

Induction of multidrug resistance associated protein 2 in tamoxifen-resistant breast cancer cells

Hoo Kyun Choi, Jin Won Yang, Sang Hee Roh, Chang Yeob Han and Keon Wook Kang

BK21 Project Team, College of Pharmacy, Chosun University, 375 Seosuk-dong, Dong-gu, Gwangju 501-759, South Korea

(Requests for offprints should be addressed to K W Kang; Email: kwkang@chosun.ac.kr)

H K Choi and J W Yang contributed equally to this work

Abstract

Acquired resistance to tamoxifen (TAM) is a serious therapeutic problem in breast cancer patients. The transition from chemotherapy-responsive breast cancer cells to chemotherapy-resistant cancer cells is mainly accompanied by the increased expression of multidrug resistance-associated proteins (MRPs). In this study, it was found that TAM-resistant MCF-7 (TAMR-MCF-7) cells expressed higher levels of MRP2 than control MCF-7 cells. Molecular analyses using *MRP2* gene promoters supported the involvement of the pregnane X receptor (PXR) in MRP2 overexpression in TAMR-MCF-7 cells. Although CCAAT/enhancer-binding protein β was overexpressed continuously in TAMR-MCF-7 cells, this might not be responsible for the transcriptional activation of the *MRP2* gene. In addition, the basal activities of phosphatidylinositol 3-kinase (PI3-kinase) were higher in the TAMR-MCF-7 cells than in the control cells. The inhibition of PI3-kinase significantly reduced both the PXR activity and MRP2 expression in TAMR-MCF-7 cells. Overall, MRP2 induction plays a role in the additional acquisition of chemotherapy resistance in TAM-resistant breast cancer.

Endocrine-Related Cancer (2007) 14 293–303

Introduction

Chemotherapy is the most widely applied tool for treating metastatic tumors. However, its success is limited by the ability of cancer cells to acquire chemoresistance to a broad range of anti-cancer agents. Multidrug resistance (MDR) in cancer is a phenomenon of resistance to a number of structurally different chemotherapeutic agents after exposure to one anti-cancer agent. One of the most important reasons for cancer chemotherapy resistance is the increased expression of the drug transporters that causes the efflux of the chemotherapeutic agents from the cancer cells (Gottesman *et al.* 2002). The ATP-binding cassette (ABC) transporter family includes ABCB1 (*P*-glycoprotein, *P*-gp, or MDR1), ABCC1 (MDR-associated protein 1, MRP1), and ABCC2 (MRP2, or cMOAT). These transporters are believed to be involved in the efficient pumping of a drug out of the cell, and there is an inverse correlation between

their functional expression and the chemotherapy response in various cancers (Kool *et al.* 1997, van Tellingen *et al.* 2003). Among the ABC transporters, MRP2 is a 190 kDa phosphoglycoprotein that is localized in the apical membrane of cancer cells and is involved in the transport of either xenobiotics including chemotherapeutic agents (e.g. cisplatin, anthacyclines, vinca alkaloids, and methotrexate) or various conjugated metabolites (Paulusma *et al.* 1996, Kawabe *et al.* 1999, Schrenk *et al.* 2001).

Breast cancer is the most common malignancy in western women. The ability to reduce breast tumor growth through the administration of anti-estrogens has played a key role in the endocrine therapy of breast cancer. A non-steroidal anti-estrogen, tamoxifen (TAM), is a representative drug that has been approved for the chemoprevention of breast cancer and is the most widely used anti-estrogen in estrogen receptor-positive breast cancer patients (Rose *et al.* 1985).

Although most patients are initially responsive, the acquisition of resistance to TAM is the main problem of anti-estrogen therapy (Clemons *et al.* 2002).

In order to establish a TAM-resistant breast cancer cell line, Gottardis & Jordan (1988) administered TAM long term to nude mice bearing MCF-7 cells, and the isolated MCF-7 cells became TAM resistant. In addition, a continuous culture of human breast cancer cell lines with TAM can cause the development of cell lines that are resistant to TAM. It was reported that a long-term culture of MCF-7 cells with TAM or 4-hydroxytamoxifen results in the formation of TAM-resistant cells (Badia *et al.* 2000, Knowlden *et al.* 2003). We have also established MCF-7-derived TAM-resistant cell line (TAMR-MCF-7 cells) by the long-term (>9 months) culture of MCF-7 cells with 4-hydroxytamoxifen.

Previous studies have shown that the transition of chemotherapy-responsive cancer cells to chemotherapy-resistant cancer cells is accompanied mainly by the increased expression of ABC transporters (Lage 2003). Considering that the next line of chemotherapy against TAM-resistant breast cancer involves a methotrexate- or cisplatin-containing regimen (Blumenschein *et al.* 1997, Assikis *et al.* 2003), a study on whether the expression levels of the ABC transporter can be changed in TAM-resistant breast cancer cells might lead to a better understanding of the pathological implications of TAM resistance. This study shows for the first time that the expression of MRP2 is higher in TAMR-MCF-7 cells when compared with those in MCF-7 cells and demonstrates that the phosphatidylinositol 3-kinase (PI3-kinase)-dependent activation of PXR is essential for the induction of MRP2 in TAM-resistant breast cancer cells.

Materials and methods

Materials

The anti-MRP2, anti-MDR1, and anti-PXR antibodies were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-aromatase antibody was purchased from Abcam (Cambridge, MA, USA). The horseradish peroxidase-conjugated donkey anti-rabbit IgG, anti-goat IgG, and alkaline phosphatase-conjugated donkey anti-mouse IgG were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). The 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium was acquired from Life Technologies. The antibodies for phosphorylated Akt and Akt antibodies were purchased from Cell Signaling Technology

(Beverly, MA, USA). Most of the reagents used for molecular studies were obtained from Sigma. The p2635-MRP2-Luc reporter plasmids containing human MRP2 promoter region (–2635 bp), p491-MRP2-Luc, and p491-MRP2-Luc-Mut were kindly provided by Dr Uchiyama T (Kyushu University, Fukuoka, Japan; Tanaka *et al.* 1999). The PXR reporter plasmids containing three copies of the pregnane X receptor (PXR)-responsive elements from the CYP3A23 gene and pGL3-MRP2-1 (rat MRP2 gene promoter) were kind gifts from Dr Edward PA (University of California Los Angeles, Los Angeles, CA, USA).

Cell culture and establishment of TAM-resistant MCF-7 cells

The MCF-7 cells were cultured at 37 °C in 5% CO₂/95% air in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 µg/ml streptomycin. The TAM-resistant MCF-7 cells (TAM-R) were established using the methodology reported elsewhere (Knowlden *et al.* 2003). Briefly, the MCF-7 cells were washed with PBS, and the culture medium was changed with phenol red-free DMEM containing 10% charcoal-stripped steroid-depleted FBS (Hyclone, Logan, UT, USA) and 4-hydroxytamoxifen (0.1 µM). The cells were continuously exposed to this treatment regimen for 2 weeks and the concentration of 4-hydroxytamoxifen was gradually increased up to 3 µM over a 9-month period. Initially, the cell growth rates were reduced. However, after exposure to the medium for 9 months, the rate of cell growth gradually increased, showing the establishment of a TAM-resistant cell line.

