Modulation of breast cancer resistance protein (BCRP/ABCG2) gene expression using RNA interference

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Abstract
Overexpression of the breast cancer resistance protein (BCRP/ABCG2) confers multidrug resistance (MDR) to tumor cells and often limits the efficacy of chemotherapy. To circumvent BCRP-mediated MDR, a common approach is the use of potent and specific inhibitors of BCRP transport such as fumitremorgin C, novobiocin, and GF120918. Here, we evaluated a new approach using RNA interference for the specific knockdown of BCRP. We designed and synthesized small interfering RNA (siRNA) using T7 RNA polymerase and showed that siRNAs markedly down-regulated both exogenous and endogenous expression of BCRP. As a functional consequence, knockdown of BCRP by siRNAs increased the sensitivity of human choriocarcinoma BeWo cells to mitoxantrone and topotecan by 10.5- and 8.2-fold, respectively. Using flow cytometry, we found that introduction of siRNAs also enhanced the intracellular accumulation of topotecan. We have previously identified an estrogen response element in the BCRP promoter and have shown that 17β-estradiol increased BCRP mRNA expression. Furthermore, in the present study, we found that expression of BCRP protein was inducible by 17β-estradiol and that this effect was ameliorated by the introduction of siRNAs. These studies indicate that siRNAs could modulate MDR in vitro and may present a new approach to overcome BCRP-mediated drug resistance. [Mol Cancer Ther 2004;3(12):1577–83]

Introduction
Successful chemotherapy is often hampered by the development of multidrug resistance (MDR) in tumor cells. The bête noire of cancer therapeutics, MDR is a phenomenon whereby tumor cells acquire cross-resistance to a variety of structurally and functionally unrelated compounds. It is commonly known that some forms of MDR arise from the overexpression of ATP-binding cassette transporters such as P-glycoprotein (P-gp), MDR-associated protein 1, and/or breast cancer resistance protein (BCRP; ref. 1). Unlike P-gp and MDR-associated protein 1, BCRP (gene symbol ABCG2) is a half-transporter of 655 amino acids that consists of only one transmembrane domain with six transmembrane segments and one nucleotide-binding site (2–4, 9, 10). BCRP is believed to function as a homodimer or as a multimer (5–8). Some examples of BCRP substrates include topotecan, SN-38, mitoxantrone, and flavopiridol (2–4, 9, 10). Acting as an efflux pump in tumor cells, overexpression of BCRP leads to reduced intracellular drug concentration and decreased cytotoxicity. Clinically, it has been reported that a correlation exists between BCRP expression and patient outcome in some hematologic and solid tumors (11, 12).

One pharmacologic approach to circumvent BCRP-mediated drug resistance involves the use of potent inhibitors of BCRP transport such as fumitremorgin C and its analogues (13–15). Fumitremorgin C, described as the first BCRP-selective inhibitor, has been shown to reverse resistance to mitoxantrone, doxorubicin, and flavopiridol in cancer cell lines expressing BCRP (14, 15). Other inhibitors that have been described include, but are not limited to, novobiocin (16–18), GF120918 (19–22), estrogen agonists and anti-estrogens (23), and the tyrosine kinase inhibitors, CI1033 and imatinib mesylate (Gleevec; refs. 24, 25). A nonpharmacologic approach involving the use of a hammerhead ribozyme directed against BCRP to modulate the MDR phenotype has also been reported (26).

More recently, an alternative strategy employing the use of small interfering RNA (siRNA) targeted against the MDR1 gene is effective in reversing MDR (27–30). Both synthetic and vector-based siRNAs markedly down-regulated the expression of MDR1 gene and restored drug sensitivity in cancer cells (27–30). Based on an evolutionarily conserved mechanism termed RNA interference (RNAi), these dsRNA molecules can direct sequence-specific degradation of homologous mRNA to cause specific knockdown of genes (31, 32).

In the present study, we have designed and synthesized siRNAs for the specific knockdown of BCRP and reversal of BCRP-mediated MDR. We found that siRNAs generated using T7 RNA polymerase effectively down-regulated the expression of both exogenous and, importantly, endogenous BCRP and increased tumor cell drug sensitivity and accumulation. We have previously identified an estrogen response element in the BCRP gene promoter (33). Here, using estrogen receptor–positive human choriocarcinoma...
BeWo cells, we found that 17β-estradiol (E$_2$) increased BCRP protein expression in a dose-dependent manner and that siRNAs were effective in ameliorating the stimulatory effect of E$_2$.

