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The role of multidrug resistance proteins MRP1, MRP2 and MRP3 in cellular folate homeostasis

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Abstract

Previously, we reported that the multidrug resistance proteins MRP1, MRP2 and MRP3 confer resistance to therapeutic antifolates by mediating their cellular extrusion. We now determined whether MRPs also play a role in controlling cellular homeostasis of natural folates. In MRP1, MRP2 and MRP3-transfected 2008 human ovarian carcinoma cells total cellular folate content was 32–38% lower than in 2008 cells (105 ± 14 pmol folate/mg protein) when grown in medium containing 2.3 μM folic acid (FA). Under these conditions cellular growth rates were not compromised. However, when cells were challenged under folate-depleted conditions with a short exposure (4 hr) to FA or leucovorin, MRP1 and MRP3 overexpressing cells were impaired in their growth. In contrast to wild-type cells, MRP1 transfected cells retained only 60% of the maximum growth when exposed to 500 nM leucovorin or 500 μM FA. For 2008/MRP1 and 2008/MRP3 cells FA growth stimulation capacity was dramatically decreased when, during a 4 hr exposure, metabolism into rapidly polyglutamatable and retainable dihydrofolate was blocked by the dihydrofolate reductase inhibitor trimetrexate. To retain growth under such conditions MRP1 overexpressing cells required much higher concentrations of FA (EC_{50} > 500 μM) compared to 2008 cells (EC_{50}: 12 μM). These results suggest that down- and up-regulation of MRP1 (and MRP3) expression can influence cellular folate homeostasis, in particular when cellular retention by polyglutamylation of folates is attenuated.

Keywords: MRP; Folic acid; Leucovorin; Folate homeostasis; Multidrug resistance; Polyglutamylation

1. Introduction

Over the past decades an increasing number of ATP-driven efflux pump families (ABC-transporters) has been identified that can confer multiple drug resistance by cellular extrusion of a wide variety of structurally unrelated drugs [1]. The best-known efflux pumps include P-glycoprotein (Pgp, ABCB1) [2] and the multidrug resistance protein (MRP1, ABCC1) [3]. More recently another transporter has been identified: the breast cancer resistance protein (BCRP, ABCG2), also known as mitoxantrone resistance associated protein (MXR) [4].

Since the first description of the MRP1 [3] many studies focused on MRP-mediated cellular extrusion of cytotoxic drugs (for reviews see [1,5,6]). The discovery of several MRP1 homologues, including MRP2 (ABCC2) and MRP3 (ABCC3), broadened this field of research [7]. These related transporters only partly share the same subset of substrates. Beside the established function of MRPs in the protection of cells against toxic compounds, there is cumulative evidence for other biological functions of MRPs [1,5,6].

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Abbreviations: RFC, reduced folate carrier; MTX, methotrexate; MRP, multidrug resistance protein; FA, folic acid; FPGS, folylpolyglutamyl synthetase; 5FU, 5-fluorouracil; LF, low-folate; LV, leucovorin; HF, high-folate; DHFR, dihydrofolate reductase; TS, thymidylate synthase.

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The folate antagonist MTX finds widespread use in cancer chemotherapy [8,9] and treatment of inflammatory diseases, e.g. in rheumatology [10]. As a potent inhibitor of folate metabolism by binding to DHFR and TS, MTX abolishes purine and thymidylate biosynthesis, thereby disrupting DNA replication.

In the past, several causes of cellular resistance against MTX and other hydrophilic folate antagonists have been discovered. Among them are (1) downregulation of antifolate uptake mediated by the RFC, (2) impaired polyglutamylation of MTX due to downregulation of FPGS, or (3) upregulation of the targets of MTX (i.e. DHFR) (for reviews see [11]). More recently, attention is being paid to efflux systems of antifolates that may contribute to cellular resistance [18–21].

Initial studies by our laboratory as well as other laboratories demonstrated that members of the MRP family, in particular MRP1, MRP2 [12], MRP3 [13,14], and MRP4 [15,16] play a role in MTX efflux. Inhibition of MRP1 efflux function by probenecid reverted the MTX-resistant phenotype *in vitro* [12] or enhanced the activity of the antifolate 10-deazaaminopterin *in vivo* [17].

Beyond a function for MRPs in antifolate resistance, we have obtained indirect evidence for a possible physiological role of MRPs in controlling cellular homeostasis of natural folates [12]. Cells transfected with MRP1 or MRP2 displayed a collateral sensitivity to the lipophilic antifolate trimetrexate (TMQ), which is indicative of a decreased intracellular folate pool status. Other evidence for a contribution of MRPs in folate homeostasis was reported by Kusuhara *et al.* [23], showing that MRP2/cMOAT was able to transport reduced folate cofactors in rats. Finally, Zeng *et al.* [14] and Chen *et al.* [16] described vesicular membrane transport of FA by MRP3 and MRP4, respectively.

