Differential Role of Organic Anion-Transporting Polypeptides in Estrone-3-Sulphate Uptake by Breast Epithelial Cells and Breast Cancer Cells

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ABSTRACT

The purpose of this study was to investigate the differential expression and function of organic anion-transporting polypeptides (OATPs) in breast epithelial and breast cancer cells. Estrone-3-sulfate (E3S), a substrate for 7 of 11 OATPs, is a predominant source of tumor estrogen in postmenopausal, hormone-dependent patients with breast cancer. Overexpression of certain OATPs (e.g., OATP1A2) reported in breast tumor tissues compared with surrounding normal tissues could contribute toward two to three times higher tumoral E3S concentration. Little is known about 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 (OATP1A2, OATP1B1, OATP1B3, OATP1C1, OATP2B1, OATP3A1, and OATP4A1) in immortalized breast epithelial cells (MCF10A), hormone-dependent breast cancer cells (MCF7), and hormone-independent breast cancer cells (MDA/LCC6-435, MDA-MB-231, and MDA-MB-468) by quantitative polymerase chain reaction and immunoblotting, respectively. Expression of solute carrier superfamily encoding for OATPs (SLCO) 1A2, 1B1, 1B3, 2B1, and 3A1 is exclusive, similar, or significantly higher in cancer cells compared with MCF10A cells. Protein expression of OATPs is found to be either exclusive or higher in cancer cells compared with MCF10A cells. Specificity of OATP-mediated E3S uptake is observed only in cancer cells, with the highest total uptake in MCF7 cells. Transport kinetics of E3S uptake demonstrates transport efficiency that is 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 postmenopausal patients, where the major source of tumor estrogen is E3S.

Introduction

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

Unlike lipophilic estrone and estradiol, which readily diffuse...
through plasma membrane, E3S, because of its hydrophilicity and net negative charge, can not readily cross the plasma membrane by diffusion (Verheugen et al., 1984; Tan et al., 1999; Purohit et al., 2011). 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 (Kis et al., 2010a; König, 2011). This suggests that OATPs are involved in E3S uptake by breast cancer cells. Furthermore, Nozawa et al. (2004, 2005) showed that inhibition of an E3S transporter results in the suppression of hormone-dependent breast cancer cell proliferation (MCF7 and T47D cells). These data suggest a potential role of OATPs in hormone-dependent breast cancer cell proliferation by facilitating E3S cellular uptake. Maeda et al. (2010) showed the role of OATP1B3 in E3S uptake in two subclones of MCF7 cells. Among the 11 human OATP isoforms identified (Hagenbuch and Meier, 2004), seven (OATP1A2, OATP1B1, OATP1B3, OATP1C1, OATP2B1, OATP3A1, and OATP4A1) were reported to recognize E3S as a substrate (Obaidat et al., 2012). Among these, 10-fold higher expression of OATP1A2 was observed in breast cancer tissues compared with their nonmalignant counterparts (Miki et al., 2006; Meyer zu Schwabedissen et al., 2008). Pizzagalli et al. (2003) reported OATP2B1 expression in luminal epithelium in invasive ductal carcinoma tissues and myoepithelial cells in the surrounding normal tissues and also demonstrated the functional role of OATP2B1 in the transport of E3S in these tissues. Recently, Kindla et al. (2011) compared the expression and localization of OATP2B1, OATP3A1, and OATP5A1 in paired samples of normal breast tissue and breast cancer tissue and reported that whereas 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. Despite all of 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 was 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 cells, and hormone-independent breast cancer cells. Although previous work reported OATP expression in hormone-dependent breast cancer cells (MCF7, T47D, and ZR75) (Nozawa et al., 2004, 2005; Maeda et al., 2010), for the first time, we compared 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 overexpressing breast cancer cells (MDA-MB-468) (Armstrong et al., 1994), and triple negative breast cancer cells (MDA-MB-231) (Gründker 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 postmenopausal 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.