MTT cell viability assay

To determine the cell viabilities, the cells were plated at 10⁴ cells/well in 96-well plates. For the cytotoxicity determination by 4-hydroxytamoxifen, MCF-7, and TAMR-MCF-7 cells were incubated in the FBS-free medium with or without 4-hydroxytamoxifen (0.1–30 µM) for 24 h. To determine the effect of aromatase inhibitor on testosterone-mediated cell proliferation, MCF-7, and TAMR-MCF-7 cells were cultured in phenol red-free DMEM containing 5% charcoal-stripped steroid-depleted FBS in the presence or absence of testosterone (0.1 µM)/4-hydroxyandrostenedione (0.1–1 µM) for 48 h. The viable adherent cells were stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; 2 mg/ml) for 4 h. The media were then removed and the formazan crystals produced were dissolved by adding 200 µl

dimethylsulfoxide/well. The absorbance was assayed at 540 nm. The cell viability was expressed as the relative ratios to the untreated control cells.

Preparation of nuclear extracts

The nuclear extracts were prepared essentially according to a previously published method (Schreiber *et al.* 1990). Briefly, the cells in the dishes were washed with ice-cold PBS. The cells were then scraped, transferred to microtubes, and allowed to swell after adding 100 μ l of a hypotonic buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). The lysates were incubated for 10 min on ice and centrifuged at 7200 *g* for 5 min at 4 °C. Pellets containing the crude nuclei were resuspended in 50 μ l of an extraction buffer containing 20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 10 mM DTT, and 1 mM PMSF and incubated for 30 min on ice. The samples were centrifuged at 15 800 *g* for 10 min to obtain the supernatants containing the nuclear fractions. The nuclear fractions were stored at –80 °C until needed.

Immunoblot analysis

After washing with sterile PBS, the MCF-7 or TAMR-MCF-7 cells were lysed in EBC lysis buffer containing 20 mM Tris–Cl (pH 7.5), 1% Triton X-100, 137 mM sodium chloride, 10% glycerol, 2 mM EDTA, 1 mM sodium orthovanadate, 25 mM β -glycerophosphate, 2 mM sodium pyrophosphate, 1 mM PMSF, and 1 μ g/ml leupeptin. The cell lysates were centrifuged at 10 000 *g* for 10 min to remove the debris, and the proteins were fractionated using a 10% separating gel. The fractionated proteins were then transferred electrophoretically to nitrocellulose paper and the proteins were immunoblotted with the specific antibodies. Horseradish peroxidase- or alkaline phosphatase-conjugated anti-IgG antibody were used as the secondary antibodies. The nitrocellulose papers were developed using 5-bromo-4-chloro-3-indolylphosphate/4-nitroblue tetrazolium or an ECL chemiluminescence system.

Calcein efflux assay

Calcein-AM is a non-fluorescent and lipophilic acetoxymethylester of calcein. In the cytoplasm, calcein-AM is metabolized by esterases yielding hydrophilic and fluorescent calcein that cannot diffuse out of the cells (Vellonen *et al.* 2004). Since calcein is a substrate of MRP1 and MRP2, but not of MDR1

(Bauer *et al.* 2003), the only route for calcein to escape from the cell is by MRP1- or MRP2-mediated efflux. MCF-7 and TAMR-MCF-7 cells were seeded in 24-well plates. After 80% confluency reached, the cells were incubated in FBS-free DMEM for 18 h. The culture medium was changed with Hank's balanced salt solution and the cells were preincubated at 37 °C for 15 min. After incubation of the cells with 2 μ M calcein-AM for 20 min, the medium was completely removed and the cells were lysed in EBC lysis buffer. The calcein fluorescence retention in cell lysates was measured using the excitation and emission wavelengths of 496 and 516 nm respectively. The fluorescence values were divided by total protein contents of each sample.

RT-PCR

The total RNA was isolated from the cells using a total RNA isolation kit (RNAgents, Promega). The total RNA (1.0 μ g) was reverse transcribed using an oligo(dT) 18-mer as a primer and M-MLV reverse transcriptase (Bioneer, Eumsung, South Korea) to produce the cDNA. PCR was performed using the selective primers for the human PXR (sense primer: 5'-TCCGGAAAGATCTGTGCTCTT-3', anti-sense primer: 5'-AGGGAGATCTGGTCCTCGAT-3'; Masuyama *et al.* 2003), human MDR1 (sense primer: 5'-TGCTCAGACAGGATGTGAGTTG-3', antisense primer: 5'-TAGCCCCTTTAACTTGAGCAGC-3') and S16 ribosomal protein (*S16r*) genes (sense: 5'-TCCAAGGGTCCGCTGCAGTC-3', antisense: 5'-CGTTCACCTTGATGAGCCCATT-3'). The PCRs were carried out for 35 (PXR) or 40 cycles (MDR1) under the following conditions: denaturation at 98 °C for 10 s, annealing at 60 °C (PXR) or 51 °C (MDR1) for 0.5 min, and elongation at 72 °C for 1 min. The band intensities of the amplified DNAs were compared after visualization using a u.v. transilluminator.

Reporter gene analysis

The promoter activity was determined using a dual-luciferase reporter assay system (Promega). Briefly, the cells (3×10^5 cells/well) were replated in 12-well plates overnight and transiently transfected with the p-MRP2-Luc plasmids/pRL-SV plasmid (Renilla luciferase expression for normalization; Promega) or the PXR reporter plasmid/pRL-SV plasmid using Genejuice reagent (Novagen, Madison, WI, USA). The cells were then incubated in the culture medium without serum for 18 h, and the firefly and R. luciferase activities in the cell lysates were measured using a luminometer (Turner Designs; TD-20, CA).

The relative luciferase activities were calculated by normalizing the promoter-driven firefly luciferase activity versus R. luciferase.

Statistical analysis

Scanning densitometry was performed using an Image Scan & Analysis System (FLA-7000, Fujifilm, Japan). One-way ANOVA was used to assess the significant differences between the treatment groups. For each significant effect of treatment, the Newman–Keuls test was used to compare the multiple group means. The criterion for statistical significance was set at either $P < 0.05$ or $P < 0.01$.

Results

Increased expression of MRP2 in TAM-resistant breast cancer cell

The acquisition of TAM resistance in TAMR-MCF-7 cells was verified using a MTT assay. 4-hydroxytamoxifen caused a concentration-dependent decrease in the cell viability of MCF-7 cells (Fig. 1A). In contrast, the cell viability of TAMR-MCF-7 cells was not changed by up to 30 μM 4-hydroxytamoxifen (Fig. 1A).

