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Lack of an effect of breast cancer resistance protein (BCRP/ABCG2) overexpression on methotrexate polyglutamate export and folate accumulation in a human breast cancer cell line

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Abstract

Accumulation of methotrexate (MTX) and its polyglutamates (PGs) has been recognized as an important factor in MTX efficacy. We have previously described a multidrug-resistant human breast cancer cell line, MCF7/MX, that exhibits reduced accumulation of total MTX as well as MTX-PGs, and that is resistant to continuous MTX exposure [Volk EL, Rohde K, Rhee M, McGuire JJ, Doyle LA, Ross DD, et al. Methotrexate cross-resistance in a mitoxantrone selected multidrug-resistant MCF7 breast cancer cell line is due to enhanced energy-dependent drug efflux. Cancer Res 2000;60:3514–21]. These cells express high levels of the breast cancer resistance protein (BCRP/ABCG2) that has been shown to actively transport MTX and short-chain MTX-PGs in vitro. However, the effect of BCRP on MTX-PG accumulation in intact cells was unclear. Here, we show that MTX transport by BCRP is required for the observed lower levels of MTX-PGs in the resistant cells. When BCRP was inhibited with fumitremorgin C, or in cells expressing a mutated form of BCRP that is unable to transport MTX, MTX-PG accumulation was similar or even higher than that in the parental cells that do not express BCRP. Concomitantly, there was increased inhibition of thymidylate synthase. It had previously been suggested that BCRP-mediated efflux of MTX-PGs contributed to the reduced MTX-PG accumulation. However, we found no evidence of BCRP-mediated efflux of MTX-PGs from intact cells, suggesting that direct efflux of MTX-PGs does not play a major role in MTX resistance. Together, these data show that BCRP overexpression can cause a reduction in total MTX accumulation as well as a reduction in the proportion of long-chain MTX-PGs. In contrast, BCRP overexpression did not affect natural folate accumulation or the relative distribution of folylpolyglutamates in the resistant, as compared to the parental, cells. Thus, it appears that BCRP overexpression affects the metabolism of the antifolate MTX, but not that of natural folates, although indirect effects cannot be excluded.

Keywords: Drug resistance; Methotrexate; Breast cancer resistance protein; Polyglutamylation; Folate metabolism

1. Introduction

Although the phenomenon of folate and antifolate efflux from cells has been recognized for many years [1–3], the molecular basis of this efflux has only recently been elucidated in some detail. The identification of the multidrug-resistance proteins of the MRP/ABCC family as organic anion transporters [4] has led to the demonstration that at least some of the members of this family, namely MRP1–MRP4, are able to actively transport methotrexate (MTX) as well as folic acid and folinic acid [5–10]. Furthermore, overexpression of these MRPs can cause resistance against MTX, at least during short-term exposure [11–13]. Recently, it was shown that a complex relationship exists between levels of these export proteins and levels of natural folates in cells. For example, ectopic overexpression of MRPs 1–3 resulted in a 32–38% lower total cellular folate content than in the corresponding untransfected cells [14]. In contrast, loss of MRP1 expression was accompanied by reduced folate efflux activity, which resulted in increased accumulation of folates and MTX [15]. Furthermore, when cells were grown in folate-

Abbreviations: BCRP, breast cancer resistance protein; FPGS, folylpolyglutamate synthetase; FTC, fumitremorgin C; gGH, gamma glutamyl hydrolase; MRP, multidrug-resistance protein; MTX, methotrexate; PG, polyglutamate; UdR, deoxyuridine

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depleted medium, they exhibited a marked decrease in MRP1 transport activity, presumably so as to prevent loss of the essential folate co-factors required for cell proliferation [16]. Similarly, when multidrug-resistant MCF7/MR cells that overexpress the breast cancer resistance protein (BCRP/ABCG2) [17] were adapted to grow in very low folate medium, they almost completely lost expression of BCRP as well as MRP1–MRP5 [18]. Finally, selection of a Chinese hamster ovary cell line resistant to the antifolate pyrimethamine [19] was accompanied by enhanced accumulation of folates as a result of the loss of MRP1 expression [20]. Thus, there is substantial evidence in the literature that altered efflux can affect intracellular folate pools. Changes in these pools in turn affect the sensitivity of the cells to various antifolate drugs [21].

BCRP, in addition to the MRPs, has recently been shown to actively transport MTX and folates [6,22,23]. Overexpression of BCRP conferred resistance to continuous MTX treatment [24], in contrast to MRP1–MRP4 overexpression that only gave resistance to short-term MTX exposure. This difference in the resistance phenotype has been explained by the fact that, at least in vitro, BCRP is also able to transport MTX polyglutamates (MTX-PGs), whereas only the unglutamylated form of MTX is a substrate for transport by the MRPs [5,6,9,22]. However, active efflux of MTX-PGs from intact cells has not been directly demonstrated. Therefore, in the present study, we have measured the efflux of MTX and its polyglutamylated forms into the medium of the multidrug-resistant MCF7/MX cells. Compared to their parental MCF7/WT cells, MCF7/MX cells are 150-fold resistant to MTX in continuous exposure, and they express very high levels of BCRP [25–27]. As a result, total MTX accumulation is reduced, and this is accompanied by a reduction in the proportion of MTX species with long polyglutamate tails. Since both MTX and natural folates are subject to polyglutamylation, we also carried out a detailed analysis of the distribution of intracellular polyglutamates of MTX and natural folates. The results presented here suggest that the mechanism(s) affecting MTX metabolism in the MCF7/MX cells appears to be rather specific to this drug, with relatively little collateral effect on the natural folates.

2. Materials and methods

2.1. Reagents

MTX was purchased from Schirks Laboratory. All radio-labeled compounds were from Moravek Biochemicals. Fumitremorgin C (FTC) was a kind gift from Dr. Susan Bates, and GF120918 was kindly provided by Dr. François Hyafil.

