Wild-Type Breast Cancer Resistance Protein (BCRP/ABCG2) is a Methotrexate Polyglutamate Transporter

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ABSTRACT

The existence of an ATP-dependent methotrexate (MTX) efflux mechanism has long been postulated; however, until recently, the molecular components were largely unknown. We have previously demonstrated a role for the ATP-binding cassette transporter breast cancer resistance protein (BCRP) in MTX resistance (Volk et al., Cancer Res., 62: 5035–5040, 2002), Resistance to this antifolate directly correlated with BCRP expression, and was reversible by the BCRP inhibitors fumitremorgin C and GF120918. Here, we provide evidence for BCRP as a MTX-transporter using an in vitro membrane vesicle system. Inside-out membrane vesicles were generated from both drug-selected and stably transfected cell lines expressing either wild-type (Arg482) or mutant (Gly482) variants of BCRP. In the presence of the wild-type variant of BCRP, transport of MTX into vesicles was ATP-dependent, osmotically sensitive, and inhibited by fumitremorgin C. In contrast, no transport was observed in vesicles containing the mutant form of BCRP. Wild-type BCRP appeared to have low affinity, but high capacity, for the transport of MTX, with an estimated $K_m$ of 680 $\mu M$ and a $V_{max}$ of 2400 pmol/mg/min. MTX accumulation was greatly decreased by mitoxantrone, a known BCRP substrate, suggesting competition for transport. Furthermore, and in contrast to the multidrug resistance-associated proteins, BCRP also transported significant amounts of polyglutamylated MTX. Although transport gradually decreased as the polyglutamate chain length increased, both MTX-Glu2 and MTX-Glu6 were substrates for BCRP. Together, these data demonstrate that BCRP is a MTX and MTX-polyglutamate transporter and reveal a possible mechanism by which it confers resistance.

INTRODUCTION

MTX3 was one of the first antimetabolite drugs developed and is used successfully for the treatment of various malignancies. Unfortunately, the efficacy of this chemotherapeutic agent is often compromised by the development of resistance in the cancer cells. Typically, MTX resistance is the result of alterations in its target enzyme, dihydrofolate reductase (1–4), reduced drug import by the reduced folate carrier (5–8), or altered polyglutamylation (9, 10). Reduced polyglutamylation results in lower drug retention, which leads to resistance.

Evidence for the existence of an energy-dependent MTX transport or efflux mechanism has accumulated over several decades. Early reports described a rapid, energy-dependent, MTX efflux from Sarcoma-180 cells (11), and a similar, azide-inhibitable, unidirectional export of MTX was also shown in leukemic L1210 cells (12). Furthermore, efflux of both mono- and diglutamates of MTX was demonstrated in a cervical carcinoma cell line (13). A series of reports throughout the last decade have further classified the cellular export of MTX as an ATP-dependent, outwardly directed mechanism that transports both MTX and its metabolites (14–18).

Despite clear biochemical evidence, the mediator(s) of MTX efflux have only recently been described at the molecular level. Several members of the MRPs or ABCC subfamily of the ABC transporters have been implicated in MTX export and resistance (19–21). These ATP-dependent efflux pumps have been shown to transport MTX in vitro and to cause low levels of MTX resistance in both transfected and drug-selected cells, but only in short-term (1–3-h) exposure assays. This limited-resistance phenotype has been attributed to the inability of the MRPs to transport MTX in its polyglutamylated form; the addition of even a single extra glutamate residue to MTX caused an almost complete abrogation of drug transport (22, 23).

In contrast, we have described high-level MTX cross-resistance to continuous (7 days) drug exposure in the mitoxantrone-selected MCF7/MX breast carcinoma cell line, which expresses large amounts of the ABC transporter BCRP (24), also known as mitoxantrone resistance protein (25), placental-specific ABC transporter (26), or ABCG2 (27). Further characterization of these cells suggested that ATP-dependent, enhanced drug efflux caused the MTX resistance. MTX resistance in MCF7/MX cells was reversible by the BCRP inhibitors GF120918 and FTC, and levels of MTX resistance directly correlated with BCRP expression in this and multiple other cell lines of varied tissue origin (28). Together, these data suggested that BCRP can function as a MTX transporter. However, resistance to MTX occurred only in the presence of the wild-type transporter, which contains an arginine at position 482, whereas cells that overexpress a mutated BCRP (R482T and R482G) remained sensitive to MTX. Here, we expand on the association between BCRP and MTX resistance by demonstrating that wild-type, but not mutated, BCRP is able to transport MTX and its polyglutamates in an in vitro membrane vesicle system.

