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REVIEW

J. H. Hooijberg · N. A. de Vries · G. J. L. Kaspers
R. Pieters · G. Jansen · G. J. Peters

Multidrug resistance proteins and folate supplementation: therapeutic implications for antifolates and other classes of drugs in cancer treatment

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Abstract Over the past decades, numerous reports have covered the crucial role of multidrug resistance (MDR) transporters in the efficacy of various chemotherapeutic drugs. Specific cell membrane-associated transporters mediate drug resistance by effluxing a wide spectrum of toxic agents. Although several excellent reviews have addressed general aspects of drug resistance, this current review aims to highlight implications for the efficacy of folate-based and other types of chemotherapeutic drugs. Folates are vitamins that are daily required for many biosynthetic processes. Folate supplementation in our diet may convey protective effects against several diseases, including cancers, but folate supplementation also makes up an essential part of several current cancer chemotherapeutic regimens. Traditionally, the folate leucovorin, for instance, is used to reduce antifolate toxicity in leukemia or to enhance the effect of the fluoropyrimidine 5-fluorouracil in some solid tumors. More recently, it has also been noted that folic acid has the ability to increase antitumor activity of several structurally unrelated regimens, such as alimta/pemetrexed and cisplatin. Moreover, studies from our laboratory demonstrated that folates could modulate the expression and activity of at least two members of the MDR transporters: MRP1/ABCC1, and the breast cancer resistance protein BCRP/ABCG2. Thus, folate supplementation may have differential effects on chemotherapy: (1) reduction of toxicity, (2) increase of antitumor activity, and (3) induction of MRP1 and BCRP associated cellular drug resistance. In this review the role of MDR proteins is discussed in further detail for each of these three items from the perspective to optimally exploit folate supplementation for enhanced chemotherapeutic efficacy of both antifolate-based chemotherapy and other classes of chemotherapeutic drugs.

Keywords Folates · Folate homeostasis · Multiple drug resistance · Multidrug resistance proteins · Breast cancer resistance protein · Antifolates · ABC transporters

Abbreviations BCRP: Breast cancer resistance protein · DHFR: Dihydrofolate reductase · FPGS: Folylpolyglutamate synthetase · GSH: Glutathione · MDR: Multiple drug resistance · MRPs: Multidrug resistance proteins · MTX: Methotrexate · TS: Thymidylate synthase · ABC: ATP binding cassette transporters

J. H. Hooijberg · N. A. de Vries · G. J. L. Kaspers
Department of Pediatric Oncology, VU University Medical Center, Amsterdam, The Netherlands

G. J. Peters (✉)
Department of Medical Oncology, VU University Medical Center, P.O. Box 7057, 1007 MB, Amsterdam, The Netherlands
E-mail: gj.peters@vumc.nl
Tel.: +31-20-4442633
Fax: +31-20-4443844

R. Pieters
Department of Pediatric Oncology, Sophia's Children Hospital, Rotterdam, The Netherlands

G. Jansen
Department of Rheumatology, VU University Medical Center, Amsterdam, The Netherlands

Present address: J. H. Hooijberg
Department of Clinical Chemistry, Medical Center Alkmaar, Alkmaar, The Netherlands

Introduction

One of the factors that can lead to chemotherapy failure is cellular drug resistance. Cellular resistance can be divided into two categories: (1) intrinsic resistance, in which tumor cells do not respond to a drug a priori, or (2) acquired resistance, in which tumor cells initially respond to the drug but become insensitive during treatment.

Cellular resistance mechanisms that comprise a broad range of structurally and functionally unrelated drugs

are referred to as 'Multiple Drug Resistance' (MDR). The underlying mechanisms may include modification of enzymes, defects in DNA repair, and alterations of chemotherapy-induced apoptosis, e.g., Bcl-2 over-expression.

One particular aspect of MDR is defined as 'the transport of a broad range of cytotoxic agents with different subcellular targets out of the cell by specific transport proteins'. Danø [1] was the first to describe the existence of a membrane transporter protein (P-glycoprotein/ABCB1) as the cause of MDR. Since then several additional MDR-transporters have been identified, of which the family of multidrug resistance-associated proteins (MRPs/ABCCs) and the breast cancer resistance protein (BCRP/ABCG2) have been implicated in anticancer drug resistance. In order to put the roles of the various MDR-transporters in perspective, e.g., their physiological role (e.g., uptake of nutrients, including folates) versus pharmacological role (extrusion of drugs of various classes such as anthracyclines, mitoxantrone, antifolates), they are described as a class and as separate entities. Special attention is given to their role in antifolate resistance. The role of normal folate uptake and metabolism is described in order to put this in perspective with the roles of MRPs and BCRP in folate homeostasis. These processes all play an important role in the complex effects of folate supplementation on transporter expression and resistance to antifolates and other therapeutic agents.

