DOI 10.1515/pterid-2013-0019 — Pteridines 2013; 24(1): 81–86

Godefridus J. Peters*, Ietje Kathmann, Clara Lemos[a], Jan Hendrik Hooijberg, Nienke Losekoot and Gerrit Jansen

Folate homeostasis of cancer cells affects sensitivity to not only antifolates but also other non-folate drugs: effect of MRP expression

Abstract: Sensitivity to antifolates can be decreased by endogenous or exogenous folates. Leucovorin protects cancer patients against toxicity of the dihydrofolate reductase inhibitor methotrexate (MTX), while folic acid is used to protect rheumatoid arthritis patients against MTX. Folates and antifolates can be effluxed from the cell by ABC transporters multidrug resistance protein 1 (MRP1), 2 and 3. We previously demonstrated in 2008 ovarian cancer cells that MRP overexpression reduced cellular folate content by 40%, while folate depletion increased expression of MRP1. As MRPs mediate resistance to several unrelated drugs, we investigated whether folate status would affect sensitivity to doxorubicin, daunorubicin, etoposide and vincristine. Ovarian cancer 2008 cells and its MRP1 transfected variant (2008/MRP1) were adapted from normal folate medium [2.3 μM; high folate (HF) cells] to short-term folate depletion (up to 7 days) (low folate cells); drugs were added after 2 days and sensitivity was tested by the MTT test after 3 additional days. The effect on folate homeostasis was evaluated by measurement of intracellular homocysteine using high-performance liquid chromatography and glutathione using a kit. MRP expression of wild-type (WT) 2008 cells did not increase homocysteine pools in 2008/MRP1 cells. Three day folate depletion increased homocysteine pools 23-fold in 2008 cells and 8.6-fold in the MRP variant. Folate depletion increased glutathione 20%–40% in 2008/WT and 2008/MRP1. In 2008 HF cells MRP1 expression did not affect sensitivity to MTX, but induced 4- to 10-fold resistance to doxorubicin, daunorubicin, etoposide and vincristine. Folate depletion decreased 50% growth inhibition (IC_{50}) for MTX in both 2008 variants 25- to 4-fold, but that to doxorubicin and daunorubicin approximately 2-fold. Sensitivity to etoposide and vincristine was not affected. In conclusion, folate depletion markedly increased homocysteine, but moderately increased glutathione. Folate depletion increased MTX sensitivity, but effects on other drugs were most pronounced in WT cells, probably because MRP expression is already high in transfected variants.

Keywords: ABCC1; anthracyclines; folate; leucovorin; methotrexate; MRP1; multidrug resistance.

[a]Current address: Max Delbruck University, Berlin, Germany.
***Corresponding author: Godefridus J. Peters,** Department of Medical Oncology, VU University Medical Center (VUMC), De Boelelaan 1117, 1007 MB, Amsterdam, The Netherlands, Phone: +31-20–444-2633, Fax: +31-20–444-3844, E-mail: gj.peters@vumc.nl
Ietje Kathmann, Clara Lemos, Jan Hendrik Hooijberg and Nienke Losekoot: Department of Medical Oncology, VU University Medical Center (VUMC), Amsterdam, The Netherlands
Jan Hendrik Hooijberg: Department of Clinical Chemistry, Slotervaartziekenhuis, Amsterdam, The Netherlands
Gerrit Jansen: Department of Rheumatology, VU University Medical Center (VUMC), Amsterdam, The Netherlands

Introduction

Folate supplementation to the diet protects against cancer development and other diseases, while folates also protect against toxicity of antifolate-based treatments. For example, leucovorin is commonly used to reduce antifolate toxicity in leukemia and some solid tumors [1, 2], while folic acid is added to treatment with the antifolate pemetrexed (PMX) with cisplatin to reduce toxicity [3]. Interestingly, folic acid not only increased the efficacy of this combination but also that of cisplatin as a single drug.

