Overexpression of Wild-Type Breast Cancer Resistance Protein Mediates Methotrexate Resistance

Erin L. Volk, Kate M. Farley, Yan Wu, Fei Li, Robert W. Robey, and Erasmus Schneider

ABSTRACT

Previously, we have reported that a multidrug-resistant, mitoxantrone (MX)-selected cell line, MCF7/MX, is highly cross-resistant to the antifolate methotrexate (MTX), because of enhanced ATP-dependent drug efflux (E. L. Volk et al., Cancer Res., 60: 3514–3521, 2000). These cells overexpress the breast cancer resistance protein (BCRP), and resistance to MTX as well as to MX was reversible by the BCRP inhibitor, GF120918. These data indicated that BCRP causes the multidrug-resistance phenotype. To further examine the role of this transporter in MTX resistance, and in particular the role of amino acid 482, we analyzed a number of BCRP-overexpressing cell lines. MTX resistance correlated with BCRP expression in all of the cell lines expressing the wild-type transporter, which contains an Arg at position 482. In contrast, little or no cross-resistance was found in the MCF7/AdVp1000 and S1-M1-3.2 and S1-M1-80 cell lines, which contain acquired mutations at this position, R482T and R482G, respectively. Concomitantly, the greatest reduction in MTX accumulation was observed in the MCF7/MX cells (BCRP<sub>Arg</sub>) as compared with cells expressing the Thr and Gly BCRP variants. Furthermore, the reduction in drug accumulation was sensitive to BCRP inhibition by GF120918. In conclusion, we have demonstrated a novel role for BCRP as a mediator of MTX resistance and have provided further evidence for the importance of amino acid 482 in substrate specificity.

INTRODUCTION

The acquisition of resistance to the antifolate drug MTX typically occurs via one of several “classical” mechanisms. Amplification or mutation of the target enzyme dihydrofolate reductase (1–4), reduced drug uptake by a mutated reduced folate carrier (5–8), or alterations in the enzymes involved in MTX polyglutamylation (9, 10) have repeatedly been shown to cause MTX resistance. We have previously reported on the MX-selected human breast carcinoma cell line, MCF7/MX, which displays high levels of MTX cross-resistance (>150-fold) when compared with its parental cells. However, MTX resistance could not be attributed to any of the classical mechanisms (11). Instead, this cell line displayed an enhanced ATP-dependent MTX efflux, which is suggestive of a role for an ABC transporter.

The presence of a MTX efflux system in cancer cells has been predicted based on biochemical evidence for many years (12–18); however, the molecular identity of the transporter has been elusive. Furthermore, few previous reports have suggested that MTX resistance might be caused by enhanced drug efflux (11, 19). More recently, several members of the MRP (or ABCC) subfamily of the ABC transporters have been implicated in MTX transport and resistance. Overexpression of MRPs 1–4 (20–22) has been shown to reduce MTX accumulation and to lead to decreased MTX sensitivity, but only in short-term (<4-h) exposure assays. In contrast, the MCF7/MX cells displayed MTX resistance to long-term (7 days) MTX exposure in the absence of MRP overexpression (11).

Another ABC transporter, BCRP (also known as MXR1, ABCP, or ABCG2), was found to be highly overexpressed in the MCF7/MX cells (23). This membrane-localized (24) half-transporter was initially found to cause MX resistance (23, 25–27), and has subsequently also been associated with topotecan, irinotecan, doxorubicin, and flavopiridol resistance (26, 28–30). In contrast, its role in MTX resistance remained unclear (11). Furthermore, it was recently shown that the amino acid at position 482 has a profound effect on substrate specificity (31, 32). Therefore, to examine a putative association between BCRP overexpression and MTX resistance as well as the role of amino acid 482, we analyzed a number of known BCRP-overexpressing cell lines for MX and MTX resistance and whether amino acid 482 determined substrate specificity.

MATERIALS AND METHODS

Cell Culture.