The MRP/ABCC multidrug resistance protein family

The family of Multidrug Resistance Proteins 'MRPs' consists of at least nine protein members/homologues [2–5]. As members of the C-subclass of the superfamily of ATP Binding Cassette-transporters 'MRPs' are currently reclassified as 'ABCCs' [7]. The founding member of the MRP family is the 190 kDa N-glycosylated membrane associated protein MRP1/ABCC1 [6].

Most members of this family are cellular pumps that extrude both hydrophobic uncharged molecules and water-soluble anionic compounds. Accordingly, they have the ability to confer cellular resistance against various anticancer agents [2–4, 8–11]. The MRP homologues can be divided into two major groups: (1) MRP1, MRP2, MRP3 and MRP6, and (2) MRP4, MRP5, MRP7 and MRP8. The MRPs 1–3 and MRP6 contain 17 transmembrane segments, while the MRP4, MRP5, MRP7, and MRP8 only have 12 transmembrane segments, lacking one of the TMD_0 regions [5]. In addition, MRP9 (ABCC12) is an ABCC member that has a topology different from the other two groups. The gene encodes two transcripts of different size lacking several transmembrane regions, including the second nucleotide-binding domain [12]. Specific aspects of MRPs have been reviewed in greater detail by Borst and Oude-Elferink [2], Kruh and Belinsky [3], Scotto [4], Bodo et al. [11], and Haimeur et al. [5].

Substrates of MRPs

The MRP1 confers cellular resistance against a wide spectrum of compounds derived from natural sources. This spectrum of relatively hydrophobic agents includes anthracyclines, vinca alkaloids, antifolates, and epipodophyllotoxins [3, 13]. In addition, MRP1 transports hydrophilic anionic compounds, including natural organic anions such as glutathione-conjugates and glucuronide-conjugates, oxidized glutathione (GSSG), several cysteinyl leukotrienes, certain steroid glucuronides, and anionic conjugates of bile salts [13–16]. Being a transporter of glutathione (GSH)-conjugates, MRP1 is placed in the category of other transporters of GSH-conjugates, which are named GS-X pumps [17]. Another interesting group of physiological substrates are the folates. These are classified as vitamins and their interactions with MRPs are described in more detail elsewhere in this review.

One other typical GS-X transporter is the canalicular multi organic anion transporter (cMOAT), which is currently known as MRP2/ABCC2. The MOAT-like proteins transport a variety of anionic compounds, such as GSH-conjugates, glucuronide-conjugates, bile salt conjugates, and heavy metals including cisplatin [18].

The MRP3 is the closest homologue to MRP1. Yet, it differs from MRP1 in that it appears to be a poor transporter of GSH and GSH-conjugates. Instead, it prefers glucuronate conjugates to GSH-conjugates [19].

The MRP4 is a transporter of cyclic nucleotides and nucleotide analogues. These organic anions are not transported by MRP1-3 nor MRP6. The MRP4 functions as a cellular efflux pump for the anti-retroviral nucleoside analogues 9-(2-phosphonylmethoxyethyl)adenine (PMEA) and azidothymidine monophosphate (AZTMP) in PMEA-resistant cells [20]. Reid et al. demonstrated that MRP4 also interacts with prostaglandins [21].

The MRP5 is able to efflux acidic organic dyes, dinitrophenylglutathione, and GSH [22]. The MRP5 mediated drug resistance was only found for 6-mercaptopurine (6MP) and 6-thioguanine (6TG), and PMEA. Resistance to 6MP and 6TG is a result of conversion of the bases into the corresponding nucleotides and extrusion of the nucleotides from the cell by MRP5. Possibly, MRP5 can also confer resistance to heavy metals [23]. In addition, MRP5 confers resistance to methotrexate and several novel antifolates [24].

Functional studies suggested that GSH conjugates and small peptides (BQ123) are transported by MRP6. Also, low levels of resistance to natural product agents were found, including etoposide, teniposide, doxorubicin, and daunorubicin [25]. The levels of resistance to anticancer drugs conferred by MRP6 are, however, modest.