In contrast to these aspects, we also demonstrated that folates induce the efflux of the anthracycline daunorubicin (DNR) via multidrug resistance protein 1 (MRP1)/ABCC1 in vitro [4]. This membrane-bound ABC transporter mediates drug resistance by effluxing a wide variety of structurally unrelated drugs [5–7] and belongs to the MRP family [5, 7]. Natural folates as well as antifolates, such as methotrexate (MTX) and PMX, are good MRP1–3 substrates [8–11]. MTX is used in the treatment of several types of cancer and inflammatory diseases [1,

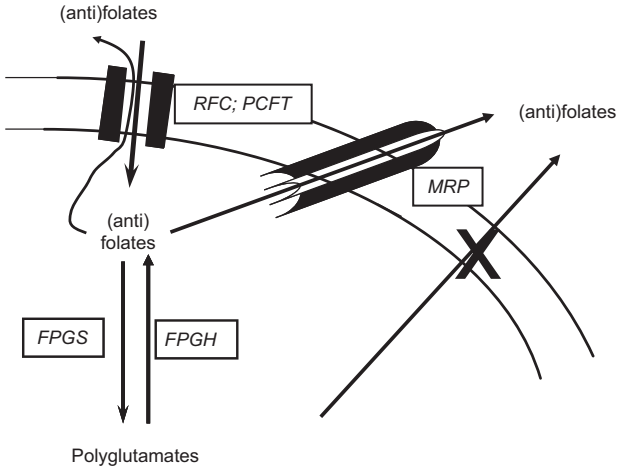

Figure 1 Role of MRP in (anti)folate efflux mechanism.
After being taken up by the reduced folate carrier (RFC) or proton-coupled folate transporter (PCFT), (anti)folates are converted to their polyglutamate form by folylpolyglutamate synthetase (FPGS), which is not a substrate for MRPs, whereas the parent monoglutamate form is.

12] and PMX for non-small cell lung cancer and mesothelioma [3]. MRP1 and other MRPs play a role in antifolate resistance and in controlling cellular homeostasis of natural folates [13] (Figure 1).

As an increase in cellular folate concentration induced MRP1-mediated efflux of DNR due to increased MRP1 transport activity, rather than an increase in expression level of MRP1 [4], we wondered whether folate supplementation or depletion (i.e., folate homeostasis) can affect MRP1-associated drug resistance. Therefore, we investigated whether folate depletion would affect sensitivity to several MRP substrates in 2008 cells transfected with MRP1. In these cells, folate homeostasis was previously investigated by measurement of folate pools. As folates reciprocally control homocysteine pools (Figure 2), we investigated the effect of folate depletion by measurement of intracellular homocysteine pools and included that of glutathione (GSH) as well, because GSH might be a co-substrate for DNR efflux [14].

