Interplay between the Nuclear Receptor Pregnan X Receptor and the Uptake Transporter Organic Anion Transporter Polypeptide 1A2 Selectively Enhances Estrogen Effects in Breast Cancer

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Abstract

The ligand-activated nuclear receptor pregnane X receptor (PXR) is known to play a role in the regulated expression of drug metabolizing enzymes and transporters. Recent studies suggest a potential clinically relevant role of PXR in breast cancer. However, the relevant pathway or target genes of PXR in breast cancer biology and progression have not yet been fully clarified. In this study, we show that mRNA expression of organic anion transporter polypeptide 1A2 (OATP1A2), a transporter capable of mediating the cellular uptake of estrogen metabolites, is nearly 10-fold greater in breast cancer compared with adjacent healthy breast tissues. Immunohistochemistry revealed exclusive expression of OATP1A2 in breast cancer tissue. Interestingly, treatment of breast cancer cells in vitro with the PXR agonist rifampin induced OATP1A2 expression in a time-dependent and concentration-dependent manner. Consistent with its role as a hormone uptake transporter, induction of OATP1A2 was associated with increased uptake of estrone 3-sulfate. The rifampin response was abrogated after small interfering RNA targeting of PXR. We then identified a PXR response element in the human OATP1A2 promoter, located ~5.7 kb upstream of the transcription initiation site. The specificity of PXR-OATP1A2 promoter interaction was confirmed using chromatin immunoprecipitation. Importantly, we used a novel potent and specific antagonist of PXR (A-792611) to show the reversal of the rifampin effect on the cellular uptake of E1S. These data provide important new insights into the interplay between a xenobiotic nuclear receptor PXR and OATP1A2 that could contribute to the pathogenesis of breast cancer and may also prove to be heretofore unrecognized targets for breast cancer treatment. [Cancer Res 2008;68(22):9338–47]

Introduction

The pregnane X receptor (PXR; SXR, NR1I2) is a member of the nuclear receptor family of ligand-activated transcription factors. This intracellular receptor has been shown to function as a xenobiotic sensor importantly involved in drug metabolism and transport. Indeed, this nuclear receptor is a major regulator of cytochrome P450 enzymes, including CYP3A4, CYP2C8, CYP2C9, and CYP2B6 (1–5), and drug efflux transporters, such as multidrug resistance protein 1 (MDR1; ABCB1) and multidrug resistance-associated protein 2 (MRP2; ABC2; refs. 6–8). The mechanistic basis of many drug-drug interactions in vivo can now be explained by the activation of this nuclear receptor and the resultant induction of hepatic and intestinal enzymes and transporters. Not surprisingly, PXR expression has been noted in several tissues which are exposed to high levels of xenobiotics, such as the liver, intestine, lung, and kidney (9, 10). Interestingly, PXR expression has also been detected in normal and neoplastic human breast tissue (11). Recently, a study by Miki and colleagues noted expression of PXR and the drug uptake transporter organic anion transporter polypeptide 1A2 (OATP1A2) in human breast cancer tissue. Importantly, OATP1A2 and PXR expression was proposed as a histopathologic marker of dedifferentiation and progression of breast cancer (12).

OATP1A2 is a member of the OATP superfamily of transporters that mediate the cellular uptake of endobiotics and xenobiotics. OATP1A2 has been shown to be expressed in hepatic cholangiocytes, the capillary endothelia forming the blood-brain barrier (13), and the apical domain of intestinal enterocytes (14). OATP1A2 expression in normal nonmalignant breast tissue has been noted to be very low especially compared with other members of the OATP family, such as OATP-B (OATP2B1), OATP-E (OATP4A1), and OATP-D (OATP3A1), although OATP1A2 expression in lactating mammary epithelium cells (MEC) is significantly greater compared with nonlactating MECs, suggesting regulated physiologic function of this transporter in breast tissue (15, 16).

Of potential significance to breast cancer, OATP1A2 is known to mediate the cellular uptake of hormone conjugates (17). Despite the known ability of this transporter to mediate the cellular uptake of biologically active hormone conjugates, such as estrone 3-sulfate (E1S) and estradiol 17β-glucuronide, the possible role of regulated OATP1A2 expression to hormone-associated progression of breast cancer has not yet been clarified. It should be noted that exposure of breast cancer cells to E1S has been shown in vitro to result in increased cellular proliferation and that this effect could be modulated by concomitant treatment with bromosulfophthalein, a known nonspecific OATP inhibitor (18, 19). However, an association of such effect to the uptake transporter OATP1A2 had not been reported. Note that E1S level is significantly greater in malignant tissue, resulting in increased levels of biologically active 17β-estradiol (20). Mechanistically, it is plausible that OATP1A2 plays a pivotal role in regulating proliferation and tumor promotion of breast tissue by enhancing the uptake of estradiol metabolites, thereby increasing intracellular levels of such hormones that activate the estrogen receptor. Importantly, molecular
mechanisms that determine OATP1A2 overexpression in breast cancer have not been defined.

