Drug Resistance Updates 15 (2012) 183–210

Contents lists available at SciVerse ScienceDirect

Drug Resistance Updates

journal homepage: www.elsevier.com/locate/drup

Antifolates in cancer therapy: Structure, activity and mechanisms of drug resistance

Nitzan Gonen, Yehuda G. Assaraf*

The Fred Wyszkowski Cancer Research Laboratory, Faculty of Biology, Technion-Israel Institute of Technology, Haifa 32000, Israel

ARTICLE INFO

Article history:
Received 25 March 2012
Received in revised form 25 June 2012
Accepted 11 July 2012

Keywords:
Folates
Folate-dependent enzymes
Folate pools
Antifolates
Cancer
Chemotherapy
Drug resistance
Influx and efflux transporters

ABSTRACT

In the past 65 years, antifolates targeting folate metabolism played a pivotal role in drug treatment of malignant, microbial, parasitic and chronic inflammatory diseases. Drug discovery of novel antifolates with improved properties and superior activities remains an attractive strategy both in academia and in the pharmaceutical industry. Among novel antifolates are pemetrexed which primarily targets thymidylate synthase as well as pralatrexate which blocks dihydrofolate reductase, and displays enhanced transport and cellular retention properties. The present review describes the evolution and pharmacological activity of antifolates and prospects for the development of the next generation antifolates. Pre-clinical and clinical studies identified a plethora of mechanisms of antifolate resistance that are a primary hindrance to curative cancer chemotherapy; these are frequently associated with qualitative and/or quantitative alterations in influx and/or efflux transporters of antifolates and in folate-dependent enzymes. Current advances including for example the deciphering of the dominant folate transporter proton-coupled folate transporter (PCFT/SLC46A1) facilitated the synthesis of experimental antifolates aimed at selectively targeting solid tumor cells, which reside in an acidic microenvironment where PCFT supposedly functions optimally. Moreover, drugs that are structurally and mechanistically distinct from folates were conjugated to folic acid (e.g. Vintafolide/EC145, a folic acid desacetylvinblastine conjugate) to facilitate endocytosis via the folate receptor (FR) which is markedly overexpressed in various solid tumors. In an alternative approach, novel antifolates selectively targeting the FR but not other folate transporters are being developed (e.g. BGC 945). Hence, targeting mechanisms of antifolate-resistance could facilitate the development of rationally-based novel antifolates and strategies that overcome chemoresistance.

1. Introduction

1.1. Cellular folate metabolism and compartmentalization

Folates are B_9 vitamins that serve as one-carbon donors in multiple crucial biosynthetic pathways including *de novo* biosynthesis of purines and thymidylate, amino acid metabolism and methylation reactions (Stockstad, 1990). Folates are composed of three chemical components: pteridine ring, *p*-aminobenzoic acid (PABA) and a glutamate residue (Fig. 1). Folates can be found in an oxidized form, folic acid, or as the naturally occurring reduced folates. Folic acid can be synthesized and reduced by the normal bacterial flora that is resident in the small intestine. Reduced folates are found as the partially reduced form 7,8-dihydrofolate (DHF) or the reduced species 5,6,7,8-tetrahydrofolate (THF). THF cofactors are the biologically active congeners of folates. In humans, the primary circulating reduced folate is 5-methyl-THF (5-CH_3-THF) (Fig. 1) which is found at low, yet sufficient physiological concentrations of ~5–30 nM in the blood (Ifergan and Assaraf, 2008). Unlike bacteria and plants that possess an autonomous biosynthetic capacity to generate their own folate cofactors, metazoan organisms including mammals lack this enzymatic capacity for folate biosynthesis. Mammals must therefore obtain their folates from the diet; green leafy vegetables serve as the major source of our dietary folate intake.

In eukaryotes, folate metabolism is compartmentalized in two main subcellular compartments: the cytosol and mitochondria (Tibbetts and Appling, 2010). 5,10-CH_2-THF is the cofactor necessary for the reductive methylation activity of thymidylate synthase (TS) that catalyzes the conversion of 2′-deoxyuridine monophosphate (dUMP) to 2′-deoxythymidine monophosphate (dTMP). The byproduct of this reaction is DHF, which is efficiently recycled to THF by dihydrofolate reductase (DHFR), an abundant key cytosolic enzyme that maintains the cellular THF cofactor pool (Fig. 2). The reduced folate cofactor, 10-CHO-THF is first used by the enzyme glycineamide ribonucleotide formyltransferase (GARFT), for the formation of the imidazole ring of purines; the second enzyme that utilizes 10-CHO-THF as a cofactor is

* Corresponding author. Tel.: +972 4 829 3744; fax: +972 822 5153.
E-mail address: assaraf@tx.technion.ac.il (Y.G. Assaraf).