Very little is currently known about MRP7 mediated drug resistance. A recent publication demonstrated a modest role of MRP7 in resistance against vinblastine and vincristine. However, more interesting was the

Table 1 The human MRP family (adapted from Refs. [3, 4])

Protein		Drug resistance profile
MRP1	ABCC1	Anthracyclines, Vinca Alkaloids, Etoposide, Camptothecins, Antifolates
MRP2	ABCC2	Anthracyclines, Vinca Alkaloids, Etoposide, Camptothecins, Antifolates, Cisplatin
MRP3	ABCC3	Etoposide, Antifolates
MRP4	ABCC4	6-Mercaptopurine, 6-Thioguanine, Antiviral drugs (AZTMP, PMEA), Methotrexate
MRP5	ABCC5	6-Mercaptopurine, 6-Thioguanine, Antiviral drugs (AZTMP, PMEA), Methotrexate
MRP6	ABCC6	Anthracyclines, Etoposide, Cisplatin
MRP7	ABCC10	Vinblastine, vincristine, docetaxel
MRP8	ABCC11	5-Fluorouracil, PMEA, Methotrexate
MRP9	ABCC12	Under investigation

Given are the classical MRP names, as well as the new ABC-transporter nomenclature.

reported resistance against docetaxel and other taxanes [26].

The MRP8 mediates the efflux of a broad range of clinically relevant nucleoside analogs (5-fluorouracil, 5-fluoro-2′-deoxyuridine, and 5-fluoro-5′-deoxyuridine, PMEA and the anti-AIDS drug 2′,3′-dideoxycytidine) as well as physiological substrates (bile acids, sulfated steroids, leukotrienes [27, 28].

In Table 1 a summary is given of recently discovered MRP homologues and their contribution to resistance against toxic agents.

Expression and localization of MRPs in human tissues and cancers

The MRP1 through six protein expression is described in approximately all normal human tissues, however, MRP2 seems to be restricted to the liver [29–32]. The tissue distribution of the more recently identified MRPs (7–9) is still under investigation.

Increased expression of MRPs in tumor cells can be responsible for in vitro cellular drug resistance against many agents. Therefore, the relation between expression of MRPs and the response of tumor cells to chemotherapy has been subject of many studies. Two major questions are whether MRPs are being overexpressed in cancer cells and tumors, and whether overexpression of MRPs is related to clinical response to (combination) therapy, and thus affect the final clinical outcome.

The broad distribution of MRPs in normal tissues suggests that they are potentially present in most forms of cancer. The levels of MRP1 in cancers are often in the same range as basal concentrations found in non-cancerous tissues. Expression of MRP1 was detected in many solid tumors, such as in lung, gastrointestinal and urothelial carcinomas, neuroblastomas, gliomas, retinoblastomas, melanomas, cancers of breast, endometrium, ovary, prostrate, and thyroid. Some malignancies have been described with high expression of MRP1, including chronic lymphocytic leukemia, esophageal squamous cell carcinoma, and in particular non-small cell lung cancer [33–39].

Upon closer inspection several studies have reported a correlation between clinical outcome and expression of MRP homologues within solid tumors and leukemias [40, 41]. Most attention has been given to MRP1, being the founding member of the MRPs. In other studies data on the expression of this protein and its prognostic value were, however, contradictory. For instance, several studies focusing on acute lymphoblastic leukemia (ALL) described differences in expression between ALL subgroups (41–49), whereas others described that there are almost no differences in expression between subgroups [41, 42, 48, 49].

Thus, there is currently no consensus on the role of MRPs in acquired resistance or their prognostic significance [3]. Yet, these studies focused on expression in the first place a priori, rather than on activity prior and during chemotherapy.

An additional interesting point is the cellular localization of these transporters. Actual location is dependent on the type of tissue in which MRPs are being expressed. The MRPs are normally located in intracellular vesicles within various tissues. In epithelial membranes, however, MRP1 and MRP3 are present in the basolateral membrane of cells. This means that these MRPs secrete their substrates into the body, rather than moving them out [2, 8]. Likewise, MRPs might enhance the uptake of natural substrates, such as folates, from the intestine into the body. In tumor cells MRPs seem to be located predominantly in the plasma membrane, thereby contributing to cellular resistance.

The BCRP/ABCG2 breast cancer resistance protein

In addition to MRPs/ABCCs, the recently identified BCRP/ABCG2 was also reported to confer resistance against multiple drugs [50–52]. Upon dimerization/oligomerization [53] this 72 kDa halftransporter induces resistance to mitoxantrone, several topoisomerase I inhibitors, several anthracyclines, flavopiridol, and methotrexate [50–58]. Interestingly, some recently introduced targeted-therapy drugs, such as STI-571 (Gleevec; imatinib mesylate), ZD1839 (Iressa; gefitinib),

and N-[4-[(3-bromophenyl)amino]-6-quinazolinyl]-2-butynamide (EKI-785) have also been reported to be substrates of BCRP [59, 60]. Also some fluorescent dyes, such as Hoechst 33342 were identified as BCRP substrates [61]. Primitive stem cells termed 'side population' stem cells (SP) that strongly efflux Hoechst 33342, were recently found to express BCRP [50,62]. Despite this apparent function of BCRP in the protection of SP cells, no clear physiological function has been described to account for it. However, recent studies by Schinkel and co-workers using BCRP knockout mice (ABCG2 −/−) indicate that BCRP does appear to have a protective role against xenotoxins [63].