In this study, we examined OATP1A2 and PXR expression in neoplastic and adjacent nonneoplastic breast tissue. In addition, expression of OATP1A2 and PXR was assessed in a panel of breast cancer tissues of different tumor stages. To assess whether a direct link between PXR activation and human OATP1A2 expression exists, cell-based reporter assays and chromatin immunoprecipitation (ChIP) were performed to identify a PXR response element in the OATP1A2 gene. In addition, using a breast cancer cell model, we were able to show OATP1A2 induction results in greater E$_S$ cellular uptake and enhanced estrogen receptor activation. Importantly, using a newly identified specific and potent PXR antagonist, we showed that inhibition of PXR activity attenuates the proliferative effects of estrogen.

**Materials and Methods**

**Chemicals.** Rifampin, tamoxifen, E$_S$, and p-nitrophenolphosphate were purchased from Sigma-Aldrich. Tritium-labeled E$_S$ was obtained from Perkin-Elmer Life Sciences. The PXR inhibitor A-792611 was provided by Perkin-Elmer Life Sciences. The PXR inhibitor A-792611 was provided by Perkin-Elmer Life Sciences. Rifampin, tamoxifen, E$_S$, and p-nitrophenolphosphate were purchased from Sigma-Aldrich. Tritium-labeled E$_S$ was obtained from Perkin-Elmer Life Sciences. The PXR inhibitor A-792611 was provided by Perkin-Elmer Life Sciences. Rifampin, tamoxifen, E$_S$, and p-nitrophenolphosphate were purchased from Sigma-Aldrich. Tritium-labeled E$_S$ was obtained from Perkin-Elmer Life Sciences. The PXR inhibitor A-792611 was provided by Perkin-Elmer Life Sciences. Rifampin, tamoxifen, E$_S$, and p-nitrophenolphosphate were purchased from Sigma-Aldrich. Tritium-labeled E$_S$ was obtained from Perkin-Elmer Life Sciences. The PXR inhibitor A-792611 was provided by Perkin-Elmer Life Sciences.

**Cell culture.** MCF-7, T47-D, HeLa, and HepG2 cells were purchased from American Type Culture Collection. MCF-7 cells were grown in EMEM supplemented with 10% fetal bovine serum (FBS), nonessential amino acids, t-glutamine, and penicillin/streptomycin. T47-D and HepG2 were maintained in RPMI and DMEM, respectively. Media and supplements were purchased from Cambrex. Cell culture was performed at 37°C with 5% CO$_2$ in a humidified atmosphere.

**Tissue samples.** Malignant and adjacent nonmalignant breast tissue samples were obtained from Vanderbilt Tissue Procurement Core. Paraffin-embedded tissue slides were prepared by the Department of Pathology at Vanderbilt University. Tissue for RNA isolation was snap frozen in liquid nitrogen and stored at −80°C. In addition, a commercially available TissueScan Real-Time Breast Cancer Disease Panel (Origene) of breast cancer tissue of different stages was used for quantification of gene expression.

**RNA isolation from tissue samples and cultured cells.** For RNA extraction, the QiAamp RNeasy kit (Qiagen) was used. Frozen breast tissue was mechanically homogenized in RLT buffered supplemented with β-mercaptoethanol. Subsequently, the thawed homogenate was centrifuged through a QiaShredder column and the eluate was loaded on purification columns. The following RNA isolation was performed, as described by the manufacturer. Briefly, phenol-chloroform extraction was performed to isolate RNA from *in vitro* experiments using Trizol (Invitrogen). The integrity and content of the RNA were determined using the Agilent Bioanalyzer (Agilent). RNA samples were stored at −80°C.

**Real-time PCR.** Total RNA was reverse transcribed in a 50-μL reaction volume containing 1.500 ng of RNA with the TaqMan Reverse Transcription kit (Applied Biosystems) using random hexamers, as described by the manufacturer. The amount of OATP1A2, PXR, CAR, and 18S were measured by SYBRgreen quantitative real-time PCR with an ABI Prism 7700 sequence detection system (Applied Biosystems). The sequences of primers used for establishing the amount of the transporter, the nuclear receptors and the 18S-rRNA.

**In silico scan for PXR response elements.** A 10-kb fragment upstream of the transcription start of the OATP1A2 gene was screened for potential PXR binding sites using the NUBiscan algorithm.

**Plasmids.** CYP3A4-XREM-Luc plasmid containing the proximal promoter (−362/+53) and distal XREM (−7836/−7208) inserted in pGL3 basic (Promega) was used as positive control for PXR response (21). OATP1A2 regulatory regions containing the basal promoter (−440 to +50) and distal fragments that contained putative PXR response elements (PXRRE) were subcloned into pGL3 basic (Promega) or vector control in 200 μL Opti-MEM (Invitrogen) using lipofectamine (Invitrogen). The amount of sample loading, the blot was reprobed with a monoclonal antiactin antibody (Sigma-Aldrich).