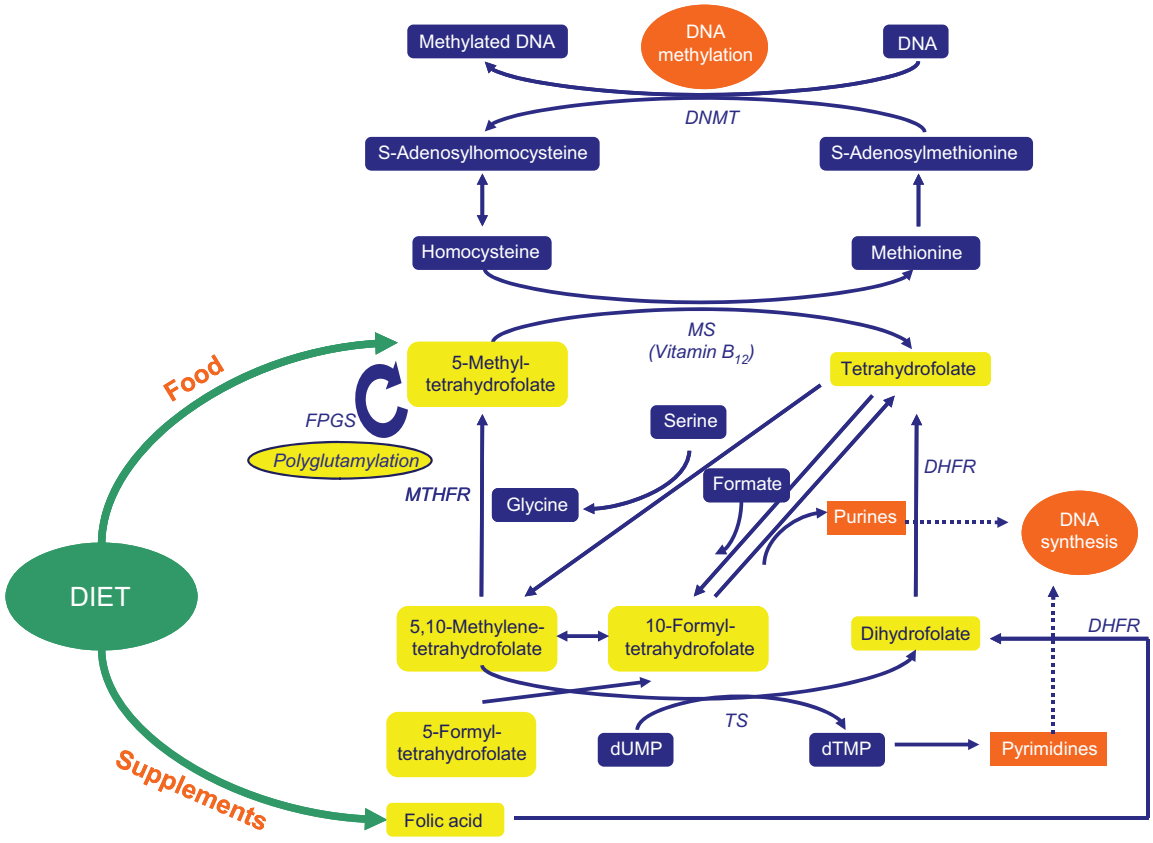

Figure 2 Schematic representation of folate supply via diet or food supplements.
Depending on the source, folates will enter the folate cycle directly (food as 5-methyl tetrahydrofolate) or indirectly (supplements, folic acid). Folic acid will be reduced by DHFR (dihydrofolate reductase) to dihydrofolate and tetrahydrofolate. An increase of 5-methyl-tetrahydrofolate will reduce homocysteine levels because the methyl group is used to convert homocysteine to methionine (catalyzed by MS with vitamin B12 as co-factor), which after conversion to S-adenosylmethionine will serve as a methyldonor for methylation reaction catalyzed by DNA methyltransferase (DNMT). Abbreviations: TS, thymidylate synthase; FPGS, folylpolyglutamate synthetase; MTHFR, methylene tetrahydrofolate reductase; MS, methionine synthase.

Materials and methods

Materials

RPMI-1640 medium (containing 2.3 μM folic acid), folate-free RPMI-1640 medium, normal fetal calf serum (FCS) and dialyzed FCS were obtained from GIBCO (Grand Island, NY, USA). Drugs and chemicals were of standard commercially available quality.

Cell lines and drug sensitivity

The human ovarian carcinoma cell line 2008, and its stable MRP1 transfectant 2008/MRP1 were kindly provided by Prof. Dr. P. Borst (The Netherlands Cancer Institute, Amsterdam, The Netherlands) [8] and cultured in standard (with folic acid) RPMI-1640 medium, supplemented with 10% FCS, 2 mM glutamine and 100 μg/mL penicillin/streptomycin. For folate deprivation, cells were cultured for 5 days in special folic acid-free RPMI-1640 medium supplemented with 10% dialyzed FCS, 2 mM glutamine and 100 μg/mL penicillin/streptomycin. After 3 days of culture in folate-free medium, sensitivity to the drugs of interest was determined using the sulforhodamine B (SRB) assay as previously described [15].

Western blotting

Western blotting for MRP1 was performed as previously described [13].

