Methotrexate Cross-Resistance in a Mitoxantrone-selected Multidrug-resistant MCF7 Breast Cancer Cell Line Is Attributable to Enhanced Energy-dependent Drug Efflux

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ABSTRACT

Cellular resistance to the antifolate methotrexate (MTX) is often caused by target amplification, uptake defects, or alterations in polyglutamyl- ation. Here we have examined MTX cross-resistance in a human breast carcinoma cell line (MCF7/MX) selected in the presence of mitoxantrone, an anticancer agent associated with the multidrug resistance (MDR) phenotype. Examination of protein expression and enzyme activities showed that MCF7/MX cells displayed none of the classical mechanisms of MTX resistance. They did, however, exhibit an ATP-sensitive accumulation defect accompanied by reduced polyglutamyl ation. Although the kinetics of drug uptake was similar between parental and resistant cells, the resistant cells exhibited increased energy-dependent drug efflux. This suggested the involvement of an ATP-binding cassette (ABC) transporter. However, cells transfected with the breast cancer resistance protein (BCRP)—the ABC transporter known to be highly overexpressed in MCF7/MX cells and to confer mitoxantrone resistance (D. D. Ross et al., J. Natl. Cancer Inst. 91: 429–433, 1999)—were not MTX resistant, which suggested that this transporter is not involved in MTX cross-resistance. Moreover, members of the MRPs protein family of transport proteins, which had previously been implicated in MTX resistance, were not found to be overexpressed in the MCF7/MX cells. Thus, our data suggest that a novel MTX-specific efflux pump may be involved in this unusual cross-resistance phenotype.

INTRODUCTION

MTX3 is a chemotherapeutic agent that is commonly used for the treatment of leukemias and lymphomas, as well as of solid tumors such as breast cancer (1). This drug is a member of the antifolate class of compounds that exerts its cytotoxicity by blocking the intracellular pathway whereby thymidylate and de novo purine biosynthesis occur. MTX enters the cell via the RFC1 (2) and, once inside the cell, becomes polyglutamylated by the enzyme FPGS (3). Polyglutamyla- tion of the drug causes its retention within the cell and, therefore, increases the likelihood that MTX will bind to its target enzyme, DHFR (4). Alterations in each of these steps in the intracellular metabolic pathway of MTX can lead to cellular resistance. MTX resistance has been shown to be caused by decreased uptake attributable to the absence (2) of or defects (5, 6) in RFC1, reduced polyglutamylation either through decreased FPGS activity and/or expression (7), or enhanced γ-GH activity and/or expression (8), and DHFR overexpression (9) or mutation (10–12).

In contrast, a putative role for drug efflux in MTX resistance, possibly mediated by an active transport mechanism, is less clear. Although evidence for an ATP-dependent MTX efflux mechanism has accumulated over the years (13–17), its molecular identity has not yet been established. Recently, loss of a folate efflux pump was implicated in resistance to the antifolate pyrimethamine, although the effect seems to be indirect through aberrations in the natural folate pools (18). No evidence for the overexpression of a MTX-specific efflux protein as the cause of MTX resistance has thus far been reported.

A large number of ABC transport proteins have been identified (19, 20). These proteins comprise an ever-growing family of efflux pumps, some of which have been implicated in the MDR phenotype. MDR occurs when cells selected for resistance to one compound develop cross-resistance to other structurally and functionally unrelated drugs. The prototype of this class of proteins, Pgp, was first identified by Juliano and Ling (21) in 1976, and was found to produce resistance to drugs such as doxorubicin, MX, Taxol, and vincristine through its action as a drug efflux pump. Overexpression of Pgp was also shown to be associated with MTX resistance in CEM/MTX cells (22), although cross-resistance to MTX does not appear to be a universal characteristic of cells that overexpress Pgp/MDR1 (23). Another group of transporters found to be involved in the MDR phenotype are the MRPs (24), some of which were shown to efflux various compounds conjugated to glutathione, glucuronide, or sulfate (25) and to cause resistance to compounds such as doxorubicin, VP-16, and vincristine (26). However, cross-resistance to MTX has not been shown. Recently, several reports have been published that demonstrate the potential for some of the MRP family members to actively transport MTX and to cause resistance (27, 28), albeit only under certain specific conditions, and, hence, their role in acquired MTX resistance remains unclear.