**Western blot analysis.** To determine the effect on OATP1A2 protein expression, T47-D cells were cultured in 10-cm dishes. After treatment for 24 h, the cells were harvested in 5 mmol/L Tris-Cl (pH 7.4). After freeze-thaw in liquid nitrogen, the cell suspension was homogenized using a Dounce Potter. Membrane fraction was collected by centrifugation at 100,000 × g at 4°C, and 50 μg of protein were separated by SDS-PAGE and electrotransferred to nitrocellulose membrane Western blotting system (Invitrogen). OATP1A2 in HeLa cells overexpressed using a virus virus, as previously described, was used as positive control (13). Total cell lysate (15 μg) of transfected cells was used. To enhance utility of the OATP1A2, antibody described above (1:1,000), for Western blot analysis, the antibody was further purified using the Melon Gel IgG purification kit (Thermo-Fisher). A horseradish peroxidase–labeled antirabbit antibody (1:2,000; Bio-Rad) was used in the secondary. The immobilized secondary antibody was detected using the ECL Plus Western Blotting Detection System (GE Healthcare) and KODAK ImageStation 4000 MM (Mandel). To normalize sample loading, the blot was reprobed with a monoclonal antiactin antibody (Sigma-Aldrich).

**Protein localization of OATP1A2 in human breast cancer tissues.** Using standard methods. The tissue sections were deparaffinized in xylol. Afterwards the tissue sections were incubated in ethanol of declining concentration from 100% to 50% for rehydration and then rinsed in distilled water. For heat-induced epitope retrieval, the tissue sections were boiled in citrate buffer (10 mmol/L, pH 6.0). After washing twice in ice-cold PBS, the slides were blocked with 5% FBS-PBS. Thereafter, the slides were incubated with diluted anti–OATP1A2 antibody (1:50) in a humidified atmosphere at 4°C overnight. After several washing steps with PBS, the sections were incubated with the fluorescent-labeled secondary antibody (Invitrogen). After washing the slides with PBS, the tissue was mounted in antifading mounting medium containing 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories). Images were obtained by fluorescence microscopy. As negative control, the primary antibody was omitted.

**Immunofluorescence.** Protein localization of OATP1A2 in human breast neoplastic and normal tissue was investigated by immunofluorescence microscopy. For OATP1A2 detection, a polyclonal antibody raised in rabbit was used (13). Paraffin-embedded tissue sections were prepared by standard methods. The tissue sections were deparaffinized in xylol. Afterwards the tissue sections were incubated in ethanol of declining concentration from 100% to 50% for rehydration and then rinsed in distilled water. For heat-induced epitope retrieval, the tissue sections were boiled in citrate buffer (10 mmol/L, pH 6.0). After washing twice in ice-cold PBS, the slides were blocked with 5% FBS-PBS. Thereafter, the slides were incubated with diluted anti–OATP1A2 antibody (1:50) in a humidified atmosphere at 4°C overnight. After several washing steps with PBS, the sections were incubated with the fluorescent-labeled secondary antibody (Invitrogen). After washing the slides with PBS, the tissue was mounted in antifading mounting medium containing 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories). Images were obtained by fluorescence microscopy.
mixture and then treated for 24 h with the tested agents. The reporter enzyme activities were assayed with a dual-luciferase reporter assay system, as described by the manufacturer (Promega). Luminescence was quantified using a plate reader (Fluoroskan Ascent FL, Thermo-Fisher). Luciferase activities in the presence of transfected PXR were expressed as a percentage of cells transfected with blank vector.

**Estrogen receptor reporter gene assays.** T47-D cells were plated in 24 wells in RPMI supplemented with 5% charcoal-stripped FBS. After 24 h, cells were transfected with 250 ng of a commercially available vector containing a secreted alkaline phosphatase gene, whose expression is controlled by an estrogen responsive element (ERE; Clontech). After pretreatment with rifampin or DMSO in RPMI without phenol red supplemented with 1-glutamine and 1% charcoal-stripped FBS (Invitrogen), the cells were exposed to E1S (10 nmol/L). After treatment, activity of alkaline phosphatase was determined by combining 30 μL of the sample with 150 μL of 5 mmol/L p-nitrophenolphosphate prepared in 80 mmol/L Tris-HCl (pH 9.6) for 60 min at 37°C. The reaction was stopped by the addition of 20 μL 0.1 mol/L NaOH. The amount of reduced p-nitrophenolphosphate was determined by UV spectroscopy at 405 nm using a plate reader (Multiskan Spectrum, Thermo-Fisher).