Homocysteine assay

Shortly, cell pellets (2–5 million cells) were suspended in 200 μL water, sonicated, followed by addition of 25 μL cysteamine as internal standard (25 μM). To release and reduce protein-bound thiols, 15 μL 10% tri-n-butylphosphine in dimethylformamide was added for 30 min at 4°C, centrifuged and deproteinized with 150 μL 10% TCA (with EDTA), and centrifuged. The supernatant (50 μL) was mixed with 20 μL sodium hydroxide, 150 μL borate buffer containing EDTA and 50 μL SBD-F solution (1 mg/mL) and incubated for 60 min at 60°C, followed by addition of 5 μL concentrated H_3PO_4 to acidify the mixture for reversed phase high-performance liquid chromatography analysis on a 5-μm Supelco LC-18-DB C 18 column (Phenomenex, Utrecht, the Netherlands; 150 mm×4.6 mm) using an isocratic elution with a 0.1 M phosphate buffer containing 5.66% acetonitrile at 1 mL/min, with fluorescence detection at 385 nm excitation and 515 emission wavelengths.

Glutathione assay

For the glutathione assay, 2–5 million cells were used, which were lysed in 500 μL phosphate-EDTA buffer. GSH was measured in this lysate with a commercial kit which is based on the reaction of the sulfhydryl group of GSH with DTNB (5,5′-dithio-*bis*-2-nitrobenzoic acid, Ellman's reagent), which produces a yellow colored 5-thio-2-nitrobenzoic acid (TNB). The mixed disulfide, GSTNB (between GSH and TNB) that is concomitantly produced, is reduced by glutathione reductase to recycle the GSH and produce more TNB. The rate of TNB production is directly proportional to this recycling reaction, which in turn is directly proportional to the concentration of GSH in the sample. Measurement of the absorbance of TNB at 412 nm provides an accurate estimation of GSH in the sample. GSH is easily oxidized to the disulfide dimer GSSG.

Results

The role of MRP1 expression on folate pools and drug sensitivity

Previously, we reported that MRP1 expression affected total and reduced folate pools, from 105±14 (means±SD) pmol/mg protein in 2008 cells to 65±15 pmol/mg protein in 2008/MRP1 cells [13]. In other MRP variants a similar decrease was found. In particular, the 10-CHO-THF pool (which was 50% of the total pool) was reduced by approximately 50%, but the other folates only slightly decreased. Hence, MRPs clearly transport reduced folates out of the cells, leading to a slightly folate-depleted cell. Folate depletion by culturing cells in folate-free medium resulted in a largely decreased MRP1-mediated cellular efflux of DNR [4], whereas in other cell lines we observed that folate depletion increased sensitivity to various antifolates [16].

The expression of MRP1 was much higher than in 2008 cells (Figure 3). However, folate depletion increased the expression of MRP1 in 2008 parent cells but barely in 2008/MRP1 cells. In wild-type (WT) cells, sensitivity to MTX was dependent on folate homeostasis drug exposure time. At a continuous exposure to MTX, no difference in sensitivity was found between 2008/WT and 2008/MRP1 cells, when cultured in normal (high folate, HF) medium. However, in low folate (LF) cells, IC_{50} values were 3- to 4-fold lower compared with HF cells, for both 2008 variants. A completely

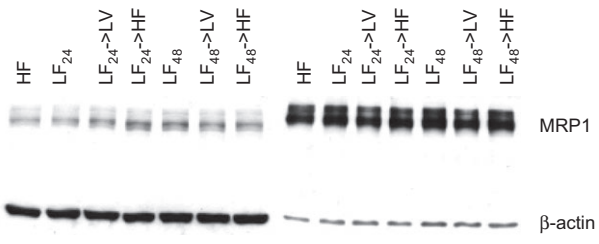

Figure 3 MRP1 expression in 2008 and 2008/MRP1 cells cultured under normal conditions (high folate, HF) and after adaptation to low folate (LF) conditions for 24 or 48 h.
Several samples were put on 5 μM leucovorin (LV) containing medium or on normal (HF) medium again for 24 h.

different picture was found at a 4 h exposure, followed by a 68-h culture in drug-free medium. 2008/WT cells were 10-fold more sensitive when cultured in LF medium, but 2008/MRP1 cells were more than 200-fold less sensitive when cultured in HF compared with LF medium.

In normal medium, 2008 cells were 2- to 3-fold less sensitive to doxorubicin and DNR, compared with LF medium. In 2008/MRP1 cells this difference was more pronounced. The large difference between 2008 and 2008/MRP1 cells in normal medium (5-fold) was not found when cultured under LF conditions. For the other tested drugs the differences were less (data not shown).