The following cell lines were cultured in improved minimal essential medium containing 10% fetal bovine serum and 0.01 g/liter ciprofloxacin at 37°C in the presence of 5% CO₂. The human breast carcinoma cell lines MCF7/WT and MCF7/Par (33); the MX-selected derivatives MCF7/MX (34), MCF7/MX100 (31), and MCF7/Mitox (35); the flavopiridol-selected MCF7/FLV1000 (36); the melphalan-selected MCF7/Mel<sup>R</sup> (37); the topotecan-selected MCF7/TPT300 (30); the Adriamycin and verapamil-selected MCF7/AdVp1000 (38); the BCRP-transfected MCF7/BCRP<sub>Arg</sub> (clones R37, R67, R95), MCF7/BCRP<sub>Gly</sub> (clones G52, G57, G70), MCF7/BCRP<sub>Thr</sub> (clone 8; Ref. 25) and MDA-MB231/BCRP<sub>Thr</sub> (clone 23; Ref. 11) cells; the central nervous system-derived tumor cell line Si295/Par and its MX-selected derivative Si295/MX50 (39); the non-small cell lung carcinoma cell line H460/Par and its MX-selected derivative H460/MX20 (39); the gastric carcinoma cell line EPG257/Par and its novantrone-selected derivative EPG257/RNOV (40); and the colon carcinoma cell lines S1 and its MX-selected derivatives S1-M1-3.2 (41) and S1-M1-80 (27), and WiDr/S and its MX-selected derivative WiDr/R (42, 43), were added to wells immediately prior to addition of the drug.

Cytotoxicity Assays.

MRPs 1–4 (20–22) have been shown to reduce MTX accumulation and to lead to decreased MTX sensitivity, but only in short-term (<4-h) exposure assays. In contrast, the MCF7/MX cells displayed MTX resistance to long-term (7 days) MTX exposure in the absence of MRP overexpression (11).

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Real-Time Quantitative RT-PCR.

RNA was extracted from each cell line using the RNeasy kit (Qiagen, Valencia, CA). One µg of RNA was reverse transcribed with Omniscript reverse transcriptase using random hexamers as primers (Qiagen). The resulting cDNAs were 2.5-fold diluted with water, and levels of BCRP and the housekeeping gene PBGD were determined by real-time quantitative PCR on the Light Cycler using the FastStart DNA Master SYBR Green I reagent kit (Roche, Indianapolis, IN) under the following conditions: 95°C, 5 s; 63°C, 5 s; 72°C, 20 s (temperature ramping 3°C/sec). Amplification products were detected by SYBR green at 81°C and 85°C, respectively, and BCRP levels were normalized to those of PBGD. The primers used were 5'-GCTTTCAAGGAGATGCTACGTCACC-3' and 5'-GCTGAAATAGCCACCATCATAAGG-3' for BCRP, and 5'-TCTGGTA-
BCRP mediates MTX resistance

ACGGCAATGGGCGC-3' and 5'-CCAGGGCATCTTCAAGCTCC-3' for PBGD.

Western Blotting. Cell membranes were prepared as previously described (11), and 50 µg of protein, dissolved in sample buffer [50 mM Tris/HCl (pH 6.8), 2% DTT], were loaded onto 10% polyacrylamide running gels overlaid with 4% stacking gels containing 8M urea. The fractionated proteins were electroelutectronically transferred to a polyvinylidene difluoride membrane at 100 V for 2 h. Nonspecific binding was blocked by incubation in Blotto (Bio-Rad, Hercules, CA) for 30 min. The membrane was then incubated overnight in the presence of anti-BCRP antibody (kindly provided by Dr. Susan Bates, National Cancer Institute, Bethesda, MD), followed by horseradish peroxidase-conjugated antirabbit secondary antibody for 1 h. After addition of chemiluminescent substrate (Pierce, Rockford, IL), bound antibody was detected by exposure of the membrane to X-ray film.

