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Tamoxifen Activates CYP3A4 and MDR1 Genes Through Steroid and Xenobiotic Receptor in Breast Cancer Cells

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Cytochrome P450 monooxygenase 3A4 (CYP3A4) and P-glycoprotein, encoded by multidrug resistance 1 (*MDR1*) gene, are responsible for the metabolism of endogenous steroids, prescribed drugs, and xenobiotics. Both genes are regulated by steroid and xenobiotic receptor (SXR), a member of nuclear hormone receptors. Various endogenous steroids and drugs function as ligands of SXR. Although CYP3A4, MDR1, and SXR are expressed mainly in the liver and the small intestine, these gene products are also expressed in breast cancer cells. Because tamoxifen (TAM) is known to be metabolized by CYP3A4 and P-glycoprotein, we investigated the effect of TAM on these SXR-targeted genes in breast cancer cells. Transient transfection-based reporter gene assays showed 4-hydroxy TAM activated the SXR-mediated transcription through CYP3A4 and MDR1 promoters in a ligand- and receptor concentration-dependent manner. We confirmed the binding of 4-hydroxy TAM to SXR by ligand binding assay. Moreover, semiquantitative RT-PCR studies revealed that 4-hydroxy TAM activated the expression of CYP3A4 and MDR1 mRNA in MCF-7 cells. These results suggest that TAM induces CYP3A4 and MDR1 gene expression through SXR, which may affect TAM metabolic pathway in breast cancer cells.

Key Words: Breast cancer; SXR; CYP3A4; MDR1; tamoxifen.

Introduction

Selective estrogen receptor modulators (SERMs) act by competing with estrogens for binding sites on estrogen receptor (ER). Tamoxifen (TAM) is the most frequently prescribed SERM, which has significant overall survival benefit in ER-positive breast cancer patients *(1–3)*.

Despite the relative safety and significant antineoplastic activities of TAM, a significant amount of initially responsive breast cancers acquire resistance to TAM *(4)*. However, it is hard for many physicians to predict the response to antiestrogens in ER (+) breast cancers *(5)*. Furthermore, many ER (+) and/or progesterone receptor (PR) (+) breast tumors are *de novo* resistant to TAM *(6)*. The mechanisms generating resistance in these cancers are not fully understood. A single mechanism or a single gene may not fully confer antiestrogen resistance. Rather, several mechanisms likely exist involving molecular, pharmacological, and immunological events *(6)*. Such events may occur in the liver, where TAM is mainly metabolized, or within breast cancer cells.

TAM is metabolized by cytochrome P450 monooxygenases (CYPs) *(7,8)*. CYP belongs to the superfamily of heme-containing monooxygenases that play an important role in the oxidative metabolism of endogenous substances, natural compounds, and xenobiotics *(9,10)*. Two different CYPs, CYP3A4 and CYP2D6, are involved in TAM metabolic pathway. CYP3A4 mainly produces *N*-desmethyl TAM *(11)*, and CYP2D6 produces 4-hydroxy TAM *(12)*. Because affinity of 4-hydroxy TAM to ER is 25–50 times higher than that of TAM *(13,14)*, 4-hydroxy TAM is considered as one of the most potent antiestrogens. Furthermore, because the concentration of 4-hydroxy TAM in serum is approx 5% of that of TAM *(13,15)*, indicating that 4-hydroxy TAM is the major compound to inhibit estrogen action through ER. On the other hand, the affinity of *N*-desmethyl TAM to ER is less than 1 % of that of TAM *(13,14)*. Thus, *N*-desmethyl TAM is a biologically weaker antiestrogen. Activation of CYP3A4 preferentially metabolizes TAM to *N*-desmethyl TAM and relatively reduces the 4-hydroxy TAM level, which may lead to TAM resistance.

TAM concentration in serum/tissue may be also modulated by P-glycoprotein (P-gp). P-gp, which is encoded by multidrug resistance (*MDR1*) gene, is a member of the ATP-

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binding cassette superfamily of active transporters and functions as an energy-dependent efflux pump that reduces the intracellular concentration of cytotoxic compounds *(16, 17)*. In the intestine, P-gp is involved in transporting substances, including TAM, back into the intestinal lumen to be absorbed or eliminated *(17)*.

Although the major organ expressing CYP3A4 and MDR1 gene product is the liver and the intestine, respectively *(9,10,17)*, recent reports have shown that these are also expressed in breast cancer cells *(18–20)*. The expression of these genes is regulated by steroid and xenobiotic receptor (SXR) *(9,19,20)*. SXR has been cloned from the liver cDNA and classified as a member of the steroid/thyroid hormone receptor superfamily, which binds to many steroids and their metabolites, prescribed drugs, and xenobiotics *(9,10)*.