Here, we investigated the cause of MTX cross-resistance in MCF7 breast cancer cells selected in the presence of 600 nM MX (MCF7/ MX), a topoisomerase II inhibitor. These cells exhibit a typical MDR phenotype in that they display an ATP-dependent drug accumulation defect and are highly resistant to not only the drug for which they were selected (MX) but also to other structurally unrelated drugs such as the camptothecin derivatives TPT and CPT-11 (29). MX resistance in MCF7/MX cells is attributable to the overexpression of the recently identified ABC half-transporter BCRP (30, 31), which has also been called MX-resistance protein (MXR; Ref. 32) or placental ABC protein (ABC-P; Ref. 33). In contrast, MTX resistance in these cells has yet to be characterized. In the present study, we further define this
cell line by examining classical mechanisms of MTX resistance as well as the potential role of ATP-dependent drug efflux as the cause of this unusual cross-resistance.

MATERIALS AND METHODS

Cell Culture and BCRP Transfection. The human breast cancer cell line MCF7/WT, its drug-resistant variants MCF7/MX (34), MCF7/MTX (35), MCF7/VP (36), and BCRP-transfected MCF7 (31), and MDA-MB231 cells were cultured in IMEM medium (Richter’s modification) supplemented with 10% fetal bovine serum and 0.01 g/liter ciprofloxacin at 37°C in a humidified atmosphere of 5% CO2. Cloning of BCRP and transfection of MCF7 cells were described previously (31). Human breast carcinoma MDA-MB231 cells were transfected as described for MCF7 cells (31). The specific clones used in this study were clone 8 (MCF7/BCRP) and clone 23 (MDA-MB231/BCRP). Note that the MCF7/WT and the MCF7 cells used for transfection are two different sublines of the MCF7 cell line that have been kept separately in tissue culture for many years.

Cytotoxicity Assay. Drugs were stored at −20°C or at 4°C as the following stock solutions: 6 mM MX in water (Sigma, St. Louis, MO), 10 mM MTX in slightly basic sodium phosphate (Sigma), 100 mM VP-16 in DMSO (Sigma), 10 mM TPT in water (Smith Kline Beecham Pharmaceuticals, King of Prussia, PA), 18 mM DAITHF in DMSO (Eli Lilly and Company, Indianapolis, IN), 54 mM tritemetrex in DMSO (provided by Drs. Fry and Jackson of Warner Lambert Laboratory, Ann Arbor, MI), 115 mM 5-fluorouracil in DMSO (Sigma), 27 mM metoprine in DMSO (Burroughs Wellcome, Research Triangle, NC), 20 mM AG337 in DMSO (Aurion), and 5.4 mM Tomudex in water (ZD1694; Zeneca Pharmaceuticals, Wilmington, DE). Trypsinated cells (0.1 ml/well) were seeded in 96-well microtiter plates at a concentration of 2,500 cells/ml for MCF7/WT; 5,000 cells/ml for MCF7/MX and MCF7/VP; and 10,000 cells/ml for MCF7/MTX, MCF7/BCRP, and MDA-MB231/BCRP. After 24 h of incubation at 37°C, cells were treated by adding 0.1 ml/well of drug solution with increasing concentration. Corresponding controls received medium instead of drug solution. Toxicity of the drugs was determined by sulforhodamine B (SRB) assay as described previously (36, 37).

Cell Membrane Preparation. Cells were seeded at a density of 1,000,000 cells/10-cm dish. After 72 h, or at 70% confluence, cells were washed twice with PBS and then scraped into PBS. Samples were sonicated (three 10-s bursts at maximal setting), followed by centrifugation for 10 min at 10,000 × g at 4°C. The supernatant was diluted 1:4 into 10 mM Tris/HCl (pH 7.5) and 250 mM sucrose, and the membrane proteins were sedimented by centrifugation at 100,000 × g for 30 min. The pellet was dissolved in 62.5 mM Tris/HCl (pH 6.8) and 1% CHAPS, sonicated (three 10-s bursts at maximal setting), and solubilized for 30 min on ice. An equal volume of 2× sample buffer [0.1 mM Tris/HCl (pH 6.8) and 4% DTT] was added. The protein concentration was measured by Bradford assay (38). Samples were dispersed into aliquots and stored at −80°C.

