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Folate concentration dependent transport activity of the Multidrug Resistance Protein 1 (ABCC1)

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Abstract

The Multidrug Resistance Protein MRP1 (ABCC1) can confer resistance to a variety of therapeutic drugs. In addition, MRP1/ABCC1 mediates cellular export of natural folates, such as folic acid and L-leucovorin. In this study we determined whether cellular folate status affected the functional activity of MRP1/ABCC1 mediated efflux of an established substrate, the anthracycline daunorubicin (DNR). As a model system we used the human ovarian carcinoma cell line 2008wt, and its MRP1/ABCC1 transfected subline 2008/MRP1. Both types of these moderate- and high-MRP1/ABCC1 expressing cells displayed efflux of DNR when maintained in standard culture media (2.3 μM folic acid). The initial total cellular DNR efflux rate in 2008/MRP1 cells was ~2-fold higher compared to 2008wt cells. This efflux consisted of MRP1/ABCC1 mediated transport, possibly non-MRP1 mediated transport, as well as passive diffusion. Benzbromarone, a specific MRP1 inhibitor, decreased the initial efflux rate in 2008/MRP1 cells (4-fold) and in 2008wt cells (2-fold). When 2008/MRP1 cells were challenged for 2 days in folate-free medium, total cellular DNR efflux was decreased to 43% of the initial efflux rate under folate-rich conditions. In 2008wt cells DNR efflux was decreased to 84% of the folate-rich conditions. Benzbromarone did not inhibit DNR efflux after the folate-free period in both cell lines. Repletion of folate by a 2–24 hr exposure to 2.5 μM L-leucovorin or folic acid resulted in a complete restoration of DNR efflux. In contrast, expression of MRP1/ABCC1 protein was not changed significantly during the folate-free period or the repletion-period, nor were cellular ATP or ADP pools. In conclusion, this study demonstrates that the cellular folate status can influence the transport activity of MRP1/ABCC1. These results have potentially important implications in the understanding of the (patho-)physiological roles of MRP1/ABCC1, and possibly other ABC transporter proteins in cellular folate homeostasis and drug resistance.

Keywords: MRP1; ABCC1; Folate; Methotrexate; L-Leucovorin; Multidrug resistance; Polyglutamylation; Daunorubicin

1. Introduction

Multidrug Resistance Proteins (MRPs) belong to the superfamily of ATP Binding Cassette (ABC) transporter proteins [1,2]. These ATP-driven efflux pumps were initially identified as membrane associated transporters that confer multiple drug resistance against a wide variety of structurally unrelated drugs by cellular extrusion [1,2]. Of all MRPs, which form the C-subclass of the ABC-transporter family, the MRP1 (ABCC1) is currently best characterized. Among established MRP1 substrates are the anthracyclines, vinca alkaloids, and epipodophyllotoxins [1,3]. One particular group of drugs, the antifolates, which are structural analogues of natural reduced folates and act as antagonists of the cellular folate metabolism [4,5], were recently also identified as potential targets for MRP associated resistance [6–13]. By inhibition of

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Abbreviations: RFC, reduced folate carrier; MTX, methotrexate; MRP1, Multidrug Resistance Protein; FPGS, folylpolyglutamyl synthetase; LF, low-folate; LV, L-leucovorin; HF, high-folate; DHFR, dihydrofolate reductase; TS, thymidylate synthase; DNR, daunorubicin.

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the folate converting key-enzymes thymidylate synthase (TS) and dihydrofolate reductase (DHFR), antifolates block DNA-synthesis [4,5]. A classical antifolate is methotrexate (MTX), used in the treatment of several types of cancer and inflammatory diseases [4,5]. Our laboratory, as well as others, demonstrated that members of the MRP family (MRP1, MRP2, MRP3, and MRP4) play a role in antifolate efflux [6–10]. Inhibition of MRP1 mediated efflux by the established MRP1 inhibitor probenecid reversed the MTX-resistant phenotype *in vitro* [6], and enhanced the activity of the antifolate 10-deazaaminopterin *in vivo* [11].