Unlike classical nuclear hormone receptors, SXR is described as "broad specificity and low affinity sensing receptor" *(21)*, owing to its wide ligand binding surface, which accounts for the binding of a variety of ligands *(22)*. SXR forms heterodimer with retinoid X receptor (RXR) on steroid and xenobiotic-response elements (SXREs), located in the promoter region of target genes *(21,23,24)*. SXR is expressed in the liver, small intestine *(9,10)*, and in a variety of breast cancer cell lines *(25)*. Although no detailed analysis has been performed *(21)*, one previous study has shown that TAM could be a ligand for SXR.

Because many substances bind to SXR, and the protein products of CYP3A4 and MDR1 genes, which metabolize TAM, are all expressed in breast cancer cells, we hypothesize that SXR in breast cancer cells may be associated with TAM resistance. Therefore, in the present study, we investigated the transcriptional regulation and mRNA expression of CYP3A4 and MDR1 genes by TAM in the presence and absence of SXR in MCF-7 breast cancer cells.

Results

TAM and 4-Hydroxy TAM Activate Transcription of CYP3A4 and MDR1 Genes in a Dose-Dependent Manner

To determine the action of TAM on SXR-mediated transcription on SXRE–containing promoter, we performed a series of transient transfection–based reporter gene assays using MCF-7 breast cancer cells and CV-1 fibroblast-derived cells. Because low levels of SXR are expressed in MCF-7 and CV-1 cells, we examined the effect of intrinsic SXR on transcription. CYP3A4-SXRE-LUC was used as SXR target reporter. In the absence of external SXR, transcription of CYP3A4 gene was slightly activated by 10^{-6} *M* rifampicin or 4-hydroxy TAM in both cell lines (Figs. 1A,B). Cotransfection of SXR induced an increase in basal transcriptional activity and the response to rifampicin or 4-hydroxy TAM. Therefore, we cotransfected SXR in the following experiments.

Because 4-hydroxy TAM is a metabolite of TAM, we examined if these two compounds possess fundamental differences on SXR-mediated transcription. Although the magnitude was different, a similar tendency of transcriptional activation was observed between TAM and 4-hydroxy TAM (Figs. 1C and 2A), suggesting that there was no essential difference between these two compounds on SXR-mediated transcription. Thus, we used 4-hydroxy TAM in the following experiments.

Next, we examined the time course of SXR-mediated transcription by 4-hydroxy TAM (Fig. 1D). 4-Hydroxy TAM–induced transcription from 24 h after adding ligand, and reached the maximum level at 36 h. Thus, 4-hydroxy TAM activated transcription in a time related manner.

To examine the effect of 4-hydroxy TAM, expression vector encoding the SXR and reporter plasmid CYP3A4-SXRE-LUC or MDR1-SXRE-LUC were cotransfected into MCF-7 (Figs. 2A,C) and CV-1 (Figs. 2B,D) cells. Transfected cells were cultured in the presence of 10^{-10}–10^{-5} *M* of 4-hydroxy TAM, and rifampicin was used as a positive control. Increasing amounts of 4-hydroxy TAM activated SXR-mediated transcription in a dose-dependent manner in MCF-7 and CV-1 cells. In CV-1 cells, significant activation of transcription through CYP3A4 promoter was seen following treatment with 4-hydroxy TAM in orders higher than 10^{-6} *M*. Whereas, in MCF-7 cells, treatment with 4-hydroxy TAM in orders as low as 10^{-8} *M* activated the transcription of CYP3A4, suggesting that MCF-7 cells are likely to be more sensitive to 4-hydroxy TAM than CV-1 cells under the same experimental condition.

Although the mechanism of differential response to 4-hydroxy TAM between two cells is not known, one possibility is the differential expression of ER. Because ERα is expressed in MCF-7 cells but not in CV-1 cells, we investigated the effect of ERα on SXR-mediated transcription through SXRE in CV-1 cells. ERα did not affect on the SXR-mediated transcription induced by 4-hydroxy TAM through SXRE (data not shown). In addition, ERα did not activate the transcription through SXRE in the presence of 4-hydroxy TAM in CV-1 cells (data not shown). These results indicated that ERα may not affect on the transcription through SXRE.

Because SXR also regulates the expression of MDR1 *(22)*, we tested the effect of 4-hydroxy TAM through the MDR1 promoter in both MCF-7 and CV-1 cells (Fig. 2C, D). 4-Hydroxy TAM activated SXR-mediated transcription driven by MDR1 promoter in a dose-dependent manner (Figs. 2C,D), similar to that seen in CYP3A4 (Figs. 2A,B).

In addition, because it has been reported that a series of steroid hormone activated SXR-mediated transcription, we examined the effect of 17β-estradiol (E2) on SXR-mediated transcription, and obtained that E2 activated the transcription through both CYP3A4 and MDR1 containing promoters as expected (data not shown).