1368-7646/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.drup.2012.07.002

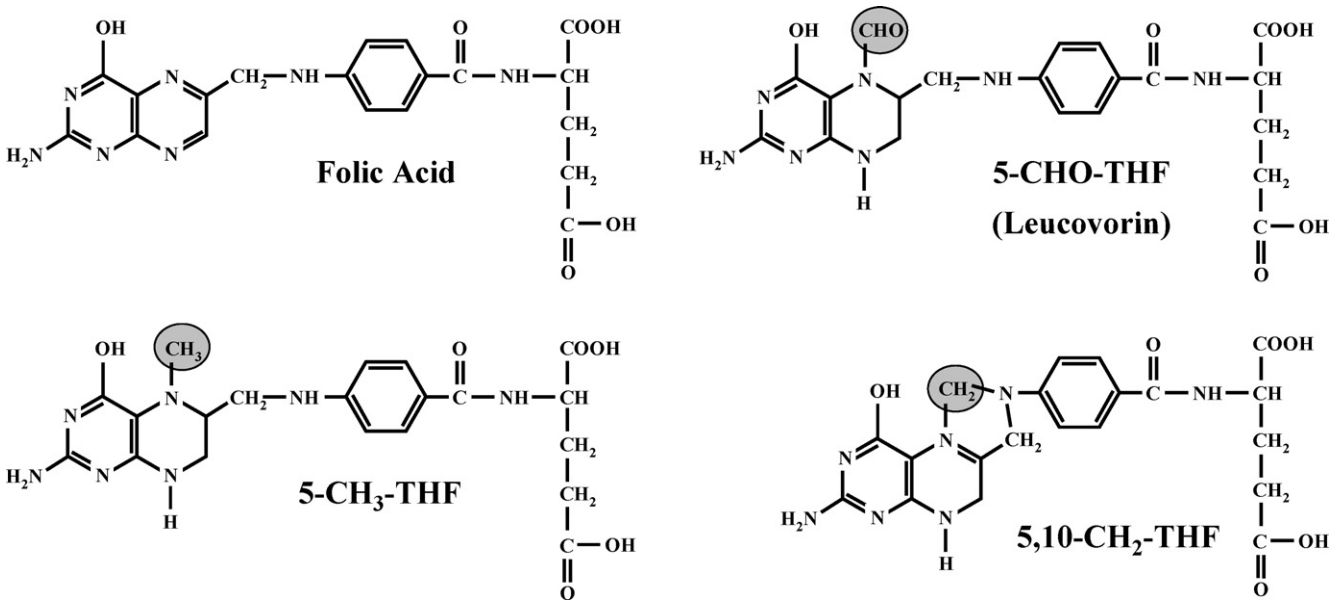


Fig. 1. Chemical structures of oxidized and reduced folates. The one-carbon units that are transferred during a multitude of folate-dependent reactions are highlighted in gray.

5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase (AICARFT), a more downstream enzyme in the purine biosynthetic pathway which eventually generates the purine intermediate inosine 5′-monophosphate (IMP) (Fig. 2). Another cellular form of THF cofactor is 5-methyl-THF (5-CH_3-THF) (Fig. 1); 5-CH_3-THF serves as a cofactor, along with vitamin B_{12}, for the catalytic activity of methionine synthase (MS) which mediates the conversion of homocysteine to methionine. ATP can be conjugated to methionine, thereby forming S-adenosylmethionine (AdoMet); the latter serves as the universal methyl group donor in multiple methylation reactions such as methylation of neurotransmitters, phospholipids, RNA, cytosine nucleotide residues within CpG islands in DNA, as well as proteins including histones (Reviewed in Fox and Stover, 2008) (Fig. 2).