2.2. Cells

The human breast cancer cell line MCF7/WT and its mitoxantrone-resistant sub-line MCF7/MX [28], and the human colon cancer cell line S1 and its mitoxantrone-resistant sub-line M1-80 [29], were grown in improved minimal essential medium (Richter's modification), supplemented with 10% FBS and 1 μg/ml ciprofloxacin. MCF7/MX cells express the wild-type form of BCRP with an arginine at amino acid position 482 (482R), whereas the M1-80 cells have acquired a mutation at this position from arginine to glycine (R482G) [30].

2.3. MTX accumulation, polyglutamylation and efflux

Cells were grown for 72 h in regular medium, followed by 24 h in folate-free medium. Then 2 μM [^{3}H]MTX (specific activity 0.05 μCi/nmol) were added, and the incubation was continued for another 24 h. The cells were then washed and drug-free medium was added. At various time-points thereafter, aliquots of cells and medium were collected and analyzed for remaining and exported total [^{3}H]MTX, respectively. In addition, we also analyzed and quantitated the individual polyglutamylated species of MTX. To this effect, the cells were extracted with water, whereas the media samples were concentrated, followed by separation of the individual MTX-PGs by HPLC and quantitation by liquid scintillation counting, as previously described [31].

2.4. FPGS and gGH activities

Folylpolyglutamate synthetase (FPGS) activity was assayed according to the method of McGuire et al. [32], and gamma-glutamyl hydrolase (gGH) activity was assayed according to the method of O'Connor et al. [33].

2.5. De novo thymidylate synthesis

Cells grown for 72 h were incubated with various concentrations of MTX for an additional 4 or 24 h, and 250 μM [6-^{3}H]deoxyuridine (UdR) (2 μCi/ml, specific activity 0.05 μCi/nmol) were added during the last hour. Cells were then washed three times with cold phosphate-buffered saline and were extracted with 0.1% SDS. Cell extracts were precipitated with 5% trichloroacetic acid, the precipitates collected on glass fiber filters (Whatman) according to the method of Duch et al. [34], and the retained radioactivity was determined by liquid scintillation counting.

2.6. Folate accumulation and folylpolyglutamates

Total cellular folates and their folylpolyglutamates were determined according to the method of Shane [35]. Briefly, cells were grown in medium containing either 2 μM

Table 1
MTX accumulation and long-chain MTX polyglutamate content

BCRP inhibitor	MCF7/WT				MCF7/MX			
	Total MTX (pmol/mg)	P	MTX-$Glu_{3–6}$ pmol/mg (% of total)	P^a	Total MTX (pmol/mg)	P	MTX-$Glu_{3–6}$ pmol/mg (% of total)	P^a
–	51.6 ± 5.9	–	25.6 ± 4.7 (48 ±4.4)	–	27.1 ± 3.6	0.007[b]	9.1 ± 1.9 (31 ± 4.5)	0.003[b]
FTC	72.4 ± 2.5	0.02[b]	43.6 ± 1.7 (61 ± 4.1)	>0.05[c]	123.8 ± 1.8	<0.001[c]	87.0 ± 1.0 (70 ± 1.1)	<0.001[c]
GF	44.5 ± 1.5	>0.05[c]	17.5 ± 1.6 (39 ± 2.5)	>0.05[c]	37.6 ± 9.9	>0.05[c]	9.8 ± 2.8 (26 ± 3.6)	>0.05[c]

	S1		M1-80	
	Total MTX (pmol/mg)	MTX-$Glu_{3–6}$ pmol/mg (% of total)	Total MTX (pmol/mg)	MTX-$Glu_{3–6}$ pmol/mg (% of total)
–	89.5 ± 0.7	70.5 ± 2.0 (79 ± 2.8)	91.8 ± 5.6	71.3 ± 5.7 (78 ± 1.5)
FTC	105.9 ± 2.3	84.6 ± 3.4 (80 ± 1.5)	114.2 ± 5.9	90.3 ± 5.7 (79 ± 0.9)

Sensitive MCF7/WT and S1 cells and resistant MCF7/MX and M1-80 cells were grown for 72 h, then incubated with 2 μM [^{3}H]MTX in the presence or absence of the respective BCRP inhibitor for another 24 h, at which time they were washed and lysed, and the intracellular MTX and MTX-PG content was analyzed by HPLC as described [31]. Values shown are the mean ± S.E. from six (Control) or three (FTC, GF) experiments for the MCF7 cell lines, and the mean from two experiments ± half the difference between the high and low values for the S1 and M1-80 cell lines.

[a] P-value is for % MTX-$Glu_{3–6}$.

[b] P-value relative to the respective control value in the parental cells.

[c] P-value relative to the corresponding control value in the same cell line.

[^{3}H]folic acid or [^{3}H]folinic acid for 96 h, followed by extraction with 0.1 M HCl. The folylpolyglutamates were then cleaved at the C_9–N_{10} bond to generate *p*-amino-benzoyl polyglutamates, which were then converted to the azo dyes of naphtylethylene diamine. The azo dye derivatives were purified by BioGel P-2 chromatography and re-cleaved to *p*-amino-benzoyl polyglutamates, which were then separated by SAX anion exchange HPLC and quantitated by liquid scintillation counting.