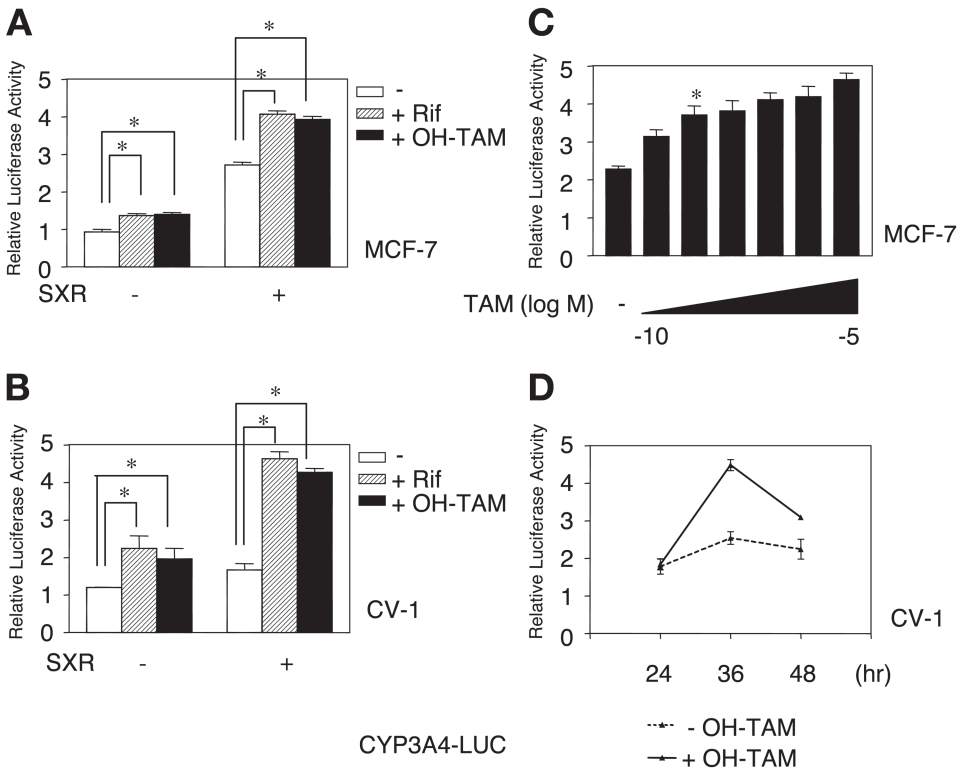

Fig. 1. TAM and 4-hydroxy TAM activated SXRE-mediated transcription. (**A,B**) Expression plasmid encoding SXR (30 ng) was cotransfected with CYP3A4-SXRE-LUC reporter plasmid (400 ng) into MCF-7 (**A**) or CV-1 (**B**) cells. Cells were grown in the absence or presence of 10^{-6} *M* of rifampicin or 4-hydroxy TAM. (**C**) Expression plasmid encoding SXR (30 ng) was cotransfected with CYP3A4-SXRE-LUC reporter plasmid (400 ng) into MCF-7 cells. Cells were grown in the absence or different amounts of TAM (10^{-10}–10^{-5} *M*). Data are the mean of at least five separate experiments in triplicate ± standard error of means (SEM). *Statistically significant ($p < 0.01$ by ANOVA) vs ligand (–) column. (**D**) Expression plasmid encoding SXR (30 ng) was cotransfected with CYP3A4-SXRE-LUC reporter plasmid (400 ng) into CV-1 cells. Cells were grown in the absence or presence of 10^{-6} *M* of 4-hydroxy TAM for 24, 36, and 48 h, respectively. *Statistically significant ($p < 0.01$ by ANOVA) vs ligand (–) column. SXR: steroid and xenobiotic receptor, Rif: rifampicin, OH-TAM: 4-hydroxy tamoxifen, CYP3A4: cytochrome P450 monooxygenase 3A4, SXRE: steroid and xenobitic response element.

4-Hydroxy TAM Activates the Transcription of CYP3A4 and MDR1 Genes Following a Dose-Dependent Increase in SXR

Reporter plasmid CYP3A4-SXRE-LUC or MDR1-SXRE-LUC were cotransfected into MCF-7 and CV-1 cells in the presence of 10^{-5} *M* of 4-hydroxy TAM. In the absence of SXR, CYP3A4 and MDR1 gene transcriptions were slightly activated. However, increasing amounts of SXR activated transcription in a dose-dependent manner (Figs. 3A,B) with essentially no difference between CYP3A4 and MDR1 gene activation in MCF-7 and CV-1 cell lines (Figs. 3C,D). These results indicate that 4-hydroxy TAM activated CYP3A4 and MDR1 promoter through SXR.

Activation of CYP3A4 Transcription by 4-Hydroxy TAM via SXR Is Induced by RXR

We further examined the effect of 4-hydroxy TAM on SXR in the presence of RXR, which forms a heterodimer with SXR. Cotransfection of RXRβ with SXR resulted in induction of 4-hydroxy TAM-activated transcription in the presence of 10^{-7} *M* 9-*cis* retinoic acid (9cRA) (Fig. 4). This result is similar to that shown with HepG2 cells *(13)*, and suggests that RXR cooperatively activates transcription with SXR in CV-1 cells.