Table 1 Effect of folate depletion on homocysteine and glutathione levels in 2008 wild-type and 2008/MRP1 transfected cells.

Cell line	Homocysteine, nmol/10^6 cells		Glutathione, nmol/10^6 cells	
	2008	2008/MRP1	2008	2008/MRP1
Time of folate depletion, days				
D=0 (HF)	0.55±0.13	0.56±0.06	0.45±0.07	0.38±0.09
D=2 (LF)	13.0±0.79	5.3±0.57	0.55±0.07	0.59±0.10

Values are mean±SEM of 3–4 separate experiments. D=0 (HF) are cells harvested from a normal cell culture in regular high folate medium; D=2 (LF) are cells harvested from a 2-day culture in folate-depleted medium.

Effect of folate depletion on homocysteine and glutathione pools

As an indicator for intracellular folate pools, we measured intracellular homocysteine levels in these cells (Table 1). In 2008 and 2008/MRP1 cells, homocysteine levels were similar when cultured under HF conditions (D=0). As expected, folate depletion increased homocysteine levels 20-fold in 2008 cells, but in 2008/MRP1 cells this increase was only 10-fold. GSH levels in 2008 HF (D=0) cells were comparable to that in LF cells. In both 2008 variants, folate depletion resulted in a 20%–50% increase.

Discussion

Since the 1970s folate supplementation is often routinely embedded in chemotherapy-based cancer treatment protocols to reduce drug toxicity [1]. More recently, it has also been exploited as a strategy to increase antitumor activity [3]. In addition, cancer patients increasingly take folate supplements as part of a healthy diet [17]. In the present study, we report, however, that high folate exposure in vitro provokes the ABC transporter MRP1, which mediates cellular drug resistance, not only for antifolates such as MTX but also doxorubicin and DNR. We describe that

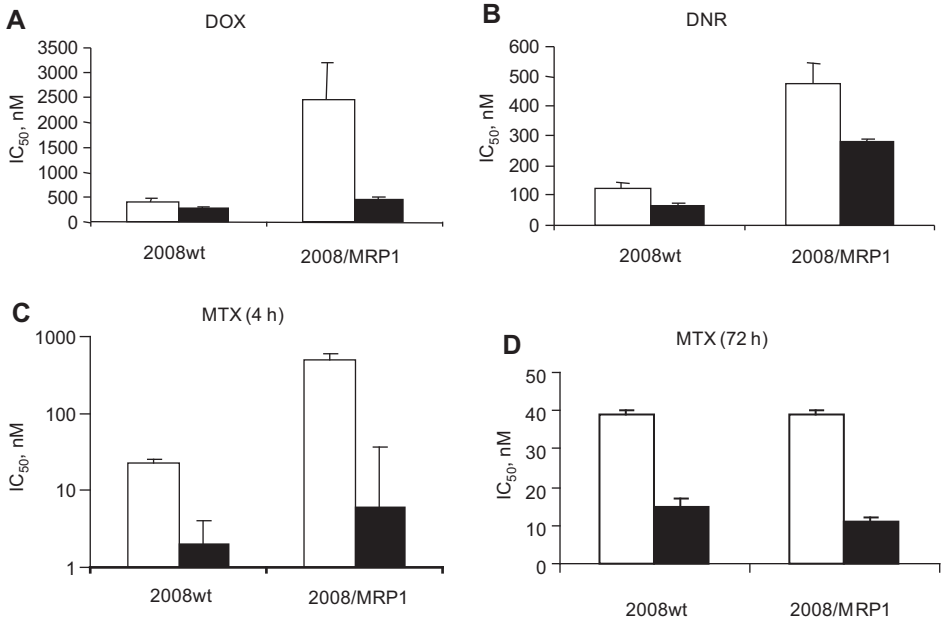

Figure 4 Influence of folate concentration on MRP1-associated drug resistance.
Growth inhibition by doxorubicin (DOX), daunorubicin (DNR) and methotrexate (MTX) was determined in 2008 and 2008/MRP1 cells. IC_{50} values for each drug were determined for a 3-day culture in either folate-rich medium (white bars) or in folate-free medium (black bars). During this period drugs were continuously present, except for MTX. The latter was present during the first 4 h and subsequently washed away to prevent polyglutamylation of MTX. IC_{50} values are given as mean±SD (n=5).

a decrease in (cellular) folate levels apparently reduces MRP1-mediated cellular efflux of its substrates and increases its sensitivity.