Recently, we obtained several lines of evidence for a (patho-)physiological role of the MRP family in controlling cellular homeostasis of natural folates: (A) Cellular folate pools were diminished to ~38–40% in MRP1 and MRP3 (ABCC3) overexpressing cells [14], and (B) MRP1 protein expression was markedly down regulated in cells selected for growth in low concentrations of folates [15]. In addition, (C) cells transfected with MRP1, MRP2 (ABCC2 or cMOAT), or MRP3 displayed a collateral sensitivity to the lipophilic antifolate trimetrexate (TMQ) [6], which is indicative for decreased intracellular folate pools. Other evidence for a contribution of MRPs in folate homeostasis was reported by Kusuhara *et al*. [12], showing that MRP2/cMOAT was able to transport reduced folate cofactors in rats. Finally, Zeng *et al*. [8] described MRP3 mediated transport of folic acid into membrane vesicles, whereas Chen *et al*. [10] showed this for MRP4.

Cellular folate status has a major impact on the sensitivity of various cell lines to antifolates. Marginal increases in the folate content of cells can have a major impact on cellular resistance/sensitivity to a series of polyglutamylation dependent antifolates [16,17]. Consistently, animal experiments with mice fed with low-folate diets showed the importance of folate status for the *in vivo* sensitivity to several novel antifolates [18,19]. MRP mediated diminishment of cellular folate levels might significantly influence the cellular sensitivity to antifolates and vice versa [13]. Also, transport of folates *via* MRPs might competitively inhibit extrusion of other, established MRP substrates. Therefore, the study of the relation between cellular folate status and MRP function is an important issue.

In this report we demonstrate that changes in cellular folate concentrations affect the MRP1 mediated efflux of the established substrate DNR. This effect might contribute to variabilities in MRP1 associated resistance to anticancer drugs, and to variations in toxicity of DNR in cancer patients.

2. Materials and methods

2.1. Materials

Folic acid, L-leucovorin (LV), benzbromarone, verapamil (Vp) and digitonin were obtained from Sigma Chemical Co. Protease inhibitor cocktail was purchased from Boehringer, NL. Normal RPMI-1640 medium (which contains 2.3 μM folic acid), folate-free RPMI-1640 medium, normal Fetal Calf Serum, and dialyzed Fetal Calf Serum were obtained from GIBCO. Daunorubicin (DNR) was from Specia. The DNA probe Syto 13 was from Molecular Probes. Radiolabelled daunorubicin hydrochloride ([^{3}H]DNR, 163 GBq/mmol) was from Dupont de Nemours.

2.2. Cell lines

The human ovarian carcinoma cell line 2008wt, and its stable MRP1 transfectant 2008/MRP1 were kindly provided by Dr. M. Kool and Prof. Dr. P. Borst (The Netherlands Cancer Institute, Amsterdam). These cell lines were cultured in RPMI 1640 medium, supplemented with 10% FCS, 2 mM glutamine and 100 μg/mL penicillin/streptomycin. This culture medium is factory-supplied with 2.3 μM folic acid. For folate deprivation, cells were cultured for 2 days in special folic acid-free RPMI 1640 medium supplemented with 10% dialyzed FCS, 2 mM glutamine and 100 μg/mL penicillin/streptomycin.

2.3. Cellular drug accumulation

The steady-state cellular drug accumulation of [^{3}H]DNR was performed as described earlier [20]. The assay was initiated by addition of 0.5 μM tritium labeled DNR, followed by a 75 min incubation at 37°. Hereafter, cells were washed in ice-cold PBS. The amount of radioactivity was determined by scintillation counting.

2.4. MRP-western blotting

Total lysates were prepared from 5×10^6 cells by lysing in 250 μL buffer containing 50 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 3–30 μg protein was loaded. For detection of MRP1, a monoclonal antibody (MRPr1) [21] was used in a dilution of 1:500 (0.5 μg/mL). As a loading control expression of β-actin was determined using anti-human β-actin (Chemicon). MRP1 expression relative to β-actin expression was measured as OD × mm^2 with densitometric scanning (Imaging Densitometer GS-800, Biorad Laboratories).