THF cofactors that originate in the cytosol can be transported into mitochondria in their monoglutamate form via the mitochondrial folate transporter (MFT/**SLC25A32**) (Lawrence et al., 2011; Titus and Moran, 2000). Inside mitochondria, folates are predominantly used for the biosynthesis of formate, which is later used for cytoplasmic one-carbon reactions, as well as for mitochondrial glycine biosynthesis (Fig. 2). Mitochondria also serve as a prominent folate reservoir accumulating as much as 40% of total cellular folates, which does not exchange with the cytosol (Fox and Stover, 2008; Lin et al., 1993). However, some communication does occur

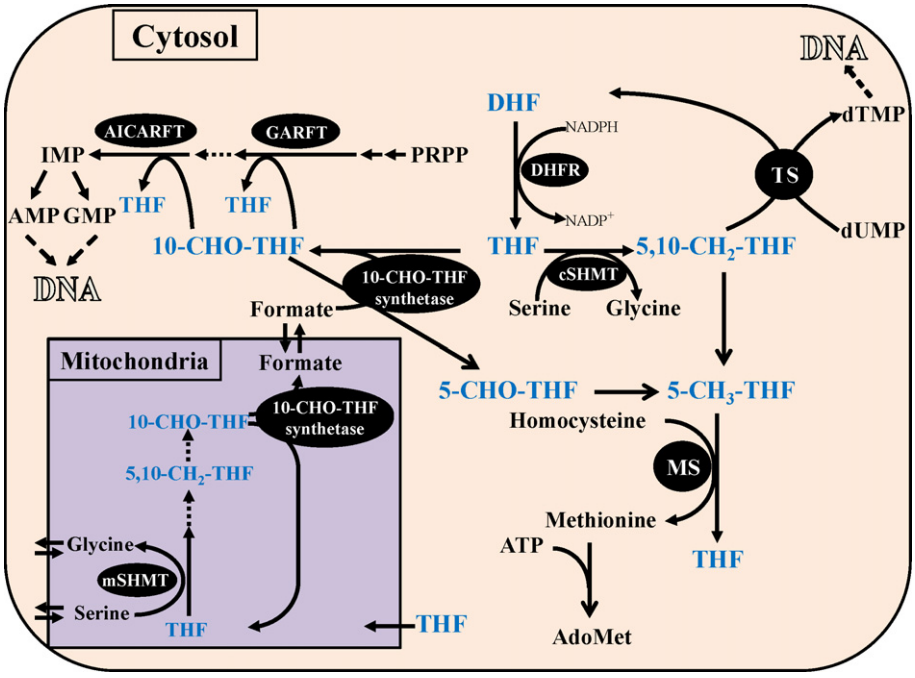


Fig. 2. Cellular folate metabolism and its compartmentalization in the cytosol and mitochondria.

between mitochondria and cytosol that is facilitated by the transport of one-carbon donor substrates such as formate, glycine and serine (Tibbetts and Appling, 2010) (Fig. 2).

1.2. Cellular folate transport and homeostasis

1.2.1. Transporters that mediate influx of folates

THF cofactors are negatively charged under physiological pH due to the ionization of the dicarboxylic glutamic acid moiety. Therefore, folate cofactors cannot cross the plasma membrane by passive diffusion and rely on specific uptake systems for their entry into the cell. Accordingly, three transport systems are currently known to accommodate folate uptake:

(a) The reduced folate carrier (RFC/SLC19A1): RFC was the first transporter to be described at the kinetic level (Goldman et al., 1968; Zhao et al., 2009a). RFC belongs to the major facilitator superfamily (MFS) of transporters, and more specifically to the solute carrier (SLC) family of facilitative carriers (Pao et al., 1998; Saier, 1999). Within the SLC19A sub-family resides RFC (SLC19A1) and its close homologues, thiamine transporters THTR1 (SLC19A2) and THTR2 (SLC19A3) (Diaz et al., 1999; Dutta et al., 1999; Fleming et al., 1999; Labay et al., 1999; Rajgopal et al., 2001). RFC is a 591 amino acids transmembrane protein with a molecular mass of ~85 kDa and 12 hydrophobic transmembrane domains (TMD) with short hydrophilic N-terminus and a long hydrophilic C-terminus, both of which reside in the cytoplasm (Cao and Matherly, 2004; Ferguson and Flintoff, 1999; Liu and Matherly, 2002). RFC contains a large cytoplasmic loop connecting TMD6 and TMD7. The human RFC is *N*-glycosylated at a single conserved consensus *N*-glycosylation site located at the loop connecting TMD1 and TMD2 (Asn58) (Wong et al., 1998). Although harboring a single *N*-glycosylation site, RFC undergoes heavy glycosylation at Asn58, which results in a ~20 kDa increase in its molecular mass (i.e. core MW of ~65 kDa vs ~85 kDa of the *N*-glycosylated form) (Wong et al., 1998).