3. Results

3.1. Accumulation of MTX polyglutamates

We have previously shown that MCF7/MX cells exhibit a reduction in the relative accumulation of long-chain MTX-PGs (MTX-G3[1] and longer) [25]. In order to elucidate a possible role of BCRP overexpression in MTX polyglutamylation, we examined the accumulation of MTX-PGs in the presence or absence of the BCRP inhibitors FTC and GF120918. After 24 h the proportion of long-chain MTX-PGs relative to the total amount of drug was significantly reduced, from 50% in the parental cells to 30% in the resistant cells (Table 1). In addition, MCF7/MX cells contained only half the amount of total MTX found in MCF7/WT cells. However, when FTC was added in order to inhibit BCRP-mediated drug efflux, the proportion of long-chain MTX-PGs in the MCF7/MX cells increased to levels that were substantially higher than those in the parental cells (Table 1 and Fig. 1A and B). Indeed, in the resistant cells in the presence of FTC the proportion of long-chain MTX-PGs more than doubled compared to that in the absence of FTC, such that at 70% these forms now represented the majority of MTX species. In addition, there was also a substantial increase in total MTX accumulation. A qualitatively similar, but smaller, effect was seen in MCF7/WT cells when they were treated with FTC. Surprisingly, though, when we used another BCRP inhibitor, GF120918, the effects on the total accumulation and on the proportion of long-chain MTX-PGs were minor and not significant. The reasons for this discrepancy are currently not known. For comparison, we also analyzed MTX and MTX-PG accumulation in the colon cancer cell line S1, and in its mitoxantrone-resistant derivative, M1-80. Similarly to MCF7/MX cells, M1-80 cells express very high levels of BCRP and are highly resistant to mitoxantrone. However, in contrast to the MCF7/MX cells, M1-80 cells are not cross-resistant to MTX [24], because their BCRP contains the R482G mutation [30] that has been shown to abolish the transport of MTX [6,22,23]. In agreement with the lack of MTX cross-resistance in the M1-80 cells, we found no difference in the total amount of MTX or in the relative amount of long-chain MTX-PGs between the S1 and M1-80 cell lines. Total drug accumulation in the presence of FTC was, however, increased by about 20–25% in both of these cell lines, although the MTX-PG distribution remained unchanged (Table 1 and Fig. 1C and D).

3.2. MTX efflux

Reduced MTX accumulation in MCF7/MX cells has been attributed to increased drug export by BCRP [24]. In order to directly demonstrate increased drug efflux, we determined the amount of drug that remained in cells, as well as the amount that was released into the medium. In both the parental and resistant cell lines, the loss of the

[1] MTX-G2, -G3, -Gn refer to MTX with 2, 3 or *n* total glutamate residues, respectively. According to this nomenclature the native MTX molecule is MTX-G1.

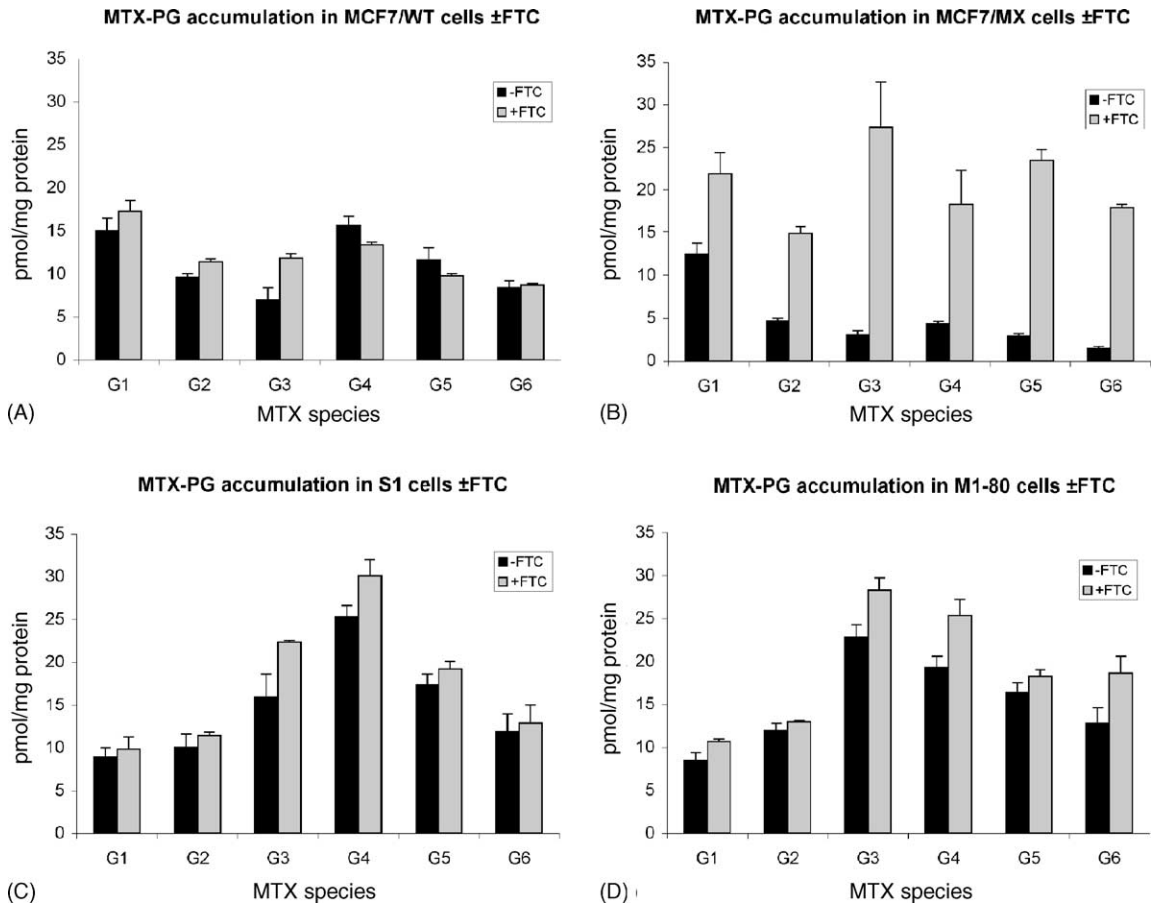

Fig. 1. Distribution of intracellular MTX polyglutamates. Cells were grown for 72 h in complete medium, followed by 24 h in folate-free medium with (grey bars) or without (black bars) 5 μM FTC, at which time 2 μM [^{3}H]MTX were added for another 24 h. Cell extracts were then analyzed for individual MTX polyglutamate species by HPLC. (A) MCF7/WT cells, (B) MCF7/MX cells, (C) S1 cells, and (D) M1-80 cells. The data shown are the mean ± S.E. from three separate experiments.

intracellular MTX in the absence of extracellular drug was accompanied by the reciprocal appearance of MTX in the medium (Fig. 2A and C). Cellular MTX decreased continuously and relatively rapidly for the first 4 h before leveling off. After 4 h in drug-free medium, MCF7/WT cells retained approximately 60% of the initial total cellular MTX, whereas in MCF7/MX cells only approximately 35% of MTX was retained. Conversely, in both cell lines there was a rapid increase in the amount of drug released into the medium, which closely corresponded to the amount lost from the cells. The loss of MTX from the cells as well as its appearance in the medium were increased by 40% in MCF7/MX cells relative to the parental cells, and this difference was completely abolished when the cells were incubated in the presence of the BCRP inhibitor FTC. In contrast, there was no difference in drug efflux between parental S1 and resistant M1-80 cells, either with or without FTC present (Fig. 2B and D), consistent with the inability of the mutated BCRP to transport MTX. These results confirmed that the increased export of MTX from the resistant MCF7/MX cells was directly mediated by the overexpression of wild-type BCRP(482R). However, approximately 50% of the drug present initially was lost from all four cell lines in an FTC-independent manner at a rate that was essentially indistinguishable between the respective parental and resistant cell lines. Together, these results confirm that the increased MTX efflux from the MCF7/MX cells is mediated by wild-type BCRP(482R), and they indicate that the majority of drug efflux must occur by a different mechanism.