**Materials and Methods**

**Cell Culture.** MCF7, MDA-MB-468, and MDA-MB-231 cells were purchased from the American Type Culture Collection (Manassas, VA). MDA435/LCC6 and MCF10A cells were kindly donated by Dr. Robert Clarke (Georgetown University School of Medicine, Washington, DC) and Dr. Fei-Fei Liu (University Health Network, Toronto, Canada), respectively. Madin-Darby canine kidney II (MDCKII) cells stably expressing OATP2B1 (MDCKII/OATP2B1) were 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) were kindly donated by Dr. Martin F. Fromm (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 were grown in Dulbecco's modified Eagle's medium. The MDCKII/OATP2B1 medium was supplemented with 1% l-glutamine and 400 μg/ml hygromycin B (Sigma-Aldrich, St. Louis, MO) to provide selection pressure for stably transfected cells (Kis et al., 2010b). HEK/OATP3A1 and HEK/OATP4A1 medium was supplemented with 800 μg/ml G-418 as described previously (König et al., 2011). Protein expression of OATP2B1 was induced by incubating the cells with 2.5 mM sodium butyrate (Sigma-Aldrich) 24 h before cell pellet collection. MDA-MB-468 cells were grown in RPMI medium (along with 10% fetal bovine serum) and transfected cells (Kis et al., 2010b). HEK/OATP3A1 and HEK/OATP4A1 medium was supplemented 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 × 10³ cells/cm².

**Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction.** Total RNA was extracted from the cells (from three different passages) by using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The concentration (absorbance at 260 nm) and purity (absorbance 260 nm/absorbance 280 nm ratio) of RNA samples was assessed by using a Beckman Coulter (Mississauga, Ontario, Canada) DU Series 700 UV/Vis Scanning Spectrophotometer. Isolated total RNA was subjected to DNase I digestion (0.1 U/ml) according to the manufacturer's instructions to remove genomic DNA. The reverse transcription on DNase-treated total RNA (2 μg) (in a final reaction volume of 40 μl) was performed by using an ABI high-capacity reverse transcription kit according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). The samples were then incubated for 10 min at 25°C, followed by 120 min at 37°C and finally 5 min at 85°C by using a Mastercycler EP Realplex 2S thermal cycler (Eppendorf Canada, Mississauga, Ontario, Canada).

The expression of the SLCO genes encoding for the OATP transporters was analyzed by qPCR on a Mastercycler EP Realplex 2S using TaqMan fluorescence detection. We purchased TaqMan Gene Expression Assays (Applied Biosystems, Warrington, UK) for seven human OATPs. To evaluate an appropriate reference gene, 18S was selected as the appropriate housekeeping gene. Multiplex quantitative real-time RT-PCR was performed in an amplification mixture with a volume of 20 μl. The target gene amplification mixture contained 10 μl of 2× TaqMan Universal PCR Master Mix, 1 μl of the appropriate gene expression assay, 1 μl of TaqMan endogenous.
control (18S), and 50 ng of template cDNA diluted in 5 μl of nuclease-
free water. Thermal cycling conditions comprised 2 min at 50°C, 2 min at 95°C followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Fluorescence generation caused by TaqMan probe cleavage by the 5’ → 3’ exonuclease activity of the DNA polymerase was measured with the Mastercycler EP Realplex 2S. All samples were amplified in triplicate. Results were imported into Microsoft (Redmond, WA) Excel for further analysis. Quantitative real-time RT-PCR was performed with the following prevalidated TaqMan Gene Expression Assays (Applied Biosystems) containing intron-
spanning primers: OATP1A2, Hs00245360_m1; OATP2B1, Hs00200670_m1; OATP1B1, Hs00472357_m1; OATP1B3, Hs00251986_m1; OATP1C1, Hs00219714_m1; OATP2A1, Hs00202518_m1; OATP4A1, Hs00245683_m1, and 18S (4310893E; Wickl et al., 2008).