2.5. Cellular drug efflux

Efflux of fluorescent drugs (DNR) was measured using an online computerized method described by Wielinga *et al*. [22]. In brief, cells were cultured on glass coverslip that fitted to the wall of a cuvette of 1 cm × 1 cm × 4 cm (width, depth, and height). To load the cells with substrate the coverslips were placed in a petridish containing culture

medium (37°), 10 μM DNR, and 150 μM Verapamil (to block MRP1 mediated efflux during the loading period) until steady-state was reached (≥2 hr). After loading, the cells were put on ice until further use. More than 98% of the cells were viable after the 2-hr DNR loading period, as determined by trypan-blue exclusion test.

For an efflux experiment a coverslip was washed twice with ice-cold medium A (RPMI medium without phenol red). The coverslip was placed in the cuvette that contained 3 mL warm (37°) medium A. The fluorescence of the effluxed substrate appearing in the medium was monitored with a spectrafluorometer (FluoroMax, SPEX Industries, Edison, NJ). Fluorescence of DNR was measured every second at excitations and emission wavelengths of 480 and 590 nm, respectively. At the end of the experiment 25 μM digitonin was added to permeabilize the cells, and to determine the total amount of intracellular DNR. Next, the cell number was determined by adding 400 nM of the DNA probe Syto 13. The DNR signals were normalized for the Syto 13 signals. The fluorescence of Syto 13 was determined at excitation wavelength of 485 nm, and emission wavelength of 520 nm.

2.6. ATP/ADP levels

Nucleotides were extracted from cells as described previously [23,24]. Separation and quantification of the normal NTPs was achieved with a gradient HPLC system consisting of two Gyncotek pumps (model 300, Separation Analytical Instruments BV, Hendrik Ido Ambacht), as described earlier [23]. The HPLC system was connected to a photo-diode array detector (Separations, model 1000S), regularly set at 254 and 280 nm. Peaks were quantitated by the data acquisition program Chromeleon 3.02.

3. Results

3.1. Effect of cellular folate concentration on MRP1 expression

Changes in cellular folate status can influence the expression of a variety of genes [25,26]. Prior to studying the effect of changes in the cellular folate status on MRP1 mediated transport of established substrates, we first determined whether the expression of MRP1 alters as a result of short-term (≤2 days) incubation in folate-free medium. To this end, we analyzed MRP1 expression in the human ovarian carcinoma cells 2008wt and in its MRP1 transfected subline 2008/MRP1. Cells were either continuously cultured in the presence of normal medium (which standardly contains 2.3 μM folic acid, further referred to as 'high-folate' conditions or 'HF'), or in folic acid free medium for a 24- or 48-hr period. The latter conditions are referred to as 'low-folate' conditions (LF).

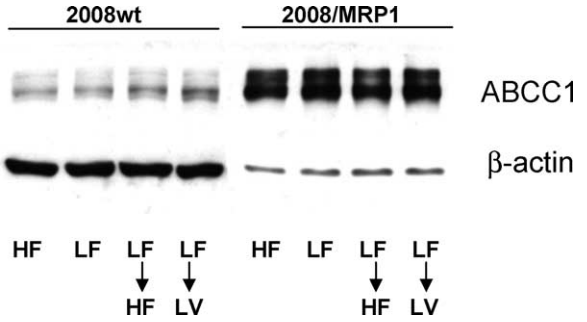

Fig. 1. Cellular expression of MRP1 under different folate conditions. The protein expression as determined by Western blotting of MRP1 in 2008wt ovarian carcinoma cells and in (transfected) 2008/MRP1 cells is shown. Different folate conditions do not affect MRP1 expression significantly. Conditions: HF: continuous high folate; LF: 24 hr low folate; LF → LV, LF followed by 24 hr leucovorin (2.5 μM); LF → HF, LF followed by 24 hr HF. Per lane 30 μg of 2008wt protein or 3 μg 2008/MRP1 protein was loaded. As a loading control β-actin levels are indicated.

