK/OATP3A1 and HEK/OATP4A1 medium is additionally supplemented with 800 µg/ml of geneticin (as previously described(Konig, et al., 2011)). Protein expression of OATP2B1 is induced by incubating the cells with 2.5mM sodium butyrate (Sigma-Aldrich), 24h prior to cell pellet collection. MDA-MB-468 cells are grown in RPMI medium (along with 10% fetal bovine serum and 1% penicillin/streptomycin). MDA435/LCC6 is grown in Minimum Essential Medium (MEM) Alpha. MCF10A cells are grown in special medium (Lonza), as

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directed under culture conditions prescribed by ATCC. All media are supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum.

All cells are maintained at 37°C in humidified 5% CO_2 with fresh medium replaced every 2 to 3 days. Cells are subcultured with 0.05% trypsin-EDTA upon reaching 95% confluence. For transport experiments, cells are seeded into 24-well plates with a cell density of 25×10^3 cells/cm^2.

B. Quantitative real-time RT-PCR: Total RNA is extracted from the cells (from three different passages) using TRIzol reagent (Invitrogen) as per the manufacturer's instructions. The concentration (absorbance at 260nm) and purity (absorbance 260 nm/ absorbance 280 nm ratio) of RNA samples is assessed using a Beckman Coulter DU Series 700 UV/Vis Scanning Spectrophotometer (Mississauga, Ontario, Canada). Isolated total RNA is subjected to DNase I digestion (0.1U/ml) according to the manufacturer's instructions in order to remove genomic DNA. The reverse transcription on DNase treated total RNA (2µg) (in a final reaction volume of 40 µl) is performed using an ABI high capacity reverse transcription kit as per the manufacturer's instructions (Applied Biosystems, Foster City, California, USA). The samples are then incubated for 10 min at 25˚C, followed by 120 min at 37˚C and finally 5 min at 85˚C using Mastercycler EP Realplex 2S thermal cycler (Eppendorf Canada, Mississauga, Ontario, Canada). The expression of the SLCO genes encoding for the OATP transporters are analysed by qPCR on a Mastercycler EP Realplex 2S using TaqMan fluorescence detection. We purchased TaqMan® Gene Expression Assays (Applied Biosystems, Warrington, United Kingdom) for seven human OATPs. In order to evaluate an appropriate reference gene, 18S is selected as the appropriate house keeping gene. Multiplex quantitative real-time RT-PCR is performed in an amplification mixture with a volume of 20 µl. The target gene amplification mixture contains 10 µl 2X

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TaqMan® Universal PCR Master Mix, 1 µl of the appropriate Gene Expression Assay, 1 µl TaqMan® endogenous control (18S), 50 ng template cDNA diluted in 5 µl nuclease free water. Thermal cycling conditions comprise two min at 50°C, two min at 95°C followed by 40 cycles of 15 s at 95°C and one min at 60°C. Fluorescence generation due to TaqMan® probe cleavage by the 5'→3' exonuclease activity of the DNA polymerase is measured with the Mastercycler EP Realplex 2S. All samples are amplified in triplicate. Results are imported into Microsoft Excel for further analysis. Quantitative real-time RT-PCR is performed with the following prefabricated TaqMan® Gene Expression Assays (Applied Biosystems) containing intron-spanning primers: OATP1A2: Hs00245360_m1, OATP2B1:Hs00200670_m1, OATP1B1: Hs00272374_m1, OATP1B3: Hs00251986_m1, OATP1C1:Hs00213714_m1, OATP3A1: Hs00203184_m1,OATP4A1:Hs00249583_m1, and 18S Part #4310893E (Wlcek, et al., 2008).

C. Transient transfection of OAT1B1, OATP1B3 and OATP1A2 cDNAs: The pEF/Amp-OATP1B1, -OATP1B3 and -OATP1A2 vectors encoding the full-length human organic anion transporting polypeptides (OATP1B1, OATP1B3 and OATP1A2) are kindly provided by Dr. Richard Kim (Department of Physiology and Pharmacology, University of Western Ontario, London, Canada). The generation of recombinant constructs was published elsewhere in detail (Tirona, et al., 2003;Sandhu, et al., 2005). The cDNAs supplied on blotting paper are eluted in TE (Tris EDTA: 10mM Tris, 1mM EDTA, pH 8.0) buffer and transformed into XL1blue competent cells according to the suppliers protocol (Sigma), and the cells are incubated overnight at 37^{o}C on Luria Bertani (LB)-Amp plate. The individual bacterial colony is propagated in LB-Amp liquid media overnight at 37^{o}C, and the recombinant plasmids are then isolated using GenEluteTM HP Plasmid Midiprep Kit (Sigma). Purified Plasmids are then

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transfected into HEK293 cells using lipofectamine following the protocol supplied with the reagent (Invitrogen). After 48h of transfection, whole cell lysates are prepared and protein overexpression is verified by immunoblotting using antibodies specific to each transporter.

D. Immunoblot analysis: Immunoblotting is performed as described previously (Kis, et al., 2010b;Ronaldson, et al., 2010) with minor modifications. Cells from three different passages are harvested and lysates are prepared as previously described. After determining the protein content using an assay kit from Bio-Rad Laboratories (Hercules, CA) and bovine serum albumin (BSA) as the standard, 10µg is loaded for the over-expressing cells (both stably and transiently transfected cells) and 50µg is loaded for each of the breast cell lines [MCF10A, MCF7, MDA435/LCC6, MDA-MB-468 and MDA-MB-231]. Samples are incubated with Laemmli buffer(Kis, et al., 2010b) and 10% β-mercaptoethanol for 10 min at 37°C. Proteins are separated on a 7% SDS polyacrylamide gel, and then transferred onto polyvinylidene fluoride transfer membranes. The blots are then blocked in 5% skimmed milk for 2h and incubated in primary antibody overnight at 4˚C. Primary rabbit polyclonal antibodies for anti-OATP1A2, anti-OATP2B1, anti-OATP3A1 and anti-OATP4A1 (Sigma Aldrich) are used in a 1:1000 dilution. The primary rabbit polyclonal antibodies for anti-OATP1B1 and anti-OATP1B3 are kindly provided by Dr. Richard Kim and are used at 1:3000 and 1:2500 dilutions(Ho, et al., 2006). All blots are also incubated with primary mouse antiactin (AC40) antibody (1:2000) as a loading control (Santa Cruz).The blots are then incubated for 1.5h with corresponding horseradish peroxidase-conjugated anti-rabbit (1:15000) or anti-mouse (1:2000) secondary antibody, respectively. Signals are enhanced by using a 1:1 mixture of Reagent A and Reagent B of chemiluminescence SuperSignal West Pico System (Thermo Fisher Scientific, Waltham, MA)

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and then detected by exposing them to an X-ray film. Densitometric analysis is performed using AlphaDigiDoc RT2 software to quantify relative protein expression.