**Transient Transfection of OAT1B1, OATP1B3, and OATP1A2 cDNAs.** The pEF/Amp-OAT1B1, -OATP1B3, and -OATP1A2 vectors encoding the full-length human organic anion-transporting polypeptides (OATP1B1, OATP1B3, and OATP1A2) were kindly provided by Dr. Richard Kim (University of Western Ontario, London, Canada). The generation of recombinant constructs has been published elsewhere in detail (Tirona et al., 2003; Sandhu et al., 2005). The cDNAs supplied on blotting paper were eluted in Tris-EDTA buffer (10 mM Tris and 1 mM EDTA, pH 8.0) and transformed into XL1blue competent cells according to the supplier’s protocol (Sigma-Aldrich), and the cells were incubated overnight at 37°C on Luria-Bertani-Amp plates. The individual bacteria colony was propagated in Luria Bertani-Amp liquid media overnight at 37°C, and the recombinant plasmids were then isolated using GenElute HP Plasmid Midprep Kit (Sigma-Aldrich). Purified plasmids were then transfected into HEK293 cells by using Lipo-
fectamine following the protocol supplied with the reagent (Invitrogen). After 48 h of transfection, whole cell lysates were prepared, and protein overexpression was verified by immunoblotting using antibodies specific to each transporter.

**Immunoblot Analysis.** Immunoblotting was performed as described previously (Kis et al., 2010b; Ronaldson et al., 2010) with minor modifications. Cells from three different passages were harvested, and lysates were prepared as described previously. After determining the protein content by using an assay kit from Bio-Rad Laboratories (Hercules, CA) and bovine serum albumin as the standard, 10 μg was loaded for the overexpressing cells (both stably and transiently transfected cells) and 50 μg was loaded for each of the breast cell lines (MCF10A, MCF7, MDA435/LCC6, MDA-MB-468, and MDA-MB-231). Samples were incubated with Laemmli buffer (Kis et al., 2010b) and 10% β-mercaptoethanol for 10 min at 37°C. Proteins were separated on a 7% SDS polyacrylamide gel, and then transferred onto polyvinylidene fluoride transfer membranes. The blots were then blocked in 5% skimmed milk for 2 h and incubated in primary antibody overnight at 4°C. Primary rabbit polyclonal antibodies for anti-OATP1A2, anti-OATP2B1, anti-OATP2A1, and anti-OATP4A1 (Sigma-Aldrich) were used in a 1:1000 dilution. The primary rabbit polyclonal antibodies for anti-OATP1B1 and anti-
OATP1B3 were kindly provided by Dr. Richard Kim and used at 1:3000 and 1:2500 dilutions, respectively (Ho et al., 2006). All blots were also incubated with primary mouse antiactin (AC140) antibody (1:2000) as a loading control (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The blots were then incubated for 1.5 h with corresponding horseradish peroxidase-conjugated anti-rabbit (1:15,000) or anti-mouse (1:2000) sec-
ondary antibody, respectively. Signals were enhanced by using a 1:1 mixture of Reagent A and Reagent B of the chemiluminescence Secondary antibody, respectively. Signals were enhanced by using 1:1 mixture of Reagent A and Reagent B of the chemiluminescence Super-
Signal West Pico System (Thermo Fisher Scientific, Waltham, MA) and then detected by exposing them to X-ray film. Densitometric analysis was performed by using AlphaDigiDoc RT2 software (Alpha Innotech, Kaywulle, UT) to quantify relative protein expression.