Southern Blotting. DNA was extracted from each cell line using the Purgene kit (Gentra Systems, Minneapolis, MN). Ten µg of each DNA were separately digested with EcoRI and BamHI (Roche) overnight at 37°C. Digenstated samples were ethanol-precipitated and separated on a 0.8% agarose gel at 40 V for 4 h. After depurination, denaturation, and neutralization, DNAs were transferred overnight onto positively charged nylon membranes (Roche) by capillary action. Membranes were UV cross-linked, prehybridized at 37°C for 2 h in DIG Easy Hyb (Roche), and hybridized overnight to a dioxygenin-labeled BCRP probe. Labeled membranes were washed twice in 2X SSC at room temperature, twice in 0.5X SSC, 0.1% SDS at 55°C, and blocked in blocking buffer (Roche) for 1 h at room temperature. The membrane was then incubated in the presence of alkaline phosphatase-conjugated anti-digoxigenin antibody for 30 min. After washing twice in washing buffer (Roche), the membrane was incubated with CSPD substrate and chemiluminescence was detected by exposure to X-ray film.

Steady-State Drug Accumulation. MCF7/WT, MCF7/MX, MCF7/Advp1000, S1, and S1-M1-80 cells were seeded in triplicate in 12-well tissue culture plates, grown for 96 h, and incubated in the presence of serum-free medium for another 24 h. Cells were exposed to either 1 µM [3H]-MX for 4 h or 2 µM [3H]-MTX for 24 h at 37°C. After drug exposure, plates were washed twice in ice-cold PBS and lysed overnight in 0.2 N NaOH. Lysates were neutralized with an equal volume of 0.2 N HCl, and the remaining radioactivity was determined using liquid scintillation counting. Counts were normalized for protein concentration that was measured by the Bradford assay (45). Accumulation was expressed as pmol drug/mg protein.

RESULTS

BCRP Inhibition. The overexpression of BCRP has been shown to cause MX resistance in many drug-selected cell lines. We have previously demonstrated that MCF7/MX cells, which overexpress BCRP, are highly resistant to MX but also display a strong cross-resistance to the antifolate MTX (11). MX resistance has been shown to be effectively reversed by the BCRP inhibitor GF120918 (46, 47). Therefore, to determine a possible role of BCRP in MTX resistance, cytotoxicity assays were performed in the presence of 5 µM GF120918. GF120918 effectively reversed MTX resistance as demonstrated by the 30-fold sensitization of MCF7/MX cells to MTX (Table 1). Similar results were also obtained with furnitremorigin C (data not shown). These data indicate that BCRP may be an important determinant of MTX resistance. As expected, GF120918 also reversed MX resistance and sensitized the resistant cells 300-fold. Only small effects of the inhibitor on drug sensitivity were found in MCF7/WT cells.

Table 1 Effect of the BCRP inhibitor GF120918 on MX and MTX resistance in MCF7/WT and MCF7/MX cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC50 (µM)</th>
<th>IC50+GF (µM)</th>
<th>DFM*</th>
<th>IC50 (µM)</th>
<th>IC50+GF (µM)</th>
<th>DFM*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF7/WT</td>
<td>0.46</td>
<td>0.13</td>
<td>3.5</td>
<td>0.10</td>
<td>0.06</td>
<td>1.6</td>
</tr>
<tr>
<td>MCF7/MX</td>
<td>863</td>
<td>2.8</td>
<td>308</td>
<td>11.3</td>
<td>0.39</td>
<td>29</td>
</tr>
</tbody>
</table>

* DFM, dose-modifying factor.

Drug Resistance. MX resistance appears to be a universal characteristic of cell lines that overexpress BCRP. To determine whether MTX cross-resistance occurs in parallel with the acquisition of MX resistance, a number of known MX-resistant cell lines were assayed for sensitivity to MTX. For comparison, we also included the MCF7/Mel8 cell line, which, although not known to overexpress BCRP, exhibits a MTX cross-resistance phenotype that resembles that of the MCF7/MX cells (48). MX and MTX resistance appeared to occur concurrently, although MX resistance was generally higher. Most of the cell lines tested were similar to the MCF7/MX cells in that they exhibited a relatively low (<30) ratio of MX/MTX resistance (MX/MTX; Fig. 1A). However, there were two exceptions: the MX-selected S1-M1-80 colon carcinoma cell line and the Adriamycin- and verapamil-selected MCF7/Advp1000 breast cancer cell line, which displayed little or no MTX cross-resistance, despite high levels of MX resistance. This is reflected in the high MX/MTX ratios of 3300 and 450, respectively, in these two cell lines. Interestingly, all of the MTX cross-resistant cell lines express the wild-type BCRP, which contains an Arg in position 482 (49), whereas the two MTX-sensitive cell lines express mutated variants of this exporter (data not shown). The BCRP variant in S1-M1-80 cells contains a Gly at position 482 (31), whereas this position is mutated to Thr in the MCF7/Advp1000 cells (31). These data suggested that MTX resistance correlates with MX resistance in the presence of wild-type BCRP, and that mutations in this transporter may abrogate this effect.