As described by our laboratory [24,33] and recently by others [25] subtle changes in the folate content of cells might result in a major increase in resistance for a series of polyglutamylation-dependent antifolates. Likewise, animal studies [23,26] showed the relevance of folate status for the *in vivo* sensitivity to several novel antifolates.

In this report we addressed the issue of whether overexpression of MRPs affects cellular homeostasis of folates due to efflux of these compounds. We show that MRP1 and MRP3 overexpression results in a diminished cellular folate content. Also, we demonstrate that MRP1/3 overexpression can hamper FA stimulated growth, especially when metabolism or cellular retention by polyglutamylation is attenuated.

2. Materials and methods

2.1. Materials

Folic acid and L-leucovorin were obtained from Sigma Chemical Co. TMQ was a gift from Warner Lambert/Parke Davis (Ann Arbor, MI). Protease inhibitor cocktail was purchased from Boehringer, NL. RPMI-1640 medium (with and without 2.3 μM FA) and (non)dialyzed fetal calf serum were obtained from GIBCO.

2.2. Cell lines

The human ovarian carcinoma cell line 2008, and its stable MRP1, MRP2 and MRP3 transfectants were kindly provided by Dr. M. Kool and Prof. Dr. P. Borst (The Netherlands Cancer Institute, Amsterdam) [28]. These cell lines 2008, 2008/MRP1, 2008/MRP2 and 2008/MRP3 were cultured in RPMI-1640 medium (containing 2.3 μM FA), supplemented with 10% FCS, 2 mM glutamine and 100 μg/mL penicillin/streptomycin. The MRP1–3 transfectants display MTX resistance following short-term drug exposure [12,13].

To deprive cells of folates they were cultured in folate-free RPMI-1640 medium supplemented with 10% dialyzed FCS, 2 mM glutamine and 100 μg/mL penicillin/streptomycin for a period of 4 days.

2.3. Folate pool analysis

The measurement of reduced folate cofactors was made using procedures described previously by Bunni *et al.* [27].

2.4. MRP-Western blot

Total lysates were prepared from 5×10^6 cells by lysing in 250 μL buffer containing 10 mM Tris–HCl (pH 7.6), 20% glycerol, 5 mM DTT, 0.5% NP-40, and a protease inhibitor cocktail 2.0% v/v (Boehringer). Lysates were sonicated at 3×5 s with 30-s interval (MSE Soniprep 150, 4°, amplitude 6–7). Western blotting was performed according to standard techniques. In each lane of a Biorad minigel system 10–50 μg protein was loaded. For detection of MRP1, MRP2, and MRP3 monoclonal antibodies (MRPr1, M2III-6, and M3II-9, respectively) [28] were used in a dilution of respectively, 1:500 (0.5 μg/mL), 1:50 (5 μg/mL), and 1:50 (5 μg/mL). As a loading control expression of β-actin was determined using anti-human β-actin (Chemicon). MRP expression was measured as OD × mm^2 with densitometric scanning (Imaging Densitometer GS-800, Biorad Laboratories).

3. Results

3.1. MRP expression in human 2008 ovarian carcinoma cells

In order to investigate whether MRPs contribute to the cellular extrusion of natural folates, we used the human ovarian carcinoma cell line 2008 and its MRP1-, MRP2-, and MRP3-transfected sublines. These cells were used