Materials and Methods

Chemicals

E$_2$ was purchased from Calbiochem (San Diego, CA) and Topotecan was a gift from SmithKline Beecham Pharmaceuticals (King of Prussia, PA). All other chemicals were purchased from Sigma (St. Louis, MO).

Cell Lines and Culture

All cell lines were obtained from American Type Culture Collection (Manassas, VA). The human choriocarcinoma BeWo cells were maintained in Ham's F-12 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 units/mL streptomycin in a humidified atmosphere of 5% CO$_2$ and 95% air. The human cervical carcinoma HeLa cells were maintained in DMEM containing 10% fetal bovine serum under similar conditions. All cell culture reagents were purchased from BioWhittaker (Walkersville, MD) unless otherwise stated.

siRNA Preparation

In vitro synthesis of siRNAs was done using T7 RNA polymerase in a previously described protocol (34). The siRNA sequence targeting BCRP lies within exon 7 and corresponds to nucleotides 904 to 923 relative to the transcription start site (Genbank accession no. NM_004827; sense: 5'-AAGATGATTGTTCGTCCCTG-3′ and antisense: 5'-TTCTCCA-TTCATCAGCCTCG-3′) and antisense: 5'-GAGAAGATGACCCAGATCATGT-3′ and antisense: 5'-ACTACAAGGGACAGCTATGAAAGT-3′). Target sequences were scrambled and used as a negative control (sense: 5'-AAGCCTTGAGTAGTCGTCTT-3′ and antisense: 5'-GAGAAGATGACCCAGATCATGT-3′ and antisense: 5'-TCGTCATACTCTCTGCTGAGCAG-3′). For Western blot analysis, cell lysates were prepared as described elsewhere (35), and 60 to 80 μg of protein per lane were loaded on a 9% gel. Following electrophoretic separation of proteins and the transfer to nitrocellulose, membranes were immunoblotted with monoclonal antibody BXP-21 (Chemicon, Temecula, CA) for human BCRP and monoclonal antibody AC-74 (Sigma) for human β-actin. A chemiluminescence detection kit from Amersham Biosciences (Piscataway, NJ) was used for detection.

Cytotoxicity Assays

BeWo cells were transfected with siRNAs as above before being seeded in 96-well plates at a density of 4,000 cells per well and were grown overnight. The cells were then treated with drugs at the concentrations indicated and incubated at 37°C for 3 to 4 days. Cytotoxicity was assessed by using the CellTiter 96 AQueous assay according to the manufacturer’s instructions (Promega, Madison, WI).

Intracellular Drug Accumulation

Topotecan, a BCRP substrate, is commonly used to test for changes in drug accumulation mediated by BCRP (23, 25, 36, 37). Here, the effect of siRNA introduction on cellular accumulation of topotecan was determined by flow cytometry as described elsewhere (37). Briefly, BeWo cells were transfected with 5 μg siRNAs as described above for 24 hours and incubated with 30 nmol/L topotecan for 15 minutes at 37°C. After washing twice with ice-cold PBS, cells were subjected to fluorescence analysis using a FACSCalibur (Becton Dickinson, San Jose, CA) with 488 nm excitation at the University of Illinois at Chicago Research Resources Center.

Results

siRNA Knockdown of Exogenous BCRP mRNA and Protein Expression

To determine whether siRNA specific to the BCRP gene sequence could down-regulate gene expression, we first tested its effectiveness in cells exogenously expressing BCRP. Both the expression plasmid encoding the wild-type,
full-length BCRP (pcDNA3-BCRP) and siRNAs targeting the coding sequence of BCRP (siBCRP) were simultaneously transfected into HeLa cells for 24 hours before being harvested for total RNA and protein. As shown in Fig 1A, levels of BCRP mRNA were decreased by at least 25% twenty-four hours after transfection. However, no change in mRNA levels was observed in HeLa cells that were simultaneously transfected with pcDNA3-BCRP and siRNAs containing target sequences that were scrambled (siBCRP-scram). On the protein level, a more dramatic decrease was observed with the introduction of siBCRP as shown in Fig. 1B. siBCRP-scram, on the other hand, did not result in any change in the levels of BCRP protein.