4-Hydroxy-TAM Bound to SXR Directly

To determine the binding affinity of TAM to SXR, GST-SXR-LBD was incubated with [^{3}H]labeled TAM in the presence or absence of increasing concentration of unlabeled TAM. Scatchard analysis revealed a single, low affinity binding site with a slope of 0.0031. Linear regression gave a K_d of 322.6 n*M* (Fig.5). Although K_d is low, we confirmed the binding of 4-hydroxy TAM to SXR.

Expression of CYP3A4 and MDR1 mRNAs in MCF-7 and Other Breast Cancer Cells

To investigate the expression of SXR-regulated gene in breast cancer cell lines, we performed semiquantitative RT-PCR studies using MCF-7 cells. After 5 d in culture with 10^{-6}–10^{-5} *M* 4-hydroxy TAM, the expression of CYP3A4 and MDR1 mRNAs was measured. GAPDH mRNA was used as an internal control. In MCF-7 cells, the level of

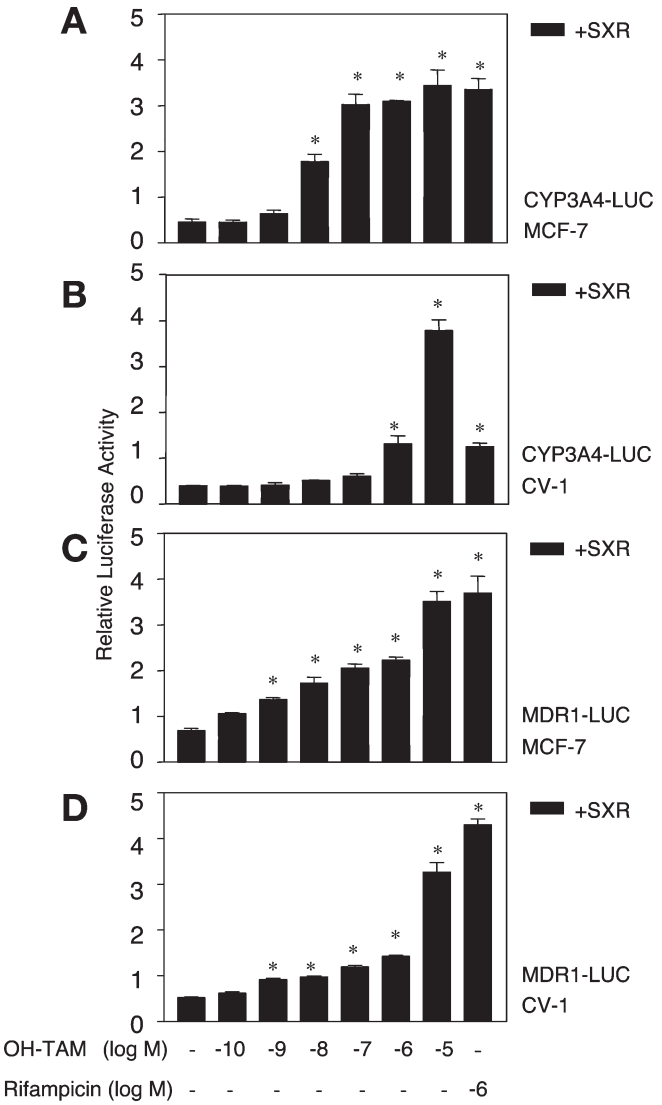

Fig. 2. 4-Hydroxy TAM activated SXRE-mediated transcription through CYP3A4 and MDR1 promoter in a dose dependent manner. Expression plasmid encoding SXR (30 ng) was cotransfected with CYP3A4-SXRE-LUC (**A,B**) or MDR1-SXRE-LUC (**C,D**) reporter plasmid (400 ng) into MCF-7 (**A,C**) or CV-1 (**B**) cells. Cells were grown in the presence or absence of different amounts of 4-hydroxy TAM. Data are the mean of at least five separate experiments in triplicate ± SEM. *Statistically significant ($p <$ 0.01 by ANOVA) vs ligand (–) column. SXR: steroid and xenobiotic receptor, OH-TAM: 4-hydroxy tamoxifen, CYP3A4: cytochrome P450 monooxygenase 3A4, MDR1: multidrug resistance 1.