**Transport Experiments.** Transport experiments were con-
ducted as described previously (Kis et al., 2010b) with minor modifications. For the time-course studies, confluent cell mono-
layers were incubated (for different times) with 20 nM E3S (Sig-
ma-Aldrich Canada, Mississauga, ON, Canada), and the incubation buffer contained 0.3 μCi/ml [3H]E3S (57.3 Ci/mmol; chemical name: 3-hydroxyestr-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 contained 0.3 μCi/ml [3H]E3S, and the cells were incubated with final concentrations of E3S ranging from 5 nM to 1 mM. The specificity of OATP-mediated uptake was demonstrated by the use of a specific transport inhibitor bromosulphophthalein (BSP) [chemical name: 3,3’-(4,5,6,7-tetrambromo-3-oxo-1(3H)-isobenzo-
furanylidene)bis(6-hydroxy-,disodium salt] at a 100 μM final concentration, which was added to the preincubation buffer and the transport buffer. To fully clarify the specificity of E3S uptake we compared the E3S uptake obtained in the presence of 100 μM BSP to the one obtained with the use of an excess concentration (i.e., 1 mM) 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 the remaining uptake is the nonspecific uptake that is most likely composed of a mixture of diffusion and nonspecific cellular binding. All buffers and Triton X-100 were purchased from Sigma-Aldrich.

**Data Analysis.** All experiments were repeated at least three times in cells pertaining to three different passages. Within an individual experiment, each data point represents triplicate tri-
als. Results are presented as mean ± S.D. or mean ± S.E.M as appropriate. All statistical analysis was performed with 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 concentration-dependent experiments, kinetic analysis for estimation of the Michaelis-Menten affinity constant (Km) and the maximal velocity (Vmax) was performed by using the following equation, which consists of one saturable Michaelis-Menten component combined with a nonsaturable component:

\[
V = \frac{V_{\text{max}}[S]}{K_m + [S]} + k_{\text{ns}} \times [S].
\]

where V is the total rate of uptake of the studied substrate, \(V_{\text{max}}\) is the maximum uptake rate, \(K_m\) is the dissociation constant, \([S]\) is the substrate concentration, and \(k_{\text{ns}}\) is the coefficient for non-
specific uptake or diffusion. In the concentration-dependent up-
take experiments performed in the different cells, the total uptake was corrected for the nonspecific binding (i.e., background radio-
activity at zero time) and the nonsaturable or diffusion compo-
nent. The fitting of data into each model was performed by non-
linear least-squares analysis for “one site-fit total and nonspecific binding” using Prism 5 software (GraphPad Software Inc.). Eadie-
Hofstee plots were performed (data not shown) 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.
Results

OATP mRNA Expression in Human Breast Epithelial Cell Lines

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 (MDA-MB-231, MDA-MB-468, and MDA-MB-231) by quantitative real-time PCR. Although 11 human OATPs have been reported (Hagenbuch and Meier, 2004), our investigation was limited to the genes that encode transporters responsible for E3S uptake into cells. We observed heterogeneity in OATP detection and expression levels among the different cell lines. We determined the relative expression of each gene with respect to the housekeeping gene 18S. As described under Materials and Methods, one-way ANOVA for test of repeated measures was used to determine statistical significance. Bars labeled with different letters (a, b, c, or d) indicate a significant difference in expression. A, a versus b, \( p < 0.001 \). B, a versus b, \( p < 0.001 \). C, a versus b, \( p < 0.001 \). D, a versus b, \( p < 0.001 \). E, a versus b, a versus c, b versus c, \( p < 0.001 \). F, a versus b, a versus c, a versus d, b versus d, c versus d, \( p < 0.001 \), b versus c, \( p < 0.05 \).