RFC is devoid of an ATP-binding domain and hence its transport activity is not driven by direct ATP hydrolysis. Instead, it is a bidirectional antiporter that facilitates the exchange of folates with organic phosphates such as adenine nucleotides as well as thiamine mono- and pyrophosphate, which are largely retained within the cell (Fig. 3) (Goldman, 1971; Henderson and Zevely, 1983; Zhao et al., 2002, 2001b). This major asymmetry in the concentrations of organic phosphates across the plasma membrane constitutes the driving force for the uphill transport of folates into the cell via RFC (Goldman, 1971; Zhao et al., 2002, 2001b). Consistent with RFC activity as a folate/organic phosphate exchanger, impaired folate efflux was demonstrated under depletion of extracellular anions. This effect could be reversed upon restoration of extracellular anions including AMP, phosphate or thiamine pyrophosphate (Henderson and Zevely, 1983). Moreover, it was recently shown that ectopic overexpression of RFC resulted in an approximately 15-fold decline in cellular viability in medium lacking folates but not in folate-containing medium, further supporting the bidirectional transport activity of RFC (Ifergan et al., 2008). As reflected in its name, RFC exhibits relatively high transport affinity for reduced folates (Km = 1–3 μM), but poor affinity for the oxidized folate, folic acid (Km = 200–400 μM) (Sirotnak, 1985).

In humans, RFC is ubiquitously expressed in a variety of normal tissues and malignant tumors, including bone marrow, breast, lung, heart, small intestine and lymphocytes (Liu et al., 2005; Matherly and Angeles, 1994; Matherly et al., 1992; Whetstine et al., 2002a). Multiple cDNA isoforms were described for the human RFC, differing in their 5′-untranslated regions (UTRs) (Gong et al., 1999; Tolner et al., 1998; Whetstine et al., 2002a). Human RFC gene expression is driven by two minimal promoters (A and B) and a linker region that consists of 3 additional binding elements (Whetstine and Matherly, 2001). Minimal promoter A is localized within a 47 bp region (positions −3912 to −3959) and is regulated by an inducible cAMP-response element (CRE)/AP1-like element (CRE/AP1). Minimal promoter B resides within a 46 bp region, located upstream to the transcription start site (positions −4508 to −4550). This promoter contains a constitutive element and is regulated by a GC-box. Apart from the constitutive GC-box and inducible CRE/AP-1 element, additional promoter elements including AP-2, myeloid zinc finger 1 (Mzf-1) and E-box are contained within or near four

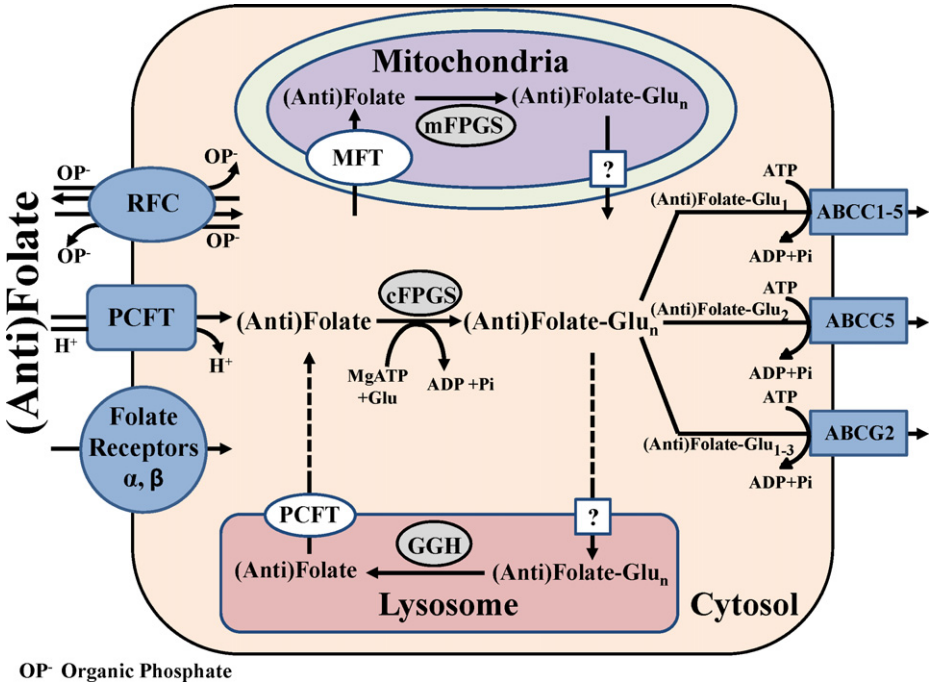


Fig. 3. Homeostasis of folates and cellular accumulation of antifolates. Influx and efflux transporters that transport (anti)folates. Once inside the cell, (anti)folates undergo polyglutamylation in the cytosol and mitochondria, whereas the counteracting process of hydrolysis occurs in the lysosome.

tandemly repeated sequences upstream of promoter A (Whetstine et al., 2002b).