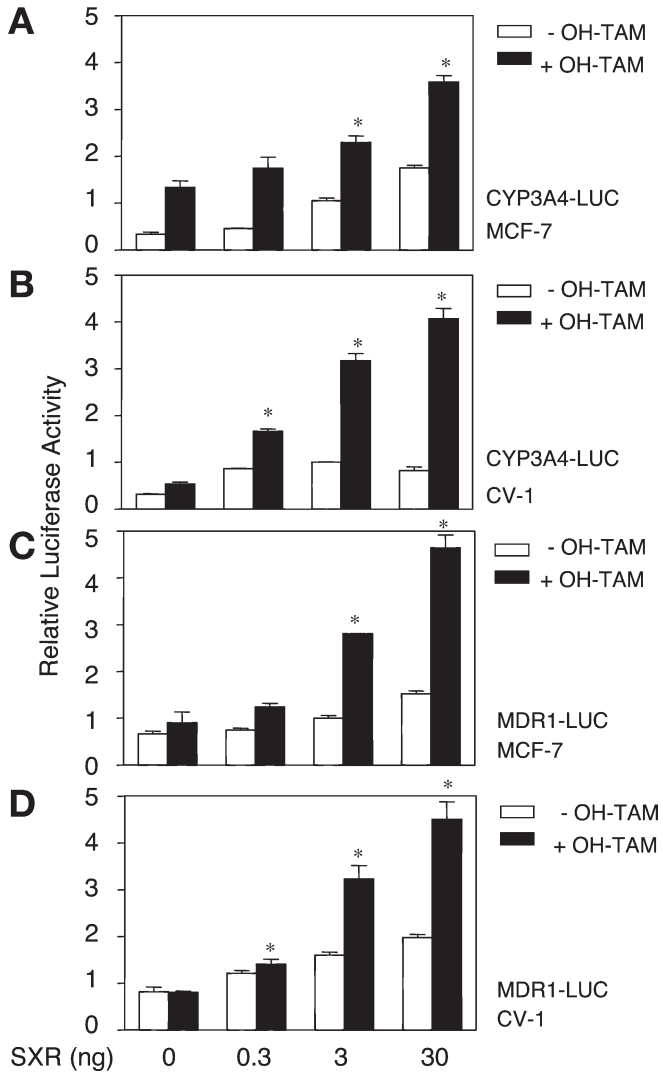

Fig. 3. 4-Hydroxy TAM activated transcription through SXR binding to CYP3A4 and MDR1 promoters. (**A,B**) Different amounts of the expression plasmid encoding SXR were cotransfected with CYP3A4-SXRE-LUC reporter plasmid (400 ng) into MCF-7 (**A**) or CV-1 (**B**) cells. Cells were grown in the presence or absence of 10^{-6} *M* of 4-hydroxy TAM. (**C,D**) Different amounts of the expression plasmid encoding SXR were cotransfected with MDR1-SXRE-LUC reporter plasmid (400 ng) into MCF-7 (**C**) or CV-1 (**D**) cells. Cells were grown in the presence or absence of 10^{-5} *M* of 4-hydroxy TAM. Total amounts of DNA for each well were balanced by adding vector pcDNA3. Data are the mean of at least five separate experiments in triplicate ± standard error of means (SEM). *Statistically significant ($p < 0.01$ by ANOVA) vs SXR (–), 4-hydroxy TAM (+) column. SXR: steroid and xenobiotic receptor, OH-TAM: 4-hydroxy tamoxifen, CYP3A4: cytochrome P450 monooxygenase 3A4, MDR1: multidrug resistance 1.

CYP3A4 mRNA was about 150% of that in 4-hydroxy TAM (–) cells (Fig. 6). Similarly, the level of MDR1 mRNA was approx 150% of that in 4-hydroxy TAM (–) cells (Fig. 6). These results indicate that 4-hydroxy TAM activates the expression of endogenous CYP3A4 and MDR1 in MCF-7 cells.

To investigate if CYP3A4 or MDR1 mRNAs are expressed in other breast cancer cell lines, we performed semiquantitative RT-PCR studies using MDA-MB231, T-47D, and ZR75-1 cells. GAPDH was used as an internal control. It has been reported that SXR is expressed in MDA-MB231 and T-47D cell lines *(25)*, and that ER is expressed in T-47D and ZR75-1 cell lines but not in MDA-MB-231 cells. CYP3A4 mRNA expression was seen in all cell lines examined. A large amount of MDR1 mRNA expression was seen

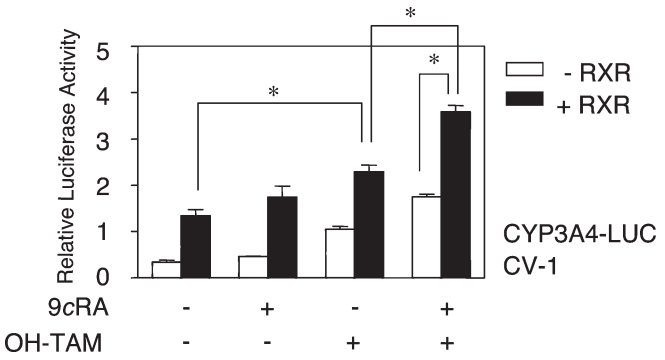

Fig. 4. Activation of CYP3A4 transcription by 4-hydroxy TAM via SXR is induced by RXR. Expression plasmid encoding SXR (30 ng) and/or RXRβ (30 ng) was cotransfected with CYP3A4-SXRE-LUC reporter plasmid (400 ng) into CV-1 cells. Cells were grown in the presence or absence of 10^{-7} *M* of 9-*cis* retinoic acid (9cRA) and/or 10^{-6} *M* of 4-hydroxy TAM. Total amount of DNA for each well was balanced by adding vector pcDNA3. Data are the mean of at least five separate experiments in triplicate ± SEM. *Statistically significant ($p < 0.01$ by ANOVA) 9cRA: 9-*cis* retinoic acid, OH-TAM: 4-hydroxy tamoxifen, CYP-3A4: cytochrome P450 monooxygenase 3A4, RXR: retinoid X receptor.