E. Transport experiments: Transport experiments are conducted as previously described (Kis, et al., 2010b) with minor modifications. For the time course studies, confluent cell monolayers are incubated (for different times) with 20nM E3S (Sigma Aldrich, Canada) and the incubation buffer contains 0.3 μCi/ml [^{3}H]-E3S [(57.3 Ci/mmol; chemical name: 3-hydroxyestra-1,3,5(10)-trien-17-one hydrogen sulfate); PerkinElmer Life and Analytical Sciences (Waltham, MA)]. For the concentration dependent studies, the incubation buffer also contains 0.3 μCi/ml [^{3}H]-E3S and the cells are incubated with final concentrations of E3S ranging from 5nM to 1mM. The specificity of OATP mediated uptake is demonstrated by the use of a specific transport inhibitor bromosulphophthalein (BSP) (chemical name: 3,3'-(4,5,6,7-tetrabromo-3-oxo-1(3H)-isobenzofuranylidene)bis(6-hydroxy-, disodium salt) at a 100μM final concentration which is added to the pre-incubation buffer and the transport buffer. In order to fully clarify specificity of E3S uptake, we have compared the E3S uptake obtained in the presence of 100μM BSP to the one obtained with the use of an excess concentration (i.e., 1mM) of E3S (data not shown). Similar E3S uptakes were observed in the presence of BSP and an excess concentration of E3S, suggesting that BSP is able to abolish the specific E3S transporter-mediated uptake component and that the remaining uptake is the non-specific uptake which is most likely composed of a mixture of diffusion and non-specific cellular binding. All buffers and Triton X-100 are purchased from Sigma-Aldrich.

F. Data analysis: All experiments are repeated at least three times in cells pertaining to three different passages. Within an individual experiment, each data point represents triplicate trials. Results are presented as mean ± SD or mean ± S.E.M as appropriate. All statistical analysis is

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performed with Graphpad InStat version 3.0 software (GraphPad Software, Inc., San Diego, CA). Statistical significance is 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. $p < 0.05$ is considered statistically significant.

Results for qPCR are presented as relative mRNA abundance ± SD of each gene of interest normalized to the housekeeping gene 18S using the comparative C_T method, where ΔC_T is equal to $C_{T\ sample}$ minus the $C_{T\ 18S}$ and relative mRNA abundance is equal to $2^{-\Delta CT}$. The relative abundance of each gene is then explored in cells from three different passages and the ΔC_T is reported as a mean ± S.E.M from all the three passages.

Results from western blots are analysed by densitometry where the expression of protein of interest is normalized to actin expression. Relative protein expression is reported as mean ± SD of three independent experiments.

In the time dependent uptake experiments, the non-specific uptake is estimated by measuring E3S accumulation in the presence of the transport inhibitor BSP (100µM). Specific uptake is 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}) is performed using the following equation, which consists of one saturable Michaelis-Menten component combined with a nonsaturable component:

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

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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 is 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 is performed by nonlinear least-squares analysis for "One site-Fit total and nonspecific binding" using Prism 5 software (GraphPad Software Inc., San Diego, CA). Eadie-Hofstee plots are performed (data not shown) in order to confirm that the rates of uptake data best fit to a single binding site as represented by the "One site-Fit total and nonspecific binding" model.

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RESULTS:

A. OATP mRNA expression in human breast epithelial cell lines: In order to determine the difference in expression of each of the genes between the normal breast epithelial cells and the breast cancer cells (both hormone dependent and independent cells), we measured the relative mRNA expression of seven OATP isoforms, SLCO1A2, SLCO1B1, SLCO1B3, SLCO1C1, SLCO2B1, SLCO3A1 and SLCO4A1, in the immortalized breast epithelial cells (MCF10A), the hormone dependent breast cancer cells (MCF7) and the hormone independent breast cancer cells (MDA435/LCC6, MDA-MB-468, MDA-MB-231) by quantitative real-time PCR. Although there are 11 human OATPs reported (Hagenbuch and Meier, 2004), our investigation was limited to the genes that encode transporters responsible for E3S uptake into cells. We observe heterogeneity in OATP detection and expression levels among the different cell lines. We determined the relative expression of each gene with respect to the house keeping gene 18S. Among all of the seven OATP isoforms investigated, SLCO1C1 encoding OATP1C1 is the only gene that is below the detection limit in all cell lines. SLCO1A2 is detected in MCF7, MDA435/LCC6 and MDA-MB-231 (Figure 1A) with highest expression in MDA435/LCC6 cells. SLCO1B1 and SLCO1B3 are primarily expressed in MDA-MB-231 cells and show significantly lower expression in MDA435/LCC6 and MCF10A (Figure 1B, 1C). Although Maeda, *et al.* reported OATP1B3 to be involved in E3S uptake in two sub-clones of MCF7 cells, we could not detect any expression of the SLCO1B3 gene in the MCF7 cells. This could possibly be explained by the use of different cell systems. SLCO2B1 is undetectable is most of the cell systems with negligible expression in MCF7 and MDA435/LCC6 cells (Figure 1D). Thus, all four of these transporters show significantly higher or exclusive expression in breast cancer cells, compared to the immortalized breast epithelial cells (MCF10A). SLCO3A1 and SLCO4A1 are

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the only transporters that show high expression in MCF10A cells. Also, these are the only two genes which show expression in all cell lines investigated. SLCO3A1 shows significantly higher expression in MDA435/LCC6 compared to MCF10A, while other cells show similar or lower expression (Figure 1E). In contrast to all other genes explored, SLCO4A1 is the only gene which shows significantly higher expression in the immortalized breast epithelial cells (MCF10A) compared to the breast cancer cells. Among the breast cancer cells, MCF7 shows significantly higher expression of SLCO4A1 in comparison to expression levels in MDA-MB-468 and MDA-MB-231 (Figure 1F).

B. OATP protein expression in human breast epithelial cell lines: We also examined protein expression for each of the transporters that are detected at the transcript level. To determine the relative transporter expression, we assessed OATP1A2, OATP1B1, OATP2B1, OATP3A1 and OATP4A1 protein expression in the breast epithelial and breast cancer cell systems by western blot analysis (OATP1B3 expression could not be explored due to lack of specific antibody). OATP1A2 is expressed in all cell lines except for MCF10A cells and hence the level of expression is significantly ($p<0.05$) greater in all breast cancer cells compared to MCF10A. The highest expression for OATP1A2 is observed in the hormone dependent breast cancer cells, MCF7 (Figure 2A). The protein expression of OATP1A2 is concordant with the gene expression of SLCO1A2. OATP1B1 is detected in MDA-MB-231 and MDA435/LCC6 cells (similar to the SLCO1B1 expression pattern) and the level of expression is three fold greater in MDA-MB-231 cells compared to MDA435/LCC6 cells (Figure 2B). We also explored OATP2B1 expression and although the MDCKII-2B1 cells (used as a positive control) shows clear protein expression, we cannot detect OATP2B1 in the immortalized breast epithelial or breast cancer cells (Figure