Western Blotting. Western blots were generated using 100 μg of whole cell or cytoplasmic cell extracts or 50 μg of cell membrane preparations. Proteins were fractionated on polyacrylamide running gels (12% for DHFR, 10% for MTP, 10% for TPT in water (Smith Kline Beecham Pharmaceuticals, King of Prussia, PA), 100,000 m M TPT in water (Smith Kline Beecham Pharmaceuticals, King of Prussia, PA), 18 mM DAITHF in DMSO (Eli Lilly and Company, Indianapolis, IN), 54 mM tritemetrex in DMSO (provided by Drs. Fry and Jackson of Warner Lambert Laboratory, Ann Arbor, MI), 115 mM 5-fluorouracil in DMSO (Sigma), 27 mM metoprine in DMSO (Burroughs Wellcome, Research Triangle, NC), 20 mM AG337 in DMSO (Aurion), and 5.4 mM Tomudex in water (ZD1694; Zeneca Pharmaceuticals, Wilmington, DE). Trypsinated cells (0.1 ml/well) were seeded in 96-well microtiter plates at a concentration of 2,500 cells/ml for MCF7/WT; 5,000 cells/ml for MCF7/MX and MCF7/VP; and 10,000 cells/ml for MCF7/MTX, MCF7/BCRP, and MDA-MB231/BCRP. After 24 h of incubation at 37°C, cells were treated by adding 0.1 ml/well of drug solution with increasing concentration. Corresponding controls received medium instead of drug solution. Toxicity of the drugs was determined by sulforhodamine B (SRB) assay as described previously (36, 37).

Western Blotting. Western blots were generated using 100 μg of whole cell or cytoplasmic cell extracts or 50 μg of cell membrane preparations. Proteins were fractionated on polyacrylamide running gels (12% for DHFR, RFC, and γ-GH and 7.5% for MRPs) with a 4% stacking gel containing 8 μl urea. Multimark (Novex, San Diego, CA) and/or broad range markers (Bio-Rad, Hercules, CA) were used as molecular weight standards. Proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA) using wet electrophoretic transfer. TBST [20 mM Tris/HCl (pH 7.5), 0.137 M NaCl, and 0.01% Tween 20] was used for washing the blot. The nonspecific binding sites were blocked with Blotto (5% nonfat milk in TBST) overnight. The membrane was then incubated with primary antibody for 2 h or overnight and with the appropriate secondary antibody for 1 h. Chemiluminescence was used for detection. The blots were exposed in stripping buffer [62.5 mM Tris/HCl (pH 6.8), 0.2% SDS, and 0.69% β-mercaptoethanol] for 30 min at 65°C, followed by brief washing in TBST. The following antibodies were obtained: anti-DHFR (Research Diagnostics Inc., Flanders, NJ); anti-γ-GH (Dr. Thomas Ryan, Wadsworth Center, Albany, NY; Ref. 39); anti-RFC1 (Dr. Jeffrey Moscow, University of Kentucky Medical Center, Lexington, KY; Ref. 40); anti-MRP1, -2, -3, and -5 (Dr. Rik Scheper, Department of Pathology, Free Hospital, Amsterdam, the Netherlands); and anti-MRP4 (Dr. John Schultz, St. Jude Children’s Research Hospital, Memphis, TN). SDS-Page and Western blots for FPGS were performed as described previously (41).

γ-GH Activity Assay. Cells were grown for 72 h and were then exposed with 0.1 μM Tris/HCl (pH 6.0) containing 0.1% Triton X-100, 0.1 mM 2-mercaptoethanol, and protease inhibitors. The supernatant was used for γ-GH assays with 100 μM MTXGlu4, (4-NH2)-10-CH3PteGlu3, as substrate and 15 min of incubation at 37°C (42). Activity is expressed as nmol/min/mg protein.

DHFR Activity Assay. DHFR activity was assayed by the modified method of Mathews and Hueneke (43). The cell extracts were incubated in the presence of 0.066 mM dihydrofolate (FH4) and 0.1 mM NADPH in 0.05 M potassium phosphate (pH 7.0) for 10 min at 37°C. The decrease of absorbance at 340 nm. One unit of DHFR activity is defined as μmol of FH4 reduced to FH2 per min.