As shown in Fig. 1 and Table 1, under HF conditions (i.e. normal growth medium) 2008wt cells expressed a low level of endogenous MRP1, as was also reported previously [27]. After depletion of folates for either 24 or 48 hr, no significant changes in MRP1 expression were observed on Western blot and densitometric scanning in both 2008wt and 2008/MRP1 cells. Repletion of high concentrations of folates after this folate-free period by a 24-hr exposure to either 2.3 μM folic acid or 2.5 μM L-leucovorin, resulted in a little increase in MRP1 expression in the wild type cells.

3.2. Effect of MRP1 overexpression on cellular accumulation and extrusion of daunorubicin

MRP1 is a transporter of several anticancer agents, among which the fluorescent anthracycline daunorubicin (DNR). To determine the effect of MRP1 (over)expression in 2008wt cells and 2008/MRP1 on the accumulation and extrusion of DNR, we first exposed cells to 0.5 μM radiolabelled daunorubicin ([^{3}H]DNR) for 1.5 hr, until steady-state was reached. Accumulation of [^{3}H]DNR in 2008/MRP1 cells was lower, i.e. 76.8 ± 3.5% (N = 3) compared to wild type cells (set at 100%).

Next, to study MRP1 transport *activity* we used an online fluorescent method [22], which allows analysis of efflux

Table 1
Expression of MRP1 in 2008wt and 2008/MRP1 under different folate conditions

Cell	HF	LF	LF → HF	LF → LV
2008wt	0.23 ± 0.08	0.59 ± 0.21	0.52 ± 0.09*	0.63 ± 0.07*
2008/MRP1	10.32 ± 2.28	14.95 ± 1.20	12.55 ± 2.25	17.45 ± 3.21

Average expression is given relative to β-actin as a mean ± SD (N = 3). HF: high folate (2.3 μM folic acid). LF: 2 days folate-free culture. LF → HF: 2 days folate-free culture, followed by 24 hr HF. LF → LV: 2 days folate-free culture, followed by 24 hr 2.5 μM leucovorin.

* Significantly different from corresponding HF value ($P < 0.05$).

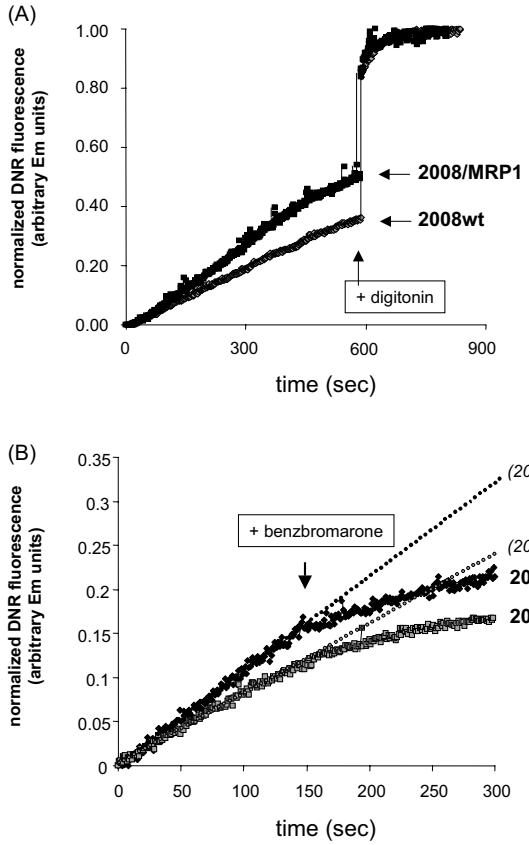

Fig. 2. MRP1 mediated efflux of DNR. Representative DNR efflux traces are shown for 2008wt cells and 2008/MRP1 cells. Cells were loaded with 10 μM DNR for 2 hr in the presence of 150 μM verapamil (to block MRP1 and non-MRP mediated extrusion during the loading period) prior to the efflux experiment. As indicated in (A), 25 μM digitonin was added to determine the total amount of DNR in cells and cuvette. In (B) benzbromarone (100 μM) was added. The slopes of efflux rates before addition of benzbromarone are given as dotted lines. The fluorescent signals are plotted after normalization with respect to the total amount of DNR in each experiment. SD between experiments (N = 4) was <15%.