Of interest is that levels of folates in human plasma, normal human tissues and solid tumors are normally low (between 5 and 20 nM) and are of the same order of magnitude as those in our folate-deprived cells in vitro [18–20]. Nutritional intake of folates via food and dietary supplements largely controls folate homeostasis [21]. Folate supplementation can result in significantly higher plasma levels and influence cellular processes that are dependent on folates and alter sensitivity to antifolates. However, the 'folate-rich' concentrations used in our study (i.e., normal culture medium, HF) are still much higher than normally found in human plasma. Yet, our results represent a proof-of-principle that can possibly be translated to the physiological situation after supplementation.

Although we show here that high folate levels result in MRP1-associated drug resistance in vitro (Figure 4), it remains to be elucidated whether this can also affect multidrug resistance in patients. Besides, low folate conditions may be responsible for other effects, such as severe toxicity in normal cells. Especially for the use of antifolates, such as MTX, this is an important issue. In our experiments, we observed an extreme reduction of IC_{50} for MTX as a function of folate deprivation, compared with the use of other drugs. This phenomenon is most likely not ascribed to MRP1 function alone. Previous publications showed that cellular folate status has a large influence on sensitivity to polyglutamylation-dependent antifolates in general [22, 23].

Folates have a striking similarity with the cellular compound GSH. GSH is a crucial factor in the MRP1-mediated transport of the cation DNR. GSH was described as (i) a co-substrate in transport of positively charged drugs [24] and (ii) to form a conjugate with positively charged drugs [14] (Figure 5). Interestingly, folates and GSH both contain a glutamate residue that might play a role in binding to a reactive site in MRP1. Accordingly, folates might induce MRP1 activity by binding to this site, as proposed for GSH by Borst et al. [25]. By contrast, in our present study we show that folates also enhance MRP1-mediated transport. Changes in cellular levels of homocysteine as a result of lowered folate status might, for example, affect catalytic activity of several proteins [26], but whether this influences MRP1 is not known. Following long-term folate deprivation (>3 months), however, MRP1 protein expression and efflux function can be markedly lost [27].

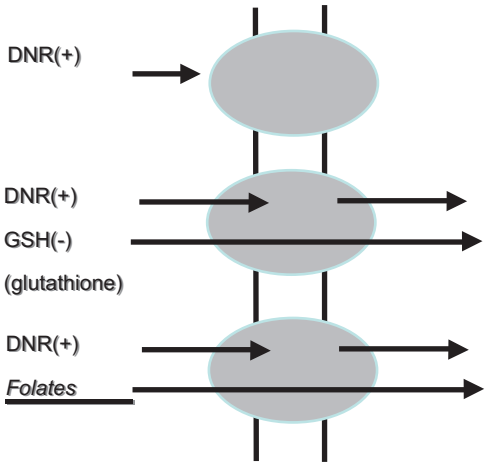

Figure 5 Model for the possible role of glutathione (GSH) in drug efflux.

In conclusion, folate supplementation can have several effects: reduction of toxicity, increase of antitumor activity, but also (as we demonstrate here) induction of MRP1-associated cellular drug resistance. An additional intriguing question is whether the effect of folates on MRP1 activity can be exploited to alter the transport of non-toxic physiological MRP1 substrates, such as leukotrienes, GSH and natural folates. This makes continued studies on the relationship between folates and MRP1 highly worthwhile and relevant. Folate intake and folate levels should be monitored more closely in cancer patients during chemotherapy than is currently the case.

Acknowledgments: This study was supported by the Dutch Cancer Society (grant NKB-VU 2000–2237).

Received February 11, 2013; accepted April 5, 2013; previously published online May 11, 2013

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