MTX Accumulation. Steady-state MTX accumulation was measured by growing MCF7 WT and MCF7 MX cells for 12 h, followed by incubation in serum-free IMEM for an additional 24 h. At this time, 2 μM [3H]MTX (specific activity 1.89 × 108 dpm/nmol; Moravek Biochemicals, Brea, CA) was added for 3, 6, and 24 h. After the cells were lysed and neutralized, total MTX accumulation was measured by LSC. Activity is expressed as nmol MTX-Glu4 formed per hour per mg protein.

MTX Efflux. Six-well plates containing cells at 70% confluence were washed in PBS and incubated for 15 min in either ATP-sustaining control medium (1 g/liter glucose in PBS) or ATP-depletion medium (50 mM deoxyglucose, 15 mM sodium azide, and 1 mM dinitrophenol). Cells were then exposed to 10 μM [3H]MTX (specific activity 2.2 × 105 dpm/nmol) in either control ATP-sustaining conditions or ATP-depleting conditions for 0, 2, 5, 10, and 30 min. Three successive washes in ice-cold PBS were used to stop drug accumulation. Cells were lysed overnight in 0.2 N NaOH and were then neutralized with 0.2 N HCl and radioactivity in the cells was quantitated by LSC. Values were normalized to total protein content to determine pmol MTX accumulated per mg cell protein. The kinetics of MTX accumulation was assayed in MCF7/WT and MCF7/MX cells as follows. Cells were grown to confluency in 6-well plates and were washed once in prewarmed PBS, followed by incubation for 2 min in [3H]MTX ranging in concentration from 0.5 to 10 μM. As previously described, uptake was terminated by washing the plates three times in ice-cold PBS, and radioactivity in the cell lysates was counted by LSC. Values were normalized to protein content, and uptake velocity was calculated as pmol/mg/min. These values along with MTX concentration were plotted as a double reciprocal Lineweaver-Burke plot, and approximate Ks and Vmax values were calculated from linear regression curves.

MTX Polyglutamylate. The long-term polyglutamate profile of MTX was measured after 24 h of exposure to 2 μM [3H]MTX. Polyglutamates were

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extracted, separated by high-performance liquid chromatography and quantitated using LSC as described previously (8).

RESULTS

Drug Resistance. MCF7/MX cells were previously shown to be highly and relatively specifically resistant to MX and TPT (29, 34), presumably because of the overexpression of BCRP (30). During our work to characterize this cell line further, we observed that MCF7/MX cells were also 150-fold cross-resistant to the antifolate MTX (Table 1), a drug not normally associated with MDR phenotypes. To elucidate the mechanism for this unusual cross-resistance, we tested several additional antifolate drugs with different modes of entry and different intracellular targets for their cytotoxic effects on MCF7/MX and parental MCF7/WT cells (Table 1). As controls, we used MTX-selected MCF7/MTX and etoposide-selected MCF7/VP cells, which are known to overexpress DHFR and MRPI, respectively (35, 36). As shown previously, MCF7/MX cells were highly resistant to the anti-topoisomerase II drug MX and the anti-topoisomerase I drug TPT and somewhat cross-resistant to another anti-topoisomerase II drug, VP-16. As expected, MCF7/MTX cells were highly resistant to MTX but, surprisingly, also showed some cross-resistance to both MX and VP-16, whereas MCF7/VP cells were resistant to VP-16 and MX. In contrast, only little, if any, cross-resistance was observed with the other antifolate drugs tested, with the exception of an approximately 5-fold resistance to AG337 in MCF7/MX cells. Thus, it seems that the mechanism responsible for the MTX cross-resistance in MCF7/MX cells is rather specific for this particular antifolate.

DHFR. Overexpression of the target enzyme DHFR has been shown to be one of the main mechanisms for MTX resistance (9). To investigate whether resistance of MCF7/MX cells to MTX could be attributable to the same mechanism, the activity of DHFR was determined (Table 2). As expected (35), 22-fold higher DHFR activity was found in MCF7/MTX cells. In contrast, no substantial difference could be measured in the DHFR activity of MCF7/MX and MCF7/VP cells compared with MCF7/WT cells. These results were confirmed by Western blot using a polyclonal anti-DHFR antibody (Fig. 1). Neither in MCF7/MX cells nor in MCF7/VP cells could a substantial difference be detected in the expression level of the 22 kDa DHFR protein compared with MCF7/WT cells, whereas MCF7/MTX cells showed clear overexpression of DHFR as expected. Thus, it seemed unlikely that MTX cross-resistance in MCF7/MX cells was attributable to DHFR overexpression.