**siRNA Knockdown of Endogenous BCRP mRNA and Protein Expression**

BeWo cells were the first human trophoblastic endocrine cell type to be maintained in continuous culture and were initiated from a malignant gestational choriocarcinoma of the placenta (38). They were also shown to express high endogenous levels of BCRP, comparable with that found in topotecan-resistant human ovarian Igrov/T8 cells (39), and thus were used to examine the effect of siRNA on the endogenous expression of BCRP. Similar knockdown of BCRP expression in both mRNA and protein levels was observed. As shown in Fig. 2A, levels of BCRP mRNA were decreased by at least 50% in BeWo cells transfected with 0–10 μg siRNAs and harvested for total RNA and protein after 24 hours as described in Materials and Methods. Total RNA (1.5 μg) was used for reverse transcription-PCR and 60 μg of protein were separated by 9% SDS-PAGE and then transferred onto nitrocellulose membrane. Membranes were immunoblotted with monoclonal antibody BXP-21 for human BCRP. β-Actin was used as a loading control. Representative of three independent experiments.

**Increase in Drug Sensitivity in siRNA-Transfected Cells**

To test whether siRNA-mediated knockdown of BCRP resulted in increased drug sensitivity, we compared IC_{50} values of mock- and siRNA-transfected BeWo cells obtained from cytotoxicity assays. As shown in Table 1 and Fig. 3A-C, the sensitivity of siBCRP-transfected BeWo cells to two BCRP substrates (i.e., mitoxantrone and topotecan) increased when compared with mock- and siBCRP-scram-transfected cells. More specifically, siRNAs caused a 10.5- and 8.2-fold sensitization to mitoxantrone and topotecan, respectively. On the other hand, the sensitivity to etoposide, a non-BCRP substrate, was not affected by siRNA knockdown of BCRP.

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**Figure 1.** siRNA knockdown of exogenous BCRP mRNA (A) and protein (B) expression in HeLa cells. HeLa cells were simultaneously transfected with 0–10 μg siRNAs and 3 μg expression vector for wild-type, full-length BCRP (pcDNA3-BCRP) using LipofectAMINE Plus according to the manufacturer’s protocol. After 24 hours, cells were harvested for total RNA and protein as described in Materials and Methods. Total RNA (1.5 μg) was used for reverse transcription-PCR and 60 μg of protein were separated by 9% SDS-PAGE and then transferred onto nitrocellulose membrane. Membranes were immunoblotted with monoclonal antibody BXP-21 for human BCRP. β-Actin was used as a loading control. Representative of three independent experiments.

**Figure 2.** siRNA knockdown of endogenous BCRP mRNA (A) and protein (B) expression in BeWo cells. Human choriocarcinoma BeWo cells were transfected with 0–10 μg siRNAs and harvested for total RNA and protein after 24 hours as described in Materials and Methods. Total RNA (1.5 μg) was used for reverse transcription-PCR and 60 μg of protein were separated by 9% SDS-PAGE and then transferred onto nitrocellulose membrane. Membranes were immunoblotted with monoclonal antibody BXP-21 for human BCRP. β-Actin was used as a loading control. Representative of three independent experiments.
Increase in Drug Accumulation in siRNA-Transfected Cells

With the introduction of siRNAs into BeWo cells, intracellular drug accumulation was enhanced (Fig. 3D). Using flow cytometry, we showed that intracellular accumulation of topotecan was increased in siBCRP-transfected BeWo cells when compared with those that were mock transfected. As expected, cells that were transfected with siBCRP-scram did not result in any change in intracellular accumulation of topotecan.

Table 1. Effect of siRNA on IC50 values of drugs in BeWo cells

<table>
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<tr>
<th>siRNA</th>
<th>Mitoxantrone IC50</th>
<th>Topotecan IC50</th>
<th>Etoposide IC50</th>
</tr>
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<tr>
<td>Mock</td>
<td>0.294 ± 0.066</td>
<td>0.164 ± 0.055</td>
<td>2.07 ± 0.095</td>
</tr>
<tr>
<td>siBCRP</td>
<td>0.028 ± 0.075</td>
<td>0.020 ± 0.077</td>
<td>1.77 ± 0.079</td>
</tr>
<tr>
<td>(10.5)</td>
<td>(8.2)</td>
<td>(1.2)</td>
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</tr>
<tr>
<td>siBCRP-scram</td>
<td>0.241 ± 0.077</td>
<td>0.158 ± 0.058</td>
<td>1.87 ± 0.113</td>
</tr>
<tr>
<td>(1.2)</td>
<td>(1.0)</td>
<td>(1.1)</td>
<td></td>
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</tbody>
</table>

NOTE: In vitro cytotoxicity assays (CellTiter 96 AQuantiBlue assay) were done as described in the manufacturer’s protocol. IC50 is the concentration (μmol/L) that produced 50% inhibition of cell growth. Results represent the mean ± SE of three experiments done in triplicate. Numbers in parentheses represent fold sensitization of drug resistance.