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2C). Negligible gene expression is observed for SLCO2B1 in MCF7 and MDA435/LCC6 which justifies the lack of OATP2B1 protein expression in these cell systems. Similar to OATP1A2, OATP3A1 can be detected in all cell lines. The expression of OATP3A1 is significantly greater in the breast cancer cell lines, MCF7 ($p<0.01$) and MDA435/LCC6 ($p<0.05$) cells compared to MCF10A. In the hormone dependent breast cancer cell line, MCF7, the expression is nearly three fold higher than in the MCF10A (Figure 2D). Although expression of SLCO3A1 gene is found to be significantly higher in MDA435/LCC6 cells, expression of OATP3A1 protein does not show significant differences between MDA435/LCC6 and MCF10A cells. Contrary to SLCO4A1 expression observed ubiquitously in all the breast cell lines, we can only detect OATP4A1 expression in MDA435/LCC6 cell lines. The afore minimal discordances observed between gene and protein expression could be due to higher sensitivity of detection of the qPCR method as compared to immunoblotting. Overall, all of the OATP transporters investigated in this study show exclusive or higher expression in breast cancer cells compared to the immortalized breast epithelial cells. Over expression of these transporters in breast cancer cells (particularly in the hormone dependent MCF7 cells) could lead to higher uptake of E3S by these cell lines.

Characterization of E3S uptake by the breast epithelial cells:

C. Specificity of OATP mediated E3S uptake: In order to determine the specificity of carrier mediated E3S cellular uptake, we performed time course experiments with transport buffer containing 20nM E3S, in the presence or absence of the transport inhibitor, 100μM-BSP. Significant differences between the total and non-specific uptake indicates the presence of a specific carrier mediated process that contributes towards E3S intracellular accumulation. The

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gene and protein expression of OATPs detected in the different cell systems strongly suggest that the OATPs have a major contribution towards the specific cellular uptake of E3S. However, as E3S is a common substrate for other uptake transporters as well (e.g. OAT3 and OAT4) (Ninomiya, et al., 2006;Ugele, et al., 2003;Asif, et al., 2005), and BSP is also an inhibitor of these transporters, there is a possibility that specific cellular uptake of E3S is contributed by OATPs along with other uptake transporters. No specific carrier mediated E3S uptake can be detected in the immortalized breast epithelial cells, MCF10A (Figure 3A) suggesting that although some OATP isoforms are expressed in the MCF10A cells, they are not functionally involved in E3S uptake. Contrary to this, specific carrier mediated E3S uptake is observed in all the breast cancer cells. This suggests that specificity of OATP mediate E3S uptake is present in the cancer cells. It is further observed that specific E3S uptake is the highest in the hormone dependent breast cancer cells, MCF7 (Figure 3B, 3C, 3D, 3E).

D. **Comparative uptake of E3S in breast epithelial cells at steady state:** After establishing the specificity of OATP mediated E3S transport, we determined the total E3S uptake in each of the cell systems by measuring intracellular concentration of tritium at steady state. Upon comparison with the immortalized breast epithelial cells (MCF10A) we observe significantly higher E3S uptake in the hormone dependent MCF7 and the hormone independent MDA435/LCC6 cells. Among the breast cancer cell lines, the hormone dependent MCF7 cells have the highest E3S uptake (Figure 3F).

E. Transport kinetics of E3S in hormone dependent and hormone independent cancer cells: In order to determine the transport kinetics of E3S uptake in each of the cell systems depicting specific OATP mediated E3S uptake, we conducted transport experiments by incubating the cells with different E3S concentrations. Determination of the transport kinetics allows further

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evaluation of the efficiency of E3S transport (expressed as a ratio of V_{max}/K_m) in each cell line (as previously described (Letschert, et al., 2004)). The K_m of 6.5±1.9μM, for E3S uptake is the lowest in the hormone dependent MCF7 cells as compared to the hormone independent breast cancer cells indicating that the transporters in MCF7 cells have the highest affinity towards E3S. This value is similar to the one previously reported for E3S uptake in MCF7 cells (Nozawa, et al., 2005). The V_{max} value is 66 +/- 8.5 pmol/mg protein/min which is also similar to previously reported values (Figure 4A). Among the hormone independent breast cancer cells, the K_m values are 68.3 +/- 7.8 μM, 203.8 +/- 41μM, 46.8 +/- 1.7μM for the MDA435/LCC6, MDA-MB-468 and MDA-MB-231 cells, respectively. The V_{max} values for E3S uptake in the same cells are 57.6 +/- 4.5 pmol/mg protein/min, 131.4 +/- 15.92 pmol/mg protein/min, and 27.3 +/- 6.2 pmol/mg protein/min, respectively (Figure 4B, 4C, 4D). All cell lines show "one site saturation binding" kinetics.

The efficiency of transport is more than ten fold greater in the hormone dependent breast cancer cells, MCF7, as compared to the hormone independent cells. No significant difference was observed among the various hormone independent cells. (Figure 5) [Table 1]

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DISCUSSION:

OATPs are expressed in various epithelial tissues and cancers (Obaidat, et al., 2011) and are known to be involved in the transport of endogenous hormones, their conjugates and several anticancer drugs (Hagenbuch and Meier, 2003;Hagenbuch and Gui, 2008;Smith, et al., 2007;van de Steeg, et al., 2009). OATPs have been reported to be upregulated (Hamada, et al., 2008;Yang, et al., 2011;Lee, et al., 2008) or down regulated(Zollner, et al., 2005) in various cancers and as a result could affect cancer progression by increasing the supply of hormones or decreasing the intra cellular concentration of anticancer agents. Although expression of OATPs has been investigated in prostate, colon, liver and gastrointestinal cancers, not much is known about their expression and function in breast cancers.

In hormone dependent cancers (e.g. prostate cancer, thyroid cancer and breast cancer), OATP expression is many fold greater than in normal tissues. Miki *et al*. and Meyer Zu Schwabedissen *et al.* reported 10 fold greater expression of OATP1A2 in breast cancer tissues as compared to surrounding normal tissues (Miki, et al., 2006;Meyer Zu Schwabedissen, et al., 2008). Pizzagalli *et al.* detected the localization of the OATP 2B1 in the luminal epithelium in invasive ductal carcinoma tissues while it was primarily confined to the myoepithelial cells in the surrounding normal tissues of the breast (Pizzagalli, et al., 2003). In prostate cancers, another hormone dependent cancer which is dependent on androgens for proliferation, OATP1B3 and OATP2B1 are over expressed (Hamada, et al., 2008;Yang, et al., 2011). Wright *et al.* further demonstrated high expression of OATP1B3 (3.6 fold) and OATP2B1 (5.5 fold) in metastatic lesions from men with castration-resistant prostate cancers as compared to untreated prostate cancers (Wright, et al., 2011). Again in colon cancer samples, OATP1B3 expression was detected to be 100 fold higher compared to adjacent normal colon tissues(Lee, et al., 2008). SLCO2B1 expression was

also reported to be significantly higher in thyroid cancers than in normal tissues(Pressler, et al., 2011). All these observations strongly suggest that various members of the OATP family have a specific role in the progression of hormone dependent cancers wherein they facilitate the uptake of various hormones and their conjugates. Therefore, these transporters could potentially serve as biomarkers of cancer progression and/or as a new therapeutic target to prevent proliferation of hormone dependent cancers. To further our understanding of the role of OATPs in E3S uptake in breast cancers, we analysed the expression and function of seven OATP isoforms that recognize E3S as a substrate in normal breast epithelial cells and hormone dependent and independent breast cancer cells.