Interestingly, a recent study suggested that RFC is crucial for the epigenetic regulation of neural crest development in *Xenopus* (Li et al., 2011). The authors demonstrated that inhibition of *Xenopus* RFC, blocked expression of a series of neural crest marker genes while overexpression of RFC or injection of 5-CH_3-THF expanded the neural crest territories. They also found that mono- and trimethyl-Histone 3-K4 levels were dramatically lower under RFC knockdown. These results provide a possible explanation for the neural tube defect phenotype seen in embryos of pregnant women consuming a folate-deficient diet. One could speculate that decreased synthesis of AdoMet could contribute to this reduced methylation effect.

(b) The proton-coupled folate transporter (PCFT/SLC46A1): Another route of folate uptake occurs via the PCFT gene product (Qiu et al., 2006) (Fig. 3). PCFT is a 459 amino acids transmembrane protein with a molecular mass of 55 kDa (Unal et al., 2008). Based on hydropathy analysis, the predicted membrane topology of PCFT reveals 12 TMDs with a large intracellular loop after TMD 6; the N- and C-termini reside in the cytoplasm. The large predicted extracellular loop between the first and second TMDs contains two consensus *N*-glycosylation sites which were further confirmed by *in vitro* experiments (Qiu et al., 2007; Unal et al., 2008). Targeted disruption of the consensus *N*-glycosylation sites decreased influx activity by 40%; however, lack of *N*-glycosylation had no effect on the plasma membrane targeting of PCFT (Unal et al., 2008).

PCFT was initially described as a heme transporter (influx Km = 125 μM) and was therefore named heme carrier protein-1 (HCP1) (Shayeghi et al., 2005). However, it was subsequently shown that the hemin transport capacity of PCFT is lower by two orders of magnitude compared to folate transport (Qiu et al., 2006); this was recently confirmed by the group of McKie who first cloned HCP-1 (Laftah et al., 2009). Hence, this gene is now referred to as PCFT due to its high transport affinity towards oxidized folates and reduced folates.

PCFT functions as a unidirectional symporter that co-transports folates along with protons into the cell (Qiu et al., 2006) (Fig. 3). The high concentration of protons in the acidic microclimate of the upper small intestine, which is partly facilitated by the activity of Na^+/H^+ exchangers, is the driving force for the uptake of folates (Zhao et al., 2009a). As predicted from its proton-coupled transport capacity, PCFT operates optimally at an acidic pH of 5.5, with folate transport activity increasing as the pH decreases, as shown in mammalian cells and *Xenopus* oocytes (Qiu et al., 2006). Unlike RFC, PCFT exhibits high affinity for both folic acid and 5-CH_3-THF at pH 5.5 (Km ~ 0.5–1 μM). Although the highest transport activity of PCFT was documented at pH 5.5, some transport activity was also observed at physiological pH (i.e. pH 7.4; Lasry et al., 2008; Zhao et al., 2009a). PCFT was recently reported to form homo-oligomers that appear to possess functional transport activity (Hou et al., 2011).

A recent study addressed the extent to which PCFT contributes to the transport and pharmacological activity of the folic acid antagonist (i.e. antifolate) pemetrexed as well as other antifolates, relative to the contribution of the ubiquitously expressed RFC at physiological pH (Zhao et al., 2008). Either PCFT or RFC cDNAs were stably transfected into a folate transporter-null HeLa cell variant to achieve transport activities similar to their endogenous function in parental HeLa cells. PCFT and RFC produced comparable increases in pemetrexed cytotoxicity in growth medium containing 40 nM 5-formyl-THF. However, PCFT had little or no effect on the activities of MTX, raltitrexed and PT523 in comparison with RFC, irrespective of the folate growth source that was either folic acid or 5-formyl-THF. Hence, when cells were grown with 5-formyl-THF as the sole folate source, RFC alone could fully restore activity to the above antifolates. In contrast, pemetrexed was the only antifolate for which PCFT fully restored pharmacological activity. However, it is possible that at the acidic microenvironment of solid tumors, PCFT may have a more significant cytotoxic effect for antifolates other than pemetrexed. It was further found that PCFT, expressed at high levels in *Xenopus laevis* oocytes and in folate transporter-competent HepG2 cells, exhibited a high affinity for pemetrexed, with remarkable influx Km values of 0.2–0.8 μM at pH 5.5. PCFT markedly increased the growth inhibitory activity of pemetrexed, but not that of the other antifolates in HepG2 cells grown with 5-formyl-THF at physiological pH. These findings illustrate the unique role that PCFT plays in the transport and pharmacological activity of pemetrexed.