Circumvention of BCRP Expression under E2 Stimulation by siRNA

We have previously identified an estrogen response element in the BCRP gene promoter and have shown that E2 enhanced expression of BCRP mRNA in estrogen receptor–positive cells (33). To determine whether BCRP protein expression is similarly inducible by E2, we first placed estrogen receptor–positive BeWo cells in phenol red–free medium supplemented with charcoal/dextran-stripped fetal bovine serum (Gemini) for 3 days before subsequent siRNAs transfection and E2 treatment. It is important to note that, with the removal of estrogens found in phenol red and fetal bovine serum, the levels of BCRP protein decreased dramatically. This is shown in Fig. 4A whereby the amount of BCRP protein in cell lysate obtained from untreated BeWo cells was much lower than that in the same amount of lysate obtained from cells cultured in phenol red–containing medium (Fig. 2B). With the addition of 1 to 100 nmol/L E2, we observed a dose-dependent increase in the levels of BCRP protein from 1.0- to 7.8-fold in BeWo cells (Fig. 4A). In contrast, when BeWo cells were transfected with siBCRP and then subjected to E2 treatment, only a marginal 1.5-fold increase was observed at maximal E2 concentration of 100 nmol/L (Fig. 4B).

Figure 3. Effect of siRNA on drug sensitivity and accumulation in BeWo cells. BeWo cells were transfected with siRNAs before being seeded in 96-well plates at a density of 4,000 cells per well and grown overnight. Cells were then treated with (A) mitoxantrone, (B) topotecan, or (C) etoposide at the concentrations indicated and incubated at 37°C for 3–4 days. Cytotoxicity was assessed by using the CellTiter 96 AQuantiBlue assay according to the manufacturer’s instructions. Representative of three independent experiments done in triplicate. D, mock- or siRNA-transfected BeWo cells were incubated with 30 μmol/L topotecan for 15 minutes at 37°C. Cellular content of topotecan was measured by fluorescence-activated cell sorting. Bold lines, shaded area, siBCRP transfected; bold lines, unshaded area, mock transfected; dotted lines, siBCRP-scram transfected. See Materials and Methods for details.
that was knocked down by the siRNAs might be infection such that BCRP mRNA was transcribed in great overwhelming with plasmid DNA during transient transfection with siBCRP. siBCRP-transfected cells were cultured for an additional 24 hours before E2 treatment. Protein (60–80 μg) was separated by 9% SDS-PAGE, and separated proteins were then transferred onto nitrocellulose membrane. Membranes were immunoblotted with monoclonal antibody BXP-21 for human BCRP. See Materials and Methods for details.

Discussion
To date, clinical success using functional modulators to reverse MDR has been modest due in part to toxicity problems and pharmacokinetic interactions (40). Whereas the search for effective small molecule inhibitors continues, molecular strategies via the use of hammerhead ribozymes and antisense oligonucleotides have also been proposed.

Although experimental results indicate that these strategies are effective in vitro, their clinical utility is limited due to difficulties in delivery, stability, and potency. With our increasing understanding of the mechanism of RNAi, a third approach of using siRNAs in combating MDR should not be left unexplored. Here, we have designed and synthesized siRNAs against human BCRP and showed that siRNAs could specifically and effectively down-regulate BCRP expression in tumor cells. We found that siRNAs transected for an optimal time of 24 hours were effective in down-regulating BCRP expression not only in HeLa cells containing enforced expression of wild-type BCRP but also in human choriocarcinoma BeWo cells displaying high endogenous expression of BCRP (39). It is interesting to note that, when the BCRP cDNA and siRNAs were simultaneously transfected into HeLa cells, a 25% decrease in mRNA levels resulted in a dramatic decrease in protein levels. This may be because the HeLa cells were overwhelmed with plasmid DNA during transient transfection such that BCRP mRNA was transcribed in great excess over the translational machinery in positively transfected cells. The relatively small proportion of mRNA that was knocked down by the siRNAs might be representative of the population of transcripts that were actually translated in the cells; hence, we observed a dramatic decrease in protein levels. On the other hand, we do not rule out the possibility that Western blotting is not sensitive enough to detect subtle changes in the protein levels. Most important, siRNAs effectively down-regulated endogenous BCRP in BeWo cells with decreases in mRNA and protein levels that were in good agreement with changes in the MDR phenotype. siRNAs sensitized BeWo cells to BCRP substrates such as mitoxantrone and topotecan and enhanced the intracellular accumulation of topotecan.