Accumulation of MTX. Reduced drug accumulation has been shown in some systems to cause MTX resistance (45), and, therefore, intracellular drug accumulation was measured. As shown in Fig. 2A, steady-state levels of total MTX in MCF7/MX cells were one-half to one-third of those in the parental cells, and short-term MTX accumulation was also decreased in MCF7/MX cells relative to MCF7/WT cells (Fig. 2B). After 20 min of MTX exposure, accumulation was approximately 30% lower in the resistant cell line. This reduction seemed to be ATP dependent; depletion of ATP in MCF7/MX cells resulted in increased drug accumulation to levels comparable with those in MCF7/WT cells, whereas little effect on MTX accumulation was seen in the parental cell line. To determine whether the differences in accumulation were caused by alterations in the initial rate of MTX uptake, cells were exposed to various drug concentrations for 2 min. Approximate $K_v$ and $V_{max}$ values were inferred from the double reciprocal plot of initial uptake rate and are as follows (Fig. 2C): the $K_v$WT = 6.8 ± 1.5 μM; $K_v$MX = 5.4 ± 2.3 μM; $V_{max}$WT = 17.0 ± 0.9 pmol/mg/min; and $V_{max}$MX = 16.6 ± 2.8 pmol/mg/min. Thus, it appears that the reduced MTX accumulation observed in MCF7/MX cells is not likely to be attributable to differences in drug uptake because parental and resistant cells exhibited very similar kinetic profiles.

This conclusion is further supported by the results from Western

Table 1 IC50 values of MCF7/WT cells and relative resistance of the selected sublines

The cell lines were exposed to the drugs for 7 days. IC50 values (means ± SE) are from three or more separate experiments, each performed in triplicate.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Target</th>
<th>Mode of entry</th>
<th>Polyglutamylation</th>
<th>Fold-resistance of MCF7 cell lines</th>
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<tr>
<td>MTX</td>
<td>μM</td>
<td>DHFR</td>
<td>RFC</td>
<td>MCF7/WT</td>
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<tr>
<td>Metoprine</td>
<td>μM</td>
<td>DHFR</td>
<td>Diffusion</td>
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<tr>
<td>Trimetrexate</td>
<td>μM</td>
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<tr>
<td>AG337</td>
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<td>5-FU</td>
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<td>Diffusion</td>
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<td>Tomudex</td>
<td>μM</td>
<td>TS</td>
<td>RFC</td>
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<td>MX</td>
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<td>VP-16</td>
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<td>TPT</td>
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<td>Topoisomerase I</td>
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* NF, relative resistance factor, obtained by dividing the IC50 values of the resistant cells by that of the parental cells.

† 5-FU, 5-fluorouracil; GAR-TF, glycinamide ribonucleotide transformylase.
blots using a polyclonal antibody against the RFC to determine the expression of RFC1 in MCF7 cells (Fig. 1). RFC1, a transporter with a molecular mass of 58 kDa, appeared slightly overexpressed in the drug-resistant MCF7 sublines tested compared with MCF7/WT and, therefore, did not seem to be involved in the reduced drug accumulation and MTX cross-resistance found in the MX-selected cells.

**MTX Polyglutamylation.** Decreased polyglutamylation leading to decreased drug retention has been demonstrated to cause MTX resistance. Therefore, we analyzed the polyglutamylation pattern of MTX at 24 h and found that the distribution of the various MTX-polyglutamate species was different in MCF7/WT and MCF7/MX cells (Fig. 3A). Although in MCF7/WT cells the amount of each of the MTX-Glu$_{2-6}$ species was approximately equal, in MCF7/MX cells, there was a gradual decrease in long-chain MTX-Glu$_{n}$ forms, with no detectable levels of MTX-Glu$_{5}$ or -Glu$_{6}$. To further examine the possible role of MTX polyglutamylation in MTX resistance, we
measured the noneffluxable MTX pools in both MCF7/WT and MCF7/MX cells. After a 24-h exposure to 2 μM MTX, the fraction of noneffluxable drug in MCF7/MX compared with MCF7/WT cells was significantly smaller (Fig. 3B). Thus, both the absence of long-chain polyglutamates as well as the decreased pool of noneffluxable MTX in the resistant relative to the parental cells are consistent with altered polyglutamylation playing a role in the accumulation defect associated with MTX resistance in MCF7/MX cells.