High expression of BCRP was reported in placenta, brain, endothelium, prostate, small intestine, testis, ovary, colon and liver. Little or no expression was found in heart, lung, skeletal muscle, kidney, pancreas, spleen, thymus, and peripheral blood leukocytes [50, 64]. In a variety of human cancers BCRP expression has been observed [50]. However, only limited studies on the potential clinical relevance of BCRP expression in cancer have been published. The outcome of these studies was not always consistent, which may be related to the different types of methodology used for assaying the BCRP activity (immunohistochemistry, Western blotting, functional activity). Similar to the data on MRP1, some investigators have reported large differences in BCRP expression, which could be correlated with cellular resistance, whereas others were not able to confirm this [65–68].

Remarkably, the existence of a mutation in the BCRP gene coding for amino acid R482 was reported to have an important effect on BCRP transport activity in vitro [69, 70]. This single nucleotide polymorphism results in a significantly decreased transport of several substrates, such as MTX, whereas the transport of mitoxantrone is unaffected [71, 72]. Whether such polymorphisms also play a role in drug-transporter function in vivo (e.g., in patients) remains to be elucidated.

MRPs, BCRP and antifolates

One particular and interesting group of drugs that are MRP and BCRP substrates are the antifolates. These compounds, such as methotrexate (MTX), are antagonists of the cellular metabolism of *natural* folates and are widely used as antineoplastic drugs. MTX is an important drug in ALL, non-Hodgkin lymphoma, osteosarcoma, head and neck cancer, choriocarcinoma, small cell lung cancer, and breast cancer [reviewed by Bertino: 73, and Peters: 74]. Novel generation antifolates, such as ALIMTA (pemetrexed) and raltitrexed (Tomudex) have been registered as anticancer agents, while OSI-7904(l) (GW1843), plevitrexed (BGC9331) and Thymitaq (nolatrexed) are being evaluated in Phase III studies [75].

The MRP1-4 play a role in the cellular extrusion and resistance to MTX (see Fig. 1) [76, 78]. Inhibition of MRP1 mediated the efflux by probenecid reverted

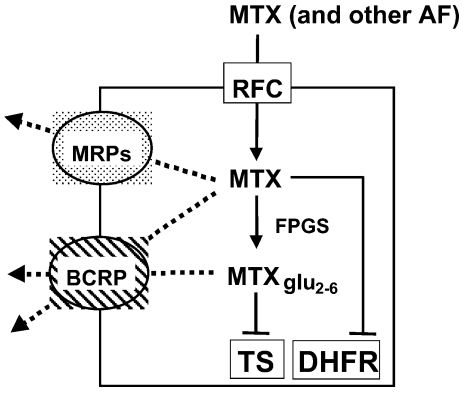

Fig. 1 Model of MRP and BCRP mediated efflux of antifolates. The reduced folate carrier (*RFC*) transports methotrexate (*MTX*) and other antifolates (*AF*) into the cell. Hereafter, MTX either inhibits dihydrofolate reductase (*DHFR*), or is polyglutamylated by folyl-polyglutamate synthetase (*FPGS*). This polyglutamylation enhances the cellular retention of MTX and results in inhibition of thymidylate synthase (*TS*). The MRPs and BCRP efflux MTX monoglutamates. The BCRP also effluxes MTX in its di- and triglutamate form

MTX-transport in vitro [76] or enhanced the activity of the antifolate 10-deazaaminopterin in vivo [77]. There is ample evidence that MRP1-4 efflux MTX preferentially in its monoglutamate form, polyglutamates are not transported. Consistent with this notion is that MRP1-4 expressing cells were more resistant to MTX after short term drug exposure, during which time polyglutamylation is rate-limiting, rather than standard long-term drug exposure assays which allow MTX polyglutamylation. This was exemplified for MRP1-3 transfected cells that displayed resistance against relatively short (4 h) exposures to MTX, and its novel analogues raltitrexed (Tomudex) [77, 79]. In contrast, after longer exposure times (72 h) MRP associated resistance was limited. Later, also MRP4 expression was shown to confer MTX resistance [80].