MATERIALS AND METHODS

Cell Culture

The following cell lines were cultured in improved MEM containing 10% fetal bovine serum and 1 $\mu g$/ml ciprofloxacin at 37°C with 5% CO2: the human breast carcinoma cell line MCF7/WT (29); its mitoxantrone-selected derivative MCF7/MX (30); transfected sublines MCF7/pcDNA3, MCF7/BCRP-Arg clone 67, MCF7/BCRP-Gly clone 52 (28); and the human colon cancer cell line S1 and its mitoxantrone-selected derivative, S1-M1–80 (25).

Vesicle Preparation

Vesicle preparation was adapted from Loe et al. (31). Cells were seeded onto 15-cm plates and grown to 70% confluency, at which time they were washed twice with PBS and scrapped into the residual PBS. Cells from 20 plates were combined and centrifuged at 500 × g at 4°C for 15 min; the pellet was frozen overnight at −80°C. Inside-out membrane vesicles were prepared by thawing the cell pellet at 4°C and resuspending the cells in 7 ml of homogenization buffer containing 50 mM Tris-HCl (pH 7.5), 250 mM sucrose, 0.25 mM CaCl2, and one EDTA-free protease inhibitor mixture tablet (Roche, Indianapolis, IN). The cell suspension was then transferred to a 50-ml round-bottomed tube and placed on ice into the nitrogen cavitation chamber (Parr Instruments, Moline, IL). Under constant stirring, cells were saturated with

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3 The abbreviations used are: MTX, methotrexate; ABC, ATP-binding cassette; BCRP, breast cancer resistance protein; FTC, fumitremorgin C; MRP, multidrug resistance-associated protein; PVDF, polyvinylidene difluoride.
nitrogen gas at a pressure of 1,250 p.s.i. for 20 min. After release of the pressure, membrane suspensions were centrifuged at 1,000 × g at 4°C for 15 min. The supernatant was overlayed on a sucrose cushion containing 35% sucrose, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and was centrifuged at 200,000 × g at 4°C for 120 min. The opaque interface was collected and diluted 1:30 into TS buffer containing 10 mM Tris-HCl (pH 7.5) and 250 mM sucrose and was centrifuged at 123,000 × g at 4°C for 40 min. The vesicle-containing pellet was resuspended in 50 mM Tris-HCl (pH 7.5) and 250 mM sucrose, and dispersed 20 times through a 27-gauge needle. Vesicle preparations were assayed for protein concentration using the Bradford method (Bio-Rad, Hercules, CA), and membrane orientation was determined by measuring the activity of the endo-enzyme glyceraldehyde-3-phosphate dehydrogenase in the absence and presence of 0.1% Triton X-100.

Western Blot Analysis

Immunodetection of BCRP in the membrane vesicles was performed as described previously (28). Briefly, 50 μg of the protein of the membrane vesicles were electrophoresed on a 10% polyacrylamide gel and were transferred to a PVDF membrane (Millipore, Bedford, MA). BCRP-specific signals were detected using a polyclonal rabbit anti-BCRP primary antibody (kindly provided by Dr. Susan Bates, National Cancer Institute, Bethesda, MD), horse-radish peroxidase-conjugated goat antirabbit secondary antibody (Bio-Rad), and enhanced chemiluminescence (Pierce, Rockford, IL).