**Cellular modulation of PXR expression with small interfering RNA.** T47-D cells were suspended to a final concentration of 1 × 10⁵ cells/mL in growth medium and incubated at 37°C. The lipid-based transfection agent siPort NeoFX (Ambion) was used for transfection of PXR small interfering RNA (siRNA; Ambion), as described by the manufacturer. The transfection was incubated for 12 h at 37°C at 5% CO₂. Afterwards, cells were treated with rifampin (1 μmol/L) or DMSO for 24 h.

**E1S uptake experiments.** T47-D cells were grown in 12 wells and pretreated with rifampin or DMSO. Cells were incubated with tritium-labeled E1S (1 μmol/L, containing 400,000 dpm/well). After 10 min of incubation at 37°C, the cells were washed twice with ice-cold Opti-MEM and lysed using 1% SDS. The cellular uptake of E1S was determined using Ultima Gold scintillation liquid and a liquid scintillation counter (Tri-Carb 2900TR, Perkin-Elmer Life Sciences).

**AlamarBlue cell proliferation assay.** T47-D or MCF-7 cells were pretreated with rifampin or DMSO and then exposed to E1S. To determine cell viability and proliferation, the AlamarBlue reagent (Biosource) was used, as described by the manufacturer. The production of the reduced product was monitored spectrophotometrically at 570 and 600 nm.

**ChIP assay.** For DNA cross-linking and ChIP, the EZ ChIP Assay (Millipore) was used, as described by the manufacturer. Briefly, T47D cells were cultured in 10-cm dishes and treated for 48 h with DMSO or rifampin (1 μmol/L). After DNA cross-linking, the cells were lysed. DNA was then sheared by sonication (Vironson). The shearing efficacy was analyzed by agarose gel electrophoresis to ensure that the average size of DNA was 1,500 kb. Thereafter, the sheared and cross-linked chromatin was incubated with 5 μg of each antibody overnight at 4°C. Two polyclonal anti-PXR sera were used (A-20 and N-16, Santa Cruz Biotechnology). An anti–acetyl-histone (H3) antibody was used as control.

The antibody-antigen-chromatin complex was gathered with protein G agarose and centrifugation. After several washing steps, the antibody-chromatin complex was eluted and bound DNA was released by incubation at 65°C overnight after adding 8 μL of 5 mol/L NaCl treated with RNase A and proteinase K, and purified. Binding of human PXR to the SLCO1A2 gene

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![Figure 1](https://example.com/figure1.png)

**Figure 1.** Expression of OATP1A2 and PXR comparing nonneoplastic and neoplastic breast tissue. OATP1A2 (A) and PXR (D) mRNA expression was assessed in neoplastic and adjacent nonneoplastic breast tissue samples of four individuals using real-time PCR, revealing significantly higher expression of both genes in the cancerous tissue. Immunofluorescent staining of the uptake transporter OATP1A2 (green) in human malignant and nonmalignant breast tissue showed an intense and restricted expression in malignant cells (C). No distinct staining pattern was detected in nonmalignant breast tissue (B). DNA chromatin was counterstained with DAPI (red). As control, the primary antibody was omitted (insert, B, C). Columns, mean; bars, SD; *, P < 0.05, t test.
was confirmed by PCR amplification of a 210-bp fragment of genomic DNA encompassing the putative PXR (DR4-1) binding site. The following primers were used in the PCR: ChIP for 5′-CACCTTTCTTCTCCAAGTT-3′, ChIP rev 5′-TCAATTTAAGCCACATTCC-3′. PCR was carried out in 50 µL volume using the AmpliTaqGold PCR system (Applied Biosystems). The annealing temperature was 54°C, followed by elongation at 72°C for 30 s. The PCR was performed at 32 cycles. For the acetyl histone H3, the PCR was performed, as described by the manufacturer, using the AmpliTaq Gold PCR system. PCR products were separated on 4% agarose gels.

**Statistical analysis.** Determination of statistical differences between group variables was determined using Student’s t test and Mann-Whitney U test (GraphPad Software, Inc.). A P value of <0.05 was taken to be the minimum level of statistical significance.

**Results**

**OATP1A2 and PXR expression in malignant and normal human breast tissue.** OATP1A2 and PXR expression was examined using real-time PCR. Both transcripts were detectible in all malignant (n = 4) and nonneoplastic (n = 4) tissue samples. Breast cancer tissue showed an 8-fold higher expression of OATP1A2 compared with the adjacent normal tissue in all samples tested (Fig. 1A). Similarly, the level of PXR expression was nearly 8-fold higher in malignant tissue compared with the adjacent normal tissue of each subject (Fig. 1D). Immunofluorescence microscopy revealed restricted expression of OATP1A2 in human malignant breast tissue using a specific antibody against OATP1A2. Distinct immunofluorescent signals were not observed in normal human breast tissue and in tumor or normal tissue when the primary antibody was omitted (Fig. 1B, C).