BCRP mRNA Expression and Drug Resistance. To further explore a possible association of BCRP with MTX resistance, we measured BCRP mRNA expression levels using real-time quantitative RT-PCR. Expression levels of BCRP mRNA in the resistant cells were normalized to levels in their respective parental cells and were correlated with the relative resistances to either MX or MTX. A generally linear relationship between BCRP overexpression and drug resistance was observed for MX (r² = 0.81; P < 0.001; Fig. 1B). Similarly, resistance to MTX also increased linearly with BCRP expression (r² = 0.90; P < 0.001; Fig. 1C), although BCRP appeared much less efficient in conferring MTX resistance. Analysis of 10 cell lines expressing wild-type BCRP revealed that there was an ~1:1 relationship between BCRP overexpression and MX resistance, whereas the corresponding relationship was ~10:1 for MTX, i.e., 100-fold overexpression of BCRP corresponded to ~125-fold MX resistance, but only to about 9-fold resistance to MTX. Interestingly, as shown in Fig. 1, B and C, there were some notable outliers, especially for MTX. Two of these, represented by the open upright triangles, are the S1-M1-3.2 and S1-M1-80 cell lines, and the third, represented by the open circle, is the MCF7/Advp1000 cell line. All three express mutated BCRP. In each of these cell lines, MTX cross-resistance is significantly lower than expected from their levels of BCRP overexpression. However, there was an excellent correlation of overexpression with MX resistance; indeed, these cells seemed even more resistant than had been expected from their amount of BCRP overexpression. Thus, these data provide further support for a direct relationship between BCRP expression and MX resistance and suggest that a similar, albeit less efficient, relationship exists for MTX, but only for wild-type BCRP. This conclusion is further supported by the fact that MCF7 cells, transfected with BCRPArg ( ), BCRPgly ( ), or BCRPThr ( ) essentially exhibit the same relationship between resistance and overexpression.

BCRP Protein Expression. To confirm that the previously observed BCRP mRNA overexpression corresponded to an increased
expression at the protein level, a Western blot analysis was performed on several representative cell lines. As expected, the parental cell lines exhibited very low-to-undetectable levels of BCRP expression, whereas the drug-selected derivatives all displayed a substantial amount (Fig. 2). Of the MCF7 cell lines examined, the greatest increase in BCRP expression was observed in the MCF7/MX, MCF7/MX100, MCF7/FLV1000, and MCF/AdVp1000 cells, whereas BCRP expression in MCF7/TPT300 cells was lower, and no protein was detected in the MCF7/MelR cells. Similarly low, but clearly detectable, protein levels in the MCF7/TPT300 cells have also been previously reported (30). Among the other cell lines analyzed, the highest levels of BCRP were found in the S1-M1-80 and EPG257/RNOV

Fig. 2. Western blot analysis of BCRP expression in various parental and drug-selected cell lines. Fifty µg of protein from membrane preparations from each cell line were electrophoresed on a polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. The membrane was incubated with an anti-BCRP antibody, and BCRP-specific signals were detected by chemiluminescence. Top two panels contain drug-selected cell lines; bottom panel contains various transfected clones together with the MCF7/WT and MCF7/MX par, run on the same gel as the two BCRP transfectants.
cells. Generally, overexpression of BCRP at the mRNA level was reflected by a corresponding increase in protein levels, and this was also true for the various BCRP-transfected clones.