Vesicular Drug Accumulation Assays

Vesicular drug accumulation assays were adapted from Saxena and Hendersson (33). All of the reaction mixtures were prepared as 20-μl samples containing the following: 0.1 mg/ml creatine kinase, 10 mM phosphocreatine (both from Sigma, St. Louis, MO), 10 mM Tris-HCl (pH 7.4), 10 mM magnesium chloride, 250 mM sucrose, 400 μM [3H]methotrexate (Moravek Biochemicals, Brea, CA), 4 mM ATP or AMP, and 5 μg of vesicles. Reactions were mixed at 37°C (0 time points were incubated at 4°C), and transport was terminated by dilution into 4 ml of ice-cold TS buffer. Vesicles were then separated from free drug by filtration of the reaction mixture through a glass microfiber filter (GF-F; Whatman, Fairfield, NJ) and rinsing five times with TS buffer. The amount of drug in the vesicles that remained on the filters was used to generate inside-out membrane vesicles from the following cell lines: parental MCF7/WT and S1 and their respective mitoxantrone-selected derivatives MCF7/MX and S1-M1–80 cells, whereas moderate levels were observed in the vesicles derived from the BCRP-transfected cells MCF7/BCRP-Arg and MCF7/BCRP-Gly, (Fig. 1). The apparent slight overexpression of BCRP in the MCF7/BCRP-Gly clone compared with the MCF7/BCRP-Arg clone is also seen in glyceraldehyde-3-phosphate dehydrogenase as a loading control. It most likely can be attributed to slight differences in the amount of protein loaded and, therefore, does not represent a true difference in the level of BCRP in these vesicles. Using a slight dilution of membranes from MCF7/MX cells, we estimated that these cells expressed approximately 300–400-fold more BCRP than the transfecteds (data not shown); this value corresponded well to that obtained by quantitative reverse transcription-PCR (28). Little or no BCRP was detected in vesicles from the parental cell lines MCF7/WT and S1, or from the control transfectants MCF7/pcDNA3. Together, these data indicated that the in vitro system was suitable for the intended studies; membrane vesicles were inverted and BCRP levels in vesicles were consistent with those observed in whole cells.

MTX Accumulation into Vesicles. To establish BCRP as an energy-dependent transporter of MTX, steady-state drug accumulation into vesicles from each cell line was measured in the presence of 4 mM either ATP or AMP. As shown in Fig. 2A, high levels of MTX accumulation were found in MCF7/MX and MCF7/BCRP-Arg vesicles, both of which express the arginine variant of BCRP, in the presence of ATP, but not in the presence of AMP. In contrast, vesicles generated from S1-M1–80 or MCF7/BCRP-Gly cells, which express the glycine variant of BCRP, displayed a significant increase in MTX accumulation in the presence of ATP compared with the increase in the presence of AMP. As expected, vesicles derived from MCF7/WT- and control-transfected cells exhibited little or no MTX transport in the presence of either ATP or AMP. Thus, it appeared that vesicles derived from cells containing the arginine variant of BCRP were able to accumulate MTX, and that accumulation was ATP dependent.

RESULTS

BCRP Expression in Membrane Vesicles. Nitrogen cavitation was used to generate inside-out membrane vesicles from the following cells: parental MCF7/WT and S1 and their respective mitoxantrone-selected derivatives MCF7/MX and S1-M1–80 and stable transfectants MCF7/pcDNA3, MCF7/BCRP-Arg, and MCF7/BCRP-Gly. The inverted orientation of the membrane in the vesicles was determined by assaying for the activity of the endo-enzyme glyceraldehyde-3-phosphate dehydrogenase in the presence and absence of detergent. All of the preparations were found to be, on average, 70% (range, 50–96%) inside-out (data not shown).

To confirm the presence of BCRP in the inside-out vesicles, a Western blot analysis was performed. As expected, high levels of BCRP were detected in the vesicles from drug-selected MCF7/MX and S1-M1–80 cells, whereas moderate levels were observed in the vesicles derived from the BCRP-transfected cells MCF7/BCRP-Arg and MCF7/BCRP-Gly (Fig. 1). The apparent slight overexpression of BCRP in the MCF7/BCRP-Gly clone compared with the MCF7/BCRP-Arg clone is also seen in glyceraldehyde-3-phosphate dehydrogenase used as a loading control. It most likely can be attributed to slight differences in the amount of protein loaded and, therefore, does not represent a true difference in the level of BCRP in these vesicles. Using a slight dilution of membranes from MCF7/MX cells, we estimated that these cells expressed approximately 300–400-fold more BCRP than the transfecteds (data not shown); this value corresponded well to that obtained by quantitative reverse transcription-PCR (28). Little or no BCRP was detected in vesicles from the parental cell lines MCF7/WT and S1, or from the control transfectants MCF7/pcDNA3. Together, these data indicated that the in vitro system was suitable for the intended studies; membrane vesicles were inverted and BCRP levels in vesicles were consistent with those observed in whole cells.