These observations were confirmed by others using inside-out vesicles uptake studies [81–84]. In these models systems, formed out of cellular membranes, the outside of the membrane faces inside, and the inside faces outside. Thus an overexpression of an efflux would lead to an increased uptake in the vesicle, due to the fact that now this pump will catalyzes uptake into the vesicle. Thus, polyglutamylation of antifolates (see later for more details) is a critical factor that diminishes MRP mediated extrusion of antifolates. In contrast to these MRPs, MRP5 is able to efflux diglutamates [24]. Like MRP5, BCRP is also capable of transporting both mono-, as di- and triglutamate forms of MTX (Fig. 1) [72, 85]. For this reason, BCRP may be a major contributor to antifolate resistance. Of considerable importance for BCRP mediated antifolate resistance may be the single nucleotide polymorphism at the site of R482 in the gene of BCRP. Based on vesicle studies, the mutant form of BCRP was not capable of transporting MTX (polyglutamates) [72]. However, recent studies

using intact cells demonstrated that expression of either the wild type R482 or the mutant G482 conferred resistance to a clinically relevant 4 h exposure to the hydrophilic antifolates, MTX, raltitrexed and GW1843, although resistance was more pronounced in cells transfected with the G482 mutation [86]. At continuous 72 h exposure to these antifolates, both variants lost all their antifolate resistance [86]. Addition of a specific BCRP inhibitor reversed resistance. Polyglutamates of MTX (both di- and triglutamates) were markedly decreased in cells transfected with either the G482 or R482 polymorphisms compared to the wild type. Thus, for the prediction of BCRP associated drug resistance, screening for both polymorphisms (at least based on in vitro models) seems relevant.

Most of these studies have been performed with MTX, being considered as the prototype of antifolates. Different results for the various MRPs were observed, possibly because each transporter would have different substrate specificity. However, in various papers describing MTX efflux, a number of analogs were tested [24, 76, 86], such as raltitrexed and GW1843, with raltitrexed being a much better substrate for FPGS as MTX, while GW1843 cannot be polyglutamylated further than the diglutamate. Although differences were observed, the tendency was as expected.

With the discovery that antifolates are an important group of MRP- and BCRP-substrates it was hypothesized that *natural* folates might also be substrates of these transporters. Circumstantial and direct evidence for a role in cellular folate efflux by MRPs and BCRP has been demonstrated for both MRPs and BCRP.

Folates

Natural reduced folates are important vitamins that cannot be synthesized in the human body. Many different plants and bacteria serve as folate resources. Folates are essential for the eukaryotic cell. As one-carbon donors and co-factors they play a role in a variety of biosynthetic reactions, including the synthesis of amino acids (methionine, serine), de novo purine synthesis, and synthesis of thymidylate for DNA synthesis [73]. Folates also regulate gene expression by providing methyl donors for DNA methylation.

Folate metabolism

Folates are present in human blood plasma primarily as methyl tetrahydrofolate (5-CH_3THF). Cellular uptake of 5-CH_3THF is mediated by at least three routes: the Reduced Folate Carrier (RFC), the Folate Receptor (FR), or a low pH folate transporter [87–97]. A model of the folate transport and metabolism is given in Fig. 2.

After entering the cell, the methyl group of 5-CH_3THF is utilized for methylation reactions, after which tetrahydrofolate (FH4) is recycled. Oxidized folic

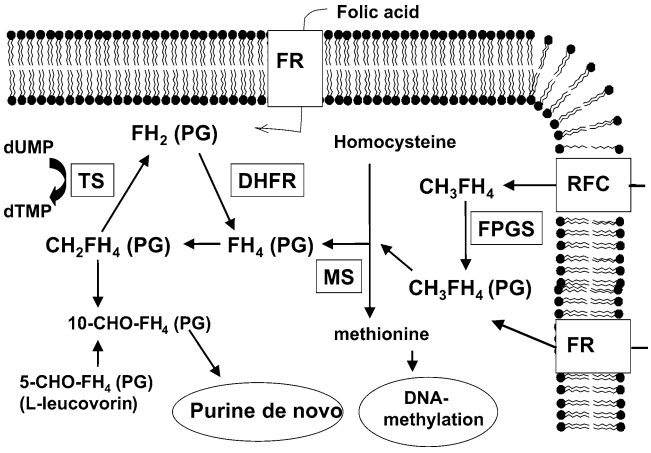

Fig. 2 Model of folate import and metabolism. Schematic depiction of the folate metabolism and uptake systems, which is described in the text

acid is preferentially taken up by the FR, or by the RFC that has low affinity for this substrate. Intracellularly, folic acid is reduced to dihydrofolate (FH2) and FH4 by dihydrofolate reductase (DHFR). Cellular retention of FH4 is regulated by its conversion to FH4-polyglutamates (PG) forms by folylpolyglutamate synthetase (FPGS) [96–101], which increases the negative charge on this compound and thereby prevents its efflux [96,102]. Thereafter, FH4 can be converted into methylene-tetrahydrofolate (CH2FH4) in a reaction that uses serine as a methyl donor. Subsequently, CH2FH4 is oxidized into FH2 by serving as a methyl-donor for the enzyme thymidylate synthase (TS). In this reaction dUMP is methylated to dTMP, required for DNA synthesis. FH2 can be regenerated to CH2FH4 by DHFR. Alternatively, CH2FH4 can be interconverted into 10-formyl-tetrahydrofolate (10-CHO-FH4), which is a precursor of purines for DNA synthesis.