Transcripts for six OATP transporters are detected in the breast cancer cell lines. SLCO1A2 is expressed in all of the cancer cell systems, i.e. MCF7, MDA435/LCC6, MDA-MB-468 and MDA-MB-231 cells while SLCO2B1 is present in negligible quantities in MCF7 and MDA435/LCC6 cells. Our observations are similar to those reported by Miki *et al* (Miki, et al., 2006;Meyer Zu Schwabedissen, et al., 2008) and Pizzagalli *et al* (Pizzagalli, et al., 2003) for OATP1A2 and OATP2B1 expression respectively. Again in accordance with the results of Pizzagalli *et al*. (Pizzagalli, et al., 2003) and Wlcek *et al*. (Wlcek, et al., 2008), we also observe SLCO3A1 and SLCO4A1 expression in all the cell lines investigated. We also detect SLCO1B1 and SLCO1B3 expression in MDA-MB-231 cells. Comparison of gene expression patterns between hormone dependent and independent breast cancer cells reflects expression of most OATPs in hormone dependent MCF7 cells. Among the seven OATP isoforms investigated, we detect expression of SLCO1A2, SLCO2B1, SLCO3A1 and SLCO4A1 in the MCF7 cells. This high expression could explain in part the high intracellular concentration of E3S in MCF7 cells.

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Since mRNA expression differences are not always reflected at the protein level, we investigated the protein expression of six OATP isoforms (except OATP1C1 as it showed no transcript expression) among the immortalized breast epithelial and cancer cell lines to verify the expression pattern of the OATP transporters encoded by the respective SLCO genes that we examined. OATP1A2, reported to be expressed in hormone dependent breast cancer cells T47D (Meyer Zu Schwabedissen, et al., 2008), shows protein expression in all the cell systems. The level of expression is significantly higher in the breast cancer cells compared to the MCF10A. Among the breast cancer cell systems, highest expression is observed in the hormone dependent MCF7 cells. OATP3A1 is the other transporter that is expressed in all the cell systems. However, unlike SLCO3A1 gene expression pattern, where we observe highest expression in the immortalized breast epithelial cells, MCF10A, OATP3A1 protein expression is highest in the hormone dependent MCF7 cells. MCF7 and MDA-MB-468 cells show significantly higher OATP3A1 protein expression than MCF10A cells. Similar to the SLCO1B1 gene expression, OATP1B1 protein is expressed in MDA-MB-231 and MDA435/LCC6 cells. Although very low SLCO2B1 gene expression is detected in some cell lines, we can not detect OATP2B1 protein expression in any cell system. Discordance between gene and protein expression is primarily observed in OATP4A1 expression. Although, SLCO4A1 gene expression is observed in all the cell systems, OATP4A1 protein is only expressed in MDA435/LCC6 cells. It is not unlikely that gene expression does not reflect into protein expression (Wang, et al., 2011). Overall, we observe significantly higher protein expression of OATPs in cancer cells, compared to the normal cells. It also appears that the OATP mediated E3S uptake in hormone dependent breast cancer cells is primarily mediated by OATP1A2 and OATP3A1. Since the expression of several OATP isoforms is observed in a single breast cancer cell line system, the total E3S uptake is

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possibly a summative contribution from all the OATPs that are expressed in the particular cell system.

To further clarify the functional role of OATPs in E3S transport in the cell systems, we conducted transport experiments, measuring E3S uptake in the presence or absence of the specific OATP transport inhibitor, BSP. While specific carrier mediated E3S uptake is demonstrated in all the breast cancer cell line systems investigated, no specific carrier mediated E3S uptake is observed in the immortalized breast epithelial cells, MCF10A.This suggests that the specificity of OATP mediated E3S uptake is mainly restricted to breast cancer cells. As we observe specific uptake in both hormone dependent and independent cancer cells, it suggests that the specificity of OATP mediated E3S uptake is independent of estrogen receptor expression. We also compared total E3S uptake among the various breast cancer cells at steady state and demonstrate that the highest E3S uptake occurs in the hormone dependent breast cancer cells, MCF7. Furthermore, the expression pattern of the transporters in the various cell lines supports their functional contribution towards E3S uptake. For example, we observe highest expression of OATP isoforms in the MCF7 cells where we have highest E3S uptake at steady state.Taken together these results may suggest that the high tumor concentrations of E3S observed in hormone dependent breast cancers in post menopausal patients could be due to an OATP mediated uptake process.

After determining the specific role of the OATPs in contributing towards the intracellular concentration of E3S in the breast cancer cells, we further compared the efficiency of E3S transport among the different breast cancer cells. We observed ten times higher transport efficiency (estimated by V_{max}/K_m) in the hormone dependent breast cancer cells, MCF7, compared to the hormone independent cells. This implies that although specific carrier mediated

E3S uptake is observed in all the breast cancer cells, the efficiency of E3S transport is highest in the hormone dependent breast cancer cells.

In summary, we observe i) significantly higher OATP gene and protein expression in breast cancer cells compared to immortalized breast epithelial cells, ii) OATP is primarily functional in breast cancer cells, and iii) OATP mediated E3S transport has greater efficiency in hormone dependent breast cancer cells. Taken together, these data suggest that OATPs could be a novel therapeutic target for hormone dependent breast cancers detected in post menopausal women. Further investigation characterizing the expression of these transporters in human breast tumor tissues are needed. Current limitations of endocrine therapies are acquired or de novo resistance caused by a loss or mutation of estrogen receptors. By targeting OATPs, we could potentially treat primary hormone dependent tumors which have lost estrogen receptor expression over the years. Further research is required to better characterize the expression of these transporters in primary hormone dependent tumours which have lost estrogen receptor expression and in turn have acquired resistance to endocrine therapy.