kinetics of fluorescent MRP1 substrates. We assessed the cellular DNR efflux in 2008wt cells and 2008/MRP1 cells under HF conditions. Extrusion of DNR was observed from both 2008/MRP1 cells and 2008wt cells (Fig. 2A), which is the sum of (1) passive DNR diffusion into the extracellular medium, (2) active transport mediated by MRP1, and (3) efflux *via* possible other (unidentified) ABC-transporters present in these cells. The initial efflux (within the first 100 s of the experiment) of 10 μM DNR in wild type cells was 2-fold lower compared to MRP1 transfected cells (Figs. 2A and 3). After 600 s of efflux 25 μM digitonin was added to release free DNR in order to determine the total amount of DNR in cells and cuvette. Benzbromarone, an established inhibitor of MRP1 mediated transport [28,29], was used to estimate the contribution of MRP1 in the total cellular DNR efflux. Fig. 2B shows inhibition by 100 μM benzbromarone, added during the efflux at 150 s after efflux initiation. From this time-point on the transfected cell line and wild type cell line showed similar DNR efflux slopes. In Fig. 3 the relative DNR efflux

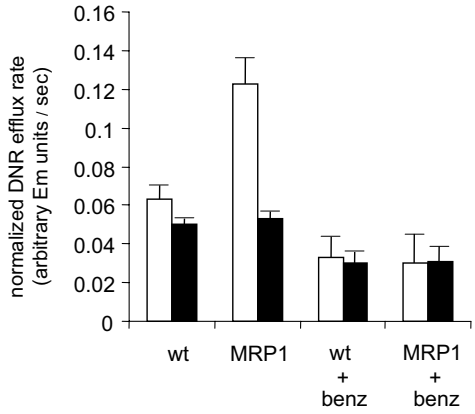

Fig. 3. Effect of folate on MRP1 mediated DNR efflux. The normalized efflux rates of 10 μM DNR from 2008wt cells (wt) and 2008/MRP1 cells (MRP1) is shown. Folate conditions used were high-folate (white bars) and low-folate (48 hr folic acid free culture) (black bars). The inhibiting effect of 100 μM benzbromarone on DNR efflux under both conditions is given (+benz). Benzbromarone was present during the entire experiment. Efflux of DNR was markedly decreased in 2008/MRP1 cells under low-folate conditions. Efflux rates are presented as means ± SD (N = 3).

rates are presented when in the continuous presence of 100 μM benzbromarone. This concentration was sufficient for maximal MRP1 inhibition, based on a titration experiment (not shown). Clearly, efflux rates in wild type cells and MRP1 transfected cells were reduced by the continuous presence of benzbromarone to a similar end-level, that reflects the passive diffusion/non-MRP component.

3.3. Effect of folate depletion on MRP1 mediated DNR efflux

The capacity of MRP1 to transport the natural reduced folates L-leucovorin and folic acid [14,15] implies that high cellular folate concentrations might competitively inhibit the MRP1 mediated extrusion of classical MRP1 substrates. In order to study this, we measured the cellular extrusion of DNR under different folate conditions. To assess whether the concentration of cellular folates influences MRP1 activity, we measured DNR efflux from 2008wt cells and 2008/MRP1 cells under different folate conditions. Cells were either continuously cultured under HF conditions, or cultured in folate-free medium for 48 hr. Figure 3 shows the effect of folate depletion on the cellular efflux of DNR. After folate depletion, DNR efflux was 2.3-fold decreased in the MRP1 transfected cells, and also lowered in the wild type cells (1.2-fold). After the folate-free period the efflux-rate of DNR from 2008/MRP1 and 2008wt was lowered to the same basal level. In addition, we determined the effect of benzbromarone on DNR efflux before, and after folate depletion. Complete inhibition of MRP1 by benzbromarone resulted in a comparable efflux rate in cells under high-folate conditions as under low-folate conditions. Benzbromarone just marginally inhibited DNR efflux under low-folate conditions, which indicates that under these (LF) conditions MRP1 activity was