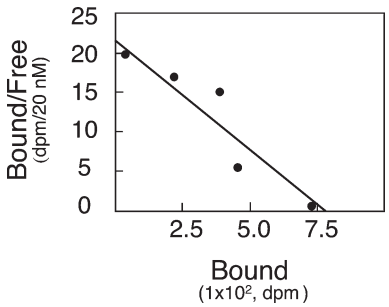

Fig. 5. Binding of tamoxifen to SXR. GST-fused SXR-LBD (57.8 μg) was incubated in triplicate with 0.76 pM of [^{3}H]4-hydroxy-tamoxifen methiodide, [*N*-methyl-^{3}H], in the presence or absence of increasing concentration (0–25.8 μ*M*) of unlabeled 4-hydroxy tamoxifen. Binding was performed end-over-end for 5 h at 37°C. Unbound ligand was removed by centrifugation, and the bound radioactivity was measured with a liquid scintillation counter.

in MDA-MB231 and ZR75-1 cell lines but a low amount in T47-D cells (Fig. 7).

Discussion

In the present study, we show for the first time that 4-hydroxy TAM, a potent antiestrogenic metabolite of TAM, activates SXR-mediated transcription through the CYP3A4 and MDR1 promoters in breast cancer cell lines. No essential difference was observed between TAM and 4-hydroxy TAM induced SXR-mediated transcription. The expression of intrinsic CYP3A4 and MDR1 gene was activated by 4-hydroxy TAM approx 1.5-fold in MCF-7 cells. We confirmed the direct binding of 4-hydroxy TAM to SXR in vitro. These results indicate that 4-hydroxy TAM activates the

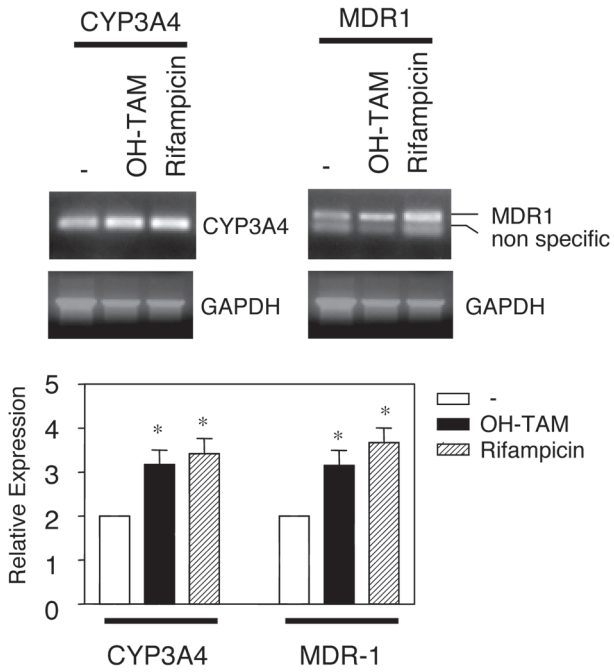

Fig. 6. 4-Hydroxy TAM induced expression of intrinsic CYP3A4 and MDR1 in MCF-7 cells. MCF-7 cells were incubated in the absence or presence of 10^{-5} *M* of 4-hydroxy TAM or 10^{-6} *M* rifampicin for 5 d. First-strand RNAs were obtained from total RNAs from each plate with reverse transcriptase and random decamer. Semiquantitative RT-PCR was performed with primers indicated in Material and Methods. Bands were obtained from increasing phase of PCR products. Each band was normalized with GAPDH expression. Each experiment was performed three times in five independent experiments. Data are the mean of at least five separate experiments in triplicate ± SEM. *Statistically significant ($p < 0.01$ by ANOVA) vs ligand (–) column. OH-TAM: 4-hydroxy tamoxifen, CYP3A4: cytochrome P450 monooxygenase 3A4, MDR1: multidrug resistance 1, GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

