Original Paper

Progestosterone Negatively Regulates BCRP in Progesterone Receptor-Positive Human Breast Cancer Cells

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Key Words
Breast cancer resistance protein • Transcriptional regulation • Progesterone • RU-486 • Breast cancer cells

Abstract

Background/Aims: Breast cancer resistance protein (BCRP) plays a crucial role in multidrug resistance (MDR). Previous studies have shown that steroid hormones, like progesterone (PROG), regulate BCRP expression. The presence of a progesterone response element (PRE) in the BCRP promoter, suggests that PROG may regulate transcription of BCRP. Methods: To investigate the role of PROG in the regulation of BCRP expression, two constructs encoding full-length BCRP driven by either an endogenous PRE promoter or a constitutive CMV promoter, were transfected into T47D cells that express the progesterone receptor (PR) or into PR-negative MDA-MB-231 cells. Results: After treatment with PROG, qPCR and Western blotting analyses indicated that BCRP mRNA and BCRP protein levels were significantly reduced in a dose-dependent manner in PR-positive cells, but PROG had no significant effect on BCRP levels in the PR-negative cells. The effect observed in PR-positive cells was reversed by co-treatment with RU-486, a specific PROG inhibitor. Cytometric analysis confirmed that BCRP-mediated drug efflux was inhibited and chemosensitivity to mitoxantrone was markedly increased by PROG treatment. Conclusion: These results suggest that PROG reverses BCRP-mediated MDR by down-regulating BCRP expression in breast cancer cells by affecting transcription from the PRE-containing BCRP promoter. Our studies suggest that breast cancer patients with BCRP-mediated MDR may be successfully treated with PROG.
Introduction

Breast cancer is the most prevalent type of cancer documented in women. Multidrug resistance (MDR) is the greatest obstacle preventing effective chemotherapy treatment in these patients. One outcome of MDR involves the expulsion of a wide range of structurally and functionally unrelated drugs being pumped out of cancer cells by ATP-binding cassette (ABC) transporter proteins [1]. Breast cancer resistance protein (BCRP) is an ABC transporter protein that was first identified in human breast cancer cells those were grown in the presence of adriamycin and vincristine. BCRP is expressed at high levels in cells isolated from various tumors[2-4]. BCRP mediates resistance to adjuvant chemotherapeutic agents such as mitoxantrone, topotecan, and SN-38 [5-8], presumably by acting as an efflux pump to inhibit intracellular accumulation and reduce cytotoxic effects [9]. Although much has been uncovered regarding the molecular roles of BCRP, a better understanding of the mechanisms regulating BCRP expression is necessary to guide development of strategies to reverse MDR in breast cancer patients.

Steroid hormones like estrogen and progesterone (PROG) regulate BCRP expression via receptor-mediated pathways. One such example is that of the estrogen receptor (ER), which regulates BCRP expression in breast cancer cells via ER elements (ERE) in the BCRP promoter. Progesterone receptor (PR) is also a steroid hormone receptor and its expression is tightly regulated by estrogen [10], as such PR levels should be reduced in ER-negative breast cancer cells. However, this is not the case given that certain ER-negative breast cancer types have high levels of PR expression. Our previous studies in isolated cells revealed that BCRP expression is down-regulated upon treatment with toremifene. Toremifene reverses BCRP-mediated MDR through TOR-ER complexes that bind to the ERE in the BCRP promoter and inhibit the transcription of BCRP [11]. The progesterone response element (PRE), which is a similar regulatory element present in the BCRP promoter, was also recently identified [12]. Little is known about the role of this novel element in the BCRP-mediated MDR pathway in breast cancer cells.

In PR transfected PR- or ER-negative breast cancer cells, DNA synthesis and cell proliferation are markedly inhibited by PROG treatment [13], suggesting that PROG may have a role in cancer growth inhibition. In support of this, megestrol acetate (MA), a synthetic form of PROG that specifically binds to the PR, has been successfully used to treat advanced breast cancer, endometrial cancer, and prostate cancer. Treatment of breast cancer cells in vitro with MA reverses resistance to adriamycin and vincristine [14-16]. However, the underlying mechanisms are not understood.