The cloning of the human PCFT gene paved the way for the discovery that inactivating mutations within the PCFT coding region are the genetic cause for the rare autosomal recessive disorder, hereditary folate malabsorption (HFM; OMIM 229050) (Atabay et al., 2010; Lasry et al., 2008; Meyer et al., 2010; Qiu et al., 2006; Shin et al., 2011, 2012; Zhao et al., 2007). HFM is a folate deficiency syndrome caused by impaired intestinal folate absorption. Patients with HFM present with low folate levels in the blood and cerebrospinal fluid (CSF). HFM manifests within the first few months of life with anemia, diarrhea, hypogammaglobulinemia, severe infections and failure to thrive. Moreover, due to the poor folate levels in the CSF and hence the impaired folate uptake into the central nervous system (CNS), neurological abnormalities and deficits occur including seizures and mental retardation. Unless the condition is diagnosed early and treated with high doses of oral folates, HFM is fatal, or neurological deficits become permanent.

Consistent with its main role in intestinal folate absorption, the highest PCFT expression can be observed in the upper small intestine, mainly in the duodenum and the upper jejunum. PCFT is also expressed in a wide variety of tissues including the kidney, liver, placenta and spleen, and to a lesser extent, colon and testis (Qiu et al., 2006). PCFT gene expression in human tumor cell lines was also examined; it was found that the colorectal cancer cell line, Caco-2, shows the highest level of PCFT gene expression. High expression was also documented in HeLa-R5 (a derivative of HeLa cells with a genomic deletion of the RFC locus), SW620 and A549 tumor cells. HeLa and MCF-7 cells exhibited moderate levels of PCFT expression. In contrast, PCFT mRNA levels in the two T-cell leukemia lines CCRF-CEM and Jurkat were essentially undetectable (Gonen et al., 2008). This has led to the identification of a dense, 1085 bp CpG island, present in the PCFT promoter and the coding region (nucleotides −600 to +485) which upon methylation plays a key role in the silencing of the PCFT gene (Gonen et al., 2008). It was specifically shown that this CpG island is densely methylated in the above human leukemia cell lines which fail to express PCFT; however, this CpG island was shown to lack methylation in PCFT-expressing cell lines (Gonen et al., 2008). Interestingly, these results were in complete concordance with a previous study which found that a spectrum of human carcinoma cell lines displays a prominent folate transport activity at acidic pH (5.5) that exceeded the influx obtained at pH 7.4 (Zhao et al., 2004b). In contrast, folate transport activity at pH 7.4 in CCRF-CEM leukemia cells exceeded the influx obtained at pH 5.5. These results suggested that whereas PCFT is expressed at substantial levels in various carcinoma cell lines, it is poorly expressed in leukemia cells. Furthermore, a recent study which examined PCFT gene expression in a wide array of 53 human tumor cell lines found similar results (Kugel Desmoulin et al., 2011). PCFT was found to be expressed in the majority of human solid tumor cell lines of different origins, however, low PCFT transcript levels exist in human leukemias, including ALL and acute myeloid leukemia (Kugel Desmoulin et al., 2011). The highest levels of PCFT transcripts were observed in Caco-2 (colorectal

adenocarcinoma), SKOV3 (ovarian carcinoma), HepG2 (hepatoma), and H69 (small cell lung cancer) cells (Kugel Desmoulin et al., 2011).