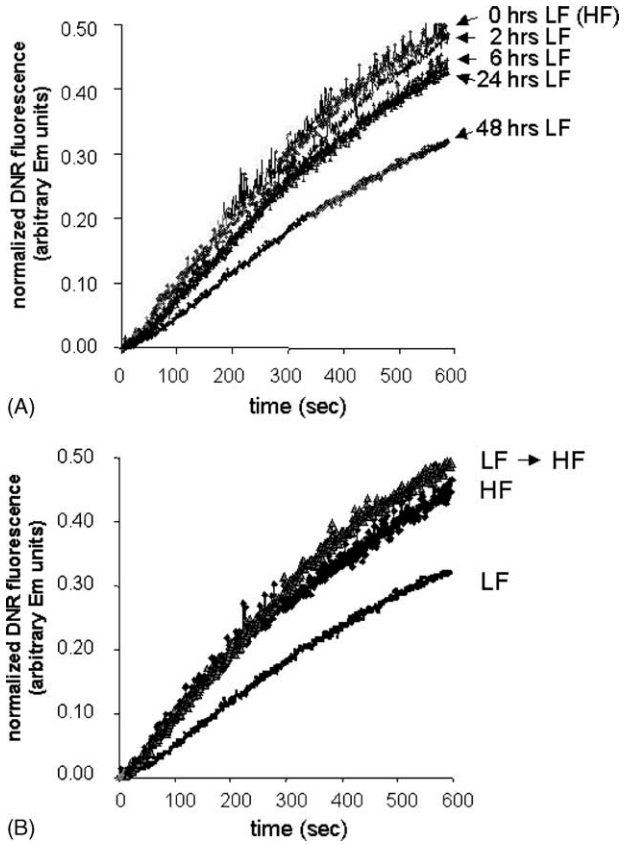

Fig. 4. Effect of altered cellular folate conditions on MRP1 mediated DNR transport. (A) Efflux of DNR from 2008/MRP1 cells after different time-periods of folate deprivation. As indicated in the figure cells were deprived of folates for 2, 6, 24, and 48 hr. As a control, cells under continuous high-folate conditions (HF) were used. (B) Effect of folate repletion on DNR efflux. 2008/MRP1 cells were folate depleted for 48 hr (LF), or subsequently re-exposed to HF medium (LF → HF, gray points) for 24 hr. As a control, HF cells were used. SD between experiments (N = 3) was <20%.

largely decreased. Thus, these results indicate that short-term folate depletion can markedly depress MRP1 mediated extrusion of DNR.

3.4. Time dependency of the influence of folate status on MRP1 transport activity

Since the above mentioned effects were observed after short folate depletion, we further fine-tuned the time-frame of folate deprivation necessary to decrease MRP1 mediated DNR efflux. For this purpose DNR efflux was measured in 2008/MRP1 cells in HF medium, and in cells cultured in folate-free medium for 2, 6, 24, and 48 hr (Fig. 4A). A time-dependent decrease of DNR efflux from 2008/MRP1 cells could be observed after 6 and 24 hr folate depletion, while the maximal decline in efflux-rate was obtained after 48 hr.

Following a 48 hr exposure to folate-free medium 2008/MRP1 cells were re-exposed to HF culture medium containing 2.3 μM folic acid. This resulted in a complete resumption of DNR efflux capacity, as observed with cells growing in HF medium (Fig. 4B).

Likewise, rather than using HF medium, the resumption of MRP1 mediated DNR efflux was observed after the addition of L-leucovorin as sole folate-source. Replenishment of folate-deprived cells (48 hr LF) with 2.5 μM L-leucovorin (to mimic HF conditions) showed a time-dependent recovery of MRP1 transport activity (Table 2). After 6 hr the DNR efflux capacity approximated that of cells continuously cultured in HF medium, while after 24 hr even an overshoot of DNR efflux capacity was observed.