**Detection of OATP1A2 mRNA in breast cancer tissue cDNA panel.** OATP1A2 expression was also assessed in a panel of different stages of breast cancer by real-time PCR. The number of OATP1A2 transcripts was greater in tumor stages I and IIa compared with stage 0 (containing samples of patients with carcinoma in situ or fibrocystic changes; OATP1A2 expression relative to stage 0, mean ± SD: stage 0, 1.23 ± 0.896 n = 4; stage I, 3.03 ± 3.37 n = 11; stage IIa, 5.10 ± 3.95, n = 15; stage IIB, 1.07 ± 0.28, n = 4; stage IIa, 0.55 ± 0.14, n = 4; stage IIIC, 0.88 ± 0.41, n = 2). It is noteworthy that OATP1A2 was undetectable in some samples of higher stages. In three of seven samples of stage IIB, in four of eight samples of stage IIa, and in one of three samples of stage IIIC, OATP1A2 expression did not reach the minimum level of detection. The absence of OATP1A2 expression was not related to estrogen receptor, progesterone receptor, or Her2 status reported by the company. PXR-mRNA was detectible in all samples, and the tumor stage–dependent expression pattern was similar to OATP1A2 (PXR expression relative to stage 0, mean ± SD: stage 0, 1.05 ± 0.35 n = 4; stage I, 2.57 ± 1.44 n = 11; stage IIa, 4.37 ± 2.03 n = 15; stage IIB, 1.82 ± 0.52 n = 7; stage IIa, 1.88 ± 2.35 n = 7; stage IIIC, 3.29 ± 1.53 n = 3).

**PXR regulates OATP1A2 expression and estrogen-mediated cell proliferation.** To examine the interaction between PXR and OATP1A2, T47-D breast cancer cells were treated with the known PXR activator rifampin. Treatment of T47-D cells was associated with a time-dependent induction of OATP1A2. Increased transporter mRNA levels were evident at 6 hours after initiation of rifampin treatment and reached maximum levels at 8 hours. OATP1A2 expression upon PXR activation was concentration-dependent (Fig. 2A). Another estrogen receptor–positive breast cancer cell line (MCF-7 cells) was used to determine if PXR expression levels differ compared with T47-D cells, resulting in changes in rifampin response. We noted no difference in PXR expression (relative PXR expression ± SE: MCF-7 cells, 1.01 ± 0.34; T47-D cells, 1.09 ± 0.57). However, the effect of rifampin on OATP1A2 expression was more pronounced in T47-D cells after treatment with rifampin for 8 hours (relative OATP1A2 expression ± SE: MCF-7 cells, 8.02 ± 0.93; T47-D cells, 13.15 ± 2.87).

One of the known effects of the OATP1A2 substrate, E1S, is the enhancement of cell proliferation *in vitro*. Cell proliferation was assessed after pretreatment of T47-D cells with rifampin (1 µmol/L) or DMSO and subsequent exposure to E1S for 24 hours (10 nmol/L) using AlamarBlue. As shown in Fig. 2B, T47-D cells treated with the PXR activator exhibited a significantly higher ability to metabolize the AlamarBlue reagent compared with cells exposed to DMSO before the E1S treatment. Similar results were obtained in MCF-7 cells (cell viability %--DMSO control, mean ± SE: pretreatment with DMSO followed by E1S, 120.31 ± 6.38; pretreatment with rifampin followed by DMSO, 94.47 ± 11.61; pretreatment with rifampin followed by E1S, 153.59 ± 25.46; data not shown).

**Effect of PXR-siRNA and OATP1A2 mediated E1S uptake in T47-D cells.** siRNA-mediated cellular knockdown of PXR was used to validate the involvement of PXR in regulating OATP1A2 expression and the resultant increase in cellular uptake of E1S. In siRNA-treated T47-D cells, a marked reduction of PXR level (Fig. 2C) was associated with the loss of rifampin-induced OATP1A2 expression (Fig. 2C) and rifampin-stimulated E1S uptake. Cells transfected with control siRNA and treated with rifampin (10 µmol/L) exhibited a 1.5-fold induction of E1S uptake compared with cells treated 24 hours with DMSO (E1S uptake %--DMSO control, mean ± SE: DMSO treated, 100.00 ± 12.45; rifampin treated, 157.18 ± 10.73), whereas cells transfected with PXR-siRNA and treated with rifampin do not exhibit an increase in E1S uptake (E1S uptake %--DMSO control, mean ± SE, DMSO treated, 99.99 ± 3.59; rifampin treated, 108.06 ± 3.31; Fig. 2D).

**Identification of functional PXR response elements in the SLCO1A2.** We next aimed to determine the molecular mechanisms involved in PXR-mediated regulated OATP1A2 expression. Hence, a 10-kb fragment of the SLCO1A2 gene was scanned using the NUBiscan algorithm (18) to reveal potential PXRRE. Included in the search were known PXR responsive DNA motifs of tandem hexameric repeats with various spacing and orientation, such as DB4, DB3, DB6, and DB8. To confirm whether the predicted binding sites were functional, fragments of the SLCO1A2 gene containing potential PXRREs were subcloned into a luciferase vector containing a basal promoter fragment of SLCO1A2 (~440 to +50 bp) and assessed in cell-based reporter gene assays.