In this study we over-expressed BCRP in PR-positive T47D and PR-negative MDA-MB-231 breast cancer cells and characterized the role of the PRE in the BCRP-mediated MDR pathway in breast cancer cells.

Materials and Methods

Chemicals, reagents, and antibodies

PROG, mifepristone (RU-486), mitoxantrone, dimethyl sulfoxide (DMSO), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). RPMI-1640 medium with L-glutamine and without phenol red or fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA, USA). The anti-BCRP antibody was purchased from Abcam (Cambridge, MA, USA), and anti-PR antibody was from ProMab (Richmond, CA, USA). The anti-β-actin antibody was from Beyotime (Haimen, Jiangsu, China).

Cells and cell culture

Human breast adenocarcinoma cell lines, T47D and MDA-MB-231, were obtained from the United States National Cancer Institute. Cells were maintained in RPMI-1640 medium supplemented with
10% (v/v) FBS, 100 U/mL penicillin, and 100 mg/L streptomycin in a humidified atmosphere with 5% CO₂ at 37 °C.

**Immunocytochemical analysis of PR in T47D and MDA-MB-231 cells**

Cells were seeded on a glass slide in 24-well plates at about 5,000 cells/well. After 48 hrs, cells were fixed with methanol for 2 hrs. After permeabilization and blocking, cells were incubated with anti-PR antibody (dilution 1:200) overnight at 4 °C. The slides were then probed with poly-HRP conjugated to secondary antibody for 25 min and visualized using DAB (PV-9000, ZSGB-Bio, Beijing, China).

**RNA extraction, PCR amplification, and plasmid construction**

Total RNA was extracted from cell lines using Trizol reagent (TaKaRa, Kyoto, Japan). cDNA synthesis and qualitative real-time PCR (qPCR) reactions were performed as described previously[17]. The primers used to amplify the human BCRP promoter region were 5'-GCC AGT GAC GGC GAC CAA-3' (sense) and 5'-AGC GCT GAC ACG AAC TTC CTA A-3' (antisense). Primers used to amplify the wild-type human BCRP cDNA were 5'-GCT GCG GCC GCG AAC TGG GTA GGA TTT AGG-3' (sense) and 5'-CCC GCG GCC GCT GAA AGA ACC CAA GAC AT-3' (antisense).

BCRP cDNA was inserted into pEGFP-C1 vector behind a CMV promoter (Invitrogen, Carlsbad, CA, USA). This created the vector we refer to as C-BCRP for expression of BCRP under control of the CMV promoter. ApaI/BamHI digestion was used to construct pEGFP/C-BCRP. To generate the pEGFP/P-BCRP construct, the BCRP cDNA was first cloned into pMD20-T (TaKaRa, Kyoto, Japan) and then sub-cloned into pEGFP/C-BCRP with SnaBI/NheI digestion to destroy the CMV promoter. The construct, named P-BCRP, allowed expression of BCRP under control of the wild-type BCRP promoter. All constructs were sequenced.

**Cell transfection, stable cell line establishment, and drug treatment**

For each construct, 8 µl of Lipofectamine (Invitrogen, Carlsbad, CA, USA) and 4 µg of construct were diluted into 250 µl of RPMI-1640 without serum. After incubating for 5-min at room temperature, samples were mixed and incubated for an additional 25 min. Cells were initially seeded in 6-well plates at 1 x 10⁶ cells per well. After 12 hrs, cells were washed three times with RPMI-1640 and then the mixture of construct and Lipofectamine was added. Six hours after transfection, the culture medium was changed to complete RPMI-1640 with 10% FBS.

For stable cell line establishment, transfected cells were switched to fresh medium and selected by growth in complete medium containing 500 µg/ml geneticin (G418; Invitrogen, Carlsbad, CA, USA) for 4 weeks. RT-PCR and Western blotting were used to confirm stable expression of BCRP. Cells stably expressing BCRP were maintained in RPMI-1640 with 10% FBS for at least 24 hrs before PROG treatment. PROG was added to the medium at final concentrations of 0.001, 0.1, or 10 µM and cells were incubated for 48 or 72 hrs. For treatment with both RU-486 and PROG, cells were first incubated with 100 µM RU-486 for 24 hrs. Culture medium was replaced with fresh medium containing 10 µM PROG and 100 µM RU-486, and cells were incubated for another 48 hrs. Cells were then harvested for mRNA and Western blotting analyses.