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AUTHORSHIP CONTRIBUTIONS:

Participated in research design: NB, CA, RB

Conducted experiments: NB

Contributed new reagents or analytical tools: RB, CA

Performed data analysis: NB, RB

Wrote or contributed to the writing of the manuscript: NB, CA, RB

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(b) This work was presented at the following meeting:

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(c) Dr. Reina Bendayan, Department of Pharmaceutical Sciences, Leslie Dan Faculty of Pharmacy, University of Toronto, 144 College Street, Toronto, ON M5S 3M2, Tel: 416-978-6979, FAX: 416-978-8511, E-mail: r.bendayan@utoronto

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FIGURE LEGENDS:

Figure 1: Relative **mRNA** expression of OATP transporters in MCF10A, MCF7, MDA435/LCC6 (MDA435), MDA-MB-468 (MDA468) and MDA-MB-231(MDA231) cells. Relative mRNA expression of **A**. SLCO1A2, **B**. SLCO1B1, **C**. SLCO1B3, **D.** SLCO2B1, **E.** SLCO3A1, **F.** SLCO4A1. Results are shown as mean relative mRNA expression +/- SEM from three separate experiments using the house keeping gene (18S). As described in Materials and Methods section, one-way analysis of variance for test of repeated measures (ANOVA) is used to determine statistical significance. Bars labelled with different letters (a, b, c or d) indicate a significant difference in expression. **A.** a vs b, $p<0.001$; **B.** a vs b, $p<0.001$; **C.** a vs b, $p<0.001$; **D.** a vs b, $p<0.001$, **E.** a vs b, a vs c, b vs c, $p<0.001$; **F.** a vs b, a vs c, a vs d, b vs d, c vs d $p<0.001$, b vs c, $p<0.05$.

Figure 2: Immunoblot and densitometric analysis of OATP transporters in breast epithelial and breast cancer cells. Protein expression of **A.** OATP1A2, **B.** OATP1B1, **C.** OATP2B1, **D.** OATP3A1 and **E.** OATP4A1 is investigated in breast cancer cells **1 (**MDA-MB-231), **2 (**MDA-MB-468), **3** (MDA435/LCC6), **4 (**MCF7) and normal breast cells **5 (**MCF10A). To determine the specificity of the respective antibody used, we included a positive control cell line over expressing the transporter of interest: (**6**) HEK293/OATP1A2, HEK293/OATP1B1, MDCKII/OATP2B1, HEK293/OATP3A1 and HEK293/OATP4A1 in each of the blots. Results of the densitometric analysis are expressed as mean ± SD of three separate experiments. Bars labelled with different letters (a, b or c) indicate a significant difference in expression. $p<0.05$ is considered to be statistically significant.

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Figure 3: Time course of [^{3}H] E3S uptake in immortalized breast epithelial and breast cancer cells (hormone dependent and hormone independent). Total uptake (closed circles) of E3S in all the cells is evaluated over 30 min at pH 7.4 and 37˚C. The non- specific (open circles) uptake is calculated by determining uptake in the presence of a transport inhibitor (BSP 100μM) as described in the Materials and Methods section. **A**. MCF10A, **B**. MCF7, **C.** MDA435/LCC6, **D.** MDA-MB-468 and **E.** MDA-MB-231 cells. Statistical significance was assessed by two-tailed Student's *t* test for unpaired experimental values. *p<0.05 is considered to be statistically significant. **F.** Total E3S uptake at steady state is measured and significant difference is observed among the cell systems. Statistical significance was calculated by one-way analysis of variance applying the test of repeated measures (ANOVA). Bars labelled with different letters (a, b, c or d) indicate a significant difference in E3S accumulation. a vs b, p<0.001; a vs c, p<0.01; b vs c, p<0.05.

Figure 4: Kinetics of E3S uptake by breast epithelial cells and breast cancer cells. Rates of uptake of [^{3}H]E3S by **A.** MCF7 cells at concentrations ranging from 5nM to 100 μM, **B.** MDA435/LCC6 cells at concentrations ranging from 5nM to 150 μM, **C.** MDA-MB-468 cells at concentrations ranging from 5nM to 1000 μM, and in **D.** MDA-MB-231 cells at concentrations ranging from 5nM to 150 μM were measured at 2 min, pH 7.4, and 37°C. The saturable (filled triangle) and nonsaturable (empty circle) components of the total (filled circle) uptake rate are determined by fitting the data into Equation 1, the Michaelis-Menten equation, as detailed in *Materials and Methods*. Saturable E3S uptake by all the above mentioned cells are fitted into a "One site-Fit total and nonspecific binding" kinetics model. The kinetic parameters K_M and V_{max} are estimated by least-square nonlinear regression analysis using Prism 5 software. The insets in

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each diagram represent specific early time points. Data represent the mean ± S.E.M. for $n = 3$ independent experiments.

Figure 5: Transport efficiency of E3S uptake by MCF7, MDA435/LLC6, MDA-MB-468 and MDA-MB-231. Transport efficiency for E3S uptake is calculated using the ratio of V_{max}/K_m as observed in each of the cells. As described under the Materials and Methods section, one-way analysis of variance for test of repeated measures (ANOVA) is used to compare the data. We observe significantly higher transport efficiency in MCF7 cells as compared to all other hormone independent breast cancer cells. *** $p<0.001$

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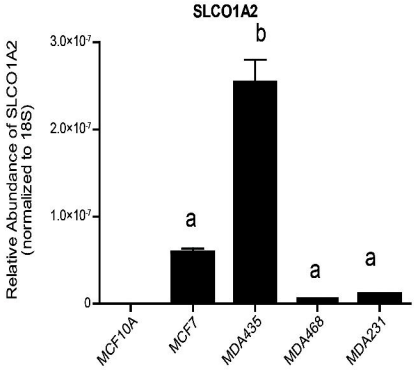
Table 1: E3S transport efficiency in breast epithelial and breast cancer cells

Cell system	V_{max} (pmol/mg protein/min)	K_m (μM)	Transport efficiency (V_{max}/ K_m) (μl/mg protein/min)	% transport efficiency
MCF7	66 ± 8.5	6.5 ± 1.8	10.21	100
MDA435/LCC6	57.6 ± 4.5	68.3 ± 7.8	0.84*	8.3
MDA-MB-468	131.4 ± 15.9	203.8 ± 41	0.6*	5.9
MDA-MB-231	27.31 ± 6.2	46.9 ± 1.7	0.58*	5.7

*$p<0.05$

Figure 1

A


SLCO1A2
Relative Abundance of SLCO1A2 (normalized to 18S)
3.0×10-7
2.0×10-7
1.0×10-7
0
a
b
a
a
MCF10A
MCF7
MDA435
MDA468
MDA231

B

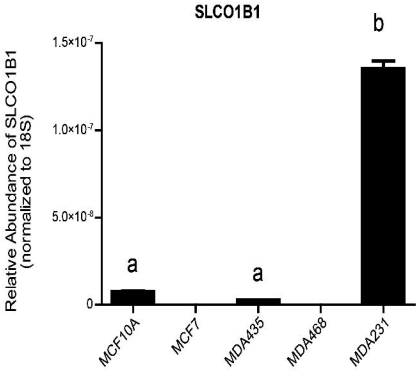
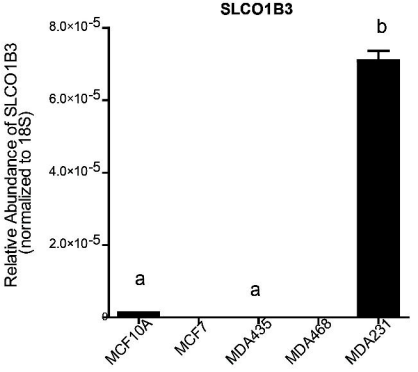