The expression levels of the ABC transporter family in the TAM-resistant breast cancer cells were determined by western blot analyses using form-specific antibodies against MDR1 and MRP2 in both TAMR-MCF-7 and control MCF-7 cells. The MDR1 protein could not be detected in either the MCF-7 or TAMR-MCF-7 cells, even though two different antibodies were used (Santacruz H-241 and Calbiochem C219). It was shown that the MDR1 mRNA and protein were not detected in the wild type MCF-7 cells (Daschner *et al.* 1999; GNF SymAtlas V1.2.4. in Genomics Institute of the Novartis Research Foundation). RT-PCR analysis showed that the MDR1 mRNA band was slightly detected in the TAMR-MCF-7 cells, but not found in the control MCF-7 cells even after 40 amplification cycles (Fig. 1B). The basal MRP2 levels were also low in the control MCF-7 cells. In contrast, the protein expression was higher in the TAMR-MCF-7 cells (Fig. 1C). The cellular retention of calcein, a substrate of MRP1 and MRP2 (Bauer *et al.* 2003), was determined to confirm the functional transport activity of MRP2. As shown in Fig. 1D, the cellular uptake ratio of calcein was 11.7-fold lower in the TAMR-MCF-7 cells, when compared with the control MCF-7 cells. There was a reasonable correlation between the cellular uptake of calcein and the MRP2 expression level in both the cell types. These suggest that the regulation of MRP2

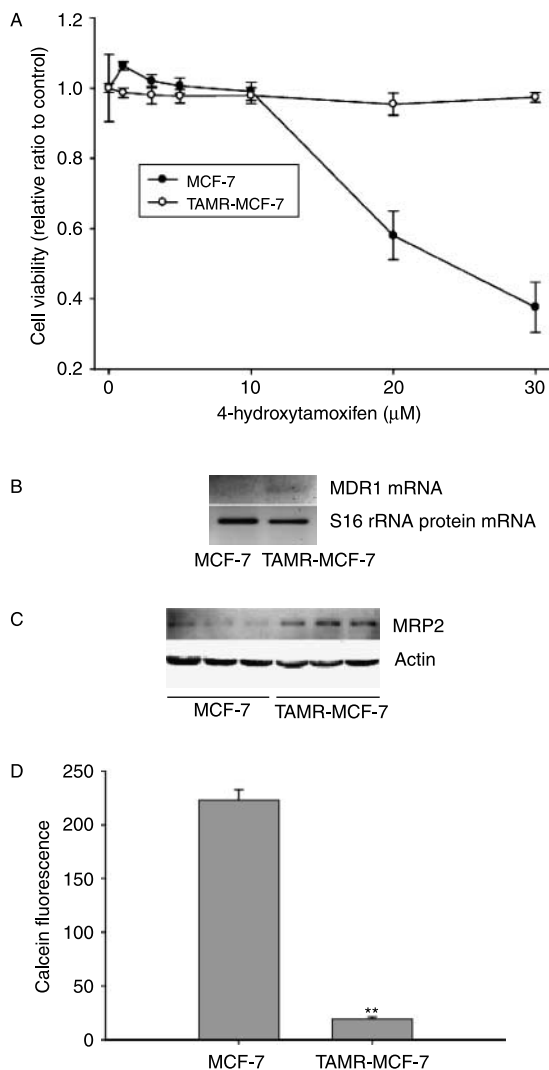
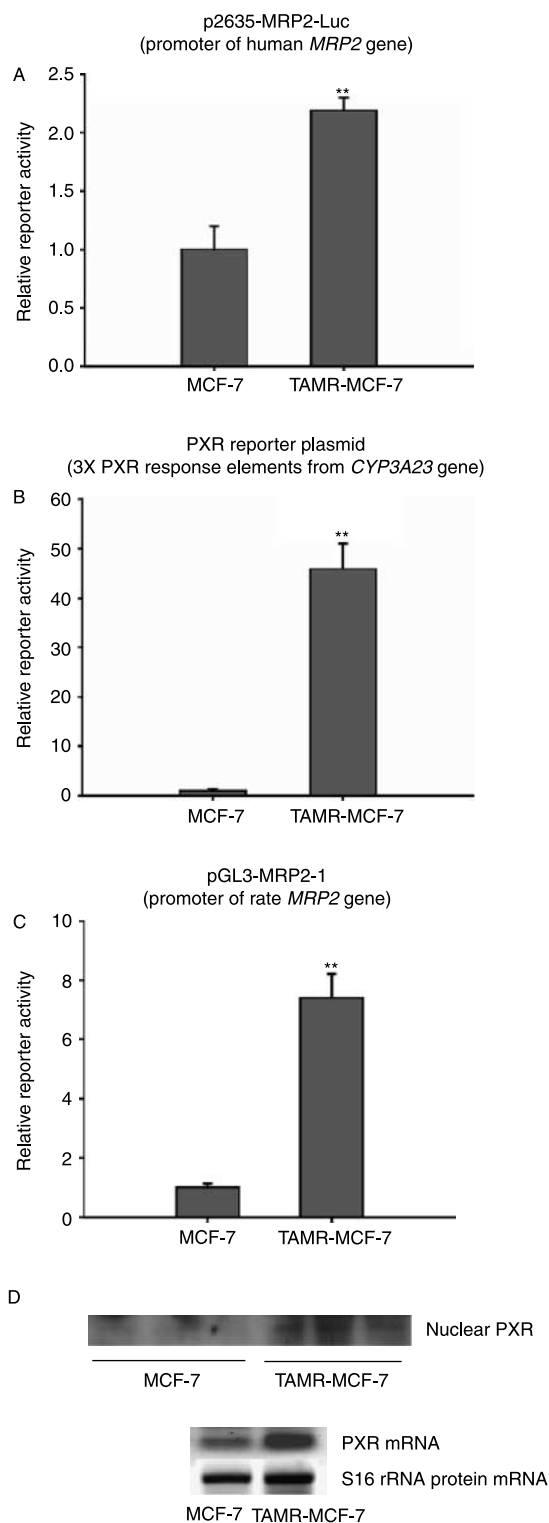


Figure 1 Overexpression of MRP2 in TAMR-MCF-7 cells. (A) Cell viability after treating with 4-hydroxytamoxifen. After treating MCF-7 and TAMR-MCF-7 cells with 4-hydroxytamoxifen (0.1–30 μM) for 24 h, cell viabilities were determined using MTT assays. Data represent the means \pm s.d. of eight separate samples. (B) MDR1 mRNA expression. MDR1 and S16 ribosomal protein mRNA expression levels were determined by RT-PCR. (C) Immunoblot analysis of MRP2. A representative immunoblot shows MRP2 protein in both MCF-7 and TAMR-MCF-7 cells serum-deprived for 24 h. Equal loading of proteins was verified by actin immunoblot. (D) Calcein retention. After incubation of the cells with 2 μM calcein-AM for 20 min, the calcein fluorescence retention in cell lysates was measured using the excitation and emission wavelengths of 496 and 516 nm respectively. The fluorescence values were divided by total protein contents of each sample. Data represent the means \pm s.d. of 12 separate samples (significant when compared with the control MCF-7 cells, ** $P < 0.01$).

expression is coupled with the process of acquiring TAM resistance, indicating that long-term exposure of breast cancer cells to TAM may cause additional changes in the uptake of chemotherapeutic agents.