**Quantitative real-time RT-PCR**

Total RNA was isolated from cell lines using Trizol reagent (TaKaRa, Kyoto, Japan) according to the manufacturer’s instructions. The first-strand cDNA was synthesized from 0.5 µg total RNA using ReverTra Ace qPCR RT Kit (TOYOBO, ShangHai, China). The BCRP mRNA was amplified with SYBR® Green Realtime PCR Master Mix (TOYOBO, ShangHai, China) as previously described[18]. The sense and antisense primers for amplification of BCRP cDNA were 5’-TGGCTGTCATGGCTTCAGTA-3’ and 5’-ACCCCCACTGAAAAAGATGA-3’, respectively. The mRNA levels of each gene evaluated were normalized to levels of 18S rRNA using the formula: ΔCt = Ct(bcrp) - Ct(18S rRNA) for the test group; ΔCt = Ct(bcrp) - Ct(18S rRNA) for the control group. Relative BCRP mRNA levels are expressed as 2-ΔΔCt.

**Protein extraction and Western blotting**

Proteins were extracted by first incubating cells with lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% Triton X-100, 2 mM EDTA (pH 8.0), 5 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride, and 10
μg/ml aprotinin) for 25 min on ice [19]. Samples were then centrifuged at 12,000 g for 15 min at 4 °C to remove cell debris. The extracted proteins were boiled at 100 °C for 5 min and then fractionated on SDS-PAGE. Proteins were transferred from the gel to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). After blocking with 5% fat-free milk in TBS at room temperature for 2 hrs, the membrane was incubated with antibodies against human BCRP or human β-actin overnight at 4 °C. The membrane was washed three times with TBST at room temperature and then incubated with a horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG secondary antibody at 37 °C for 1 hr. Finally, the membranes were visualized using an enhanced chemiluminescence kit (Millipore, Billerica, MA, USA) according to the manufacturer’s instruction.

**Flow cytometric analysis**

To detect transport mediated by BCRP, mitoxantrone was used as a fluorescence tracer. After trypsinization, cells (1×10⁶) were re-suspended in complete RPMI-1640 with 10% FBS as a control, or re-suspended and incubated in complete medium containing 20 μM mitoxantrone at 37 °C for 30 min in 5% CO₂ and then allowed to efflux for 1 hr. Cells were then washed with ice-cold PBS. A FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) equipped with a 635 nm red diode laser and 670 nm band pass filter was used to detect mitoxantrone fluorescence.

**Cytotoxicity assay**

MTT assays were performed as previously reported [20]. Briefly, 1×10⁴ cells in 0.1 ml RPMI-1640 supplemented with 10% FBS were plated each well of a 96-well plate and maintained at 37 °C for 24 hrs. Cells were first treated with RU-486 (100 μM) for 24 hrs, and then incubated with fresh medium containing PROG (10 μM) for 7 hrs. Mitoxantrone was then added at indicated concentrations, and cells were incubated for another 48 hrs. Cells were then stained with 20 μl of sterile MTT per well (5 mg/ml) for 4 hrs at 37 °C. Subsequently, culture medium was discarded and 150 μl of DMSO was added, and samples were mixed for 10 min. Absorbance at 490 nm was measured using an automatic multi-well spectrophotometer (Bio-Rad, Hercules, CA, USA). The relative drug resistance was determined by comparing the IC₅₀ (drug concentration causing 50% inhibition of cell growth) with growth inhibition curves.

**Statistical analyses**

Data analysis was performed with SPSS software version 18.0. Results are presented as means ± SD. Comparisons between groups were made using one-way analysis of variance (ANOVA) followed by Student’s paired t-test to determine significance. Values of P<0.05 were considered significant.