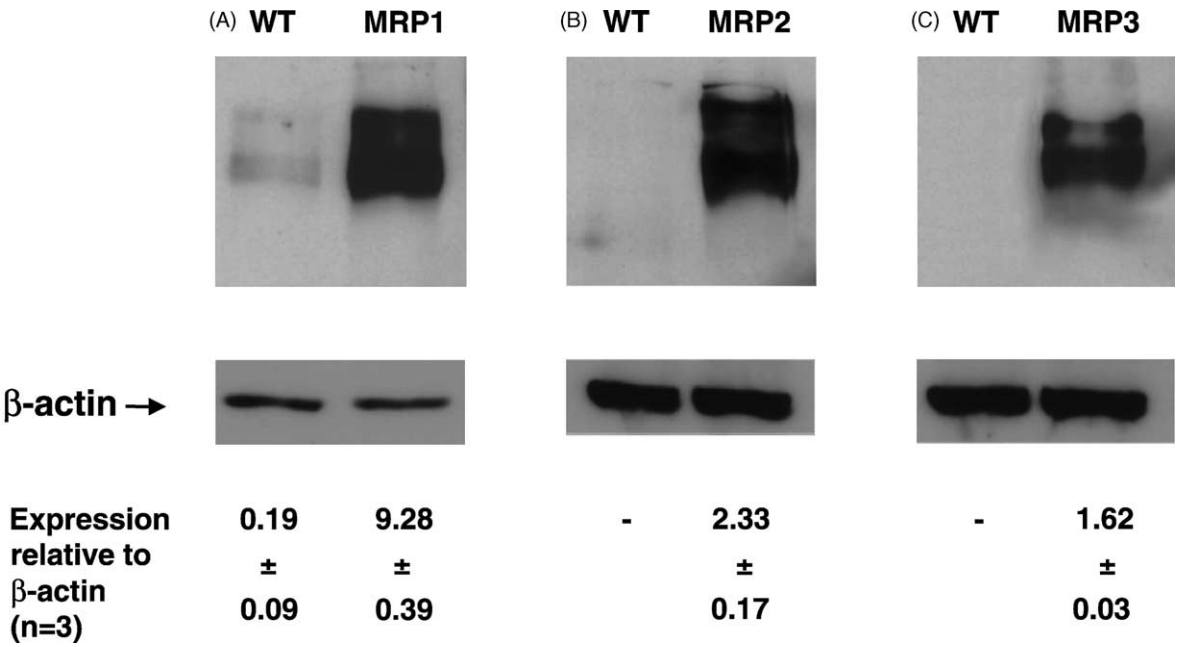

Fig. 1. Expression of MRP1, MRP2, and MRP3 in (transfected) 2008 ovarian carcinoma cells. Expression of MRP1 (A), MRP2 (B), and MRP3 (C) in 2008 (WT), and 2008/MRP1–3 was analyzed by Western blotting, as detailed in Section 2. Per lane 10 μg (A) or 50 μg (B and C) protein was loaded. As a loading control β-actin levels are indicated. Average expression of MRP1, MRP2, or MRP3 relative to β-actin ± SD (N = 3) is given.

previously by our laboratory and others to analyze MRP1-, MRP2-, and MRP3-mediated transport of MTX [6,12,13,28].

We assessed the expression levels of MRP1, MRP2, and MRP3 in transfected 2008 cells by means of Western blotting. Fig. 1A, B and C show the expression of MRP1, MRP2, and MRP3 in wild-type 2008 and 2008/MRP1–3 transfectants. Wild-type 2008 cells contain a low expression of endogenous MRP1. MRP1-transfected 2008 cells expressed 48-fold more MRP1 than wild-type 2008 cells. In wild-type 2008 cells no MRP2 or MRP3 could be detected. As calculated relative to β-actin MRP2 expression in 2008/MRP2 was 2.33-fold. The level of MRP3 in 2008/MRP3 was 1.62-fold relative to β-actin (see Fig. 1).

3.2. Effect of MRP overexpression on cellular levels of folates

Based on the earlier reports that MRPs mediate the transport of FA [13,15], we wanted to analyze whether MRP1, MRP2, or MRP3 overexpression affect the cellular folate content. Total folate pools were analyzed in wild-type 2008 cells and their MRP1, MRP2, and MRP3 transfectants at mid-log phase of growth in standard RPMI-medium, which contained 2.3 μM FA. The MRP1–3 transfectants had 32–38% diminished total folate pools relative to wild-type 2008 cells, which contained ∼105 ± 14 pmol folate/mg protein (Fig. 2). When discriminating between different reduced folate co-factors we observed that the diminishment in cellular folate was mainly due to a 35–45% decrease in the pool of 10-formyltetrahydrofolate (10-$CHOFH_4$), rather than 5-CH_3FH_4, CH_2FH_4 or FH_4 (see Fig. 2).

3.3. Effect of MRP overexpression on cellular growth

Despite the lower intracellular folate content, cellular growth rates of MRP overexpressing cells were not affected in a cell culture medium containing 2.3 μM folates (not shown). The latter folate levels are, however, far above