Involvement of PXR in the enhanced *MRP2* expression in TAM-resistant breast cancer cell

Reporter gene analyses were performed using p2635-*MRP2*-Luc reporter plasmid, which contained the luciferase structural gene and a -2.6 kb human *MRP2* promoter, in order to determine whether transcriptional activation is involved in the over-expression of *MRP2* gene in TAMR-MCF-7 cells. In addition, an attempt was made to identify the transcription factors involved in *MRP2* gene transactivation. In comparison with the control MCF-7 cells, a 2.2-fold increase in the luciferase activity was observed in TAMR-MCF-7 cells transfected with p2635-*MRP2*-Luc plasmid (Fig. 2A), which suggests that the induction of *MRP2* in the TAM-resistant breast cancer cells is mediated through the transcriptional activation of the *MRP2* gene. Since PXR is believed to be an essential transcription factor for the induction of *MRP2* (Teng & Piquette-Miller 2005), and the roles of PXR in the inducible expression of *MRP2* have been examined extensively (Kast *et al.* 2002, Anapolsky *et al.* 2006), this study compared the reporter activities in both MCF-7 and TAMR-MCF-7 cells using the PXR reporter plasmid (three copies of the PXR-responsive elements from *CYP3A23* gene; Kast *et al.* 2002). The reporter activity was significantly higher in the TAMR-MCF-7 cells than in the control MCF-7 cells (Fig. 2B). In addition, the luciferase reporter gene of rat *MRP2* gene promoter,

Figure 2 PXR-dependent transactivation of *MRP2* gene. (A) Induction of luciferase activity in TAMR-MCF-7 cells transiently transfected with p2635-*MRP2*-Luc which contained -2635 bp promoter region of human *MRP2* gene and luciferase cDNA. Dual luciferase reporter assay was performed on the lysed cells co-transfected with p2635-*MRP2*-Luc (firefly luciferase) and pRL-SV (*Renilla luciferase*; a ratio of 100:1). Activation of the reporter gene was calculated as a relative change to *R. luciferase* activity. Data represent the means \pm s.d. with six different samples (significant when compared with MCF-7 cells, ** $P < 0.01$; control level = 1). (B) PXR reporter gene assay. MCF-7 and TAMR-MCF-7 cells were transfected with PXR reporter plasmid containing three copies of the PXR-responsive elements from *CYP3A23* gene, and reporter gene analysis was performed as described in the legend of panel (A). Data represent the means \pm s.d. of six different samples (significant when compared with MCF-7 cells, ** $P < 0.01$; control level = 1). (C) Induction of luciferase activity in TAMR-MCF-7 cells transiently transfected with pGL3-*MRP2*-1 which contained -1034 bp promoter region of rat *MRP2* gene and luciferase cDNA. Reporter gene analysis was performed as described in the legend of panel (A). Data represent the means \pm s.d. of six different samples (significant when compared with MCF-7 cells, ** $P < 0.01$; control level = 1). (D) Upper, nuclear levels of PXR. PXR2 was immunoblotted with anti-PXR antibody in the nuclear fraction prepared from both cells. Lower, PXR mRNA expression. PXR and S16 ribosomal protein mRNA expression levels were determined by RT-PCR.

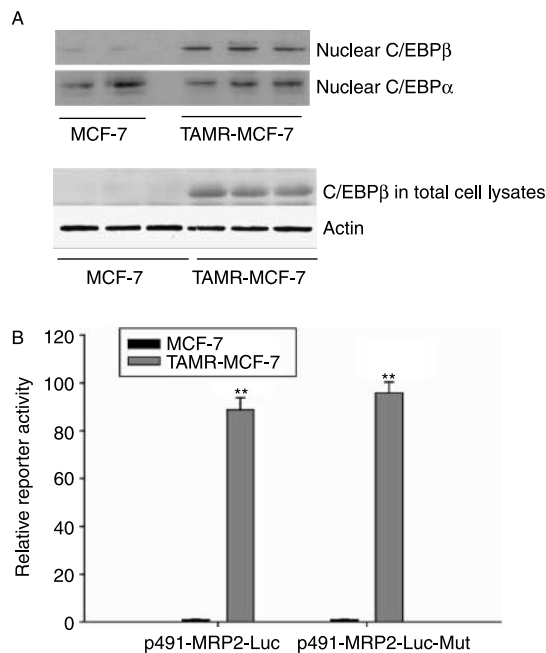


Figure 3 No role of C/EBP β activation in transactivation of *MRP2* gene in TAMR-MCF-7 cells. (A) Western blot analysis of C/EBP β in the total cell lysates and nuclear fractions. Nuclear fractions and total cell lysates were obtained from both MCF-7 and TAMR-MCF-7 cells serum-starved for 24 h, and β and α forms of C/EBP in each fraction were immunoblotted with the respective antibody. (B) Reporter activities of p491-MRP2-Luc and p-491-MRP2-Luc-Mut (–491 bp human MRP2 promoter *PXR* reporter gene assay). MCF-7 and TAMR-MCF-7 cells were transfected with p491-MRP2-Luc (491 bp human MRP2 promoter) or p-491-MRP2-Luc-Mut plasmid (491 bp human MRP2 promoter with a specific mutation in the C/EBP-binding site) and reporter gene analysis was performed as described in the legend of Fig. 2, panel (A). Data represent the means \pm s.d. of six different samples (significant when compared with MCF-7 cells, ** $P < 0.01$).

which contains farnesoid X-activated receptor (FXR)/PXR-binding region, was 7.4-fold induced in the TAMR-MCF-7 cells (Fig. 2C).

The nuclear levels of PXR in both MCF-7 and TAMR-MCF-7 cells were also measured in order to determine if the increased PXR reporter activity in the TAM-resistant breast cancer cells was associated with an increase in the PXR level. The nuclear PXR levels were higher in the TAMR-MCF-7 cells than in the control MCF-7 cells (Fig. 2D). Consistent with the western blot analysis result, RT-PCR analysis showed that the PXR mRNA level was also increased in the TAMR-MCF-7 cells (Fig. 2D). The mRNA level of the S16 ribosomal protein was comparable in all samples (Fig. 2D). Therefore, PXR activation might be one possible mechanism for the higher MRP2 expression levels in TAMR-MCF-7 cells.