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Calculation of Net Accumulation. Unless otherwise indicated, all values shown correspond to net accumulation. Net MTX accumulation was determined by measurement of drug uptake in the presence of ATP or AMP at 4°C, and subtracting these background values (T₀) from each respective measurement taken in the presence of ATP or AMP at 37°C, at time points ranging from 1 to 60 min. For example:

\[
\text{Net accumulation at 60 min} = (T_{60}\text{ATP} - T_{0}\text{ATP}) - (T_{60}\text{AMP} - T_{0}\text{AMP})
\]

MTX has an integral glutamate and, therefore, corresponds to MTX-Glu₄.
dependent. In contrast, no accumulation was observed in vesicles containing BCRP with a glycine at position 482. These results were consistent with the previously observed energy-dependence of MTX efflux and the position-482 substrate specificity of BCRP in whole cells (24, 28, 34, 35).

There was a significant difference in the absolute amount of MTX accumulation in the drug-selected MCF7/MX vesicles relative to the BCRP-transfected MCF7/BCRP-Arg vesicles. Steady-state levels of MTX were ~8-fold lower in vesicles derived from the transfected cells, which is commensurate with the reduced levels of BCRP expression in the MCF7/BCRP-Arg cells relative to the MCF7/MX cells. In contrast, despite very high levels of BCRP in vesicles from S1-M1–80 cells, no accumulation of MTX was detected.

To further examine MTX transport via BCRP, we measured time-dependent drug accumulation in vesicles derived from both drug-selected and stably transfected cell lines. The MCF7/MX vesicles displayed an ATP-dependent accumulation of MTX that increased with time and was linear for at least two min (Fig. 2B). In contrast, no transport was detected in vesicles derived from either S1-M1–80 or parental cells, even after a 60-min drug exposure. The S1-M1–80 vesicles were, however, capable of ATP-dependent mitoxantrone accumulation (data not shown), indicating that the vesicles were functional. This provides further evidence that the amino acid at codon 482 is critical for BCRP substrate specificity. Similarly, MTX accumulation increased over time in the vesicles derived from the MCF7/BCRP-Arg cells, but no drug uptake was observed in either MCF7/BCRP-Arg vesicles or vesicles from control transfectants (Fig. 2C).

Together, these data demonstrated that MTX transport was both energy and time dependent and appeared to occur only in the presence of the wild-type (Arg482) variant of BCRP.

Osmotic Sensitivity. To assess whether the ATP-dependent MTX accumulation was caused by drug import into the lumen of the vesicles or was attributable to nonspecific binding to the membranes, we determined its osmotic sensitivity. ATP-dependent uptake of MTX into the MCF7/MX vesicles was measured under various osmotic conditions. Drug accumulation was linear, relative to the reciprocal of the sucrose concentration in the presence of ATP, whereas no drug accumulation was observed in the absence of ATP under otherwise identical reaction conditions (Fig. 3). These data demonstrated that the apparent intravesicular drug accumulation observed was, indeed, attributable to transport into the lumen of the membrane vesicles, rather than to nonspecific binding.

Inhibition of MTX Transport. FTC has been shown to inhibit BCRP (36–38). Therefore, to confirm that the MTX transport into
vesicles was mediated by BCRP, MTX accumulation into vesicles derived from MCF7/MX and MCF7/BCRP-Arg cells was measured in the presence of 5 μM FTC. Essentially complete inhibition of MTX accumulation was observed in both sets of vesicles (Fig. 4), indicating that BCRP is the transporter of MTX in this system.

**Kinetics of MTX Transport by BCRP.** To determine the kinetic parameters of MTX transport via BCRP, concentration-dependent uptake of MTX was measured in the MCF7/MX-derived vesicles. Accumulation was measured after a 2-min exposure to MTX in concentration that ranged from 0 to 2000 μM, in the presence of either ATP or AMP. As shown in Fig. 5, the velocity of substrate transport increased according to Michaelis-Menten kinetics in the presence of increasing levels of drug. MTX transport by BCRP was found to have a $K_m$ of 681 ± 280 μM and a $V_{max}$ of 2384 ± 445 pmol/mg/min. Thus, BCRP appeared to be a low-affinity, high-capacity transporter of MTX.

**Substrate Competition.** Mitoxantrone is the primary substrate for BCRP; almost all BCRP-overexpressing cell lines display high levels of mitoxantrone efflux and resistance (39, 40). Therefore, to further investigate the role of BCRP in MTX transport, we assayed MTX accumulation in the presence of mitoxantrone. MTX uptake into MCF7/MX vesicles was found to decrease with increasing concentrations of mitoxantrone (Fig. 6). The inhibition of MTX transport ranged from 12%, in the presence of 10 μM mitoxantrone, to nearly 60%, in the presence of 100 μM mitoxantrone. These data suggested competition between mitoxantrone and MTX and provided further evidence that MTX was indeed a substrate for BCRP.