Fig. 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 SLCO1A2 (A), SLCO1B1 (B), SLCO1B3 (C), SLCO2B1 (D), SLCO3A1 (E), and SLCO4A1 (F) is shown. Results are shown as mean relative mRNA expression ± S.E.M. from three separate experiments using the housekeeping gene 18S. As described under Materials and Methods, one-way ANOVA for test of repeated measures was used to determine statistical significance. Bars labeled with different letters (a, b, c, or d) indicate a significant difference in expression. A, a versus b, \( p < 0.001 \). B, a versus b, \( p < 0.001 \). C, a versus b, \( p < 0.001 \). D, a versus b, \( p < 0.001 \). E, a versus b, a versus c, b versus c, \( p < 0.001 \). F, a versus b, a versus c, a versus d, b versus d, c versus d, \( p < 0.001 \), b versus c, \( p < 0.05 \).
SLCO1B3 gene in the MCF7 cells. This could possibly be explained by the use of different cell systems. SLCO2B1 was undetectable is most of the cell systems with negligible expression in MCF7 and MDA435/LC6 cells (Fig. 1D). Thus, all four of these transporters showed significantly higher or exclusive expression in breast cancer cells, compared with the MCF10A immortalized breast epithelial cells. SLCO3A1 and SLCO4A1 were the only transporters that showed high expression in MCF10A cells. In addition, they were the only genes that showed expression in all cell lines investigated. SLCO3A1 showed significantly higher expression in MDA435/LC6 cells compared with MCF10A cells, whereas other cells showed similar or lower expression (Fig. 1E). In contrast to all other genes explored, SLCO4A1 was the only gene that showed significantly higher expression in the immortalized breast epithelial cells (MCF10A) compared with the breast cancer cells. Among the breast cancer cells, MCF7 cells showed significantly higher expression of SLCO4A1 in comparison with expression levels in MDA-MB-468 and MDA-MB-231 cells (Fig. 1F).

**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 because of a lack of specific antibody). OATP1A2 was expressed in all cell lines except MCF10A cells; hence, the level of expression was significantly ($p < 0.05$) greater in all breast cancer cells compared with MCF10A cells. The highest expression for OATP1A2 was observed in the MCF7 hormone-dependent breast cancer cells (Fig. 2A). The protein expression of OATP1A2 was concordant with the gene expression of SLCO1A2. OATP1B1 was detected in MDA-MB-231 and MDA435/LC6 cells (similar to the SLCO1B1 expression pattern), and the level of expression was 3-fold greater in MDA-MB-231 cells compared with MDA435/LC6 cells (Fig. 2B). We also explored OATP2B1 expression, and although the MDCKII-2B1 cells (used as a positive control) showed clear protein expression, we could not detect OATP2B1 in the immortalized breast epithelial or breast cancer cells (Fig. 2C). Negligible gene expression was observed for SLCO2B1 in MCF7 and MDA435/LC6 cells, which justifies the lack of OATP2B1 protein expression in these cell systems. Similar to OATP1A2, OATP3A1 could be detected in all cell lines. The expression of OATP3A1 was significantly greater in the breast cancer cell lines, MCF7 ($p < 0.01$) and MDA435/LC6 ($p < 0.05$), compared with MCF10A cells. In the hormone-dependent MCF7 breast cancer cell line, the expression was nearly 3-fold higher than in the MCF10A cells (Fig. 2D). Although the expression of the SLCO3A1 gene was found to be significantly higher in MDA435/LC6 cells, the expression of OATP3A1 protein did not show significant differences between MDA435/LC6 and MCF10A cells. Contrary to SLCO4A1 expression observed ubiquitously in all of the breast cell lines, we could detect OATP4A1 expression only in MDA435/LC6 cell lines. The minimal discordances observed between gene and protein expression could be caused by the higher sensitivity of detection of the qPCR method compared with immunoblotting. Overall, all of the OATP transporters investigated in this study showed exclusive or higher expression in breast cancer cells compared with the immortalized breast epithelial cells. Overexpression 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**