Here, we showed that the sensitivity to etoposide in BeWo cells remained unchanged with the introduction of siRNAs. Various levels of cross-resistance to etoposide have been observed in several drug-selected cell lines overexpressing BCRP/Bcrp1 (14, 21, 41, 42), although there is no conclusive evidence that this phenomenon could be attributed solely to BCRP/Bcrp1 activity, because the cells in those studies were heavily selected for drug resistance. The contribution of other mechanisms of resistance, such as changes in topoisomerase II and overexpression of other drug transporters, may confound those results and should therefore be taken into account. BeWo cells, however, express high levels of endogenous BCRP and, more importantly, were not selected for resistance with drugs. Instead of evaluating substrate specificities and transport properties of a specific transporter by using cell lines with a gain-of-function, the present study shows that RNAi is also useful in performing this task by the specific knockdown of individual transporter genes (i.e., loss-of-function).

We reported previously the presence of an estrogen response element in the BCRP promoter and showed that E2 was able to activate BCRP transcription in estrogen receptor–positive breast and ovarian cancer cells (33). BCRP is highly expressed in the placenta (43) where steroid hormones such as estrogens are produced and our earlier findings suggested that estrogens may induce the endogenous expression of BCRP in the placenta. Here, using estrogen receptor–positive BeWo cells derived from the placenta, we found that E2 was also able to induce BCRP expression at the protein level. This is shown by a noticeable decrease in BCRP protein levels in BeWo cells on removal of estrogens in the culture system by using phenol red–free medium containing charcoal-stripped serum. Following treatment with 1 to 100 nmol/L E2, the levels of BCRP protein levels in BeWo cells then increased dramatically to 7.8-fold at the maximal E2 concentration. However, with the introduction of siRNAs, we found that the effect was ameliorated with only 1.5-fold induction of BCRP expression at 100 nmol/L E2. These results further confirm that siRNAs are effective in modulating the functional phenotype mediated by BCRP.

Given the exploitation of a naturally occurring machinery that is both efficient and specific, RNAi seems to be more potent than the other types of RNA strategies (44) and may have an impact as a therapeutic tool. As with antisense and gene therapy approaches, the major challenge in
translating RNAi into useful therapeutic strategy is the delivery of siRNAs. However, considerable progress has recently been made in this field and several genes have been successfully down-regulated by RNAi in in vivo models due to improved delivery systems (45–47). Given sufficient research into delivery methods, siRNA may indeed become a viable treatment modality.

To our knowledge, this is the first report describing evidence of silencing the human BCRP gene by siRNAs in cancer cells. Several studies employing the same strategy against the MDR1 gene have been published recently (27–30). Although selection for drug resistance in cell lines usually results in predominance of a single ATP-binding cassette transporter, such a situation is not observed in clinical samples. For example, in some cases of adult leukemias, combined overexpression of P-gp and MDR-associated protein 1 have been implicated in the MDR phenotype (48), and in others, even when P-gp-positive blasts were eliminated, a non-P-gp mechanism of resistance emerged in relapsed patients (49). It is also suggested that BCRP may have a role in recurrent tumors, especially in hematologic malignancies in which patients have been concurrently treated with chemotherapy and with inhibitors of P-gp (50). Taken together, it may be feasible to use siRNAs therapeutically in combination against the repertoire of ATP-binding cassette transporters to combat MDR.

In conclusion, siRNAs effectively down-regulated BCRP expression in tumor cells and modulated their functional phenotype. These findings may present a new approach in combating BCRP-mediated drug resistance.

Acknowledgments

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References