No role of CCAAT/enhancer-binding protein β (C/EBP β) activation in MRP2 induction

It has been also suggested that the C/EBP β -binding region (–356 to –343 bp) in human MRP2 promoter is involved in the basal expression of the *MRP2* gene (Tanaka *et al.* 1999). C/EBP β is mainly located in the cytoplasmic fraction and is translocated to the nucleus upon activation. The nuclear C/EBP β levels were measured by subcellular fractionation and western blot analysis in order to determine whether C/EBP β activation is essential for the enhanced MRP2 expression in TAMR-MCF-7 cells. The nuclear levels of C/EBP β were higher in the TAM-resistant breast cancer cells than in the control cells (Fig. 3A). In contrast, C/EBP α was not altered by the long-term exposure of MCF-7 cells to TAM (Fig. 3A). The total amount of C/EBP β in the total cell lysates from TAMR-MCF-7 cells was also higher than those from the control MCF-7 cells (Fig. 3A). Therefore, the nuclear accumulation of C/EBP β in TAMR-MCF-7 cells may be due to the increased expression. A promoter mutation study was performed to clarify the role of C/EBP β activation in the transactivation of MRP2 in TAMR-MCF-7 cells. The reporter activities of both p491-MRP2-Luc (–491 bp human MRP2 promoter) and p491-MRP2-Luc-Mut constructs were compared. The p491-MRP2-Luc-Mut construct contains a promoter sequence with a specific mutation in the C/EBP-binding site (AACTTTGAAAGCC (–365 to –343 bp) was converted to AACTT-TAACCGCC; Tanaka *et al.* 1999). The intensity of the luciferase activity by p491-MRP2-Luc-Mut in TAMR-MCF-7 cells was similar to that by p491-MRP2-Luc. This suggests that C/EBP β activation does not play an important role in the induction of MRP2 in TAM-resistant breast cancer cells (Fig. 3B).

Role of PI3-kinase in PXR-mediated MRP2 induction in TAM-resistant breast cancer cell

The physiological function of MRP2 is coupled with phase II conjugation enzymes because the conjugated metabolites can be pumped out through the ABC transporters. A series of our experiments demonstrated that PI3-kinase plays a key role in the induction of the phase II conjugation genes (Kang *et al.* 2001a,b). A recent study also suggested that PI3-kinase is involved in the transcriptional regulation of the *MRP2* gene in Caco-2 cells (Jakubicova *et al.* 2005). The levels of phosphorylated Akt (a downstream kinase of PI3-kinase) were measured in order to determine whether PI3-kinase is activated in TAM-resistant breast cancer cells. PI3-kinase was persistently activated in the

TAMR-MCF-7 cells, while minimal levels of phosphorylated Akt were observed in the MCF-7 cells (Fig. 4A). The role of PI3-kinase in the induction of MRP2 in TAMR-MCF-7 cells was then assessed using specific chemical inhibitor. Pretreatment of these cells with LY294002 (20 μ M) significantly blocked the induction of MRP2 in TAMR-MCF-7 cells (Fig. 4B).

Given the roles of PXR in the induction of MRP2 in these cells, this study also examined the effect of the inhibition of PI3-kinase on the PXR reporter activity. As shown in Fig. 4C, pretreating the cells with LY294002 (20 μ M) almost completely inhibited the PXR reporter activity in TAMR-MCF-7 cells (Fig. 4C). To confirm the precise role of PI3-kinase, the PXR reporter activity was monitored in cells overexpressing p85 regulatory subunit of PI3-kinase

(Fig. 4C). Overexpression of the p85 subunit suppressed PXR-driven reporter activity in TAMR-MCF-7 cells, whereas pCMV5 overexpression (Mock transfection) failed to alter the reporter activity. These results suggest that the PI3-kinase pathway plays a key role in controlling the PXR activity and PXR-dependent MRP2 expression in TAM-resistant breast cancer cells.

Aromatase expression is not altered in TAM-resistant breast cancer cell

Clinical application of aromatase inhibitors in adjuvant therapy for hormone-sensitive breast cancer has rapidly grown over the past few years. Unlike TAM, which antagonizes estrogen receptor and subsequently inhibits the transcription of estrogen-responsive genes, aromatase inhibitors block synthesis of estrogens through the inhibition of aromatase catalyzing the conversion of C₁₉ androgens to estrogens (Smith & Dowsett 2003). We further determined the effect of an aromatase inhibitor, 4-hydroxyandrostenedione (Formestane), on the cell proliferation for 48 h in testosterone-containing culture medium. The inhibition percentage of cell proliferation by 4-hydroxyandrostenedione appeared to be comparable in both the MCF-7 and TAMR-MCF-7 cells (Fig. 5A). Cell proliferation in the presence of testosterone was ~70% inhibited by 1 μ M 4-hydroxyandrostenedione in both the cell types (Fig. 5A). We also found that the protein expression level of aromatase in TAMR-MCF-7 cells was similar to that in control MCF-7 cells (Fig. 5B). These results show that transcriptional or translational regulation of aromatase is not altered during the acquisition of TAM resistance and imply