SLCO1B1
Relative Abundance of SLCO1B1 (normalized to 18S)
1.5×10-7
1.0×10-7
5.0×10-8
0
a
a
b
MCF10A
MCF7
MDA435
MDA468
MDA231

Figure 1

C


SLCO1B3
Relative Abundance of SLCO1B3 (normalized to 18S)
8.0×10-5
6.0×10-5
4.0×10-5
2.0×10-5
0
a
a
b
MCF10A
MCF7
MDA435
MDA468
MDA231

D

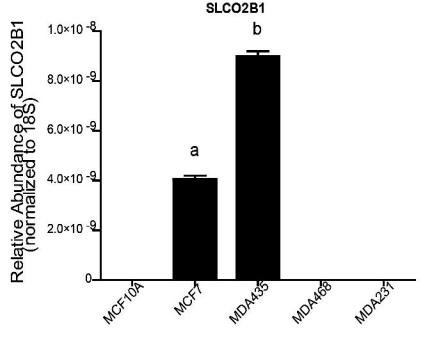
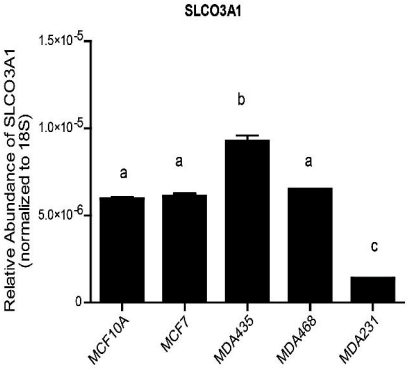

SLCO2B1
Relative Abundance of SLCO2B1 (normalized to 18S)
1.0×10-8
8.0×10-9
6.0×10-9
4.0×10-9
2.0×10-9
0
a
b
MCF10A
MCF7
MDA435
MDA468
MDA231

Figure 1

E


SLCO3A1
Relative Abundance of SLCO3A1 (normalized to 18S)
1.5×10-5
1.0×10-5
5.0×10-6
0
a
a
b
a
c
MCF10A
MCF7
MDA435
MDA468
MDA231

F

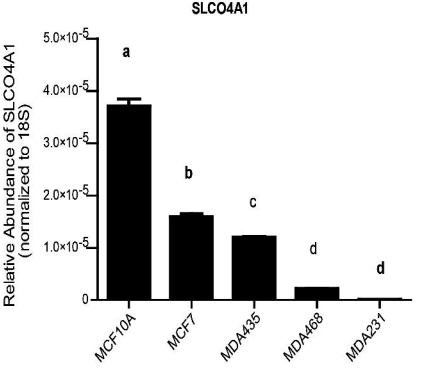

SLCO4A1
Relative Abundance of SLCO4A1 (normalized to 18S)
5.0×10-5
4.0×10-5
3.0×10-5
2.0×10-5
1.0×10-5
0
a
b
c
d
d
MCF10A
MCF7
MDA435
MDA468
MDA231

Figure 2

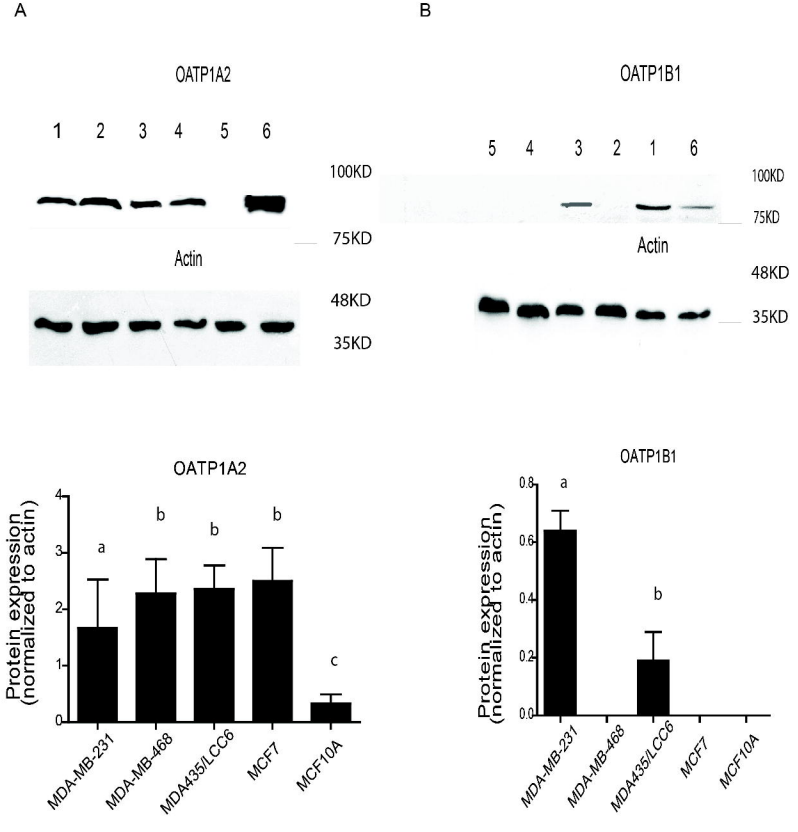

A
OATP1A2
1 2 3 4 5 6
100KD
75KD
Actin
48KD
35KD
B
OATP1B1
5 4 3 2 1 6
100KD
75KD
Actin
48KD
35KD
OATP1A2
Protein expression (normalized to actin)
a
b
b
b
c
MDA-MB-231
MDA-MB-468
MDA435/LCC6
MCF7
MCF10A
OATP1B1
Protein expression (normalized to actin)
a
b
MDA-MB-231
MDA-MB-468
MDA435/LCC6
MCF7
MCF10A