The promoter constructs were tested by cotransflecting PXR into T47-D cells. Addition of the known PXR activator rifampin (10 µmol/L) resulted in PXR-dependent enhanced luciferase activation of the construct containing the −5120 to −6600 bp fragment of the SLCO1A2 gene. Rifampin-stimulated, PXR-dependent reporter activity was not observed in other fused reporter constructs tested (Fig. 3A). However, a slight, but statistically significant, activation of the basal promoter construct was seen in rifampin-treated cells. These results are in agreement with previous findings testing the PXR-dependent activation of CYP3A4, showing that activation of the basal promoter is significantly increased by a distal enhancer module containing the actual PXRRE of CYP3A4 (22, 23). We observed similar findings performing the experiment using hepatocellular carcinoma (HepG2) cells or using the PXR activator tamoxifen (10 µmol/L; data not shown). Because another reporter construct containing overlapping sequences from −5790 to −6600 bp was unresponsive to PXR...
Within this region, NUBIscan analysis exposed five potential PXR binding sites (Fig. 3, insert). These putative PXRREs were systematically mutated using a site-directed mutagenesis approach and tested in reporter assays. Interestingly, mutation of the DR4-1 DNA motif (localized at -5786 to -5801) resulted in the complete loss of rifampin-stimulated, PXR-dependent, luciferase reporter activity in HepG2 cells (Fig. 3B) and T47-D cells (data not shown). These findings suggest that this DR4 element in the SLC10A2 promoter is transactivated by agonist-bound PXR.

ChIP using anti-PXR antibodies. To further confirm the role of the DR4-1 element in the inductive regulation of OATP1A2 expression, we used antibody-mediated immunoprecipitation of PXR bound to chromatin. T47-D cells were treated with rifampin (1 μmol/L) or DMSO, and ChIP analysis was performed on formaldehyde cross-linked DNA with subsequent immunoprecipitation with two different PXR antibodies (A-20 and N-16). PCR amplification of a 210-bp fragment of the OATP1A2 gene surrounding the putative DR-1 PXRRE from immunoprecipitated DNA revealed specific signals in cells treated with rifampin, but not in vehicle-treated cells using both antibodies (Fig. 3C). These results show that rifampin-activated PXR binds to the OATP1A2 promoter and strongly suggest that the DR4-1 motif is a key element responsible for OATP1A2 transactivation by PXR.

Pharmacologic modulation of OATP1A2 expression and function by PXR antagonism. Recently, we have identified a novel potent and selective PXR antagonist A-792611 (24). As expected, addition of A-792611 abolished the PXR activation of the OATP1A2 reporter construct (Fig. 4A). Importantly, the inductive effect of rifampin on OATP1A2 expression could be abolished by treatment of T47-D cells with A-792611 (Fig. 4B). Similar results were obtained when OATP1A2 expression was assessed using Western blot analysis. T47-D cells treated with rifampin exhibited significantly higher OATP1A2 protein levels compared with those cells treated with rifampin and A-792611 (Fig. 4C). Cotreatment with A-792611 abolished the increased cellular accumulation of E1S associated with rifampin treatment (Fig. 5A).

Stimulated estrogen receptor signaling with OATP1A2 induction is attenuated by PXR antagonism. To determine whether OATP1A2-mediated E1S transport is associated with increased estrogenic effects, estrogen receptor–positive T47-D cells were transfected with an alkaline phosphatase reporter construct driven by an ERE and then treated with rifampin. When the cells were exposed to E1S and alkaline phosphatase activity was determined, estrogen receptor activation was significantly higher

Figure 2. Association of PXR with OATP1A2 expression and activity in T47-D cells. T47-D cells were treated with different concentrations of rifampin for 8 h, and the OATP1A2 expression was determined showing a concentration-dependent effect on transporter mRNA expression (A). To assess the effect of the transporter-associated uptake of E1S on the cell viability of T47-D cells, those breast cancer cells were pretreated with rifampin 1 μmol/L for 24 h, respectively, and then exposed to E1S (10 nmol/L). After 24 h of incubation, cell proliferation was determined using the AlamarBlue assay reagent (B). T47-D cells were transfected with siRNA-PXR to validate the interplay between PXR activation and OATP1A2 expression and function. The efficacy of the cellular knockdown of PXR was assessed by real-time PCR in cells transfected with siRNA-PXR (black) or siRNA-negative control (white, C). The level of OATP1A2 and PXR mRNA expression in cells transfected with the PXR-siRNA was determined by real-time reverse transcription–PCR (RT-PCR) after 8 h of incubation with rifampin (1 μmol/L; C). To assess the effect of the cellular knockdown on the rifampin-modulated OATP1A2 function, cells were transfected with the siRNA and treated with rifampin; subsequently, the cellular accumulation of [3H]E1S was determined (D). Columns, mean; bars, SD (n = 3). * P < 0.05, t test.
after pretreatment with the PXR activator rifampin. This increase of rifampin-stimulated estrogen receptor activity could be reduced by cotreatment with the PXR inhibitor A-792611 (Fig. 5B).