**Specificity of OATP-Mediated E3S Uptake.** To determine the specificity of carrier-mediated E3S cellular uptake, we performed time-course experiments with transport buffer containing 20 nM E3S in the presence or absence of the transport inhibitor, 100 μM-BSP. Significant differences between the total and nonspecific uptake indicated the presence of a specific carrier-mediated process that contributes toward E3S intracellular accumulation. The gene and protein expression of OATPs detected in the different cell systems strongly suggests that the OATPs have a major contribution toward the specific cellular uptake of E3S. However, because E3S is a common substrate for other uptake transporters as well (e.g., OAT3 and OAT4) (Ugele et al., 2003; Asif et al., 2005; Ninomiya et al., 2006), 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 MCF10A immortalized breast epithelial cells (Fig. 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 was observed in all of the breast cancer cells. This suggests that the specificity of OATP-mediated E3S uptake is present in the cancer cells. It was further observed that specific E3S uptake was the highest in the MCF7 hormone-dependent breast cancer cells (Fig. 3, B-E).

**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 MCF10A immortalized breast epithelial cells we observed significantly higher E3S uptake in the hormone-dependent MCF7 cells and the hormone-independent MDA435/LC6 cells. Among the breast cancer cell lines, the hormone-dependent MCF7 cells had the highest E3S uptake (Fig. 3F).

**Transport Kinetics of E3S in Hormone-Dependent and Hormone-Independent Cancer Cells.** 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 allowed further evaluation of the efficiency of E3S transport (expressed as a ratio of $V_{max}/K_m$) in each cell line as described previously (Letscher et al., 2004). The $K_m$ of 6.5 ± 1.9 μM for E3S uptake was the lowest in the hormone-dependent MCF7 cells compared with the hormone-independent breast cancer cells, indicating that the transporters in MCF7 cells have the highest affinity toward E3S. This value is similar to the one previously reported for E3S uptake in MCF7 cells (Nozawa et
The $V_{\text{max}}$ value was $66 \pm 8.5$ pmol/mg protein/min, which was also similar to previously reported values (Fig. 4A). Among the hormone-independent breast cancer cells, the $K_m$ values were $68.3 \pm 7.8$, $203.8 \pm 41$, and $46.8 \pm 1.7$ $\mu$M for the MDA435/LCC6, MDA-MB-468, and MDA-MB-231 cells, respectively. The $V_{\text{max}}$ values for E3S uptake in the same cells were $57.6 \pm 4.5$, $131.4 \pm 15.92$, and $27.3 \pm 6.2$ pmol/mg protein/min, respectively (Fig. 4, B-D). All cell lines showed one-site saturation binding kinetics.

The efficiency of transport is more than 10-fold greater in the MCF7 hormone-dependent breast cancer cells compared with the hormone-independent cells. No significant difference was observed among the various hormone-independent cells. (Fig. 5; Table 1).

**Discussion**

OATPs are expressed in various epithelial tissues and cancers (Obaidat et al., 2012) and are known to be involved in the transport of endogenous hormones, their conjugates, and several anticancer drugs (Hagenbuch and Meier, 2003; Smith et al., 2007; Hagenbuch and Gui, 2008; van de Steeg et al., 2009). OATPs have been reported to be up-regulated (Hamada et al., 2008; Lee et al., 2008; Yang et al., 2011) 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 intracellular 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. (2006) and Meyer zu Schwabedissen et al. (2008) reported 10-fold greater expression of OATP1A2 in breast cancer tissues compared with surrounding normal tissues. Pizzagalli et al. (2003) detected the localization of OATP2B1 in the luminal epithelium in invasive ductal carcinoma tissues, whereas it was confined primarily to the myoepithelial cells in the surrounding normal tissues of the breast. In prostate cancers, another hormone-dependent cancer that depends on andro-
gens for proliferation, OATP1B3 and OATP2B1 are overexpressed (Hamada et al., 2008; Yang et al., 2011). Wright et al. (2011) further demonstrated high expression of OATP1B3 (3.6-fold) and OATP2B1 (5.5-fold) in metastatic lesions from men with castration-resistant prostate cancers compared with untreated prostate cancers. In colon cancer samples, OATP1B3 expression was detected to be 100-fold higher compared with 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 of 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 the proliferation of hormone-dependent cancers. To further our understanding of the role of OATPs in E3S uptake in breast cancers, we analyzed 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.