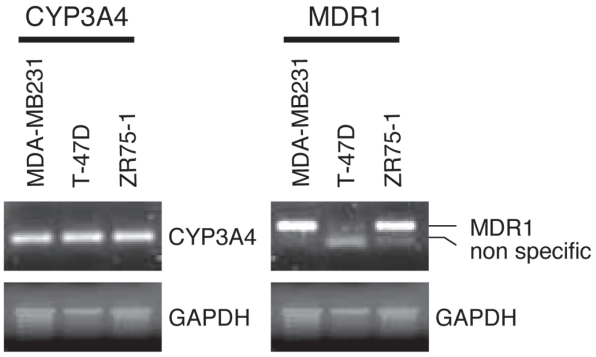

Fig. 7. Expression of CYP3A4 and MDR1 mRNA in breast cancer cell lines. MDA-MB231, T-47D, and ZR75-1 cells were incubated in the absence or presence of 10^{-5} *M* of 4-hydroxy TAM or rifampicin for 5 d. First-strand cDNAs were obtained from total RNAs from each plate with reverse transcriptase and random decamer. Semiquantitative RT-PCR was performed with primers indicated in Material and Methods. Bands were obtained from increasing phase of PCR products. Each band was normalized with GAPDH expression. Each experiment was performed at least six times and we obtained an identical result. CYP3A4: cytochrome P450 monooxygenase 3A4, MDR1: multidrug resistance 1, GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

transcriptions of CYP3A4 and MDR1 genes by binding to SXR.

As mentioned above, TAM is metabolized by two CYPs. Because of much higher affinity of 4-hydroxy TAM to ER than that of TAM and *N*-desmethyl TAM, 4-hydroxy TAM has been considered as the most potent antiestrogen. Activation of CYP3A4 preferentially produces *N*-desmethyl TAM. Furthermore, both 4-hydroxy TAM and *N*-desmethyl TAM are metabolized by CYP3A4 *(26)*. Thus, activation of CYP3A4 may greatly influence the TAM metabolic pathway by facilitating the metabolism of TAM and 4-hydroxy TAM, and by preferentially producing *N*-desmethyl TAM instead of 4-hydroxy TAM. The present study demonstrates that 4-hydroxy TAM and TAM by binding to SXR can facilitate the transcription of CYP3A4 and may result in decrease the active TAM concentration. We showed the binding of 4-hydroxy TAM to SXR-LBD using ligand binding assay, although binding affinity was low. TAM is metabolized mainly in the liver, catalyzed by the enzymes, CYP3A4 and CYP2D6, which are predominantly expressed in this organ *(26)*. However, recent studies indicate that CYP3A4 as well as SXR are also expressed in breast cancer cells *(18,25,27,28)*. Furthermore, the present study demonstrates that the effect of 4-hydroxy TAM is greater in MCF-7 cells than in CV-1 cells, and that CYP3A4 expression in MCF-7 cells is induced by TAM treatment. These results suggest that a metabolic pathway for TAM exists in breast cancer cells, which could influence its local action. In addition, the transcription of MDR1 is also regulated by TAM through SXR as shown in MCF-7 cells in the present study. Apparently, the MDR1 is expressed in breast cancer cells, which includes MCF-7 cells *(18)*. Because P-gp, a protein product of *MDR1* gene, eliminates many substances including TAM from cells *(29)*, activation of *MDR1* gene in breast cancer cells may also play an important role in altering local TAM concentration. Overall, the activation of SXR-mediated transcription by TAM in breast cancer cells could alter the sensitivity to E2, which could lead to local TAM resistance. Furthermore, because CYP3A4 and MDR1 are regulated by intrinsic steroid, many xenobiotics, and prescribed drugs, activation of these genes may lead to multi-drug resistance. For instance, we reported that the endocrine-disrupting chemical, polychlorinated biphenyl, activated the SXRE-containing promoter in MCF-7 cells (Miyazaki et al. abstract in The 87th Annual Meeting of The Endocrine Society, The forum on Endocrine Disrupting Chemicals, 2005).

In breast cancer cells, numerous coactivators such as AIB1/SRC-3/TRAM1 *(30–32)*, SRA *(33)*, and AIB3/ASC-2/TRBP *(34–36)* are overexpressed. Transfection of these coactivators further activates SXR-mediated transcription by 4-hydroxy TAM (data not shown). The effect of 4-hydoxy TAM was greater in MCF-7 than in CV-1 cells. We confirmed that ERα did not affect on the SXR-mediated transcription induced by 4-hydroxy TAM through SXRE (data not shown). These results indicate that ERα may be excluded for the differential sensitivity of TAM between two cells. Although the mechanism for this difference is unknown, we speculate that numerous coactivators in cells may contribute to the differential TAM action between the two cell lines.

SXR forms a heterodimer with RXR to interact with coactivators to induce transcription. Thus, we examined the effect of RXR on SXR-mediated transcription. The SXR–RXR heterodimer is considered to be non-permissive for RXR activation and SXR are known to be activated themselves by RXR-selective compounds and 9cRA in HepG2 cells. The data shown in Fig. 4 may suggest that cotransfection of RXR and SXR leads to a higher amount of productive heterodimers and hence reporter activation than simply transfecting SXR, and that 9cRA acts additively with OH-TAM in activating SXR.

To date, the expression of SXR in breast cancer cells has not been considered as a critical factor for prognosis. However, as shown in the present study, differential expression of SXR may alter the TAM metabolism, which could account for the variable response against TAM treatment. Thus, during pathological diagnosis of breast cancer, in addition to examination of estrogen and progesterone receptors, quantitative or histochemical examination of SXR could be useful to select appropriate therapies. Trials to examine SXR expression in surgically removed breast tumor tissue are currently underway.