Determination of responsiveness of the OATP1A2 promoter fragment to constitutive and activated CAR. To determine whether the identified PXRRE (DR4-1) in the OATP1A2 promoter fragment is activated by CAR, a nuclear receptor which shares the same DNA motif as PXR (25), we performed a reporter gene assay on the responsive OATP1A2 promoter element. In fact, expression of CAR in HepG2 cells resulted in a significant induction of luciferase activity, which increased with treatment of the known CAR ligand, CITCO (10 μmol/L). This effect was not seen using the OATP1A2 promoter fragment harboring the mutation in the DR4-1 motif (Fig. 5C).

Expression of CAR in nonmalignant and malignant breast tissue. Assessing CAR mRNA expression by performing real-time PCR in human breast cancer tissue revealed significantly higher expression of CAR in nonmalignant compared with malignant breast tissue (CAR expression relative to nonmalignant tissue, mean ± SD; nonmalignant tissue, 1.01 ± 0.72 n = 4; malignant tissue, 0.21 ± 0.74 n = 4; t test, P < 0.05; data not shown). However, in T47-D and MCF-7 cells, detection of CAR did not reach the minimum level of detection, and this is in agreement with the analysis of OATP1A2 expression in T47-D cells after 24 hours of treatment with CITCO (1 μmol/L), which failed to result in the induction of OATP1A2 expression (Fig. 5D).

Discussion

The mammary gland is widely recognized as an estrogen target tissue. Estrogens are thought to act through the intracellular estrogen receptors to direct normal lobular development, regulate epithelial cell growth, and increase the expression of steroid hormone metabolizing enzymes (26, 27). In addition to those physiologic processes, the relationship between estrogens and the development and progression of breast cancer has been widely studied. Starting with the findings from George Beatson in 1896 reporting improved outcome from breast cancer in a postmenopausal woman after removal of her ovaries, subsequent studies

**Figure 3.** Luciferase reporter gene assays of OATP1A2 promoter fragments. Luciferase reporter gene assay of subcloned OATP1A2 promoter fragments revealed increased activity of luciferase in T47-D cells transfected with the −5120 to −6600 OATP1A2 promoter fragment after treatment with rifampin (10 μmol/L; A). In silico analysis of the responsive fragment revealed several potential DNA motifs (insert). Subsequent mutation of those motifs revealed a loss of transactivation mediated by rifampin (B) in HepG2 transfected with a construct containing a mutated DR4 motif. To validate the link between OATP1A2 transactivation by PXR, a ChIP was performed using different anti-PXR antibodies (A-20 and N-16 supplied by Santa Cruz). The PCR detecting a DNA fragment, which includes the identified DR4 motif, suggests that this DNA motif is involved in the PXR-mediated induction of OATP1A2 (C). Columns, mean; bars, SD (n = 3). * P < 0.05, t test.
have established a clear link between estrogens and the pathogenesis of breast cancer (28). Several studies have focused on plasma levels of estradiol and breast cancer risk. However, such studies have been inconsistent in linking breast cancer risk with increased plasma levels of estradiol (29, 30). Interestingly, endogenous E1S levels have been linked with a greater risk of breast cancer (29). E1S is a major circulating estrogen metabolite with a long half-life in humans (~9 hours), is found at high plasma levels in women (280 pg/mL), and is thought to be one of the major precursors of active estrogen in postmenopausal women (31). Due to the hydrophilic nature of this compound, it is likely that an uptake transporter is needed to facilitate the transmembrane entry into tissues, such as breast.

In this report, we show a tumor-specific overexpression of the uptake transporter OATP1A2 in breast tissue. Importantly, we show a PXR-mediated induction of this transporter can markedly enhance the extent of E1S uptake in breast cancer cells and significantly increase estrogen receptor–mediated gene transcription. Regulation of gene expression by PXR has been studied for a few Oats/OATPs. For example, activation of PXR in mouse causes hepatic up-regulation of Oatpl1a4 (Oatp2; ref. 32). In rats, Oatp2 (Oatpl1a4) gene expression is induced by phenobarbital and pregnenolone-16α-carbonitrile, well-known activators of CAR and PXR (33).