3.3. Efflux of MTX polyglutamates

Previously, we and others, have shown that, in addition to MTX, in vitro BCRP was also able to transport MTX-PGs, especially the forms MTX-G2 and MTX-G3 [6,22]. Thus, it is possible that this ability might cause the observed reduction in long-chain MTX-PG accumulation (Table 1). However, active efflux of MTX-PGs has not been directly shown in intact cells. Therefore, we analyzed the individual MTX-PG species that had been exported into the medium. As expected, the majority of MTX released into the medium was MTX-G1, whereas only approximately 5% were released as MTX-G2, and even less as MTX-G3 (Fig. 3). Surprisingly, however, although the fraction of MTX-G1 released from the resistant cells

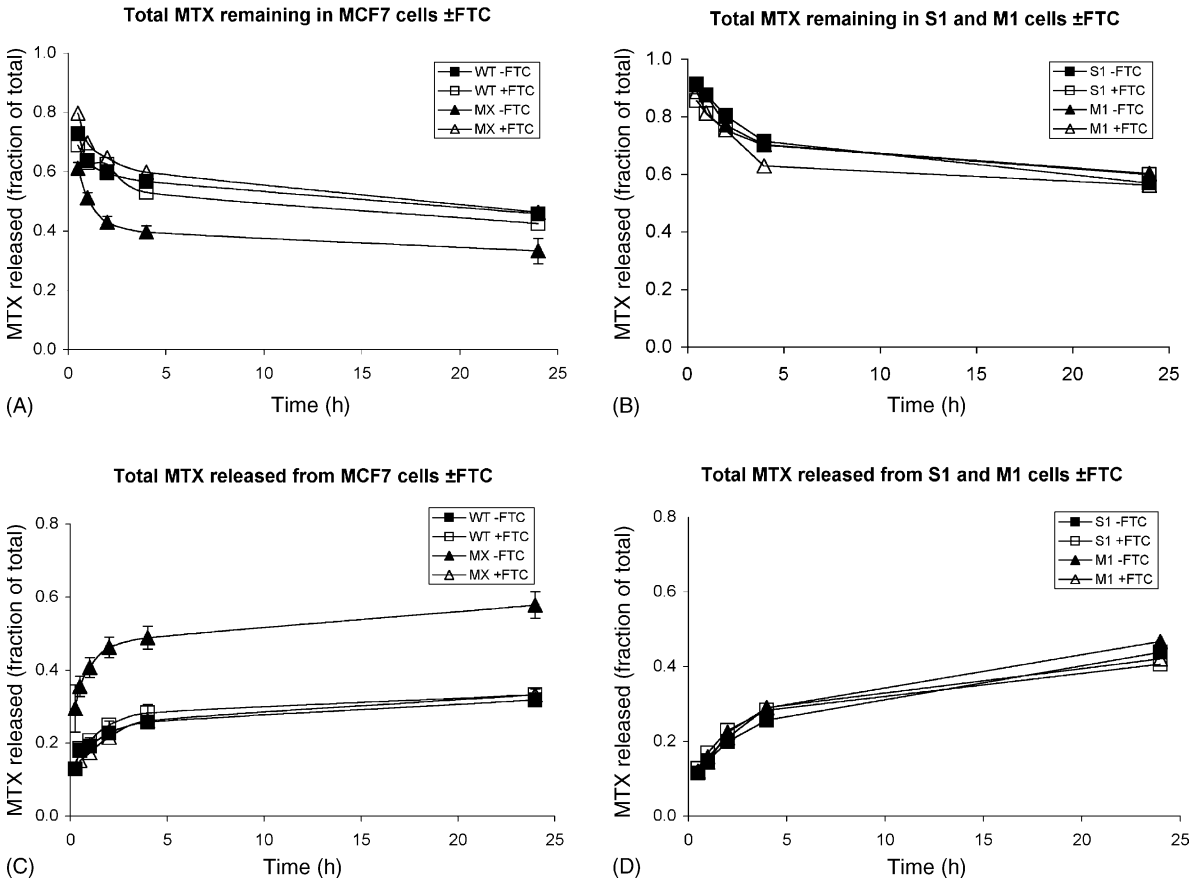

Fig. 2. MTX efflux from MCF7/WT, MCF7/MX, S1, and M1-80 cells. Cells were incubated with 2 μM [^{3}H]MTX with or without 5 μM FTC for 24 h as described in the legend to Fig. 1. The cells were then washed twice and placed into folate- and serum-free medium in the absence of MTX, but in the continuous presence (open symbols) or absence (closed symbols) of FTC. At various time points thereafter, total remaining intracellular (A, B) and released extracellular (C, D) MTX was determined by liquid scintillation counting. The data shown are relative to the total amount of intracellular MTX at time zero. (A, C) MCF7/WT (■, □) and MCF7/MX (▲, △) cells; (B, D) S1 (■, □) and M1-80 (▲, △) cells. Data for MCF7/WT and MCF7/MX cells are the mean ± S.E. from four to five separate experiments; those for S1 and M1-80 cells are the average of two experiments. For clarity, only selected error bars are shown.

was more than twice that from the sensitive cells, export of MTX-G2 and MTX-G3 was essentially identical. Thus, the clearly demonstrated transport of these MTX-PG species in vitro is not reflected in vivo. There were also small amounts of MTX-G4 to MTX-G6 detected in the medium from the resistant, but not the sensitive, cells. While it is tempting to speculate that this was specific for the resistant cells and contributed to their phenotype, we cannot exclude the possibility that the release of the longer-chain MTX-PGs in the resistant cells was due to non-specific leakage. However, since less than 10% lactate dehydrogenase was released after 24 h from either cell line, it appears unlikely that a difference in the number of cells with impaired membrane integrity would account for the observed presence of long-chain MTX-PGs in the medium from the resistant cells.