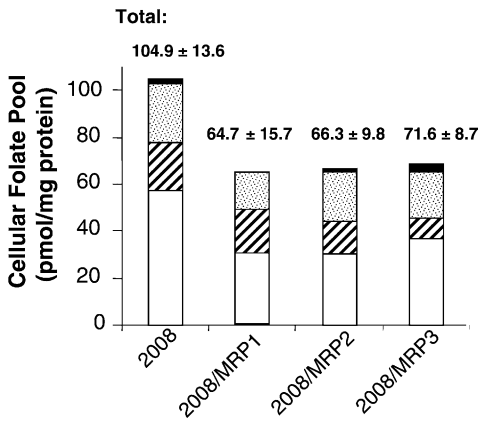

Fig. 2. Total cellular folate pools in 2008 and 2008/MRP cells. Total folate pools in 2008 cells and several MRP-transfectants were determined in their mid-log phase of growth, when grown in standard RPMI-1640 medium containing 2.3 μM folates, and supplemented with 10% FCS. Total cellular folate pools have been subdivided into FH_2 (black bars), 5-CH_3FH_4 (spotted bars), CH_2FH_4 + FH_4 (striped bars) and 10-$CHOFH_4$ pools (blank bars). Results are the means ± SEM of three separate experiments.

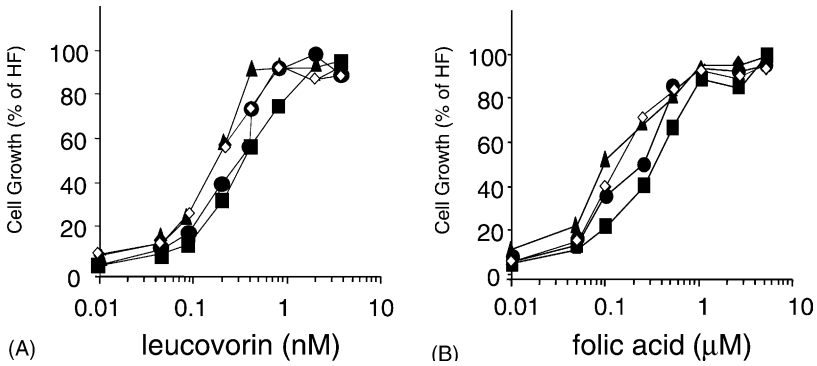

Fig. 3. Effect of MRP overexpression on folate-dependent cellular growth (72 hr exposure). Cell growth was measured when cells were cultured in folate-free RPMI-1640 medium, containing either leucovorin (A) or folic acid (B) as the sole folate source. Cell lines studied were 2008 (□), 2008/MRP1 (■), 2008/MRP2 (▲) and 2008/MRP3 (●). Exposure time of folates was 72 hr. Cell growth is given as percent of growth obtained under high folate conditions (HF). Results are representative experiments of three independent experiments. Standard deviations (SD) between experiments were <19%.

physiologically relevant concentrations (human plasma levels 5–20 nM). We therefore also analyzed the folate growth requirement of MRP overexpressing cells that were first depleted from folates by a 4-day growth in folate-free medium.

Fig. 3 shows the growth of 2008 and 2008/MRP cells after folate depletion and subsequent growth in medium replenished with either FA or the reduced folate cofactor LV. After continuous exposures of FA or LV for 72 hr minor shifts were noted for 2008/MRP1 and 2008/MRP3 cells (Fig. 3). Cell growth nearly attained levels of counterpart cultures grown continuously in high folate medium. Apparently, during such long (72 hr) exposures (even after folate depletion prior to the exposure) MRP overexpression in these cells was not able to hamper cellular growth significantly.

3.4. Effect of MRP overexpression on cellular growth under impaired folate-metabolizing conditions

Polyglutamylation of folates diminishes their MRP-mediated transport, as shown earlier by our laboratory [12] and Zeng *et al.* [14]. Thus, it might be assumed that due to the presence of folate polyglutamates after long (72 hr) exposures MRPs will not be able to diminish cellular folate pools completely. We therefore analyzed the effect of