The promoter of the PCFT gene was characterized using a sequential deletion analysis which identified a 271 bp fragment upstream to the first ATG that drives the same promoter activity obtained with a large 3.1 kb fragment. Further refinement showed that the minimal PCFT promoter localizes to only 157 bp (Stark et al., 2009a); these results were corroborated in an independent study (Diop-Bove et al., 2009). The basal promoter was shown to be rich in functional GC-box sites which play an important role in regulation of PCFT gene expression (Stark et al., 2009a). PCFT was also found to be regulated by a more selective transcription factor in certain tissues. In this respect, a vitamin D(3) and vitamin D receptor (VDR) response element was shown to increase intestinal PCFT gene expression, resulting in enhanced cellular folate uptake (Eloranta et al., 2009). Moreover, treatment with vitamin D(3) resulted in increased PCFT mRNA levels, both *in vitro* and *ex vivo*. The VDR response element in the PCFT promoter was identified and localized to nucleotides −1694 to −1680 (Eloranta et al., 2009).

Another interesting level of regulation of PCFT gene expression emerged with the discovery that PCFT is regulated by Nuclear Respiratory Factor-1 (NRF-1), the dominant transcription factor regulating mitochondrial biogenesis and respiration (Gonen and Assaraf, 2010). In this study, three functional NRF-1 binding sites residing within the basal promoter of the PCFT gene were characterized. These consensus NRF-1 binding sites proved to be functional as NRF-1 was found to bind to these sites. Targeted mutational inactivation of these NRF-1 binding sites resulted in 60% decrease in promoter activity. Consistently, overexpression of NRF-1 or a constitutively active NRF-1 VP-16 construct resulted in increased reporter activity and increased PCFT mRNA levels. Conversely, introduction of a dominant-negative NRF-1 construct markedly repressed reporter activity and PCFT mRNA levels; likewise, introduction of NRF-1 siRNA duplexes to cells resulted in decreased PCFT transcript levels. These findings provide a molecular and functional linkage between mitochondria biogenesis and folate metabolism, thereby revealing that PCFT is an NRF1-responsive gene that behaves like a mitochondrial gene.

(c) Folate receptors (FRs): FRα, FRβ and FRγ: The third route of folate uptake proceeds via the family of folate receptors (FRs). FRs are high-affinity folate-binding membrane glycoproteins, encoded by three different genomic loci including FRα, FRβ and FRγ (Fig. 3). There is ~70–80% amino acid homology between the FRs and they contain 245–257 amino acids as well as several *N*-glycosylation sites (Reviewed in Elnakat and Ratnam, 2004, 2006). FRα and FRβ are glycosylphosphatidylinositol (GPI)-anchored cell surface glycoproteins, involved in folate transport, whereas FRγ is a secreted protein (Shen et al., 1995).

Among folate transport routes, FRα and FRβ display the highest documented binding affinity for folic acid (Kd = 0.1–1 nM) (Wang et al., 1992). Folate uptake mediated by FRs proceeds via receptor-mediated endocytosis (Kamen et al., 1988; Lu and Low, 2002; Rothberg et al., 1990; Salazar and Ratnam, 2007). In this respect, it was recently suggested that PCFT plays a role in FRα-mediated endocytosis by exporting folates from acidic folate-containing endosomes into the cytoplasm (Zhao et al., 2009b). From a physiological point of view, it is less clear what the role of FRs is when co-expressed with RFC and/or PCFT as the folate transport rate via RFC is 100-fold faster than that via FRs. It is possible that under folate-deficiency conditions, or in tissues with low RFC and PCFT expression, the activity of FRs becomes more significant.

FRα (FOLR1) expression is mostly observed in epithelial cells of the uterus, placenta, choroid plexus, retina and kidney (Parker et al., 2005; Ross et al., 1994; Salazar and Ratnam, 2007). FRα is also expressed in a variety of cancers, mainly of epithelial origin, including adenocarcinomas of the ovary, cervix, uterus, endometrium, kidney, lung, breast, bladder and pancreas (Elnakat and Ratnam, 2004, 2006; Parker et al., 2005; Ross et al., 1994). The FRα gene is regulated by two promoter regions, one that resides upstream to exon 1 and is named P1, whereas the second, located upstream of exon 4 is termed P4 (Elwood et al., 1997; Kelemen, 2006; Saikawa et al., 1995). *in vitro* studies have shown that FRα is positively regulated by extracellular folate depletion (Kane et al., 1988) and elevated levels of homocysteine (Antony et al., 2004). In addition, steroid hormone regulation of FRα gene expression was also demonstrated; in this respect, FRα was shown to be down-regulated by the estrogen receptor (Kelley et al., 2003; Rochman et al., 1985), while being up-regulated by both retinoic acid (Bolton et al., 1999) and the glucocorticoid receptor (Kelemen, 2006; Tran et al., 2005). Similarly, FRβ (FOLR2) also displays a restricted pattern of tissue-specific gene expression in placenta, thymus, spleen and various malignant cells of myelomonocytic lineage (Elnakat and Ratnam, 2004; Ratnam et al., 1989; Ross et al., 1994, 1999; Shen et al., 1994). Unlike FRα, the FRβ gene is regulated and expressed from a single promoter that encodes a single transcript, by the regulation of Sp1 and GA-binding protein (GABP) (Sadasivan et al., 1994). It was demonstrated that FRβ can be highly up-regulated (20-fold) by all-trans retinoic acid in a dose-dependent and reversible manner in the absence of terminal differentiation or cell growth inhibition (Wang et al., 2000). Furthermore, the molecular mechanism of transcriptional induction of FRβ by all-trans retinoic acid was also established (Hao et al., 2003).