In summary, we have shown TAM as a ligand for SXR, which induces transcription of CYP3A4 and MDR1 in MCF-7 cells through SXRE in their promoters. Because SXR, CYP3A4, and MDR1 are expressed in breast cancer cells, therefore, TAM could activate one or several of these factors to facilitate its own metabolism. The expression of SXR in breast cancer cells could be associated with the alteration of the metabolic pathway in breast cancer. Thus, SXR should be taken into account when selecting therapies for breast cancer.

Materials and Methods

Reagents and Chemicals

Rifampicin and 4-hydroxy tamoxifen were obtained from Sigma (St. Louis, MO). Tamoxifen was purchased from Wako Pure Chemical Industries (Osaka, Japan).

Plasmids

The human SXR in pCDG1, the expression vector RXRβ/pcAMP, and the CYP3A4-SXRE-luciferase (LUC) reporter construct, containing the enhancer (nucleotides –7836 to –7208) and the promoter (nucleotides –362 to +53) of human CYP3A4 driving luciferase expression have been described elsewhere *(37)*. The MDR1 promoter fragment (–7975 to –7013) containing the cluster of SXRE *(14)* was amplified by PCR from the BAC clone CTB-60P12 (Invitrogen, Carlsbad, CA; GenBank™ accession number AC002457) with a

forward primer (5'-CGC GGA TCC TCT GCT AGC AGT GTT TCT TGT ATA-3') containing an artificial *BamH*I site and a reverse primer (5'-TAC GGG GTA CCC ATA TAA GGC AAC TGT TTT GTT-3') containing an artificial *Kpn*I site. The PCR fragment was then ligated between *BamH*I/*Kpn*I sites of pT109-LUC vector *(38)*, which contains the thymidine kinase (TK) minimum promoter to create MDR1-SXRE-LUC.

Transient Cotransfection Experiments

MCF-7 cells were grown in RPMI 1640 medium with 10% fetal bovine serum. CV-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM), containing 10% fetal bovine serum. The serum was stripped of hormones by constant mixing with 5% (w/v) AG1-X8 resin (Bio-Rad, Hercules, CA) and powdered charcoal before ultrafiltration. Cells were maintained without antibiotics. Cells were transiently transfected using the calcium phosphate coprecipitation method or LipofectoAmine 2000 (Invitrogen) in 12-well plates. CMV-β-galactosidase plasmid was cotransfected as an internal control. Cells were grown for 24–48 h in the absence or presence of ligands and then harvested. Cell extracts were analyzed for both luciferase and β-galactosidase activity to correct for transfection efficiency as described previously *(37,39)*. All transfection studies were repeated at least five times in triplicate and confirmed the consistency of results. The results are shown as mean ± standard error of means (SEM) of experiments performed in triplicate. Statistical comparisons were made by ANOVA. Post-hoc comparisons were made using the Bonferroni test.

Ligand-Binding Assay

GST protein of SXR-LBD (57.8 μg) was incubated in triplicate with 0.76 p*M* of [^{3}H]4-hydroxy tamoxifen methiodide, [N-methyl-^{3}H] (specific activity 85 Ci/mmol, American Radiolabeled Chemicals Inc., St. Louis, MO), in the presence or absence of increasing concentration (0–25.8 μ*M*) of unlabeled tamoxifen (4-hydroxytamoxifen, Sigma, St. Louis, MO). Binding was performed end-over-end for 5 h at 37°C. Unbound ligand was removed by centrifugation, and the bound radioactivity was measured with a liquid scintillation counter.

Semiquantitative RT-PCR

The primers used for RT-PCR are as follows: CYP3A4-sense, 5'-CTT CAT CCA ATG GAC TGC ATA AAT-3'; CYP3A4-antisense, 5'-TCC CAA GTA TAA CAC TCT ACA CAG ACA A-3'; MDR1-sense, 5'-CCC ATC ATT GCA ATA GCA GG-3'; MDR1-antisense, 5'-GTT CAA ACT TCT GCT CCT GA-3'; GAPDH-sense, 5'-ATG GGG AAG GTG AAG GTC GGA GTC A-3'; GAPDH-antisense, 5'-CTA CTC CTT GGA GGC CAT GTG GGC C-3'. Total RNA was obtained from MCF-7 cells using TRIsol (Invitrogen) following the manufacturer's instructions. To obtain the first-strand cDNA, RETROSCRIPT kit was used following the manufacturer's protocol (Ambion, Austin, TX). Expressed bands were confirmed by sequencing and the intensity of ethidium bromide–stained band was analyzed by Kodak Digital Science EDAS 290 system. The intensity of CYP3A4 and MDR1 band was normalized with that of GAPDH as an internal standard. A linear relationship between the inverse in band intensity and the number of PCR cycles was plotted. The PCR cycles were then determined from the plot as 45 cycles for CYP3A4 and MDR1, and 30 cycles for GAPDH.

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