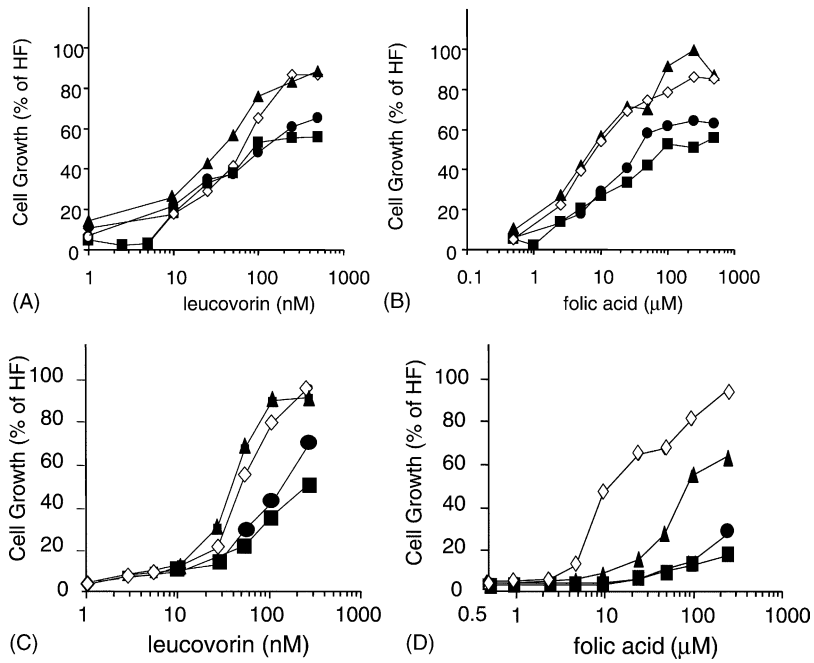

Fig. 4. Effect of MRP overexpression on folate-dependent cellular growth (4 hr exposure). Cell growth was measured when cells were cultured in folate-free RPMI-1640 medium, containing either leucovorin (A and C) or folic acid (B and D) as the sole folate source. Cell lines studied were 2008 (□), 2008/MRP1 (■), 2008/MRP2 (▲) and 2008/MRP3 (●). Exposure time of leucovorin or folic acid was 4 hr after which the cells were washed and cultured in folate-free medium (A and B). In other experiments 1 μM TMQ was also added during the 4 hr exposure time to prevent rapid metabolism of folic acid (C and D). After the incubation period cells were cultured in folate-free medium for another 68 hr. Cell growth is given as percentage of growth obtained under high folate conditions. Results presented are representative experiments of six independent experiments. Standard deviations (SD) between experiments were <24%.

MRP overexpression in cells that had been exposed to FA or leucovorin for a much shorter period of time to minimize FPGS-mediated polyglutamylation.

Fig. 4 shows that when cells were exposed to FA for only 4 hr (after a 4-day folate-free period), followed by a 68-hr period in folate-free medium, a marked effect of MRP1 and MRP3 overexpression on cellular growth was observed (Fig. 4A and B) (for leucovorin only minor shifts were noted in MRP1 and MRP3 overexpressing cells). The FA concentration at which the cells enhanced their growth to 50% of their maximal level (EC_{50}) was ~10 μM for 2008 wild-type cells compared to ~35 μM for 2008/MRP3 and ~100 μM for 2008/MRP1 cells (Fig. 4B). Moreover, in the presence of the DHFR inhibitor TMQ (to prevent metabolism of FA) even larger differences were observed in the cell growth ability. The 2008/MRP1 and 2008/MRP3 cells displayed a ~5-fold increase in LV growth requirement (EC_{50}'s of ~250 nM for 2008/MRP1 and ~150 nM for 2008/MRP3 compared to ~40 nM for 2008 wild-type). But most pronounced increases in folate requirement were observed for FA. Even at the highest FA concentration tested (250 μM), 2008/MRP1 and 2008/MRP3 cells resumed only 20 and 35% of control cell growth, respectively, and the estimated EC_{50}'s for these cell lines were >500 μM compared to ~12 μM for wild-type 2008 cells (Fig. 4D).

4. Discussion

In the present study we investigated the role of the multidrug resistance proteins MRP1, MRP2 and MRP3 in cellular extrusion of natural folates. We show that these MRPs contribute to a lowering of cellular natural folates, based on several lines of evidence: (1) diminished cellular folate pools in MRP overexpressing cells, and (2) decreased cellular growth of MRP overexpressing cells after short-term (4 hr) folate exposure. Earlier, MRP-mediated transport of FA in MRP1- and MRP3-containing membrane vesicles was described [14].

Overexpression of MRP1, MRP2 or MRP3 decreased total folate pools in transfected 2008 ovarian carcinoma cells by 32–38%. This lower level was mainly accounted for by a diminished pool of 10-$CHOFH_4$. Whether this implies that 10-$CHOFH_4$ is a preferred substrate for extrusion by MRPs remains to be established. Studies from our laboratory [12,13] and others [14,16] showed that polyglutamylation of (anti)folates, mediated by FPGS is a determining factor for (anti)folates to be available substrates for MRP-mediated efflux. In fact, conversion to diglutamate forms of MTX or FA was sufficient to markedly diminish the substrate affinity for MRP3 [14]. The substrate specificity of MRPs for other natural folate cofactors and their polyglutamate forms has not been documented. The 10-$CHOFH_4$ *per se* is known to be a good substrate for FPGS (K_m: 2–4 μM) [31], which may suggest that diminished 10-$CHOFH_4$ pools in MRP-overexpressing cells could also arise from altered metabolic interconversions due to efflux of other folate intermediates.