**Results**

**Expression of PR by T47D and MDA-MB-231 cells**

The expression of PR was detected by immunocytochemistry in the two breast cancer cell lines in this study. Nuclei of T47D cells were positive for PR, and nuclei were negative for PR in MDA-MD-231 cells (Fig. 1).

**PROG treatment decreased BCRP mRNA transcription driven by a PRE-containing promoter**

To examine whether PROG regulates BCRP expression in a PRE-dependent manner, we evaluated BCRP mRNA levels in PROG-treated PR-positive T47D cells and PR-negative MDA-MB-231 cells that stably express BCRP driven either by a CMV promoter (C-BCRP) or the wild-type promoter containing the PRE sequence (P-BCRP). PROG treatment decreased BCRP mRNA levels in a dose-dependent manner in T47D cells that stably expressed P-BCRP (Fig. 2A). PROG had no significant effect on levels of BCRP mRNA in cells in which expression was driven by the CMV promoter (Fig. 2B). The effect of 10 μM PROG was almost completely abolished by co-treatment of cells with 100 μM RU-486 (Fig. 2C, P<0.01). PROG plus RU-486 had no significant effect on BCRP mRNA levels in C-BCRP expressing T47D cells (Fig. 2D). No significant changes were detected following PROG treatment in MDA-MB-231 cells stably
expressing either P-BCRP or C-BCRP (data not shown). These results indicate that PRE is necessary for PROG-induced BCRP transcriptional activation.

**PROG treatment inhibited BCRP protein expression in PR-positive cells**

To further evaluate the effect of PROG on exogenous BCRP protein expression, we treated T47D cells expressing either P-BCRP or C-BCRP with various concentrations of PROG (from 0 to 10 μM) for 72 hrs. Proteins were then extracted for Western blotting analysis. In P-BCRP-expressing cells, PROG treatment inhibited BCRP expression in a dose-dependent manner (P<0.01) (Fig. 3A). In cells containing the BCRP gene driven by the CMV promoter, treatment with 0.001 μM or 0.01 μM PROG induced a statistically significant increase in BCRP; but treatment with 10 μM had little impact on BCRP protein levels (Fig. 3B). We then pre-treated cells stably transfected with P-BCRP with 100 μM RU-486 prior to the addition of 10 μM PROG. Under these conditions, BCRP protein levels increased compared to both control and PROG alone groups (Fig. 3C; P<0.01). In T47D/C-BCRP cells, both PROG alone and RU-486 combined with PROG induced expression of BCRP to levels significantly higher
Fig. 3. PROG treatment inhibited BCRP protein expression from the exogenous gene with the PRE-containing promoter. (A and B) Cells expressing BCRP from either (A) the P-BCRP construct or (B) the C-BCRP construct were treated with PROG at the indicated concentrations for 72 hrs, cells were harvested, and proteins were separated by SDS-PAGE followed by Western blotting with anti-BCRP antibody and anti-β-actin antibody. Densitometric analysis was then performed. (C and D) Cells expressing BCRP from either (C) the P-BCRP construct or (D) the C-BCRP construct were first treated with 100µM RU-486 for 24 hrs and then 10µM PROG was added, and cells were incubated for 48 hrs before harvest. Total proteins were subjected to SDS-PAGE followed by Western blotting with anti-BCRP antibody and anti-β-actin antibody. Densitometric analysis was then performed.

than those in the control cells (Fig. 3D; P<0.05). Western blotting for BCRP revealed two specific bands that likely resulted from different levels of glycosylation of BCRP. To obtain more objective results, the intensities of the two bands were combined in densitometry analyses. We conclude that the regulation of BCRP protein levels in the two cell lines resulted from differences in the promoters.