We also analyzed the remaining MTX-PG species in the corresponding cell extracts (Fig. 4). While no overall tendency over time was obvious in the amounts of individual MTX-PG species remaining, the presence of FTC in the MCF7/MX cells resulted in a clear shift from predominantly short-chain MTX-G2 and MTX-G3 forms to an increase in long-chain MTX-PGs (compare panels B and D in Fig. 4). Thus, it appears that although FTC prevented the efflux of MTX-G1, there was no build-up of this form in the cells. Rather, it appeared that there was a relative depletion of short-chain MTX-PG forms. In contrast, there was no significant difference between S1 and M1-80 cells in the distribution of MTX-PGs, either in cells or in medium, and FTC had no effect (data not shown). Together, these data suggest that BCRP-mediated MTX transport contributes to the apparent MTX-PG imbalance in the MCF7/MX cells.

3.4. FPGS and gGH

The amount of MTX-PG is the result of the balance between polyglutamylation by FPGS and deglutamylation by gGH. However, consistent with our previous observation [25] there were no differences in the activities of either enzyme between the two cell lines, and FTC did not inhibit gGH activity (data not shown). Thus, it is highly unlikely that the differences in MTX-PG accumulation between the parental and resistant cells are caused by altered FPGS and/or gGH per se.

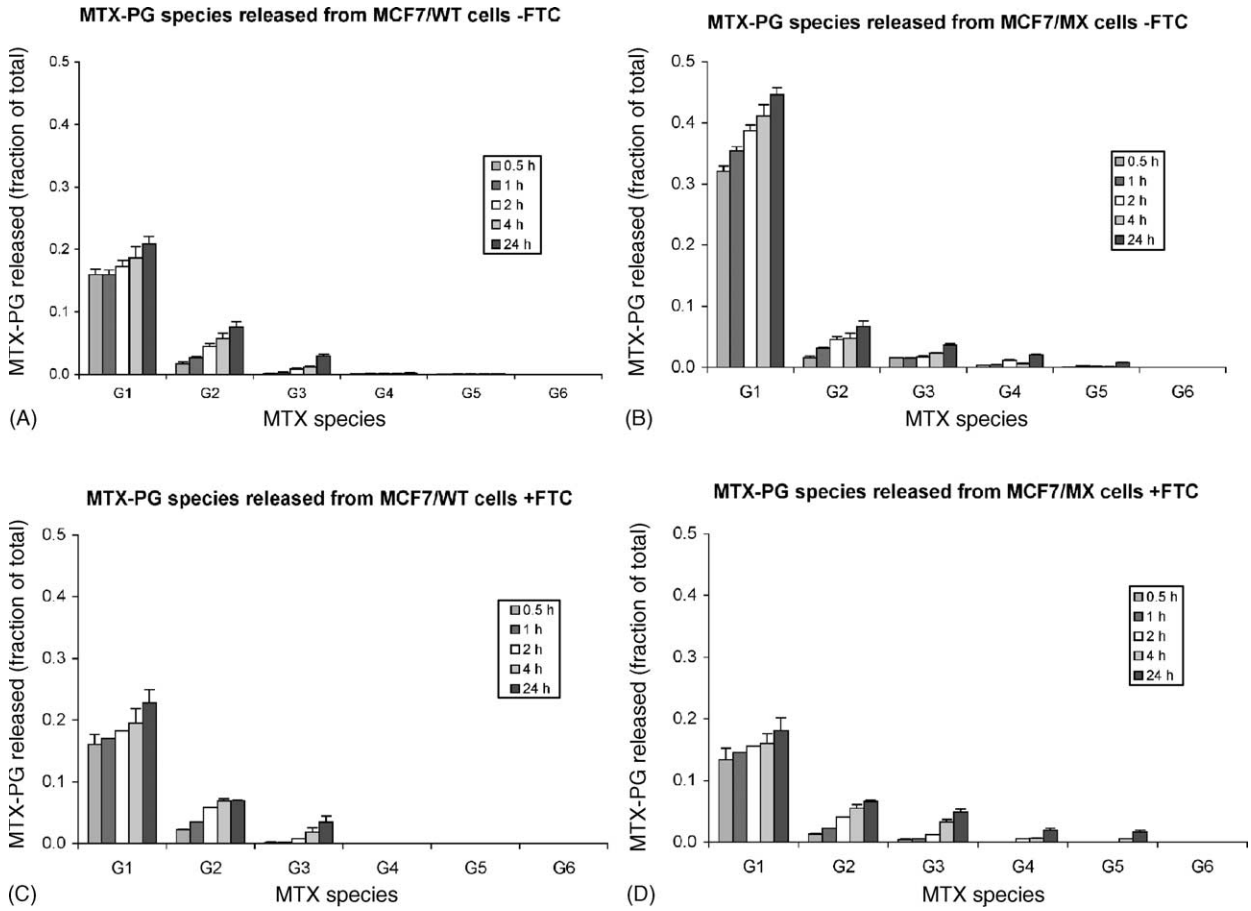

Fig. 3. Efflux of MTX polyglutamates into the medium. Cells were grown and incubated with 2 μM [^{3}H]MTX, then processed for drug efflux as described for Fig. 2. At the indicated times, the conditioned media were collected, lyophilized, reconstituted with 200 μL buffer, and analyzed for individual MTX-PG species by HPLC. Each value was expressed as the fraction of the total cellular MTX content at time zero and represents the mean ± S.E. from three experiments. (A) Medium from MCF/WT cells without FTC; (B) medium from MCF/MX cells without FTC; (C) medium from MCF/WT cells with FTC; and (D) medium from MCF/MX cells with FTC. Groups of bars from left to right along the *x*-axis represent time points at 0.5, 1, 2, 4, and 24 h.