Table 2
Effect of L-leucovorin on MRP1 mediated efflux of DNR in 2008/MRP1 cells

Exposure time to LV	Initial DNR efflux (arbitrary Em units × s^{-1})
0 (LF)	0.054 ± 0.0034
4 hr LV	0.081 ± 0.0102
6 hr LV	0.116 ± 0.0096
24 hr LV	0.134 ± 0.0028
HF	0.122 ± 0.0115

2008/MRP1 cells were cultured for 48 hr in folate-free medium (LF), and subsequently exposed to 2.5 μM L-leucovorin (LV) for time periods indicated. Alternatively, cells were continuously exposed to HF. Initial efflux rates (within the first 100 s of the experiment) are presented as means ± SD (N = 3).

3.5. Cellular nucleotide levels as function of cellular folate status

Efficient transport activity of MRP1 relies on a sufficient intracellular concentration of ATP. In order to exclude the

Table 3
Cellular ATP and ADP levels under different folate conditions

Cell	Folate status	ATP (pmol/10^6 cells)	*P*-value (N = 3)	ADP (pmol/10^6 cells) (N = 3)	*P*-value (N = 3)
2008wt	HF	3741 ± 812	–	2077 ± 1019	–
2008wt	LF	3962 ± 2461	0.99	2770 ± 1247	0.05
2008wt	LF → HF	4006 ± 1953	0.85	1359 ± 1520	0.96
2008/MRP1	HF	3492 ± 1743	–	2320 ± 892	–
2008/MRP1	LF	3794 ± 923	0.31	2612 ± 1705	0.69
2008/MRP1	LF → HF	5182 ± 536	0.22	2246 ± 1566	0.18

HF: continuous high-folate. LF: 2 days folic acid free culture medium. LF → HF: 2 days folic acid free culture medium, followed by 24 hr HF. Values given are means ± SD (N = 3). No statistical differences from corresponding HF cell lines ($P < 0.05$) were observed in unpaired two-tailed Student's *t*-tests.

possibility that alterations in MRP1 transport activity at various folate conditions were caused by fluctuations in intracellular ATP, we determined cellular nucleotide pools under the different folate conditions used in this study. As shown in Table 3, no major differences in ATP levels, as well as in ADP levels, were observed as function of altered folate status. Only in 2008/MRP1 cells after HF repletion a small trend in increased ATP was seen, but this was not significant.

4. Discussion

In the present study we report that changes in cellular folate status can affect MRP1 mediated cellular efflux of the anthracycline daunorubicin (DNR). In cells deprived of folates for a relatively short period of 2 days, MRP1 transport activity was markedly decreased. During such a folate-depletion period, the expression level of MRP1 protein was not affected, nor were there any significant changes in cellular pools of ATP and ADP.

Cellular response mechanisms to decreased folate levels include: (a) upregulation of RFC expression to enhance folate influx [30,31], (b) increased folylpolyglutamyl synthetase (FPGS) activity to enhance cellular retention of folates [32], and (c) decreased levels of MRP1 to prevent efflux of folates [14,15]. The 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 [25,26]. In the present study, however, we showed that a 2-day incubation in folate-free medium did not significantly alter the MRP1 protein expression in both 2008wt cells and 2008/MRP1 cells. The human MRP1 gene does not contain CpG islands in its promoter region [33]. Thus, it is expected that folate conditions do not rapidly affect MRP1 expression by a methylation process. Recently, however, Assaraf *et al.* reported that MRP1 protein expression and folate efflux function are markedly lost following a long-term folate deprivation [15]. This could be explained by a selection process, in which a decreased MRP1 expression is physiologically beneficial under folate restricted conditions. Under high-folate conditions and after a folate replenishment period, a progressive upregulation of MRP1 protein expression occurred. In the present study too a 24 hr replenishment with folates resulted in a slight upregulation of MRP1 protein expression (in wild type cells).