**BCRP-mediated Transport of Long-Chain Polyglutamates of MTX.** MTX transport by several members of the MRP family has been shown to be largely restricted to MTX, with little or no transport observed when even a single extra glutamate was added (22, 23). To address whether BCRP-mediated transport of MTX was similarly limited, we measured accumulation of MTX and MTX-Glu2 to MTX-Glu4 into vesicles derived from MCF7/MX (Fig. 7) and MCF7/BCRP-Arg (data not shown) cells. As expected, drug uptake into vesicles derived from both cell lines decreased as the MTX polyglutamate chain length increased; however, in contrast to the MRPs, significant amounts of MTX-Glu2 and MTX-Glu3 were still transported. Furthermore, MTX polyglutamate transport was sensitive to the BCRP inhibitor FTC, indicating that BCRP, indeed, was transporting MTX as well as its polyglutamylated metabolites.
BCRP TRANSPORTS MTX

DISCUSSION

A substantial body of evidence exists to indicate the presence of one or more energy-dependent MTX efflux mechanism(s). For example, studies by Saxena and Henderson (18), using inside-out vesicles from L1210 cells, suggested the presence of two distinct, relatively low-efficiency ATP-dependent MTX efflux systems. In contrast, Schlemmer and Sirotnak (16) reported kinetic data for MTX transport in L1210 cells that suggested a single transporter with a somewhat higher affinity and capacity for MTX. However, neither group identified the actual protein involved. Other studies have shown that MTX is also a substrate for some MRPs. Low MTX uptake was observed into inside-out vesicles from MRP3-overexpressing LLC-PK1 cells (19), and from MRP1-overexpressing S19 (41) and GLC4/ADR (42) cells, whereas MTX was more efficiently transported by MRP2-containing S19 membrane vesicles (41). Furthermore, it was shown that rat mrp2 effectively excreted i-MTX into the bile (43). Finally, Chen et al. and Zeng et al. (22, 23) demonstrated in vitro that MRP1–4 were all able to transport MTX, albeit with a low affinity, but with considerable capacity. Thus, there is clear evidence for ATP-dependent MTX transport by multiple ABC proteins. However, none of those described thus far seems to be able to also transport the polyglutamylated MTX metabolites.

In contrast, we present evidence that BCRP transports not only MTX but also MTX-polyglutamates. Using an in vitro inside-out vesicle system, we have demonstrated ATP-dependent transport of MTX into vesicles the membranes of which contain BCRP. MTX transport was osmotically sensitive and followed Michaelis-Menten kinetics. The $K_m$ and $V_{max}$ values of 680 μM and 2400 pmol/min/mg, respectively, suggest that BCRP is a low-affinity, high-capacity transporter. Furthermore, there was also substantial transport of polyglutamylated forms of MTX, MTX-Glu2, and MTX-Glu3. Accumulation of MTX-Glu2 and MTX-Glu3 was 60 and 40% of that observed with MTX. Finally, transport of all MTX species was sensitive to inhibition by the BCRP inhibitor FTC. Thus, the data presented clearly establish BCRP as a transporter of MTX and its polyglutamates.

Interestingly, MTX transport was only detected in vesicles with wild-type BCRP, which contains an arginine at amino acid position 482. In contrast, no uptake of MTX was observed into vesicles containing BCRP with an R482G mutation. This finding was consistent with the phenotype displayed by the cells from which the vesicles were derived, MCF7/MX (BCRP-Arg) and S1-M1–80 (BCRP-Gly), respectively. Whereas MCF7/MX cells are highly resistant to MTX, only minimal cross-resistance to MTX is observed in S1-M1–80 cells (28). Thus, it appears that the R482G mutation resulted in a dramatic change in substrate specificity for BCRP. In agreement with this conclusion, Honjo et al. (34) have demonstrated that cells expressing the arginine variant were resistant to mitoxantrone and CPT-11 but remained largely sensitive to Adriamycin. In contrast, cells expressing the glycine variant exhibited enhanced levels of mitoxantrone resistance as well as high levels of Adriamycin resistance, both of which were accompanied by significantly reduced drug accumulation. Furthermore, cellular rhodamine 123 accumulation was drastically reduced in cells with BCRP-Gly but unaffected in cells with BCRP-Arg (34). Similar effects were also observed with mouse bcrp when the corresponding arginine was mutated to either serine or methionine (35). Thus, it seems clear that the identity of the amino acid at position 482 has a profound effect on the specificity of BCRP-mediated drug transport. However, additional studies are needed to elucidate the exact mechanism behind this observation.