In breast tissues and breast cancer cell lines, including T47-D and MCF-7 cells, E1S is efficiently converted to bioactive estrone due to high steroid sulfatases (STS) activity (34, 35). Moreover, in breast cancer, there is a compelling association of tumor size and risk of recurrence with STS expression (36). In addition, pharmacologic targeting of STS is considered to be one of the potential strategies of breast cancer treatment. In fact, therapeutic reduction of STS activity resulted in a significant reduction in tumor growth when assessed using an animal model (37). Estrone is assumed to be converted to highly biological active 17β-estradiol by 17β-hydroxysteroid dehydrogenase type 1 (HSD1). Expressed level of this enzyme between nonmalignant and malignant tissue revealed not to differ (38). However, recent findings suggest that gene duplications of HSD1 may result in increased risk of breast cancer recurrence among estrogen receptor–positive breast cancer cases (39).

Of relevance to breast cancer biology, it has been shown that estrone and 17β-estradiol are moderate but sufficient activators of PXR (40, 41). In addition, several antiestrogens, such as tamoxifen, are PXR activators. Our data, which show direct functional

![Figure 4](image-url)

**Figure 4.** Inhibition of the PXR effect on OATP1A2, using a specific PXR inhibitor cell-based reporter gene assay in HepG2 cells using the reporter vector containing the −5120 to −6600 fragment of the OATP1A2 gene, was performed to determine the ability of A-792611 (10 μmol/L) to inhibit the rifampin (10 μmol/L)–associated PXR-mediated transactivation (A). T47-D cells were treated with rifampin (10 μmol/L) in the presence of different concentrations of A-792611 to determine the modulation of OATP1A2 expression using real-time RT-PCR (B). Western blot analysis was performed to evaluate the effect of rifampin (1 μmol/L) in the presence and absence of the PXR inhibitor A-792611 (1 μmol/L) on OATP1A2 expression in T47-D cells (C). Cell lysates of HeLa cells transfected with OATP1A2-pEF6 or pEF6-vector only were used as positive and negative controls. Actin expression was assessed in the same blot to visualize equivalent sample loading for the cell lysates (C).
interaction of PXR with promoter elements in SLCO1A2 would suggest that a feed forward regulation of OATP1A2 by PXR likely results in greater uptake of E1S. Accordingly, pharmacologic inhibition of PXR activation may have potential therapeutic effects by modulating breast cancer progression. Assuming that estrogen metabolites, such as E1S, are important driving forces in the development of breast cancer, the effect of uptake transporters in governing the accumulation of this steroid precursor in estrogenic target tissues should also be considered as an important pathophysiologic determinant. As shown in Fig. 6, the link between PXR activation and E1S accumulation may be of clinical relevance because estrone and 17β-estradiol are activators of PXR and, therefore, are likely to increase their own cellular uptake in responsive tissues.

It is of interest to note recent reports describing beneficial effects of HMG-CoA reductase inhibitors in terms of breast cancer risk (42–44). Efficacy of statins in breast cancer prevention is assumed to be related to the pleiotropic effects, including antiproliferative, proapoptotic, anti-invasive, and radiosensitizing properties of statins (45). In fact, it has been shown that lipophilic statins (simvastatin and fluvastatin) inhibit the growth of mammary tumor cells inoculated in neuTg mice. This reduction seemed to be due to statin-associated reduction in tumor proliferation and increased apoptosis (46). It should be noted that OATP1A2 not only transports E1S but also statins (47). Therefore, one plausible mechanism for their apparent beneficial effect to breast cancer proliferation might relate to reduced OATP1A2-mediated E1S uptake due to competition for uptake by statins.

It should be noted that PXR has also been linked to antiapoptotic effects in several cell types, including hepatocytes and prostate cancer cells. Recently, constitutive and pharmacologic activation of PXR was associated with antiapoptotic effects in colon cancer cells. Specifically, PXR activation resulted in increased expression of antiapoptotic genes, including BAG3, BIRC2, and MCL-1, thereby counteracting the effects of proapoptotic compounds, such as deoxycholic acid, adriamycin, staurosporine, and dimethylhydracine (48). Accordingly, if such an antiapoptotic effect is extended to our findings in relation to breast cancer, higher...
expression of PXR in neoplastic tissue would translate into a higher proliferative activity due to PXR-mediated activation of antiproliferative genes.

In summary, we have shown that OATP1A2 expression was highly increased in malignant human breast cancer tissues. Expression of the uptake transporter was associated with similarly elevated expression of PXR in malignant tissue, suggesting an interplay between the nuclear receptor PXR and OATP1A2. We show direct transactivation of SLC01A2 by PXR at a distal PXRRE and reduced PXR expression via siRNA technology or PXR function using a novel PXR antagonist significantly reduced the E3S-associated estrogen receptor signaling in a breast cancer cell line. In conclusion, the current finding suggests an important role of the xenobiotic nuclear receptor PXR to the regulated expression of the drug/hormone uptake transporter OATP1A2 and to the pathophysiology of breast cancer; therefore these proteins may be novel therapeutic targets for intervention.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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