Figure 2

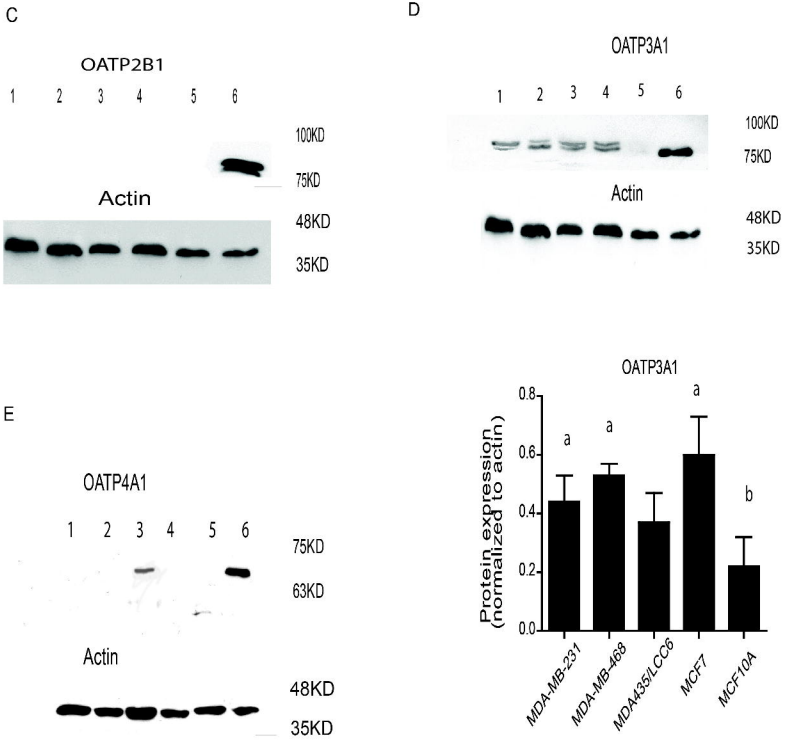

C
OATP2B1
1 2 3 4 5 6
100KD
75KD
Actin
48KD
35KD
D
OATP3A1
1 2 3 4 5 6
100KD
75KD
Actin
48KD
35KD
E
OATP4A1
1 2 3 4 5 6
75KD
63KD
Actin
48KD
35KD
OATP3A1
Protein expression (normalized to actin)
0.8
0.6
0.4
0.2
0.0
a
a
a
b
MDA-MB-231
MDA-MB-468
MDA435/LCC6
MCF7
MCF10A

Figure 3

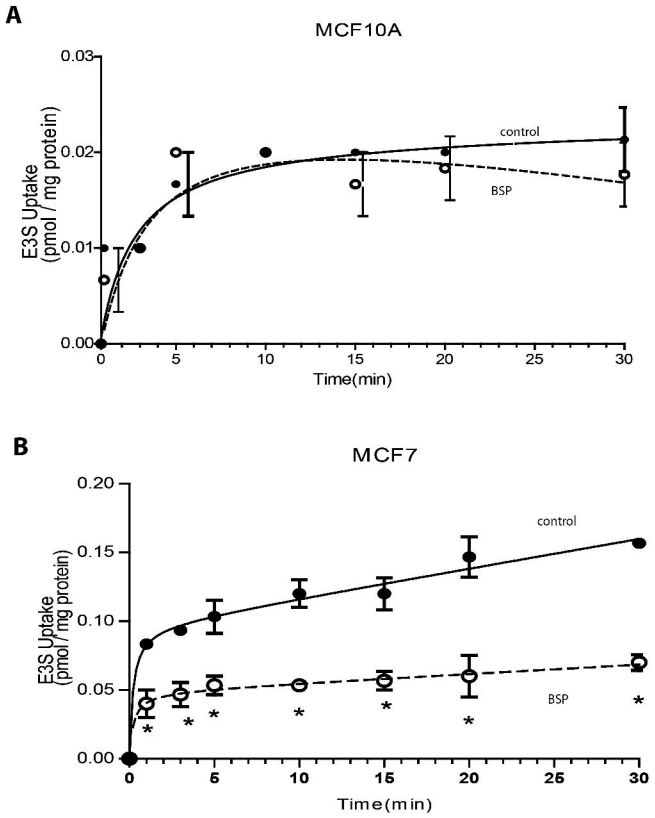

A
MCF10A
E3S Uptake
(pmol / mg protein)
0.03
0.02
0.01
0.00
0
5
10
15
20
25
30
Time(min)
control
BSP
B
MCF7
E3S Uptake
(pmol / mg protein)
0.20
0.15
0.10
0.05
0.00
0
5
10
15
20
25
30
Time(min)
control
BSP
*

Figure 3

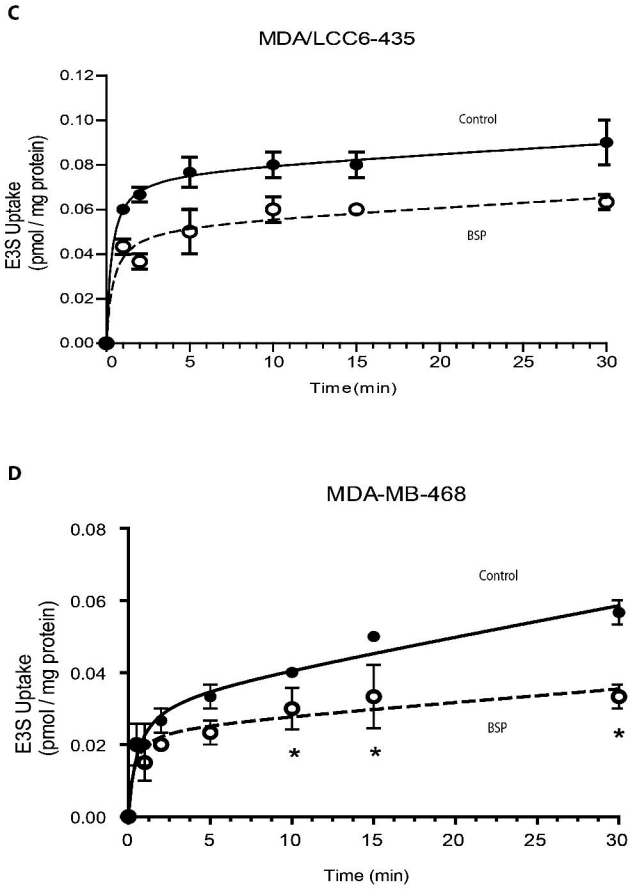
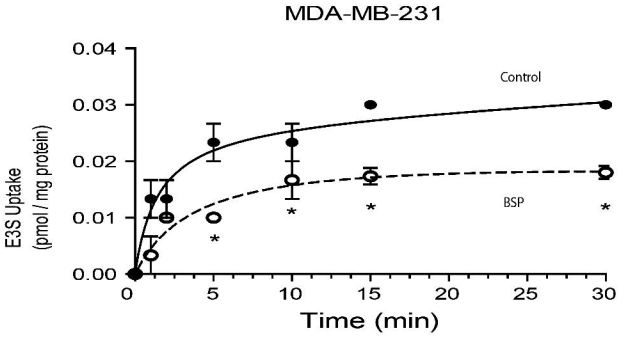

C
MDA/LCC6-435
E3S Uptake (pmol / mg protein)
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Control
BSP
0
5
10
15
20
25
30
Time (min)
D
MDA-MB-468
E3S Uptake (pmol / mg protein)
0.08
0.06
0.04
0.02
0.00
Control
BSP
*
*
*
0
5
10
15
20
25
30
Time (min)

Figure 3

E


MDA-MB-231
Control
BSP
E3S Uptake
(pmol / mg protein)
0.04
0.03
0.02
0.01
0.00
0
5
10
15
20
25
30
Time (min)
*