**Drug Accumulation.** All MTX-resistant cell lines overexpress the wild-type BCRP, which contains Arg at position 482, whereas the MTX-sensitive cell lines overexpress variants that contain either Thr or Gly at this position. To examine the impact of these variants on steady-state drug accumulation, accumulation of both MX and MTX was measured in a representative cell line of each variant (MCF7/MXArg, MCF7/AdVp1000 Thr, S1-M1-80 Gly). As expected for these highly MX-resistant cell lines, all showed a significant reduction in the accumulation of MX (Fig. 3) relative to their respective parental cell lines. Furthermore, each cell line also displayed a reduction in the accumulation of MTX, and this reduction was abrogated by the BCRP inhibitor GF120918. This indicated that the reduction in drug accumulation was mediated by BCRP. For both drugs, the reduction in accumulation was biggest in the MCF7/MX cells (BCRPArg) with <40% of the amount seen in the parental cells and the smallest amount in the S1-M1-80 cells (BCRP Gly), in which the reduction was only 20–30%. Thus, it appeared that MTX resistance roughly correlated with drug accumulation in each BCRP variant cell line. The BCRPArg-expressing MCF7/MX cells exhibited the greatest reduction in MTX accumulation and were highly resistant, whereas the BCRP Gly-expressing S1-M1-80 cells displayed only a small reduction in MTX accumulation and showed little resistance. The BCRP Thr-expressing MCF7/AdVp1000 cells fall in between those two extremes. In contrast, the observation that MX accumulation followed a similar pattern was somewhat unexpected, considering that both MCF7/AdVp1000 and S1-M1-80 cells are more highly MX-resistant than are MCF7/MX cells.

**BCRP Amplification.** Overexpression of BCRP in several of the MTX-resistant cell lines, including MCF7/MX, has been attributed to amplification of the corresponding ABCG2 gene (23, 50). The enhanced expression of BCRP in the MTX-sensitive S1-M1-80 cells, however, occurs in the absence of gene amplification (50). Thus, it was possible that MTX resistance was associated with BCRP amplification. To examine this possibility, DNA from each of the BCRP-expressing cell lines was analyzed by Southern blot (Fig. 4). As expected, ABCG2 was found to be amplified in the MCF7/MX cell line. Additionally, low levels of amplification were also seen in the MCF7/MX100, MCF7/Mitox, MCF7/FLV1000, and SF295/MX50 cells. However, amplification was not universal and, therefore, did not appear to correlate with MTX resistance.

**DISCUSSION**

The data presented confirm the relationship between MX resistance and BCRP expression and provide evidence for a novel role for BCRP as a mediator of MTX resistance. The relationship between BCRP expression and resistance to both MX and MTX was linear, at least in the presence of the wild-type form of the transporter, which contains an Arg at position 482. However, there were outliers with respect to both drugs. It appeared that the Thr and Gly variant cell lines MCF7/AdVp1000, S1-M1-3.2, and S1-M1-80 were more resistant to MX, and less resistant to MTX, than expected from their BCRP levels. These data are consistent with recent observations that mutations at...
position 482 significantly impact substrate specificity (31, 32). Honjo et al. (31) showed that, when compared with the wild-type containing Arg, the Gly variant of BCRP conferred slightly higher levels of resistance against MX, lower levels of topotecan resistance, and high cross-resistance to Adriamycin. Furthermore, rhodamine 123 was a substrate for BCRP Gly, but not for BCRP Arg. Similar results were also seen in murine Bcrp1 R482M and R482S mutants (32). In the present study, resistance to both MX and MTX increased linearly with higher levels of expression in cells transfected with BCRP Arg, whereas BCRP Gly- and BCRP Thr-transfectants displayed only enhanced MX resistance but lacked MTX cross-resistance. The 20- to 40-fold over-expression of exogenous BCRP corresponded to the 20- to 40-fold resistance against MX, in agreement with the −1:1 relationship between BCRP expression and resistance predicted from the drug-selected cells. Similarly, the −1:1 relationship between BCRP expression and MTX resistance may account for the modest 2-to 3-fold levels of MTX resistance observed in BCRP Arg transfectants. In contrast, no changes in MTX sensitivity were observed in BCRP Gly- or BCRP Thr-transfected cells, despite levels of exogenous BCRP expression similar to those seen in the BCRP Arg transfectants. Overall, the results obtained with the transfected cells mirror and confirm the findings from the drug-selected cells and demonstrate that BCRP overexpression confers, at least in part, MX and MTX resistance.