The decrease in total intracellular folate concentration in MRP overexpressing cells did not interfere with their optimal cell growth in medium containing 2.3 μM folates. Studies by Sirotnak *et al.* [30] and recently by Mauritz *et al.* [29] showed that the minimal folate growth requirement was achieved with cellular folate pools as low as 1–5 pmol/mg protein. Hence, total folate pools in 2008/MRP cells exceeded by far this folate concentration. However, upon folate depletion, and impaired intracellular metabolism and polyglutamylation of folates, 2008/MRP cells revealed a marked MRP-related effect on folate growth dependency. After a 4 hr exposure to FA, which diminishes FPGS-mediated conversion of folate cofactors into folate-polyglutamates, the folate growth requirement was markedly higher for 2008/MRP1 and 2008/MRP3 cells as compared to parental 2008 cells. This was even more pronounced during co-incubation with TMQ, which blocks FA reduction to FH_2 and FH_4 by DHFR. In contrast, under similar folate growth permissive conditions a 72 hr exposure to FA displayed only minor differences in FA growth dependency between MRP-transfectants and parental 2008 cells. This suggests that in folate uptake- and polyglutamylation competent cells, rapid metabolism of folates into FH_2 and FH_4 and to their polyglutamate forms occurs, which prevents folate efflux via MRP-routes. FH_2 is known to be one of the best substrates for FPGS (K_m: 0.8 μM), rather than FA, which is only a moderate substrate for FPGS (K_m: 60 μM) [31]. Once folates have been converted into the FH_2 form, it is therefore very unlikely that MRP is able to largely influence their cellular extrusion. In conclusion, our results imply that predominantly in cells deficient in FA uptake and/or FPGS activity, MRPs may play an important role in folate efflux.

Previously, response mechanisms to altered folate conditions were described, which include upregulation of RFC expression [32] or increased FPGS activity [33]. In general, cellular folate status can influence indirectly the regulation of gene expression through methylation of CpG islands in the promoter region of a variety of genes [34,35]. An effect of MRP overexpression on cellular folate levels might, therefore, influence cell function in a broad way such as on the level of gene expression. Otherwise, changes in cellular folate might affect MRP expression, although no methylation sensitive CpG islands in the MRP1 promotor region were reported [36]. Yet, a recent study by Worm *et al.* [36] showed that the demethylating agent 5-aza-2′-deoxycytidine induced indirect upregulation of MRP1, MRP2 and MRP3 mRNA expression in RFC-transport defective human breast cancer cells. Clearly, additional studies to determine the relation between MRP expression and cellular folate concentration as well as cell function have to be carried out. Cellular folate status is considered an important determinant in the efficacy of folate-based chemotherapeutic drugs and 5FU/LV combinations. Previously, our

laboratory and others showed that augmented levels of intracellular folates largely abrogated the activity of polyglutamylation-dependent antifolates because of competition at the level of FPGS [11,22,25,33]. In addition, it has been shown that at physiological folate levels sensitivity to antifolates is greatly enhanced while that to the fluoropyrimidine 5FU can be decreased [26,37]. The latter is due to the fact that inhibition of the target enzyme of 5FU, thymidylate synthase, can only be optimal when sufficient reduced folate cofactors are present intracellularly. Nutritional intake of folates via food and vitamin supplements can affect folate homeostasis significantly and thereby affect cellular processes dependent on folates but also the antiproliferative activity of anticancer drugs [38]. The pharmacological role of MRPs in cellular extrusion of anticancer drugs is well established [4]. Data presented in this study support a physiological role of MRP1, as well as MRP3 (in close relation to FPGS activity) in controlling cellular folate homeostasis. Further research should be directed towards elucidating the mechanistic basis of MRP1 and MRP3 functional activity (and of other members of the MRP family), in response to alterations in extra/intra-cellular folate status.