3.5. Effect of MTX on de novo thymidylate synthesis

MTX-PGs, but not MTX, are known to inhibit thymidylate synthase. Therefore, in order to determine whether the reduced accumulation of MTX-PGs in the resistant cells was reflected in a decreased inhibition of TS, we examined the inhibition of de novo thymidylate synthesis in actively dividing cells by measuring deoxyuridine (UdR) incorporation into DNA. As shown in Fig. 5, MTX effectively inhibited UdR incorporation in MCF7/WT and MCF7/MX cells, with IC_{50} values of 0.2 and 3.2 μM, respectively. Interestingly, in MCF7/MX cells, complete inhibition of UdR incorporation was not achieved under these conditions, even at 25 μM MTX. In order to achieve complete inhibition, the treatment time with MTX had to be extended from 4 to 24 h and 2 μM drug were required (data not shown). In contrast, only 0.2 μM MTX were required to achieve the same effect in the sensitive cells. Consistent with its effect on MTX-PG accumulation, the addition of FTC resulted in a substantially greater inhibition of thymidylate synthase by MTX in the resistant cells, but little additional inhibition in the sensitive cells (data not shown).

3.6. Total folate and folylpolyglutamates in MCF7/WT and MCF7/MX cells

Since both MTX and natural folates are subject to polyglutamylation/deglutamylation it was possible that the mechanism(s) that led to a reduction in long-chain MTX-PG accumulation also affected the natural folates. Therefore, we examined the accumulation and the efflux of the natural folates folic acid and folinic acid, as well as their respective folylpolyglutamate species. As shown in Table 2, there appeared to be a small but consistent reduction in folate accumulation in the resistant cells. However, results from two additional repeat experiments at 96 h with 2 μM folinic acid, each in duplicate, revealed that the difference, although reproducible, was statistically not significant (mean values 141.5 ± 8.7 versus 134.1 ± 13.8 pmol/mg; $N = 6$; $P = 0.66$). These results suggested that BCRP overexpression did not have a major

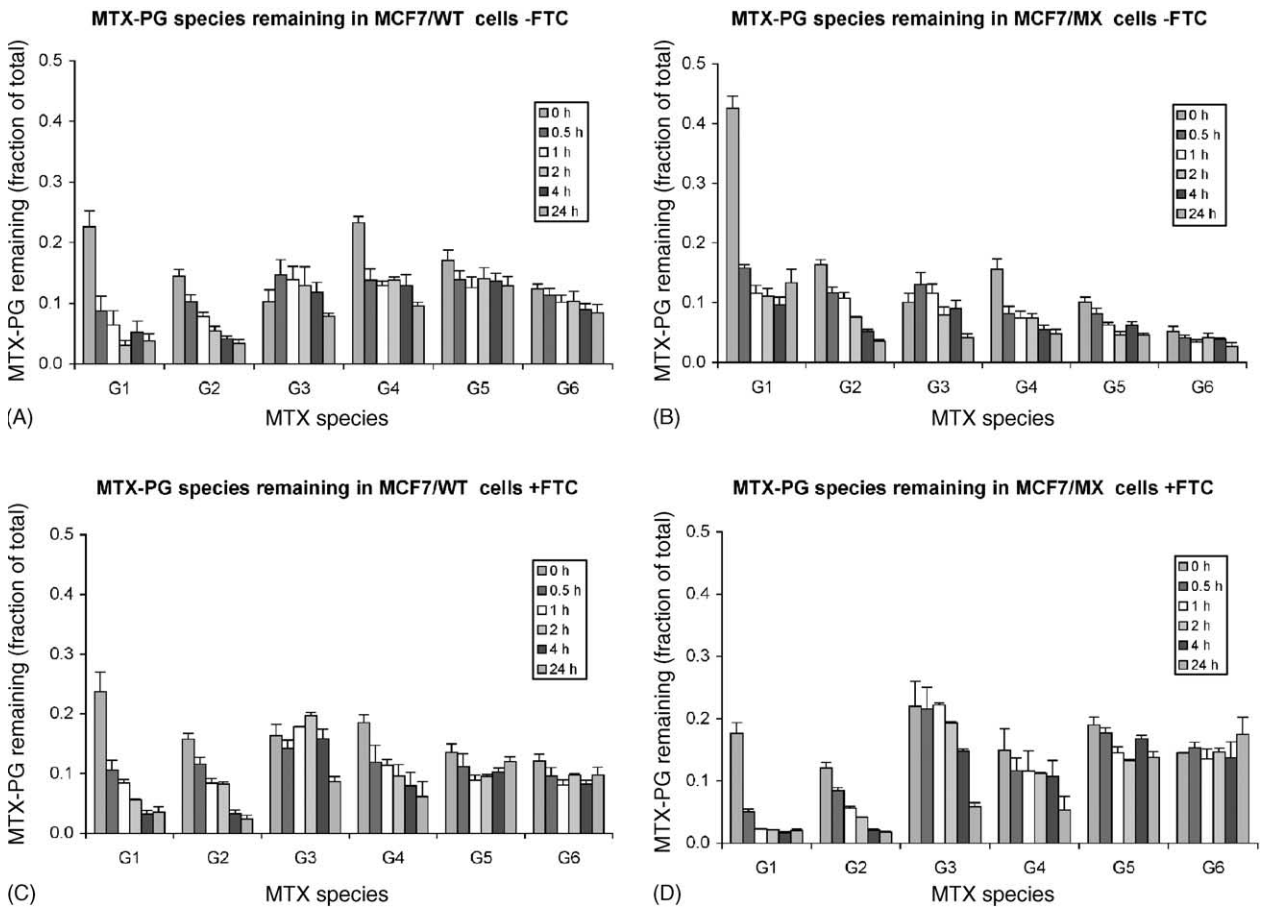

Fig. 4. Intracellular MTX polyglutamate species remaining during efflux. The cells from which the conditioned medium for the data in Fig. 3 was collected were analyzed for individual remaining MTX-PG species by HPLC. Each value was expressed as the fraction of the total cellular MTX content at time zero and represents the mean ± S.E. from three experiments. (A) MCF7/WT cells without FTC; (B) MCF7/MX cells without FTC: (C) MCF7/WT cells with FTC; and (D) MCF7/MX cells with FTC. Groups of bars from left to right along the *x*-axis represent time points 0, 0.5, 1, 2, 4, and 24 h.