Several members of the MRP family of ABC transporters have been shown to transport MTX, and their kinetic parameters have been determined. Reported $K_m$ values for MRP 1, 3, and 4 are 2150, 620, and 220 μM, respectively, and $V_{max}$ values are 2050, 2930, and 240 pmol/mg/min, respectively (22, 23). These values are comparable with those determined for BCRP in the present study. However, in contrast to what was observed for BCRP, the addition of even a single extra glutamate to MTX resulted in essentially complete abrogation of transport by the MRPs. Thus, although the affinities and capacities are in the same range, the ability to transport MTX-Glu2 and MTX-Glu3 makes BCRP clearly distinct from the MRPs, and unique among the ABC proteins reported to transport MTX.

It is tempting to speculate that the ability of BCRP to transport polyglutamylated MTX is responsible for the distinct MTX-resistance pattern of BCRP-overexpressing cells. Cells that overexpress BCRP are resistant against MTX in continuous exposure assays when polyglutamylation is likely to occur, suggesting that transport of polyglutamylated MTX may be an important requirement for resistance. In contrast, cells that overexpress MRPs are only resistant to short-term (<3 h) MTX exposure, when it is unlikely that MTX has undergone polyglutamylation (20, 21, 23). Thus, despite the low affinity of BCRP for MTX, its capacity to also transport MTX-polyglutamates may account for the high levels of resistance in long-term assays. It further suggests that MTX-polyglutamates can be exported even as they are formed, as opposed to only after degradation, which is consistent with the observed lower steady-state levels of MTX-polyglutamates in MCF7/MX cells (24).

The physiological significance of a $K_m$ as high as that observed for BCRP is unclear because it is doubtful whether the intracellular free MTX concentration will ever reach levels of that order of magnitude in vivo. For example, total MTX concentrations in the order of 500-3500 pmol/10⁶ cells or 2–15 μM, have been found in pediatric leukemia cells (44–46), which is two to three orders of magnitude below the $K_m$ for BCRP as well as for the MRPs. However, plasma MTX levels of 100 μM and greater have been measured 42 h after an initial high-dose MTX infusion (44). Furthermore, it is conceivable that the local concentration on the cytoplasmic side of the plasma membrane may be higher than the average intracellular concentration. Nevertheless, despite the low affinity, when the overexpression of BCRP is sufficiently high, it can apparently cause enhanced MTX efflux and concomitant resistance (28).

BCRP was originally identified and described as a mitoxantrone transporter. Therefore, we determined the effect of mitoxantrone on MTX transport. These studies have demonstrated that the presence of mitoxantrone reduced the transport of MTX. Increasing concentrations of mitoxantrone resulted in decreasing MTX accumulation into MCF7/MX vesicles, indicating that the presence of mitoxantrone interferes and possibly competes with MTX transport. These results further support the idea that BCRP is a MTX transporter. However, the data do not allow for further characterization of the type of competition between the two substrates.

Previously, MTX resistance in the MCF7/MX cells had been attributed to an ATP-dependent efflux mechanism, and it was shown that resistance was reversible with BCRP inhibitors (24, 28). Furthermore, MTX cross-resistance was not unique to the MCF7/MX cells; multiple BCRP-overexpressing cell lines exhibited similar cross-resistance, suggesting that it was caused by BCRP. Here, we extended this association and directly demonstrated that BCRP functions as a MTX efflux pump. BCRP appears to be unique among the MTX exporters described thus far in that it is also able to transport polyglutamylated forms of MTX. The efflux of MTX-polyglutamates may be a major contributor to MTX resistance under long-term exposure conditions.
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Note added in Proof

After this manuscript was accepted for publication, two reports appeared that inde-
pendently came to essentially the same conclusions. (1) Z. S. Chen, ev al. Transport of
Methotrexate, Methotrexate Polyglutamates, and 17-Estradiol 17-(β-D-glucuronide) by
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27. BCRP TRANSPORTS MTX

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