**FPGS and γ-GH.** The formation of MTX polyglutamates depends on the cellular level of substrates and the function of two enzymes, FPGS and γ-GH. FPGS catalyzes the addition of glutamates, whereas γ-GH catalyzes their removal. Thus, reduced FPGS and/or increased γ-GH activity could be responsible for the low levels of long-chain MTX-Glu formation. Therefore, the activities of both FPGS and γ-GH were measured (Table 2). Cell extracts from MCF7/MX cells showed no significant difference in either FPGS or γ-GH activity compared with MCF7/WT cells. In contrast, γ-GH activity was slightly increased (1.7-fold) and FPGS activity was slightly decreased in the MTX resistant cell line MCF7/MTX, whereas γ-GH activity in the MCF7/VP cells was substantially lower. These results were confirmed by Western blot using polyclonal antibodies against the respective human proteins (Fig. 1). MCF7/MX and MCF7/WT cells showed little difference in the expression levels of FPGS and γ-GH, whereas there was a slight increase in the level of γ-GH in MCF7/MX cells, in agreement with the activity data. Interestingly, there appeared to be a clear reduction in γ-GH protein in MCF7/VP cells, which was also reflected in the activity. Thus, it seems unlikely that the reduced formation of MTX polyglutamates in MCF7/MX cells was attributable to an alteration in these enzymes.

**MTX Efflux.** To examine whether the decreased accumulation of MTX observed in MCF7/MX cells was attributable to enhanced efflux, cells were preloaded with radiolabeled MTX, washed, and incubated for 0, 2, 5, 10, 20, or 30 min. As shown in Fig. 4, MCF7/MX cells exhibited increased MTX efflux relative to MCF7/WT cells. To determine whether this was an energy-dependent phenomenon, the cells were depleted of ATP before measuring drug efflux. As shown in Fig. 4, in the absence of ATP, MCF7/MX cells retained more MTX than in the presence of ATP, whereas MCF7/WT cells were essentially unaffected by energy depletion. Under these conditions, there was no difference in drug efflux between the two cell lines. Thus, these data suggested the presence of an ATP-dependent efflux mechanism, possibly an ABC transporter.

**BCRP.** Because the ABC transporter BCRP is known to be highly overexpressed in MCF7/MX cells and is presumably responsible for the resistance to MX (30, 31), its possible role in MTX resistance was examined. Two sets of BCRP- and control-transfected human breast cancer cells were used. Overexpression of BCRP in the transfected cells was approximately 5- to 10-fold as compared with the respective control transfectants (Ref. 31 and data not shown). By comparison, BCRP expression in the MCF7/MX cells is at least 100-fold higher than in MCF7/WT cells. To explore its potential role in MTX resistance, cytotoxicity assays were performed with BCRP-transfected cells. As shown in Table 3, the BCRP transfectants were not cross-resistant to MTX; MCF7/BCRP (clone 8) cells were 0.71-fold resistant relative to the pcDNA3 control transfectants, whereas MDA-MB231/BCRP (clone 23) cells were 1.45-fold resistant, differences that were not statistically significant. As expected, transfected MCF7 and MDA-MB231 cells were 23- and 89-fold, respectively, resistant to MX. Interestingly, BCRP transfection seemed to have only a modest effect on TPT sensitivity, an effect that did not quite reach statistical significance, which raised the question of whether BCRP can indeed confer cross-resistance to this drug. In conclusion, these data suggest that BCRP overexpression alone is not sufficient for MTX cross-resistance in MCF7/MX cells.