Folates also serve as methyl donors in amino acid synthesis, in particular for the synthesis of methionine from homocysteine by methionine synthase (MS). Methyl groups are supplied to the methylation cycle [103], in which methyltransferases methylate a wide variety of substrates, such as proteins, DNA and RNA, lipids, and hormones. Via the methylation of CpG islands in the promoter region of genes, gene expression is being silenced [104, 105].

Folate deficiencies and folate fortification

Many studies, starting in the 1920s, showed that deficiencies of folate co-factors in the food resulted in a variety of anemias and damage of the nervous system [103, 106–110]. In particular, tissues with a high turnover of cells demonstrated a vulnerability to folate deficiency. For example, the known role of folates in DNA synthesis and cell replication explains the strong influence of folates on the biosynthesis of cells within the

bone marrow, such as red blood cells. Consequently, folate deficiencies have been described as an important factor in the cause of anemias originating from the bone marrow [108]. In addition other effects have been reported on enterocytes resulting in atrophy of the microvilli of the intestine, and on cell formation within the skin. Furthermore, via the inverse relationship of high homocysteine levels and low (intracellular) folate levels, this parameter can be associated with higher cardiovascular risk [108, 110, 111].

Although the exact mechanism is still a matter of debate, several studies have shown that increased folate intake can be associated with a reduced risk for cancer development (colon and breast) [103, 112–116], as well as other age-related diseases [111, 117]. In 1998, the Food and Drug Administration (USA) issued regulations to allow the enrichment of the diet with folic acid to lower the incidence of neural tube defects occurring in newborns. In other countries, such as several in Europe, folate fortification of foods is currently not permitted [118]. However, many individuals both in USA and Europe take vitamin supplements on a daily basis, mostly over the counter multi vitamin supplements, which usually contain folic acid [119].

Antifolates and antifolate resistance

Antifolates inhibit key-enzymes in folate metabolism, such as TS and DHFR. Polyglutamylation of most antifolates by FPGS is an important determining step for cellular sensitivity. This is not only because it enhances the intracellular retention of antifolates [73, 120–122], but also because of an increased target enzyme inhibition as compared to the monoglutamate derivates.

The antifolate MTX is widely used in the treatment of a variety of cancers, including adult/childhood acute lymphoblastic leukemia. Currently, second and third generation antifolates are registered for neoplastic or non-neoplastic diseases, such as raltitrexed for colorectal cancer in Europe, and ALIMTA (pemetrexed) for mesothelioma and non-small cell lung cancer in both Europe and the USA. Other novel generation antifolates are being evaluated in clinical trials, such as nolatrexed (Thymitaq), OSI-7904(L), and plevitrexed (BGC 9331, ZD9331) [75, 122]. These types of antifolate novel drugs were designed for a more efficient uptake via RFC and/or a better polyglutamylation via FPGS. Plevitrexed cannot be polyglutamylated.

In the past several mechanisms of cellular resistance against antifolates have been described. Among them are up-regulation of DHFR and TS, down-regulation of FPGS, resulting in decreased polyglutamylation, and impaired cellular uptake via the RFC [87, 120, 122–132].

As described above, overexpression of the Multidrug Resistance Proteins ABCC1-4 as well as of the BCRP ABCG2 confers resistance to several antifolates [76–82, 84–86]. Since MRP1-4 exclusively efflux monoglutamate forms of antifolates, activity of FPGS is an important cofactor that determines the level of resistance. In fact, in cells with a low FPGS activity, the contribution of MRP in the resistant phenotype will be more pronounced than in cells with a high FPGS activity.

Influence of cellular folate concentration on antifolate sensitivity

The cellular folate concentration is a major determining factor in the sensitivity of cells to antifolates. In vitro studies have revealed that small increases in cellular folate pools result in major changes in antifolate resistance [129–136]. Likewise, animal studies have shown the relevance of folate status for the in vivo sensitivity to several novel antifolates [137, 138]. For example, the antitumor effect of the novel antifolates raltitrexed and pemetrexed was attenuated in mice having a high folate intake, compared to the effect on mice kept on a folate-restricted diet [138]. In a clinical setting, an increased folate status in cancer patients appeared to be an important determinant for the (decreased) activity and observed (decreased) toxicity of antifolates like pemetrexed [139, 140]. As reviewed by Peters and Jansen [141], nutritional intake of folates via food and food supplements can affect folate homeostasis, and thereby influence either cellular process dependent on folates and/or sensitivity to antifolates.