PROG treatment suppressed BCRP-mediated drug efflux

Mitoxantrone is a specific substrate of BCRP that can be used to measure BCRP transport activity. To investigate whether PROG treatment alters BCRP efflux activity, we used a previously described mitoxantrone efflux assay [21] to examine intercellular drug accumulation in T47D cells, in T47D cells that express BCRP under control of the PRE-containing promoter, and in T47D cells that express BCRP under control of the CMV promoter after treatment with 10 µM PROG with or without 100 µM RU-486. As shown in Figure 4, after PROG treatment, the fluorescence peak was shifted rightward in T47D/P-BCRP cells compared to negative control T47D cells indicating inhibition of mitoxantrone efflux. When the cells were treated with PROG and RU-486, the PROG-mediated efflux of mitoxantrone was greatly abrogated, suggesting that PROG inhibited the BCRP efflux activity and RU-486 reversed this effect. Interestingly, in T47D/C-BCRP cells, treatment with PROG resulted in a shift in the fluorescence peak to the left, which implied that efflux via BCRP was decreased, and this effect was not abolished by RU-486 treatment. The same phenomena were detected in both MDA-MB-231/P-BCRP and MDA-MB-231/C-BCRP cells (data not shown).
PROG treatment enhanced chemosensitivity of BCRP-expressing cells

The observation that PROG inhibited BCRP-mediated drug efflux implies that PROG treatment may increase the sensitivity of cancer cells to chemotherapy (Fig. 5). To validate this hypothesis, we analyzed survival of T47D cells treated with mitoxantrone that stably express BCRP under the control of the PRE-containing promoter and the CMV promoter. The \( IC_{50} \) values were 0.06 (±0.02) \( \mu \)M in T47D cells, 1.36 (±0.36) \( \mu \)M in T47D/P-BCRP cells, and 1.40 (±0.17) \( \mu \)M in T47D/C-BCRP cells. Treatment of T47D/C-BCRP cells with PROG did not significantly change the \( IC_{50} \), which was 1.25 (±0.26) \( \mu \)M. In contrast, the \( IC_{50} \) in T47D/P-BCRP cells treated with PROG decreased to 0.26 (±0.03) \( \mu \)M. Compared with T47D cells, the sensitivity of T47D/C-BCRP cells to mitoxantrone increased ~24 fold (\( P<0.01 \)). The sensitivity of T47D/P-BCRP cells was ~25 fold higher than that of the control cells (\( P<0.01 \)). After treatment with PROG, the sensitivity was ~5 fold higher than that of the control cells.
an effect that did not reach statistical significant (Table 1). These results suggest that PR-positive cells that express BCRP under control of a promoter containing PRE are significantly chemosensitized upon treatment with PROG.

Discussion

BCRP was first identified in MCF-7/AdrVp human breast cancer cell lines [22, 23] and is over-expressed in placental syncytiotrophoblasts, canalicular membranes of the liver, small intestine epithelium, endothelial cells of microvessels, and mammary epithelial cells. The localization of BCRP in normal tissues suggests that it plays a protective role [24, 25]. BCRP is highly expressed in many types of tumors, and its efflux activity confers resistance to a variety of chemotherapeutic agents including mitoxantrone, topotecan, and anthracyclines [26, 27].

Nuclear receptors, especially ER, are important determinants of BCRP expression levels in tumor cells. Imai et al. reported that both estrogens and anti-estrogens restrain BCRP-mediated drug resistance. Furthermore, they found that physiological levels of estradiol (E2) reduce BCRP expression by a post-transcriptional mechanism in ERα-positive cells [28-30]. Our group reported that 17β-E, regulates the level of BCRP mRNA at the transcriptional level [11]. We found that down-regulation of BCRP gene expression and the reversal of multi-drug resistance by tamoxifen are mediated by a novel transcriptional mechanism. Specifically, the TOR-ER complex binds to the ERE in the BCRP promoter and associates with components of the transcription machinery to inhibit transcription activation of BCRP [31]. However, the effect of PR on the regulation of BCRP in breast cancer was not clear.

PR may be regulated by ER, and the presence of PR suggests that ER is not only present but also functional in various types of cancer cells [32]. However, about 10% of primary breast cancers are negative for ER but positive for PR. A recent retrospective analysis of the breast cancer patients treated with tamoxifen or with aromatase inhibitors showed that patients with ER+/PR- tumors are more likely to be hormone responsive, whereas patients with ER+/PR+ tumors are less likely to benefit from tamoxifen therapy than those with ER-/PR- tumors. Importantly, patients with ER+/PR- tumors had a poorer disease-free and overall survival compared with patients with ER+/PR+ or ER-/PR- tumors [33, 34]. Thus, PR may have ER-independent functions in breast cancer.