**MRP Expression.** MTX has recently been shown to be a substrate for transport by several members of the MRP family of ABC transporters (27, 28, 46–48). The overexpression of an MRP protein, therefore, might confer MTX resistance in MCF7/MX cells. To examine this possibility, Western blot analyses of MRPs 1–5 were performed (Fig. 5). However, no evidence for overexpression was found for any of the MRP proteins in MCF7/MX cells compared with MCF7/WT cells (Fig. 5). Thus, it seems unlikely that these transport proteins are responsible for MTX resistance in MCF7/MX cells.

**DISCUSSION**

Cellular resistance to MTX is known to occur at various steps in the intracellular metabolic pathway of this antifolate. Intracellular access of MTX is mediated by the RFC; mutant or absent carriers have been...
associated with MTX resistance (1). Qualitative defects in RFC1 attributable to various point mutations leading to single amino acid substitutions have been shown to produce 12- to 200-fold resistance in Sarcoma 180 cells and the human leukemia cell line CCRF-CEM (5, 6, 45). Also, a generally direct relationship was observed between RFC1 levels and MTX sensitivity (49, 50). Loss of RFC1 expression resulted in increased MTX resistance that was reversible upon transfection of a cDNA encoding mRFC1 into carrier deficient, MTX-resistant ZR-75–1 breast cancer cells (2). MTX resistance has also been shown to occur at the drug target level; both overexpressed and/or altered DHFR can cause resistance. A direct correlation has been made between the acquisition of MTX resistance and the number of DHFR copies present in various cell lines, such as the MTX-resistant AT-3000 and MCF7/MX cell lines (9, 35). In addition, DHFR mutations that result in an enzyme with reduced affinity for MTX have been observed in multiple MTX-resistant cell lines (12, 51).

Another important feature of MTX metabolism that is involved in cellular resistance is polyglutamylation (7, 18). The addition of glutamate residues onto MTX causes the drug to be retained within the cell at cytotoxic concentrations. Thus, the balance between the enzyme that catalyzes the addition of glutamates, FPGS, and the enzyme that functions in the reverse reaction to cleave those glutamate residues from MTX, γ-GH, determines the level of MTX polyglutamylation. Both enzymes have been directly linked to MTX resistance. Decreased FPGS activity and protein expression were shown to cause folate analogue resistance in L1210 leukemic cell variants (7). Conversely, H35D cells, a rat hepatoma cell line selected for DDATHF resistance, were found to exhibit increased activity of γ-GH relative to sensitive parental cells (8). Thus, there are multiple mechanisms by which tumor cells can acquire MTX resistance.

In the present report, we describe MTX cross-resistance in the MX-selected cell line, MCF7/MX, that does not appear to be caused by any of the “classical” mechanisms described above. Overproduction of the target enzyme DHFR does not seem to play a role in MTX resistance in MCF7/MX cells, because both enzyme activity as well as protein expression showed no significant differences between MCF7/MX and MCF7/WT cells. This conclusion is further supported by the lack of cross-resistance to other antifolates that target DHFR, such as metoprine (1.4-fold) and trimetrexate (1.2-fold). Interestingly, MCF7/MTX cells were moderately hypersensitive to trimetrexate, despite the overexpression of DHFR. We have not, however, further investigated the cause for this observation.

The major differences between MCF7/MX and MCF7/WT cells, besides reduced MTX sensitivity, are the 3-fold reduction in steady-state accumulation accompanied by reduced formation and accumulation of long-chain MTX polyglutamates. This suggests that lower intracellular drug concentration is responsible for MTX resistance. Reduced accumulation could be attributable to either a quantitative or qualitative defect in uptake via RFC1, whereas reduced polyglutamylation could be attributable to alterations in FPGS and/or γ-GH. However, neither of these mechanisms seems to play a role.

The RFC is expressed at approximately equal levels in both the MTX-resistant and -sensitive cell lines. In further support of an intact and functional carrier is the lack of cross-resistance of MCF7/MX cells to other RFC1-dependent antifolate drugs such as DDATHF (0.6-fold) and Tomudex (0.2-fold). Moreover, MCF7/MX cells are not more sensitive to trimetrexate, a lipophilic antifolate that does not require RFC1 to gain cellular access. Cells that are MTX-resistant because of RFC1 defects have been shown to be hypersensitive to trimetrexate (50). The accumulation kinetics data also provide evidence against a role of reduced uptake in MTX resistance because MCF7/WT and MCF7/MX cells have approximately the same transport affinity and uptake velocity for MTX. The slight differences observed between the two cell lines may be attributable to the commencement of efflux activity in the resistant cells.