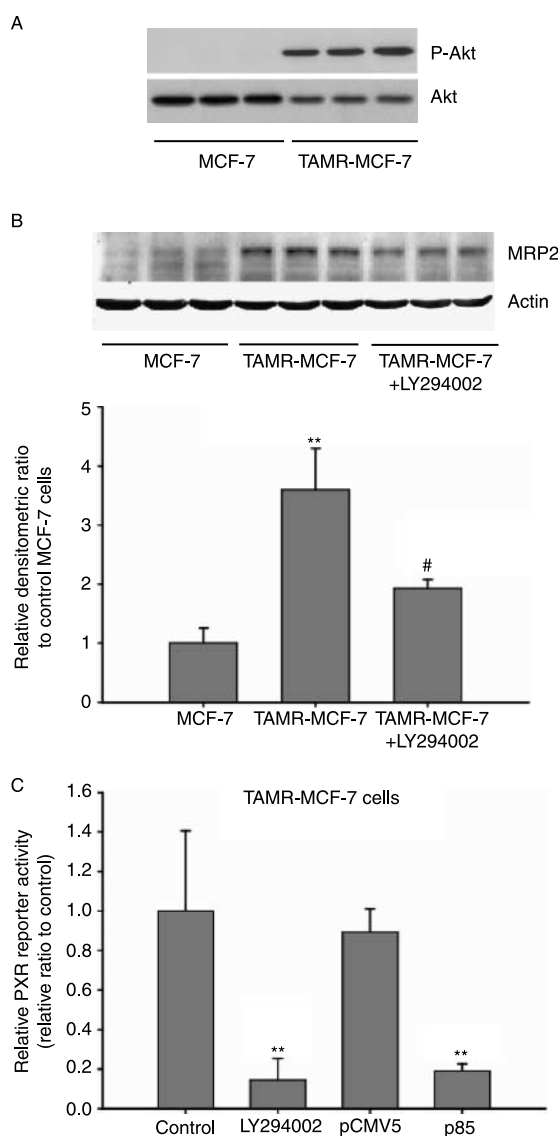


Figure 4 PI3-kinase-dependent MRP2 expression in TAMR-MCF-7 cells. (A) Akt phosphorylation. The extent of PI3-kinase activation was assessed by immunoblotting of phosphorylated forms of Akt. (B) The effects of PI3-kinase inhibitor on the expression of MRP2 in TAMR-MCF-7 cells. Expression of MRP2 was measured in TAMR-MCF-7 cells treated with PI3-kinase inhibitors (LY; LY294002 20 μ M) for 24 h. The relative change in MRP2 was assessed by scanning densitometry. Data represent the means \pm s.d. with three separate samples (significant when compared with MCF-7 cells, ** P <0.01; significant when compared with TAMR-MCF-7 cells, ## P <0.01). (C) Inhibition of PXR reporter gene activation in TAMR-MCF-7 cells by PI3-kinase suppressions. TAMR-MCF-7 cells were treated with PI3-kinase inhibitor (LY; LY294002 20 μ M), 10 min after transfection of cells with PXR reporter plasmid and then the luciferase activity was measured 18 h after inhibitor treatment. For the p85 subunit overexpression, cells were co-transfected with PXR reporter gene/pRL-SV (100:1) in combination with p85 overexpressing vector or pCMV5 vector at a ratio of 1:4. Reporter gene analyses were performed as described in the legend of Fig. 2, panel (A). Data represent the means \pm s.d. of 6–7 different samples (significant when compared with TAMR-MCF-7 cells, ** P <0.01).

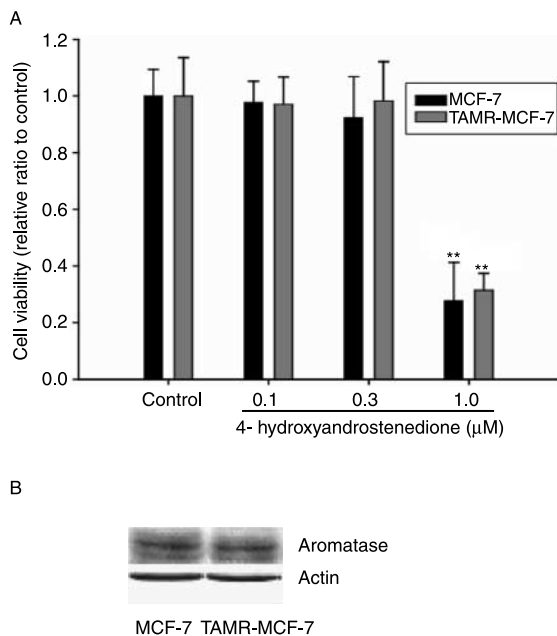


Figure 5 Expression of aromatase in TAMR-MCF-7 cells. (A) Effect of aromatase inhibitor on the cell proliferation stimulated by testosterone. MCF-7 and TAMR-MCF-7 cells were cultured in phenol red-free DMEM containing 5% charcoal-stripped steroid-depleted FBS in the presence or absence of testosterone (0.1 μM)/4-hydroxyandrostenedione (0.1–1 μM) for 48 h. Cell proliferation were determined using MTT assays. Data represent the means \pm S.D. of eight separate samples (significant when compared with the untreated control cells, ** $P < 0.01$).

that aromatase inhibitor may be active to inhibit the growth of TAM-resistant breast cancer cells.

Discussion

TAM resistance is a major challenge in the management of breast cancer patients. Despite the initial response to such therapy, the majority of patients will ultimately relapse during long-term treatment (Ali & Coombes 2002). This study showed that MRP2 expression was strongly enhanced in TAMR-MCF-7 cells established by long-term exposure to TAM. MRP2 is normally expressed on the apical membrane of several cancer cells where it transports a variety of chemotherapeutic agents into extracellular space such as methotrexate and cisplatin. Hence, MRP2 overexpression may add chemoresistance to TAM-resistant breast cancer cells. It was shown that TAM and its metabolites reverse the MDR1-mediated MDR in several types of cancer cells (Kirk et al. 1993, Hotta et al. 1996), which is related to the direct suppression of the anti-estrogen by the activity of MDR1 (De Vincenzo et al. 1996). Although there is a great deal of evidence suggesting that TAM

affects the MDR1 transporter activity, the effect of TAM on the MRP2 transporter is unclear.

A number of studies have been carried out on the changes in MRP2 expression under pathological conditions. MRP2 expression was significantly lower in obstructive cholestasis and chronic inflammation (Denson et al. 2002, Teng & Piquette-Miller 2005). Higher levels of MRP2 expression has been frequently observed in several types of cancer tissues including human colorectal carcinoma (Hinoshita et al. 2000), hepatoma (Nies et al. 2001) and lung cancers (Young et al. 1999), which might be associated with their resistance to chemotherapeutic agents. Since MRP2 is involved in the efflux of various chemotherapeutic agents, these findings indicate that breast cancer cells exposed to TAM long term may acquire additional resistance to other anti-cancer agents through the increased MRP2 activity.

The transcriptional regulation of *MRP2* gene is not completely understood. The *C/EBPβ*-binding region in the human *MRP2* promoter is essential for the transactivation of the *MRP2* gene (Tanaka et al. 1999). This study found that the long-term exposure of MCF-7 cells to TAM results in the upregulation of *C/EBPβ*. However, the promoter reporter activity was not reduced in the TAMR-MCF-7 cells transfected with p-491-MRP2-Luc-Mut containing a specific mutation in the *C/EBPβ*-binding site, which supported the hypothesis that *C/EBPβ* activation in TAM-resistant breast cancer cells is not related to the induction of MRP2. In the rat *MRP2* promoter, FXR binds to an unusual 26 bp sequence, which contains an inverted repeat of the AGTTCA motif (ER-8; Kast et al. 2002). It has been found that this sequence also interacts with PXR and the constitutive androstane receptor. Indeed, most of the PXR ligands such as rifampicin and hyperforin have been shown to upregulate MRP2 expression in humans, mice, and rats (Fromm et al. 2000, Kast et al. 2002, Anapolsky et al. 2006). Excess glucocorticoid concentrations increase the MRP2 mRNA levels, which implicates glucocorticoid-inducible PXR activation (Kliwer et al. 1998). It was found in this study that PXR was persistently activated in TAMR-MCF-7 cells. The reporter activity of ER-8 containing the rat *MRP2* promoter in TAMR-MCF-7 cells was also higher than that in the control MCF-7 cells (Fig. 2C). Therefore, MRP2 overexpression in TAM-resistant breast cancer cells might result from PXR activation. Although MDR1 expression could not be detected in the control MCF-7 and TAMR-MCF-7 cells, the reporter activity of MDR1 promoter was considerably higher in the TAMR-MCF-7 cells after transfection with the reporter containing the human MDR1 promoter region (data not shown). The most important