Folate supplementation to reduce toxicity and improve efficacy

High-dose MTX as used in acute lymphoblastic leukemia treatment is routinely followed by supplementation of folates (leucovorin) to reduce toxicity. Leucovorin, a reduced folate precursor can bypass the DHFR blockage, resulting in rescue of host cells of the bone marrow and gut [142]. Although there is still much unknown about the importance of folate supplementation, it appears that in the development of novel antifolate-based therapies folate supplementation may play an important role. For example, in initial clinical studies with lometrexol, delayed toxicity was observed that could be prevented by co-administration of either folic acid or leucovorin [139, 140]. Also, in the subsequent development of pemetrexed, observed toxicity was found to be related to high homocysteine levels, which is indicative for folate deficiency [143]. Therefore, development of pemetrexed-based therapies were re-designed with the addition of folic acid and vitamin B12 to all treatment protocols [143].

Interestingly, in a randomized study in which the efficacy of pemetrexed/cisplatin was compared with that of cisplatin as single drug, it was shown that mesothelioma patients also receiving folic acid supplementation had less toxicity compared to the historical patients (in the first part of this trial) receiving no folate supplementation [144]. Even more intriguing in the same study

was the higher antitumor effect of cisplatin itself in combination with folic acid supplementation compared to cisplatin alone without folic acid supplementation. A biochemical rationale for these (partly unexpected) folate effects is not yet clear [144]. These interesting observations formed the basis for another (phase II) study with the Gemzar and cisplatin combination in advanced esophagogastric cancer with or without folate supplementation, the results of which are pending completion of the investigation.

MRPs, BCRP, and cellular folate homeostasis

The MRPs not only transport antifolates, but also *natural* reduced folates out of the cell. Direct and indirect evidence for a role of MRPs in cellular folate export was obtained by several investigators [80, 81, 145, 146]. 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 [76]. Other evidence for a contribution of MRPs in folate efflux was reported by Kusuhara et al. [137], who showed that MRP2/cMOAT transport reduced folate cofactors in rats. In addition, Zeng [81] and Chen [80] showed that folic acid and L-leucovorin are transported by MRPs, using an inside-out vesicles uptake system. The hypothesis that MRPs have a role in cellular homeostasis of folates [147] was based on several lines of evidence: (1) diminished cellular folate pools (~30%) were found in MRP overexpressing cells; (2) MRP overexpression increased the folate dependent cellular growth after short-term (4 h) folate exposure, and (3) long-term (but not short-term) selection of cell lines in folate-restricted conditions resulted in a downregulation of MRP1 expression. The latter suggests that cells with diminished MRP1 expression have a survival advantage in folate-restricted conditions.

With respect to BCRP, Chen et al. [72] demonstrated leucovorin and folic acid transport into inside-out vesicles, whereas Ifergan et al. [147] described that folate deprivation markedly down regulated the protein expression and functional activity of BCRP. Similar to MRP1, these results may suggest that down regulation of BCRP expression and efflux function are essential components of cellular survival under conditions of folate deficiency.

Folates and MRP/BCRP associated drug resistance

The ability of MRPs and BCRP to mediate the cellular extrusion and homeostasis of folates implies that these transporters also influence cellular sensitivity for antifolates in a collateral way. It was shown that loss of MRP1 function resulted in an expansion of the cellular folate pool and diminished antifolate activity [145, 148]. Thus, whether MRPs and BCRP contribute to antifolate resistance or enhance their activity is highly dependent on (a) polyglutamylation efficiency of the antifolates, and (b) the cellular concentration of natural folates.

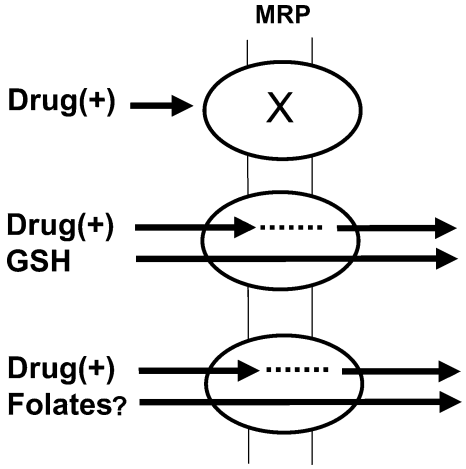

Fig. 3 Hypothetical model of the interaction of folates with MRPs. Positively charged drugs (*plus*) are not transported by MRPs themselves. Glutathione (GSH) enables MRP mediated transport of such cationic drugs, possibly by its binding to a G-site in MRP. Likewise, folates may enhance MRP transport activity