F

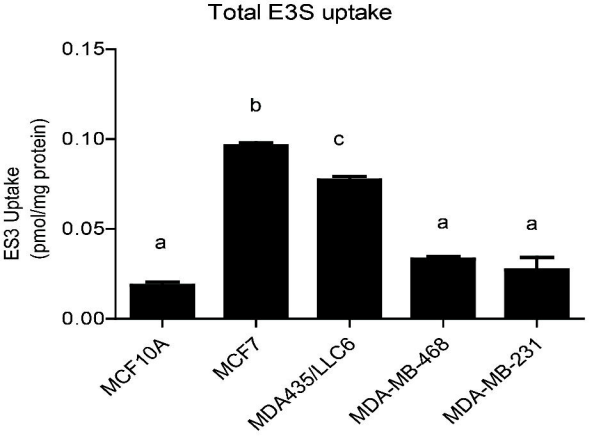
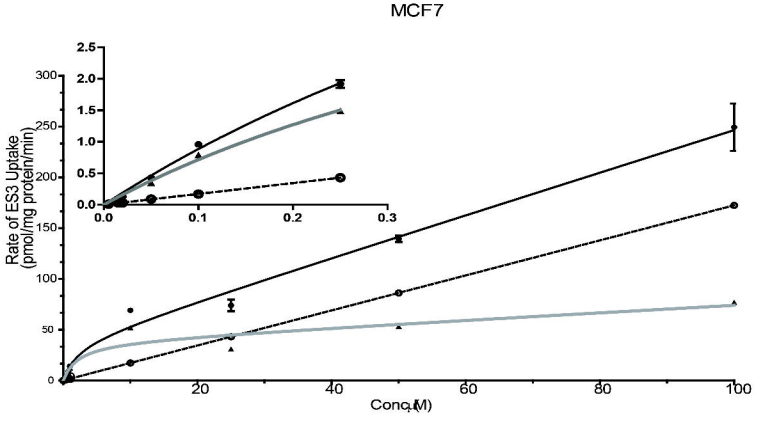

Total E3S uptake
ES3 Uptake
(pmol/mg protein)
0.15
0.10
0.05
0.00
a
b
c
a
a
MCF10A
MCF7
MDA435/LLC6
MDA-MB-468
MDA-MB-231

Figure 4

A


MCF7
Rate of E3S Uptake (pmol/mg protein/min)
Conc (μM)

B

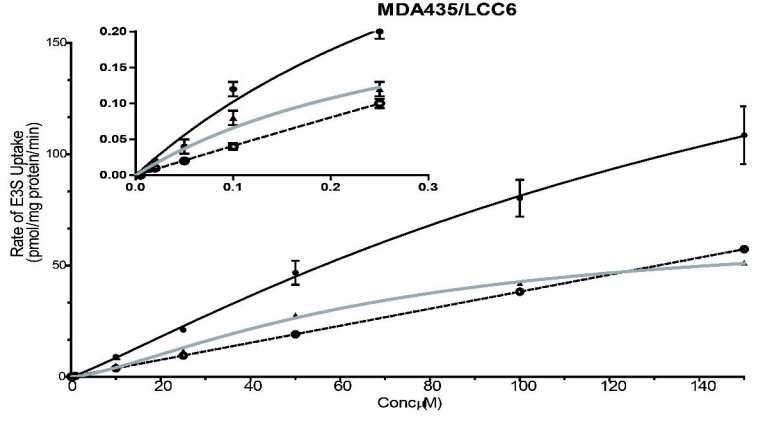

MDA435/LCC6
Rate of E3S Uptake (pmol/mg protein/min)
Conc (μM)

Figure 4

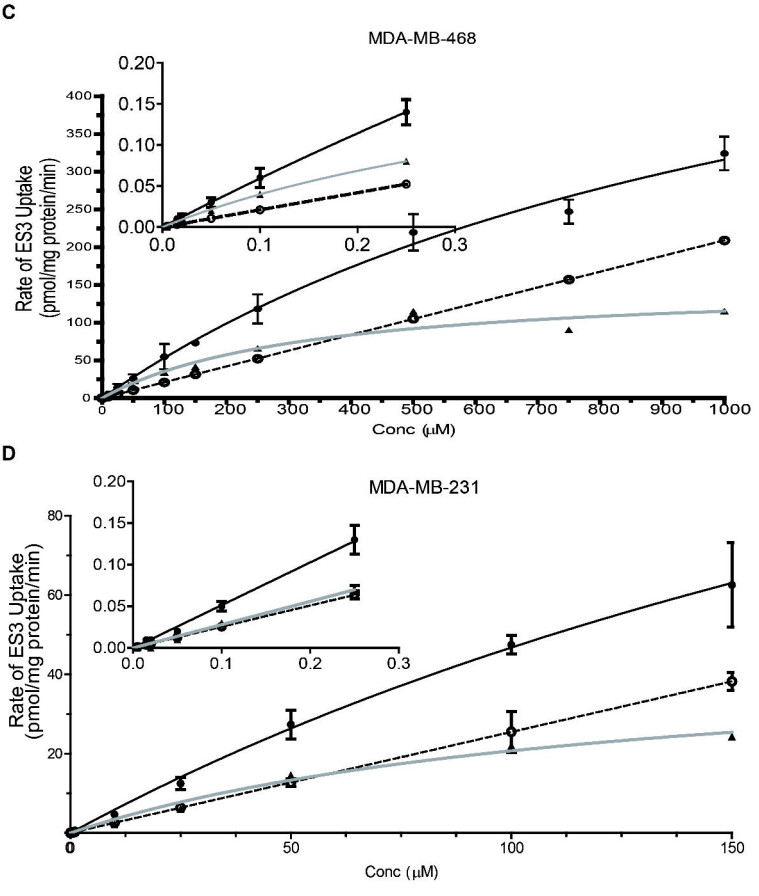
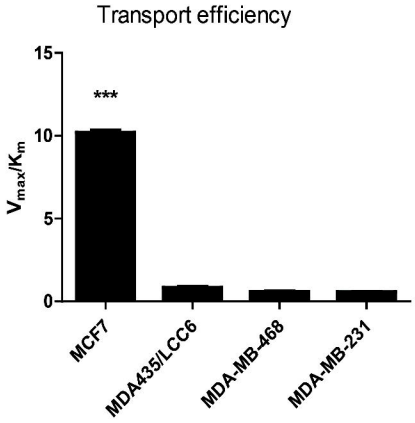

C
MDA-MB-468
Rate of ES3 Uptake (pmol/mg protein/min)
Conc (μM)
D
MDA-MB-231
Rate of ES3 Uptake (pmol/mg protein/min)
Conc (μM)

Figure 5


Transport efficiency
15
10
5
0
***
V_{max}/K_m
MCF7
MDA435/LCC6
MDA-MB-468
MDA-MB-231