transcriptional event for the expression of *MDR1* gene is PXR binding to the DR4 region (Geick *et al.* 2001). This shows that PXR activation during the acquisition of TAM resistance is the main regulator of the genes encoding MRP2 and MDR1, which contributes significantly to the additional chemoresistance. It has been reported that endocrine-disrupting chemicals activate PXR-mediated transcription, presumably through an interaction with co-activators (Masuyama *et al.* 2000). The long-term exposure of breast cancer cells to TAM might cause persistent PXR activation in a similar manner.

In this study, the increase ratio of MRP2 reporter activity by –2635 promoter region in TAMR-MCF-7 cells was obviously decreased in comparison with that by the –491 promoter. This would imply that there is some very potent repressor element between –2635 and –491 promoter region of human *MRP2* gene. Tanaka *et al.* (1999) previously showed that the reporter activity of –1659 MRP2 promoter decreased in HepG2 cells when compared with that of –491 MRP2 promoter and they suggested that a putative silencer element was located in the –1659 ~ –491 promoter region. Possible transcription factors bind to the region are C/EBP α , HNF-3 β , Evi-1 and AP-1; one of these proteins may function as a repressor for the transcription of human *MRP2* gene.

During the development of the TAM resistance phenotype, the cell survival signaling pathways such as PI3-kinase and ERK can be activated, which might be one of the key issues in the transcriptional regulation of various MRPs. Jakubikoba *et al.* (2005) recently showed that PI3-kinase, but not ERK, was essential for the expression of isothiocyanate-inducible MRP2. In this study, the PI3-kinase was potently activated in TAMR-MCF-7 cells and either chemical inhibition or transfection with the p85 subunit significantly suppressed the expression of MRP2 in TAM-resistant breast cancer cells. Considering the findings that the percentage inhibition of the PXR reporter activity by LY294002 or p85 overexpression was >80% and PI3-kinase inhibition did not cause complete loss of MRP2 expression in TAMR-MCF-7 cells, it would be possible that distinct kinase(s) and transcription factor(s) might also be involved in the regulation of MRP2 expression in TAM-resistant breast cancer cells.

Overall, this study showed that PXR is activated in TAMR-MCF-7 cells, and that the persistently activated PI3-kinase plays a role in activating PXR and PXR-dependent MRP2 expression. These observations might have clinical implications in highlighting the possibility of patients undergoing long-term TAM therapy acquiring additional chemoresistance.

Acknowledgements

This study was supported by the Korean Ministry of Science and Technology and the Korean Science and Engineering Foundation through the Research Center for Resistant Cells. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References

- Ali S & Coombes RC 2002 Endocrine-responsive breast cancer and strategies for combating resistance. *Nature Reviews. Cancer* **2** 101–112.
- Anapolsky A, Teng S, Dixit S & Piquette-Miller M 2006 The role of pregnane X receptor in 2-acetylaminofluorene-mediated induction of drug transport and -metabolizing enzymes in mice. *Drug Metabolism and Disposition* **34** 405–409.
- Assikis V, Buzdar A, Yang Y, Smith T, Theriault R, Booser D, Valero V, Walters R, Singletary E, Ames F *et al.* 2003 A phase III trial of sequential adjuvant chemotherapy for operable breast carcinoma: final analysis with 10-year follow-up. *Cancer* **97** 2716–2723.
- Badia E, Duchesne MJ, Semlali A, Fuentes M, Giamarchi C, Richard-Foy H, Nicholas JC & Pons M 2000 Long-term hydroxytamoxifen treatment of an MCF-7-derived breast cancer cell line irreversibly inhibits the expression of estrogenic genes through chromatin remodeling. *Cancer Research* **60** 4130–4138.
- Bauer B, Miller DS & Fricker G 2003 Compound profiling for P-glycoprotein at the blood–brain barrier using a microplate screening system. *Pharmaceutical Research* **20** 1170–1176.
- Blumenschein GR, DiStefano A, Caderao J, Fristenberg B, Adams J, Schweichler LH & Drinkard L 1997 Multi-modality therapy for locally advanced and limited stage IV breast cancer: the impact of effective non-cross-resistance late-consolidation chemotherapy. *Clinical Cancer Research* **3** 2633–2637.
- Clemons M, Danson S & Howell A 2002 Tamoxifen ('Nolvadex'): a review. *Cancer Treatment Review* **28** 165–180.
- Daschner PJ, Ciolino HP, Plouzek CA & Yeh GC 1999 Increased AP-1 activity in drug resistant human breast cancer MCF-7 cells. *Breast Cancer Research and Treatment* **53** 229–240.
- Denson LA, Bohan A, Held MA & Boyer JL 2002 Organ-specific alterations in RAR alpha:RXR alpha abundance regulate rat *Mrp2* (*Abcc2*) expression in obstructive cholestasis. *Gastroenterology* **123** 599–607.
- De Vincenzo R, Scambia G, Benedetti Panici P, Fattorossi A, Bonanno G, Ferlini C, Isola G, Pernisico S & Mancuso S 1996 Modulatory effect of tamoxifen and ICI 182,780 on adriamycin resistance in MCF-7 human breast-cancer cells. *International Journal of Cancer* **68** 340–348.