Previously, our laboratory and others reported that MRP1, as well as several MRP homologues, are capable of transporting unpolyglutamylated forms of (anti)folates [6–10]. Additional experiments showed that this MRP mediated transport of natural folates could play a role in controlling cellular folate homeostasis [14,15]. Since folates are substrates for MRP1 our initial hypothesis was that these compounds might act as competitive inhibitors of MRP1 mediated transport of other MRP1 substrates, including DNR. In the current study, however, we observed that MRP1 mediated efflux of DNR from both the 2008wt cell line and from the MRP1 transfected 2008/MRP1 cell line was markedly (4-fold) decreased upon cellular folate depletion. Moreover, short repletion with a high concentration of reduced folate co-factors, such as with L-leucovorin, rapidly restored the transport rate capacity. As compared with inhibition by the classical MRP1 blocker benzbromarone, folate depletion largely abrogated MRP1 mediated DNR transport. Hence, cellular folate status is an important regulating factor in the transport mechanism of MRP1.

Interestingly, our experiments revealed that the loss of DNR efflux activity upon folate depletion was apparent only after at least 6–24 hr of incubation in folate-free medium, and was maximal after 48 hr. This may imply that depletion of existing cellular pools of folate polyglutamates [34] might be of relevance for the delayed response. Polyglutamylation of reduced folate co-factors contributes to their cellular retention. A substantial decrease in cellular folate pools requires an average time-period of days rather than hours [35]. In contrast, we observed that repletion of folates by exposure to L-leucovorin affected MRP1 transport activity much faster, which may be explained by the fact that L-leucovorin is rapidly taken up by cells *via* the reduced folate carrier (RFC). The relatively rapid induction of MRP1 activity after L-leucovorin exposure underlines that the downregulation of MRP1 activity was predominantly associated with an altered folate status. This was supported by the repletion experiment using folic acid (high-folate medium), which is taken up much slower by cells than L-leucovorin, but which gave essentially similar results after 24 hr of replenishment with L-leucovorin.

The (in)direct mechanism by which folates influence MRP1 transport activity has not been established, although a number of options are open for consideration. Earlier studies described a role for (de)phosphorylation in the induction of the activity of MRP1 [36]. Whether cellular folate status plays a role in MRP1 phosphorylation is presently unclear. Alternatively, changes in cellular levels of homocysteine as a result of lowered folate status, might affect catalytic activity of several enzymes [37]. Whether this influences MRP1 is also not known. Alternatively, changes in cellular ATP levels as a result of altered cellular folate status, e.g. *via* formation of adenosine, might influence the ATPase activity of MRP1. In this study we demonstrated, however, no significant changes in cellular nucleotide pools upon short-term folate changes. Moreover, regarding the relatively rapid 'all-or-none' effect of L-leucovorin repletion on MRP1 activity (already after 4 hr), a role for altered ATP levels in this respect is not very likely. Finally, folates might play a similar role as the extensively studied compound glutathione, for which its presence is needed for the transport of many positively charged MRP1 substrates. Both folates and glutathione share a glutamate residue, which might play a role in

binding to a reactive site in MRP1. An induction of DNR transport by binding of folates to MRP1, similarly as proposed for glutathione by Borst *et al*. [38], or by acting as a co-substrate [39,40] is, however, at present only speculative.

From a (patho-)physiological point of view it is of interest that the levels of the natural folate 5-methyltetrahydrofolate in human plasma are normally between 5 and 20 nM [41]. Likewise, we recently found similar levels of folates in normal human tissues and in solid tumors ([42], and unpublished data). This nanomolar range is by far lower compared to the concentrations of folic acid present in standard cell culture media (∼2.3 μM). Thus, the low-folate conditions as used in our present study are much more representing the physiological situation than the HF culture conditions. Additionally, as reviewed by Peters and Jansen [43] nutritional intake of folates *via* food and food supplements can affect folate homeostasis, and thereby influence either cellular processes dependent on folates and/or sensitivity to antifolates. It remains to be elucidated whether (dietary) folates can affect MRP1 transport activity at the level of modulation of multidrug resistance. Also, beside an increase in antiproliferative effect of DNR, low folate conditions may be responsible for more severe toxicity on normal cells. Further, the question arises whether the effect of folates on MRP1 mediated transport can be exploited to alter the extrusion of (other) physiological MRP1 substrates. Therefore, the study of the relationship between folate status and MRPs function remains highly worthwhile and relevant.

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