Acknowledgments

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References

[1] Borst P, Elferink RO. Mammalian abc transporters in health and disease. Annu Rev Biochem 2002;71:537–92.
[2] Gottesman MM, Pastan I. Biochemistry of multidrug resistance mediated by the multidrug transporter. Annu Rev Biochem 1993; 62:385–427.
[3] Cole SP, Bhardwaj G, Gerlach JH, Mackie JE, Grant CE, Almquist KC, Stewart AJ, Kurz EU, Duncan AM, Deeley RG. Overexpression of a transporter gene in a multidrug resistant human lung cancer cell line. Science 1992;258:1650–4.
[4] Litman T, Brangi M, Hudson E, Fetsch P, Abati A, Ross DD, Miyake K, Resau JH, Bates S. The multidrug-resistant phenotype associated with overexpression of the new ABC half-transporter, MXR (ABCG2).. J Cell Sci 2000;113:2011–21.
[5] Borst P, Evers R, Kool M, Wijnholds J. A family of drug transporters: the multidrug resistance-associated proteins. J Natl Cancer Inst 2000;92:1295–302.
[6] Leslie EM, Deeley RG, Cole SPC. Toxicological relevance of the multidrug resistance protein 1 MRP1 (ABCC1) and related transporters. Toxicology 2001;167:3–23.
[7] Kool M, De Haas M, Scheffer G, Scheper RJ, Van Eijk MJT, Juin JA, Baas F, Borst P. Analysis of cMOAT (MRP2), MRP3, MRP4 and MRP5, homologues of the multidrug resistance-associated protein gene (MRP1), in human cancer cell lines. Cancer Res 1997;57:3537–47.
[8] Bertino JR. Ode to methotrexate. J Clin Oncol 1993;11:5–14.
[9] Rots MG, Pieters R, Kaspers GJL, Veerman AJP, Peters GJ, Jansen G. Classification of *ex vivo* methotrexate resistance in acute lymphoblastic and myeloid leukemia. Br J Haematol 2000;109:791–800.
[10] Cronstein BN. Methotrexate and its mechanism of action. Arthritis and Rheumatism 1996;39:1951–60.
[11] Jackman AL. Antifolate drugs in cancer therapy. New Jersey, USA: Humana Press Inc.; 1999.
[12] Hooijberg JH, Broxterman HJ, Kool M, Assaraf YG, Peters GJ, Noordhuis P, Scheper RJ, Borst P, Pinedo HM, Jansen G. Antifolate resistance mediated by the multidrug resistance proteins MRP1 and MRP2. Cancer Res 1999;59:2532–5.
[13] Kool M, Van der Linden M, De Haas M, Scheffer GL, De Vree ML, Smith AJ, Jansen G, Peters GJ, Ponne N, Scheper RJ, Oude Elferink RPV, Baas F, Borst P. MRP3, a new organic anion transporter able to transport anti-cancer drugs. Proc Natl Acad Sci USA 1999;96:6914–8.
[14] Zeng H, Chen ZS, Belinsky MG, Rea PA, Kruh GD. Transport of methotrexate (MTX) and folates by multidrug resistance protein (MRP) 3 and MRP1: effect of poly-glutamylation on MTX transport. Cancer Res 2001;61:7225–32.
[15] Lee K, Klein-Szanto AJ, Kruh GD. Analysis of the MRP4 drug resistance profile in transfected NIH3T3 cells. J Natl Cancer Inst 2000;92:1934–40.
[16] Chen ZS, Lee K, Walther S, Blanchard Raftogianis R, Kuwano M, Zeng H, Kruh GD. Analysis of methotrexate and folate transport by multidrug resistance protein 4 (ABCC4): MRP4 is a component of the methotrexate efflux system. Cancer Res 2002;62:3144–50.
[17] Sirotnak FM, Wendel HG, Bornmann WGB, Tong WP, Miller VA, Scher HI, Kris MG. Co-administration of probenecid, an inhibitor of a cMOAT/MRP-like plasma membrane ATPase, greatly enhanced the efficacy of a new 10-deazaaminopterin against human solid tumors *in vivo*. Clin Cancer Res 2000;6:3705–12.
[18] Henderson GB, Zevely EM. Inhibitory effect of probenecid on the individual transport routes which mediate the influx and efflux of methotrexate in L1210 cells. Biochem Pharmacol 1985;34:1725–9.
[19] Henderson GB, Tsjui JM, Kumar HP. Characterization of the individual transport routes that mediate the influx and efflux of methotrexate in CCRF-CEM human lymphoblastic cells. Cancer Res 1986;46: 1633–8.
[20] Sirotnak FM, O'Leary DF. The issues of transport multiplicity and energetics pertaining to methotrexate efflux in L1210 cells addressed by an analysis of *cis* and *trans* effects of inhibitors. Cancer Res 1991;51:1412–7.
[21] Dembo M, Sirotnak FM, Moccio DM. Effects of metabolic deprivation on methotrexate transport in L1210 leukemia cells: further evidence for separate influx and efflux systems with different energetic requirements. J Membr Biol 1984;78:9–17.
[22] Zhao R, Seither R, Brigle KE, Sharina IG, Wang PJ, Goldman ID. Impact of overexpression of the reduced folate carrier (RFC1), an anion exchanger, on concentrative transport in murine L1210 leukemia cells. J Biol Chem 1997;272:21207–12.
[23] Kusuhara H, Han Yhm Shimoda M, Kokue E, Suzuki H, Sugiyama Y. Reduced folate derivatives are endogenous substrates for cMOAT in rats. Am J Physiol 1998;275:G789–96.
[24] Jansen G, Mauritz R, Drori S, Sprecher H, Kathmann I, Bunni M, Priest DG, Noordhuis P, Schornagel JH, Pinedo HM, Peters GJ, Assaraf YG. A structurally altered human reduced folate carrier with increased folic acid transport mediates a novel mechanism of antifolate resistance. J Biol Chem 1998;273:30189–98.
[25] Zhao R, Gao F, Liu L, Goldman ID. Marked suppression of the activity of some but not all antifolate compounds by augmentation of the folate cofactor pools within tumor cells. Biochem Pharmacol 2001; 61:857–65.
[26] Van der Wilt CL, Backus HH, Smid K, Comijn L, Veerman G, Wouters D, Voorn DA, Priest DG, Bunni MA, Mitchell F, Jackman AL, Jansen G, Peters GJ. Modulation of both endogenous folates and thymidine enhance the therapeutic efficacy of thymidylate synthase inhibitors. Cancer Res 2001;61:3675–81.
[27] Bunni M, Sirotnak FM, Otter GM, Priest DG. Disposition of leucovorin and its metabolites in the plasma, intestinal epithelium, and