- Fromm MF, Kauffmann HM, Fritz P, Burk O, Kroemer HK, Warzok RW, Eichelbaum M, Siegmund W & Schrenk D 2000 The effect of rifampin treatment on intestinal expression of human MRP transporters. *American Journal of Pathology* **157** 1575–1580.
- Geick A, Eichelbaum M & Burk O 2001 Nuclear receptor response elements mediate induction of intestinal MDR1 by rifampin. *Journal of Biological Chemistry* **276** 14581–14587.
- Gottardis MM & Jordan VC 1988 Development of tamoxifen-stimulated growth of MCF-7 tumors in athymic mice after long-term antiestrogen administration. *Cancer Research* **48** 5183–5187.
- Gottesman MM, Fojo T & Bates SE 2002 Multidrug resistance in cancer: role of ATP-dependent transporters. *Nature Reviews. Cancer* **2** 48–58.
- Hinoshita E, Uchiumi T, Taguchi K, Kinukawa N, Tsuneyoshi M, Maehara Y, Sugimachi K & Kuwano M 2000 Increased expression of an ATP-binding cassette superfamily transporter, multidrug resistance protein 2, in human colorectal carcinomas. *Clinical Cancer Research* **6** 2401–2407.
- Hotta T, Tanimura H, Yamaue H, Iwashita M, Tani M, Tsunoda T, Tamai M, Noguchi K, Mizobata S, Arai K et al. 1996 Tamoxifen circumvents the multidrug resistance in fresh human gastrointestinal cancer cells. *Journal of Surgical Research* **66** 31–35.
- Jakubikova J, Sedlak J, Mithen R & Bao Y 2005 Role of PI3K/Akt and MEK/ERK signalling pathways in sulforaphane- and erucin-induced phase II enzymes and MRP2 transcription, G2/M arrest and cell death in Caco-2 cells. *Biochemical Pharmacology* **69** 1543–1552.
- Kang KW, Cho MK, Lee CH & Kim SG 2001a Activation of phosphatidylinositol 3-kinase and Akt by tert-butylhydroquinone is responsible for antioxidant response element-mediated rGSTA2 induction in H4IIE cells. *Molecular Pharmacology* **59** 1147–1156.
- Kang KW, Ryu JH & Kim SG 2001b Activation of phosphatidylinositol 3-kinase by oxidative stress leads to the induction of microsomal epoxide hydrolase in H4IIE cells. *Toxicology Letters* **121** 191–197.
- Kast HR, Goodwin B, Tarr PT, Jones SA, Anisfeld AM, Stoltz CM, Tontonoz P, Kliewer S, Willson TM & Edwards PA 2002 Regulation of multidrug resistance-associated protein 2 (ABCC2) by the nuclear receptors pregnane X receptor, farnesoid X-activated receptor, and constitutive androstane receptor. *Journal of Biological Chemistry* **277** 2908–2915.
- Kawabe T, Chen ZS, Wada M, Uchiumi T, Ono M, Akiyama S & Kuwano M 1999 Enhanced transport of anticancer agents and leukotriene C4 by the human canalicular multispecific organic anion transporter (cMOAT/MRP2). *FEBS Letters* **456** 327–331.
- Kirk J, Houlbrook S, Stuart NS, Stratford IJ, Harris AL & Carmichael J 1993 Selective reversal of vinblastine resistance in multidrug-resistant cell lines by tamoxifen, toremifene and their metabolites. *European Journal of Cancer* **29** 1152–1157.
- Kliewer SA, Moore JT, Wade L, Staudinger JL, Watson MA, Jones SA, McKee DD, Oliver BB, Willson TM, Zetterstrom RH et al. 1998 An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. *Cell* **92** 73–82.
- Knowlden JM, Hutcheson IR, Jones HE, Madden T, Gee JM, Harper ME, Barrow D, Wakeling AE & Nicholson RI 2003 Elevated levels of epidermal growth factor receptor/c-erbB2 heterodimers mediate an autocrine growth regulatory pathway in tamoxifen-resistant MCF-7 cells. *Endocrinology* **144** 1032–1044.
- Kool M, de Haas M, Scheffer GL, Scheper RJ, van Eijk MJ, Juijn JA, Baas F & Borst P 1997 Analysis of expression of cMOAT (MRP2), MRP3, MRP4, and MRP5, homologues of the multidrug resistance-associated protein gene (MRP1), in human cancer cell lines. *Cancer Research* **57** 3537–3547.
- Lage H 2003 ABC-transporters: implications on drug resistance from microorganisms to human cancers. *International Journal of Antimicrobial Agents* **22** 188–199.
- Masuyama H, Hiramatsu Y, Kunitomi M, Kudo T & MacDonald PN 2000 Endocrine disrupting chemicals phthalic acid and nonylphenol activate pregnane X receptor-mediated transcription. *Molecular Endocrinology* **14** 421–428.
- Masuyama H, Hiramatsu Y, Kodama J & Kudo T 2003 Expression and potential roles of pregnane X receptor in endometrial cancer. *Journal of Clinical Endocrinology and Metabolism* **88** 4446–4454.
- Nies AT, Konig J, Pfannschmidt M, Klar E, Hofmann WJ & Keppler D 2001 Expression of the multidrug resistance proteins MRP2 and MRP3 in human hepatocellular carcinoma. *International Journal of Cancer* **94** 492–499.
- Paulusma CC, Bosma PJ, Zaman GJ, Bakker CT, Otter M, Scheffer GL, Scheper RJ, Borst P & Oude Elferink RP 1996 Congenital jaundice in rats with a mutation in a multidrug resistance-associated protein gene. *Science* **271** 1126–1128.
- Rose C, Thorpe SM, Andersen KW, Pedersen BV, Mouridsen HT, Blichert-Toft M & Rasmussen BB 1985 Beneficial effect of adjuvant tamoxifen therapy in primary breast cancer patients with high oestrogen receptor values. *Lancet* **1** 16–19.
- Schreiber E, Harshman K, Kemler I, Malipiero U, Schaffner W & Fontana A 1990 Astrocytes and glioblastoma cells express novel octamer-DNA binding proteins distinct from the ubiquitous Oct-1 and B cell type Oct-2 proteins. *Nucleic Acids Research* **18** 5495–5503.
- Schrenk D, Baus PR, Ermel N, Klein C, Vorderstemann B & Kauffmann HM 2001 Up-regulation of transporters of the MRP family by drugs and toxins. *Toxicology Letters* **120** 51–57.

- Smith IE & Dowsett M 2003 Aromatase inhibitors in breast cancer. *New England Journal of Medicine* **384** 2432–2442.
- Tanaka T, Uchiumi T, Hinoshita E, Inokuchi A, Toh S, Wada M, Takano H, Kohno K & Kuwano M 1999 The human multidrug resistance protein 2 gene: functional characterization of the 5'-flanking region and expression in hepatic cells. *Hepatology* **30** 1507–1512.
- van Tellingen O, Buckle T, Jonker J, Valk MAVD & Beijnen J 2003 P-glycoprotein and mrp1 collectively protect the bone marrow from vincristine-induced toxicity *in vivo*. *British Journal of Cancer* **89** 1776–1782.
- Teng S & Piquette-Miller M 2005 The involvement of the pregnane X receptor in hepatic gene regulation during inflammation in mice. *Journal of Pharmacology and Experimental Therapeutics* **312** 841–848.
- Vellonen KS, Honkakoski P & Urtti A 2004 Substrates and inhibitors of efflux proteins interfere with the MTT assay in cells and may lead to underestimation of drug toxicity. *European Journal of Pharmaceutical Sciences* **23** 181–188.
- Young LC, Campling BG, Voskoglou-Nomikos T, Cole SP, Deeley RG & Gerlach JH 1999 Expression of multidrug resistance protein-related genes in lung cancer: correlation with drug response. *Clinical Cancer Research* **5** 673–680.