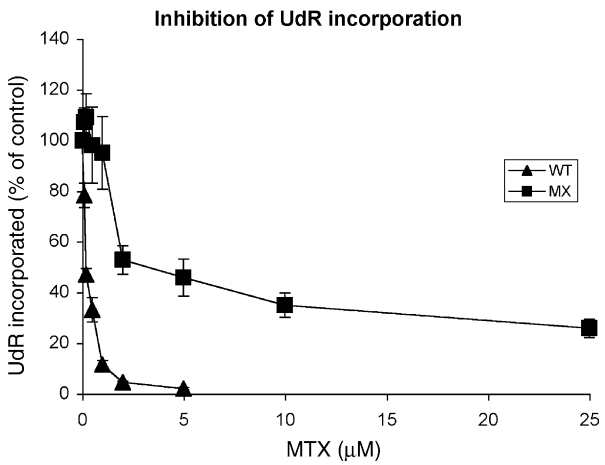

Fig. 5. Effect of MTX on UdR incorporation. Cells were incubated with various MTX concentrations for 4 h, and 250 μM [6-^{3}H]UdR were added for the last hour. Cells were then extracted with 0.1% SDS and precipitated with 5% trichloroacetic acid. Total radioactivity in the precipitates was determined by liquid scintillation counting and was normalized to total cell protein. The data are expressed as the percentage of untreated control and represent the mean ± S.E. from three experiments. (▲) MCF7/WT and (■) MCF7/MX.

effect on folate accumulation, and thus BCRP may not actually export natural folates. To further confirm this, we directly determined the efflux of folinic acid from both MCF7 cell lines in the absence or presence of FTC (Fig. 6). After loading of the cells for 30 min with 10 μM folinic acid, there was an equally rapid loss of folinic acid from the two cell lines to approximately 50% within 1 h, which was insensitive to FTC. Similarly, there was also no difference in efflux between the two cell lines after 96 h incubation with 2 μM folinic acid. However, under these conditions most of the folates had become non-exportable, such that only approximately 20% were lost after 4 h. Thus, BCRP overexpression apparently did not affect either the accumulation or the efflux of natural folates. Furthermore, even after 96 h in either 2 μM folic acid or 2 μM folinic acid the amounts and lengths of the natural folylpolyglutamate species in the two cell lines were essentially the same (data not shown). In particular, there was no apparent deficiency of long-chain folylpolyglutamates in the resistant cells. Thus, it appears that the decrease in MTX-PG accumulation was specific for MTX and did not affect the natural folates.

Table 2
Cellular folate accumulation in MCF7/WT and MCF7/MX cells

Time (h)	Concentration (μM)	Folic acid		Folinic acid	
		MCF7/WT (pmol/mg)	MCF7/MX (pmol/mg)	MCF7/WT (pmol/mg)	MCF7/MX (pmol/mg)
4	1	13.5	12.5	34.1	30.1
	10	64.9	59.7	146.8	129.1
24	1	22.2	25.2	63.7	57.2
	10	106.9	100.8	196.5	196.8
96	0.2	ND	ND	70.5	59.2
	1	ND	ND	100.2	93.0
	2	ND	ND	124.9	118.0
	10	ND	ND	262.8	233.9

Cells were grown in folate-free medium containing 100 μM thymidine and hypoxanthine for 72 h and then in various concentrations of either [^{3}H]folic acid or [^{3}H]folinic acid for 4, 24, or 96 h as indicated. Cells were extracted with 1 N NaOH, and radioactivity was quantitated by liquid scintillation counting. The results shown are the means from duplicate experiments. ND, not done.

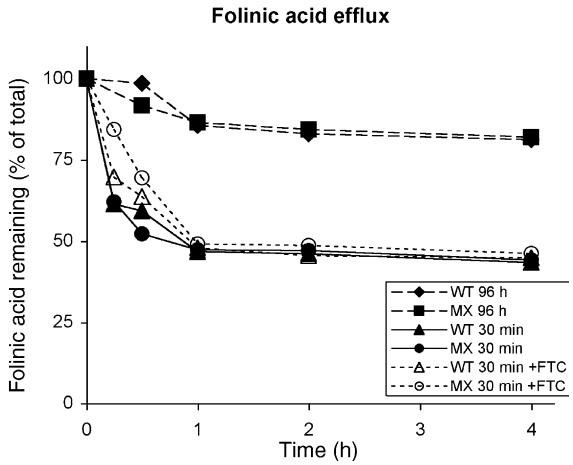

Fig. 6. Efflux of folinic acid. Cells were incubated in 10 μM [^{3}H]folinic acid with (open symbols, dotted line) or without (closed symbols, continuous line) FTC for 30 min, or in 2 μM [^{3}H]folinic acid for 96 h without FTC (closed symbols, dashed line), and then placed into folate-free medium that contained the same amount of FTC as the uptake medium. At various time points thereafter, the cells were extracted and total remaining radioactivity was determined by liquid scintillation counting and normalized to the amount of total cellular protein. Data are expressed as the fraction of the total amount of folinic acid present at time zero and represent the mean from duplicate experiments. (◆) MCF7/WT, 96 h; (■) MCF7/MX, 96 h; (●, ○) MCF7/WT, 30 min; and (▲, △) MCF7/MX, 30 min.