Fig. 4. Kinetics of E3S uptake by breast epithelial cells and breast cancer cells. Rates of uptake of [3H]E3S by MCF7 cells at concentrations ranging from 5 nM to 100 μM (A), MDA435/LCC6 cells at concentrations ranging from 5 nM to 150 μM (B), MDA-MB-468 cells at concentrations ranging from 5 nM to 1000 μM (C), and MDA-MB-231 cells at concentrations ranging from 5 nM to 150 μM (D) were measured at 2 min, pH 7.4, and 37°C. The saturable (●) and nonsaturable (○) components of the total (●) uptake rate were determined by fitting the data into eq. 1, the Michaelis-Menten equation, as detailed under Materials and Methods. Saturable E3S uptake by all of the cells was 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 using Prism 5 software. Insets, specific early time points are represented. Data represent the mean ± S.E.M. for $n = 3$ independent experiments.

Fig. 5. Transport efficiency of E3S uptake by MCF7, MDA435/LCC6, MDA-MB-468, and MDA-MB-231 cells. Transport efficiency for E3S uptake was calculated by using the ratio of $V_{max}/K_M$ as observed in each of the cells. As described under Materials and Methods, one-way ANOVA for test of repeated measures was used to compare the data. We observed significantly higher transport efficiency in MCF7 cells compared with all other hormone-independent breast cancer cells. *** $p < 0.001$.

with untreated prostate cancers. In colon cancer samples, OATP1B3 expression was detected to be 100-fold higher compared with 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 of 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 the proliferation of hormone-dependent cancers. To further our understanding of the role of OATPs in E3S uptake in breast cancers, we analyzed 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, whereas SLCO2B1 is present in negligible quantities in MCF7 and MDA435/LCC6 cells. Our observations are similar to those reported previously by Miki et al. (2006) and Meyer zu Schwabedissen et al. (2008) for OATP1A2 expression and by Pizzagalli et al. (2003) for OATP2B1 expression. In accordance with the results of Pizzagalli et al. (2003) and Wlcek et al. (2008), we also observed SLCO3A1 and SLCO4A1 expression in all of the cell lines investigated. We also detected SLCO1B1 and SLCO1B3 ex-
pression in MDA-MB-231 cells. Comparison of gene expression patterns between hormone-dependent and -independent breast cancer cells reflected expression of most OATPs in hormone-dependent MCF7 cells. Among the seven OATP isoforms investigated, we detected 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.

Because mRNA expression differences are not always reflected at the protein level, we investigated the protein expression of six OATP isoforms (except OATP1C1 because 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 SLCO genes that we examined. OATP1A2, reported previously to be expressed in T47D hormone-dependent breast cancer cells (Meyer zu Schwabedissen et al., 2008), showed protein expression in all of the cell systems. The level of expression was significantly higher in the breast cancer cells compared with the MCF10A cells. Among the breast cancer cell systems, highest expression was observed in the hormone-dependent MCF7 cells. OATP3A1 was the other transporter that was expressed in all of the cell systems. However, unlike the SLCO3A1 gene expression pattern, where we observed the highest expression in the MCF10A immortalized breast epithelial cells, OATP3A1 protein expression was highest in the hormone-dependent MCF7 cells. MCF7 and MDA-MB-468 cells showed significantly higher OATP3A1 protein expression than MCF10A cells. Similar to the SLCO1B1 gene expression, OATP1B1 protein was expressed in MDA-MB-231 and MDA435/LCC6 cells. Although very low SLCO2B1 gene expression was detected in some cell lines, we could not detect OATP2B1 protein expression in any cell system. Discordance between gene and protein expression was observed primarily in OATP4A1 expression. Although SLCO4A1 gene expression was observed in all of the cell systems, OATP4A1 protein was expressed only in MDA435/LCC6 cells. It is not unlikely that gene expression does not reflect protein expression (Wang et al., 2012). Overall, we observed significantly higher protein expression of OATPs in cancer cells compared with the normal cells. It also seems that the OATP-mediated E3S uptake in hormone-dependent breast cancer cells was mediated primarily by OATP1A2 and OATP3A1. Because the expression of several OATP isoforms was observed in a single breast cancer cell line system, the total E3S uptake was possibly a summative contribution from all of the OATPs that were 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. Although specific carrier-mediated E3S uptake was demonstrated in all the breast cancer cell line systems investigated, no specific carrier-mediated E3S uptake was observed in the MCF10A immortalized breast epithelial cells. This suggests that the specificity of OATP-mediated E3S uptake is restricted mainly to breast cancer cells. Because we observed 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 E3S uptake among the various breast cancer cells at steady state and demonstrated that the highest E3S uptake occurred in the MCF7 hormone-dependent breast cancer cells. Furthermore, the expression pattern of the transporters in the various cell lines supports their functional contribution toward E3S uptake. For example, we observed the highest expression of OATP isoforms in the MCF7 cells where we had the 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 postmenopausal patients could be caused by an OATP-mediated uptake process.