Wang et al. confirmed that the progesterone responsive element in the BCRP promoter is similar to the ERE [12, 35], suggesting that the response element might interact with both ER and PR. Reports on the role of PROG in the treatment of breast cancer are controversial. Synthetic progestin regulates transcription differently in normal cells than in breast cancer cells [36]. Although progestin agents appear to stimulate tumor growth under certain conditions [37], one study found that the use of progesterone alone increases breast cancer risk in women [38]. Li et al. demonstrated that nomegestrol acetate reverses multi-drug resistance in MCF-7/ADR cells by down-regulating both mRNA and proteins levels of P-gp and markedly increases intracellular adriamycin accumulation [39]. However, the role of PR in BCRP expression in breast cancer remains unknown.

We have reported that the PROG/PR complex binds to the PRE in the BCRP promoter and regulates BCRP expression in PR-positive breast cancer cells [40]. In the current study, we sought to clarify the molecular mechanisms by which PR regulates BCRP expression. We constructed plasmids containing BCRP cDNA driven by the wild-type PRE promoter (P-BCRP) or a constitutive CMV promoter (C-BCRP). We then established T47D (PR+) and MDA-MB-231 (PR-) cells that stably expressed the two genes and investigated the regulation of PROG on BCRP-mediated drug resistance [41]. We observed that PROG negatively regulated exogenous BCRP gene expression at the transcriptional level in T47D/P-BCRP cells at typical concentrations of circulating hormones. Furthermore, the suppression of PROG was abolished by the addition of RU-486 at a dose equal to a ten-fold molar ratio excess to that of PROG. In T47D/C-BCRP cells, in which BCRP is constitutively expressed, PROG had little
effect on \( BCRP \) expression. Moreover, in MDA-MB-231 cells expressing either BCRP construct, treatment with PROG induced little change in \( BCRP \) mRNA levels. We hypothesize that PROG binds to the PRE in \( BCRP \) promoter via PROG-PR complexes and interacts with components of transcriptional mechanism to decrease \( BCRP \) gene expression [12]. PROG inhibits \( BCRP \) mRNA expression from the gene driven by the endogenous promoter, and this effect can be reversed by RU-486, a specific PROG inhibitor, suggesting that the repression we observed was indeed mediated by PR. When expression was driven by the CMV promoter, however, \( BCRP \) mRNA levels were not significantly responsive to PROG and the slight activating effect was not abolished by RU-486. PR might regulate \( BCRP \) expression in these cells by interacting with other transcription factors in a PR-PRE-independent manner. In T47D cells that stably express \( BCRP \) under the control of the endogenous promoter, flow cytometric analysis showed that PROG suppressed the drug efflux mediated by BCRP and increased the sensitivity of cells to mitoxantrone. Collectively, these results suggest that PROG can reverse BCRP-mediated MDR in PR+ breast cancer cells by regulating \( BCRP \) expression through PRE.

There are three types of receptor candidates for mediating progesterone actions: membrane progesterin receptors (mPRs), progesterin receptor membrane components (PGRMCs), and nuclear progesterin receptors (nPRs) [42]. In this article, we only addressed classical nPRs and not the rapid activation of intracellular signaling pathways mediated by extra-nuclear PR. Further research will be necessary to clarify the contributions of the three types of receptors in the regulation of \( BCRP \) expression. In contrast to our results in T47D cells, Wang et al. reported that PROG up-regulated \( BCRP \) expression in BeWo cells [43]. In our study, \( BCRP \) expression was driven by the endogenous promoter containing a consensus PRE. In contrast, a Harvey retroviral long terminal repeat promoter drove expression in BeWo cells in the study by Wang et al. Although this difference in promoter may result in the difference in expression observed in the two cell types, the regulation of \( BCRP \) expression and function by PROG may occur through different mechanisms in different cell types. For example, further studies are required to explore whether the two PR isoforms, PR-A and PR-B, have different functions.

Conflict of Interest

The experiments comply with the current laws of China and the authors have no conflicts of interest to declare.

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