Both FPGS and γ-GH proteins were expressed at similar levels relative to sensitive cells, and, based on activity measurements, there was no evidence for either decreased FPGS activity or hyperactivity of γ-GH. Thus, the reduced formation of MTX polyglutamates in MCF7/MX compared with MCF7/WT cells does not appear to be attributable to an altered function of either of those enzymes. This is further supported by the absence of MCF7/MX cross-resistance to Tomudex (0.2-fold), a TS inhibitor that is dependent on the glutamylation activity of FPGS for cytotoxicity.

Taken together, these data indicate that both MTX import and metabolism are intact and functional in MCF7/MX cells. Nevertheless, our data indicate that the critical factor in the MTX resistance of MCF7/MX cells is reduced drug accumulation accompanied by decreased polyglutamylation. This observation is reminiscent of one previously reported by Moscow et al. (52), who showed that melphalan-selected Mel8MCF7 cells displayed MTX cross-resistance attributable to a lack of polyglutamylation associated with a decreased drug accumulation. We show here that the reduced MTX accumulation found in MCF7/MX cells could be attributable to an enhanced efflux that is sensitive to ATP levels, analogous to what has been shown for various natural product drugs. Although MTX is not a classical MDR-drug, our data support the hypothesis that the presence of an ABC transporter that may be specific for MTX is the cause of the cross-resistance in the MCF7/MX cell line. An enhanced efflux mechanism is also consistent with reduced polyglutamylation; if the drug is rapidly removed from the cell, it does not become available as a substrate for FPGS. Here, we demonstrate a rapid drug efflux in which 50% of the drug has been exported within the first 2 min of efflux. Although the rate of efflux is faster than seen for many natural product...
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REFERENCES


3. Sirotnak, F., and O’Leary, D. The issues of transport multiplicity and energetics...ine restitutive form of MTX was shown, whereas its polyglutamylated derivatives did not appear to be substrates for the various MRPs studied. Thus, there are several ABC protein candidates that could cause MTX resistance. The obvious candidate in our cells is BCRP, which was recently shown to be highly overexpressed in MCF7/MX cells and to be responsible for MX resistance (31, 58). However, whereas MCF7 and MDA-MB231 cells that were transfected with BCRP are 23-fold and 89-fold resistant, respectively, to MX, these cells did not display the same MTX cross-resistance pattern that was observed in MCF7/MX cells. This suggests that BCRP is not responsible for MTX cross-resistance, although the possibility that the MCF7/MX cells contain a variant form of BCRP cannot be excluded. This is currently under active investigation in our lab. A survey of MDR1 (34) and MRP1–5 expression gave, however, no evidence that any of these proteins are overexpressed in MCF7/MX cells. Furthermore, MTX resistance in these cells was also seen when cells were continuously exposed to the drug for 7 days, conditions under which neither MRP1 nor MRP3 were able to confer resistance (27, 28). It is presently unclear why those MRP proteins were able to confer resistance only to short-term MTX exposure, whereas MCF7/MX cells are also resistant to long-term treatment. One possibility is that the efflux pump responsible for the export of MTX in MCF7/MX cells is a more efficient transporter than the aforementioned MRP proteins, such that the majority of the drug has been extruded before polyglutamylation occurs. Furthermore, additional, not yet identified, factors may also be involved. Thus, there is no evidence that any of the known MRP or Pgp proteins cause MTX resistance in the MCF7/MX cells.

In conclusion, MX-selected MCF7/MX cells display an unusual cross-resistance pattern specific to the antifolate MTX. These cells exhibit an accumulation defect caused by an enhanced ATP-dependent efflux. The highly overexpressed BCRP protein does not seem to be involved, and, thus far, we have been unable to associate any of the other transport proteins that have been implicated in MTX transport with resistance. Studies are under way to identify the putative transporter involved, which is expected to provide valuable insight into the cause of MTX resistance in tumor cells.

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Methotrexate Cross-Resistance in a Mitoxantrone-selected Multidrug-resistant MCF7 Breast Cancer Cell Line Is Attributable to Enhanced Energy-dependent Drug Efflux

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