intraperitoneal L1210 cells of methotrexate-pretreated mice. Cancer Chemother Pharmacol 1994;34:455–8.

[28] Scheffer GL, Kool M, Heijn M, de Haas M, Pijnenborg AC, Wijnholds J, van Helvoort A, de Jong MC, Hooijberg JH, Mol CA, van der Linden M, de Vree JM, van der Valk P, Elferink RP, Borst P, Scheper RJ. Specific detection of multidrug resistance proteins MRP1, MRP2, MRP3, MRP5, and MDR3 P-glycoprotein with a panel of monoclonal antibodies. Cancer Res 2000;60:5269–77.

[29] Mauritz R, Peters GJ, Priest DG, Assaraf YG, Drori S, Kathmann GA, Noordhuis P, Bunni MA, Rosowsky A, Schornagel JH, Pinedo HM, Jansen G. Multiple mechanisms of resistance to methotrexate and novel antifolates in human CCRF-CEM leukemia cells and their implications for folate homeostasis. Biochem Pharmacol 2002;63:105–15.

[30] Sirotnak FM, Goutas LJ, Mines LS. Extent of the requirement for folate transport by L1210 cells for growth and leukemogenesis *in vivo*. Cancer Res 1985;45:4732–4.

[31] Chen LQH, Korenberg J, Garrow TA, Choi YJ, Shane B. Purification and properties of human cytosolic folylpoly-gamma-glutamate synthetase and organization, localization, and differential splicing of its gene. J Biol Chem 1996;271:13077–87.

[32] Jansen G, Westerhof GR, Jarmuszewski MJA, Kathmann I, Rijksen G, Schornagel JH. Methotrexate transport in variant human CCRF-CEM leukemia cells with elevated levels of the reduced folate carrier. J Biol Chem 1990;265:18272–7.

[33] Jansen G, Barr HM, Kathmann I, Bunni M, Priest DG, Noordhuis P, Peters GJ, Assaraf YG. Multiple mechanisms of resistance to polyglutamatable and lipophilic antifolates in mammalian cells: role of increased folylpolyglutamylation expanded folate pools, and intralysosomal drug sequestration. Mol Pharmacol 1999;55:761–9.

[34] Zingg JM, Jones PA. Genetic and epigenetic aspects of DNA methylation on genome expression evolution mutation and carcinogenesis. Carcinogenesis 1997;18:869–82.

[35] Jhaveri MS, Wagner C, Trepel JB. Impact of extracellular folate levels on global gene expression. Mol Pharmacol 2001;60:1288–95.

[36] Worm J, Kirkin AF, Dzandzhugazyan KN, Guldberg P. Methylation-dependent silencing of the reduced folate carrier gene in inherently methotrexate-resistant human breast cancer cells. J Biol Chem 2001;276:39990–40000.

[37] Backus HH, Pinedo HM, Wouters D, Padron JM, Molders N, van Der Wilt CL, van Groeningen CJ, Jansen G, Peters GJ. Folate depletion increases sensitivity of solid tumor cell lines to 5-fluorouracil and antifolates. Int J Cancer 2000;87:771–8.

[38] Peters GJ, Jansen G. Folate homeostasis and antiproliferative activity of folates and antifolates. Nutrition 2001;17:737–8.