This conclusion appears in direct contradiction to that in our previous report, in which we concluded that BCRP was unlikely to confer MTX resistance, based on the lack of MTX resistance in BCRP-transfected cells (11). However, the two BCRP-transfected clones used in the present study (represented by the open diamonds in Fig. 1, B and C) were obtained using cDNA derived from the MCF7/AdVp cells, before it was known that these cells contain the R482T mutation. Thus in light of the present findings of the importance of the Arg at position 482, it is not surprising that we did not observe MTX resistance at the time.

A notable exception to the general pattern is the MCF7/Mel18 cell line (indicated by an asterisk (*) in Figs. 1, B and C), which appears to be less resistant against MX and more resistant against MTX than expected from the 30-fold overexpression of wild-type BCRP mRNA.

Whereas the exact reasons for this apparent discrepancy are unclear, it appears that the higher mRNA levels are not translated into elevated protein levels, which may explain the lack of MX resistance. In contrast, it was previously shown that MTX resistance in these cells was caused by decreased drug uptake rather than by enhanced efflux (48) and, therefore, appears unrelated to BCRP overexpression. Low levels of BCRP protein were also observed in MCF7/TPT300 cells. However, these cells follow the general relationship between BCRP overexpression and drug resistance, which suggests that BCRP in these cells is functional. Furthermore, no evidence for either MDR1 or MRP1 overexpression was found (30). Thus, it appears that MX and MTX resistance in these cells is primarily attributable to BCRP overexpression.

In further support of a relationship between MTX resistance and BCRP Arg expression, we found that the reduction of drug accumulation in an Arg variant was greater than in either a Thr or Gly variant. Surprisingly, we found a similar accumulation pattern for MX, with the lowest drug accumulation in the MCF7/MX cells, which express the BCRP Arg variant, whereas the greatest level of MX accumulated in the BCRP Gly-expressing S1-M1-80 cells. Accumulation in the MCF7/AdVp1000 cells, which express the BCRP Thr variant, was at a level between that of the other two. These data appear to contradict the pattern of relative resistances in these cell lines, because both MCF7/AdVp1000 and S1-M1-80 cells were more resistant against MX than were MCF7/MX cells. However, it is possible that the vesicular accumulation of MX that has been observed in the S1-M1-80 cells (26), may sequester the drug from its target and, thus, reduce its effective concentration, without reducing its overall accumulation.

It is theoretically possible that another gene whose product contributes to MTX resistance is co-amplified with ABCG2 (BCRP). However, the Southern blot analysis did not reveal a correlation between amplification and MTX resistance, and, thus, the involvement of a co-amplification product is unlikely. Surprisingly, the MCF7/AdVp1000 cells were not found to contain multiple copies of ABCG2, in contrast to the highly amplified MCF7/AdVp3000 cell line (50). It is possible that amplification occurred only during the highest level of selection.

In conclusion, we have revealed a novel function for BCRP as a mediator of MTX resistance. This resistance is reversible by known BCRP inhibitors, and a number of cell lines that express BCRP all display a similar cross-resistance pattern. Although several members of the MRP family of ABC transporters have also been shown to confer MTX resistance, BCRP is the only one that does so in continuous exposure assays. Whether this is because BCRP-mediated MTX transport is not restricted to the monoglutamylated form, as was shown for the MRPs (51), or whether additional factors are involved, is currently unknown and under active investigation. Additionally, we found that the R482T and R482G mutations in BCRP abolish MTX resistance. These data provide further evidence that position 482 is critical for substrate recognition by BCRP.

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