After determining the specific role of the OATPs in contributing toward the intracellular concentration of E3S in breast cancer cells, we further compared the efficiency of E3S transport among the different breast cancer cells. We observed 10 times higher transport efficiency (estimated by $V_{\text{max}}/K_m$) in the MCF7 hormone-dependent breast cancer cells compared with the hormone-independent cells. This implies that although specific carrier-mediated E3S uptake was observed in all of the breast cancer cells, the efficiency of E3S transport was highest in the hormone-dependent breast cancer cells.

In summary, we observed: 1) significantly higher OATP gene and protein expression in breast cancer cells compared with immortalized breast epithelial cells; 2) OATP is primarily functional in breast cancer cells; and 3) 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 postmenopausal women. Further investigations characterizing the expression of these transporters in human breast tumor tissues are needed. Current limitations of endocrine therapies are acquired or de novo resistance, and are caused by a loss or mutation of estrogen receptors. By targeting OATPs, we could potentially treat primary hormone-dependent tumors that have lost estrogen receptor expression in the years. Further research is required to better characterize the expression of these transporters in primary hormone-dependent tumors that have lost estrogen receptor expression and in turn have acquired resistance to endocrine therapy.

**TABLE 1**

<table>
<thead>
<tr>
<th>Cell System</th>
<th>$V_{\text{max}}$</th>
<th>$K_m$</th>
<th>Transport Efficiency, $V_{\text{max}}/K_m$</th>
<th>Transport Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol/mg protein/min</td>
<td>μM</td>
<td>μl/mg protein/min</td>
<td>%</td>
</tr>
<tr>
<td>MCF7</td>
<td>66 ± 8.5</td>
<td>6.5 ± 1.8</td>
<td>10.21</td>
<td>100</td>
</tr>
<tr>
<td>MDA435/LCC6</td>
<td>57.6 ± 4.5</td>
<td>68.3 ± 7.8</td>
<td>0.84*</td>
<td>8.3</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>131.4 ± 15.9</td>
<td>203.8 ± 41</td>
<td>0.6</td>
<td>5.9</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>27.31 ± 6.2</td>
<td>46.9 ± 1.7</td>
<td>0.58*</td>
<td>5.7</td>
</tr>
</tbody>
</table>

* $p < 0.05.
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Authorship Contributions

Participated in research design: Banerjee, Allen, and Bendayan. Conducted experiments: Banerjee.

Contributed new reagents or analytical tools: Allen and Bendayan. Performed data analysis: Banerjee and Bendayan.

Wrote or contributed to the writing of the manuscript: Allen, Bendayan.

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Role of OATPs in Breast Cancers