Upon long-term folate depletion (weeks to months) and the resulting decrease in MRP1 and BCRP, a decline in cellular resistance against drugs such as daunorubicin, methotrexate and mitoxantrone can occur. As reported by Ifergan et al. BCRP mediated drug resistance to mitoxantrone in vitro was dramatically reduced upon folate depletion, whereas folate supplementation resulted in an induction of BCRP associated resistance [147].

Intriguingly, transport activity of MRP1 was also reported to increase rapidly after short (hours to days) exposure to high concentrations of folic acid or L-leucovorin [149]. However, during such short periods the protein expression of MRP1 was shown to remain unaltered. Apparently, a direct interaction of folates with the MPR1 protein results in the activation of transport abilities. This might be explained in a model in which folates have a similar role as GSH conjugates in the mechanism of activation of MRP1 (see Fig. 3). The GSH conjugates are a crucial factor in the MRP1 mediated transport of cationic drugs, such as DNR. Anions (e.g., calcein), are regular MRP1 substrates that do not need GSH for transport. Borst et al. [8] proposed that GSH might enable MRP1 transport activity by binding to a reactive site in the MRP1 protein, termed G-site. Since folates and GSH share some common structural similarities based on the glutamate side chain, it is conceivable that this part of both molecules harbors a common side of interaction to the G-site of MRPs and hence activation of transport activity.

From a biological point of view the following hypotheses can be suggested to explain the purpose of a direct influence of folates on MRP activity to sustain folate homeostasis:

1. Folate rich conditions provoke cells to up-regulate MRPs and BCRP mediated folate efflux as a regulatory mechanism to control folate concentrations and folate-dependent cellular processes. However, up-regulation of these MRPs and BCRP proteins increases efflux of drug xenotoxins, subsequently leading to drug resistance.
2. Conversely, in folate-restricted conditions it is necessary to give priority to shutting down efflux activity of BCRP and MRPs as a means to retain cellular folates. This also results in a diminished role of these transporters in drug resistance and protection against xenotoxins.

Conclusions

In this review we have summarized several recent aspects of MDR transporters and their interaction with folates. Folate supplementation is often routinely embedded in chemotherapy protocols to reduce toxicity. It is also exploited as a strategy to increase antitumor activity for several agents. Recent studies have revealed, however, that expression levels and transport activity of MRPs and BCRP can vary significantly in relation to changes in the cellular folate concentration. As a consequence, exposure to folates can induce MRP- and BCRP-associated drug resistance in vitro. Folate supplementation meant to improve chemotherapy might, therefore, result in clinical drug resistance via induction of MRPs and BCRP in cancer cells, as proposed in Fig. 4. These observations warrant further exploration of the relationship between individual folate supplementation, the functional activity of MDR-transporters, its implications for toxicity and cellular resistance to different classes of anticancer drugs. To develop a therapeutic window for folate supplementation as a part of chemotherapy, MDR-transporters should be taken into account.

In conclusion, the existence of MDR transporters implies that folate supplementation is a double-edged sword, which should be handled with care.

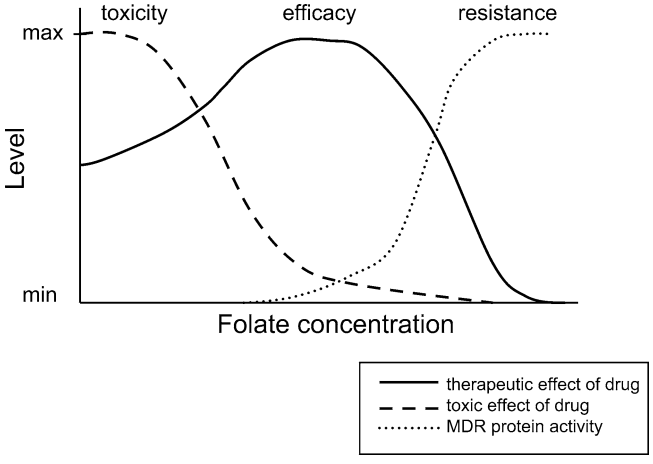

Fig. 4 Therapeutic window for folate supplementation. With increasing concentration of folates administered during chemotherapy the efficacy of single drugs or drug combinations may be improved. Simultaneously, drug toxicity decreases. Overdose of folates, however, can induce multiple drug resistance, which decreases drug efficacy

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