4. Discussion

Resistance to antifolates such as MTX has been attributed to a number of factors, including altered transport into and out of the cells, altered interactions with the respective target enzymes, and altered polyglutamylation. For example, in pediatric leukemia patients, it was shown that decreased MTX accumulation together with a lower proportion of long-chain MTX-PGs was associated with lower MTX sensitivity [21]. However, the exact mechanism(s) responsible for the reduced MTX accumulation and polyglutamylation has not been fully elucidated, especially with regard to the role that active drug export plays in this process. We previously described a multidrug-resistant MCF7 cell line, MCF7/MX, that is resistant to continuous MTX exposure and which, when compared to parental MCF7/WT cells, exhibits reduced MTX accumulation that is accompanied by a lower proportion of long-chain MTX-PGs [25]. Subsequently, we demonstrated that MTX accumulation was affected by active drug export due to the overexpression of BCRP [24], and that BCRP is able to transport MTX, as well as MTX-G2 and MTX-G3 [22], at least in vitro. In the present study, therefore, we directly determined in vivo the amount of each species of MTX-PG that was exported into the medium, as well as the amount that remained inside the cells. The results demonstrated that the majority of MTX was exported as MTX-G1, whereas less than 5% was secreted as MTX-G2 or longer PGs. Surprisingly, the amounts of MTX-G2 and MTX-G3 that were exported into the medium were similar between MCF7/MX and MCF7/WT cells, and FTC did not inhibit the appearance of these forms of MTX in the medium. This suggests that direct export of polyglutamylated species of MTX by BCRP does not play a major role in MTX resistance. While the exact reasons for this apparent discrepancy between the in vitro and in vivo results are not known, one possibility is that the intracellular concentration of the MTX-PGs was too low for export to occur with any efficiency, since BCRP has a relatively low affinity for even the monoglutamylated MTX, with a K_m in the high μM to low mM range [6,22,23]. Alternatively, we cannot exclude the possibility that polyglutamylated MTX is hydrolyzed by gGH that is secreted from the cells [36], although we found no evidence that this was occurring (data not shown).

It had previously been speculated that the ability of BCRP to also transport MTX-PGs was responsible for conferring high-level resistance to continuous MTX exposure in cells that overexpress BCRP [22,24], and that this mechanism also contributes to the relative depletion of long-chain MTX-PGs. In contrast, cells that overexpress one of the MRPs 1–4 are primarily resistant to short-term MTX exposure and only show very low levels of resistance to continuous drug exposure [11–13,37]. This difference was attributed to the fact that the MRPs cannot transport MTX-PGs [5,10], although their affinities for MTX are in

the same range as that of BCRP [5,10,38]. In agreement with this conclusion, an etoposide-selected cell line, MCF7/VP, that overexpresses MRP1 [39] was only 3.7-fold cross-resistant to continuous MTX treatment compared to its parental counterpart [25], and the proportion of long-chain MTX-PGs was identical to that in the parental cells (Rhee and Schneider, unpublished results). Similarly, cells transfected with MRP1 or MRP3 showed only 1.7–1.9-fold resistance to MTX compared to their untransfected parental cells [12,37]. Thus, despite similar in vitro transport characteristics of these ABC proteins for MTX, MTX export by BCRP and by MRPs appears to have different effects on intracellular MTX metabolism and MTX resistance. However, the lack of a demonstrable difference in the efflux of polyglutamylated MTX forms between the sensitive and the resistant MCF7 cells used here raises the question of whether direct efflux of MTX-PGs is indeed the deciding factor for the different resistance phenotypes.

In an attempt to better understand the cause for the reduction in the proportion of long-chain MTX-PGs in the resistant cells, we performed a detailed analysis of the various MTX-PG species over time under drug efflux conditions. However, it is rather difficult to evaluate efflux of MTX-PGs by examining the amount of each species that is remaining inside the cells, since polyglutamylation by FPGS and deglutamylation by gGH take place continuously. Further complicating the analysis is the fact that the products from the initial deglutamylation of long-chain MTX-PGs themselves become substrates for further deglutamylation [40,41]. Thus, the results in Fig. 4 must be interpreted with caution. Nevertheless, an analysis of the time course of the loss of long-chain MTX-G4–MTX-G6 species suggests that, in the absence of extracellular drug, rapid degradation of these forms occurred at a rate that was somewhat faster in the resistant cells than in the parental cells (70% versus 50% of the initial amount lost after 1 h in the resistant versus sensitive cells, respectively). At the same time, there was a rapid, yet transient, increase in the amount of MTX-G3 present in the cells. Interestingly, in the resistant cells, FTC seemed to cause a rapid depletion of MTX-G1 and MTX-G2, despite preventing their efflux by BCRP. This somewhat paradoxical effect was accompanied by an increase in longer-chain MTX-PG species. While it is difficult to fully explain these findings with the available data, one possibility is that FTC somehow was blocking the conversion of long-chain PGs into short-chain species. Whether this occurred by direct inhibition of, for instance, transport of MTX-PG into the lysosomes, by negative feedback of the hydrolysis reaction, or by some other, yet unknown, mechanism cannot be determined from the present results and needs further investigation.

MTX is an antifolate that is glutamylated by the same enzymes as the natural folates. Therefore, we determined whether the mechanism(s) that resulted in reduced MTX accumulation and polyglutamylation also affected the natural folates. However, despite the close structural relationship of MTX and the natural folates, there were no substantial differences between the sensitive and resistant MCF7 cell lines in the accumulation and efflux of natural folates, the degree of their polyglutamylation, or in the relative amounts of the various folylpolyglutamate species, independently of whether the cells were incubated in folic acid or folinic acid, or whether FTC was present. Together, these results suggest that BCRP does not play a major role in the transport and/or the metabolism of the natural folates, at least not in cells where it is overexpressed, and when the cells are grown in medium with high folate concentrations. In contrast, it was recently reported that, when cells were adapted to grow in medium with low folate concentrations, the expression of BCRP was downregulated, presumably to prevent unnecessary loss of the essential folates [18]. Thus, there may be a difference depending on the concentration of extracellular folate present.

In conclusion, the data presented here show that BCRP overexpression plays a major role in the accumulation of MTX and in its polyglutamylation, whereas BCRP overexpression seems to have little effect on the transport and the accumulation of the natural folates and folylpolyglutamates. However, it is not clear whether the effect of BCRP is as simple as preventing the accumulation of sufficient substrate for FPGS or whether some as yet unknown secondary or indirect mechanisms are involved.

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