The restricted pattern of tissue-specific expression of both FRα and FRβ renders them attractive candidates for diagnostic purposes and selective delivery vehicles of therapeutic agents in malignant and non-malignant disorders, hence minimizing toxic side effects in non-target healthy tissues, as will be discussed below (Hilgenbrink and Low, 2005; Paulos et al., 2004; Xia and Low, 2010; for further FR-based applications see Section 4).

1.2.2. Transporters that mediate folate efflux

Forty nine ATP-binding cassette (ABC) transporters exist in humans, all of which are transmembrane proteins that couple ATP hydrolysis to the transport of endogenous and exogenous substrates across cellular membranes. ABC transporters are classified into seven families, one of which is the important family of multidrug resistance (MDR) proteins (MRP/ABCC), that is comprised of 13 members (reviewed in Borst and Elferink, 2002; Deeley et al., 2006; Gottesman et al., 2002; Szakacs et al., 2006). Members of the MRP/ABCC family confer MDR to various hydrophobic and hydrophilic cytotoxic compounds by acting as low affinity, high capacity ATP-dependent drug efflux pumps (Reviewed in Assaraf, 2006).

Folates are among the various transport substrates of MRP1-5 (Fig. 3); it has been demonstrated that these efflux pumps have the ability to export THF, 5-CH_3-THF, 5,10-CH_2-THF and 10-CHO-THF out of the cell (Assaraf, 2006; Chen et al., 2002; Hooijberg et al., 2003; Kusuhara et al., 1998; Zeng et al., 2001, 2000). MRP1-5 are also capable of exporting antifolates (Fig. 4 and Table 1). Overexpression of MRP1-5 may result in antifolate resistance as will be elaborated in section 3 (Assaraf, 2006). Another ATP-driven efflux transporter that is capable of exporting folates and antifolates is the breast cancer resistance protein (BCRP/ABCG2), which also belongs to the ABC superfamily of efflux pumps (Fig. 3). Like the MRPs, BCRP functions as a high capacity, low-affinity exporter of multiple large hydrophobic or amphiphilic substrates, harboring some positive or negative charge. Among its substrates are anticancer drugs including anthracyclines as mitoxantrone, irinotecan (SN-38),

topotecan, antifolates as well as vitamins such as folates and riboflavin (reviewed in Assaraf, 2006; Polgar et al., 2008).

1.3. Cellular retention of folates

Following cellular uptake of folates via the influx routes described above, THF cofactors and antifolates undergo a unique metabolism known as folylpolyglutamylation catalyzed by folylpoly-γ-glutamate synthetase (FPGS) (Fig. 3). This enzyme catalyzes the MgATP-dependent, sequential addition, of multiple equivalents of glutamic acid to the γ-carboxyl chain of THF cofactors and glutamate-containing antifolates (Baugh et al., 1973; McBurney and Whitmore, 1974; McGuire et al., 1980; Moran, 1999; Moran et al., 1976).

FPGS has three known functions in mammalian cells; (a) enhanced cellular retention; polyglutamate congeners of THF and antifolates are polyanions and therefore are incapable of traversing the lipid bilayer by passive diffusion. Additionally, long chain (>3) polyglutamate derivatives are no longer substrates of efflux transport systems including the ATP-driven efflux transporters of the MRP/ABCC family (MRP1-5) (Wielinga et al., 2005; Zeng et al., 2001) and ABCG2 (ABCG2/BCRP) (Volk and Schneider, 2003); consistently, polyglutamate conjugates of THF and antifolates are not transport substrates of RFC (Matherly and Goldman, 2003). Consequently, polyglutamate conjugates of THF cofactors and antifolates are efficiently retained within cells. (b) FPGS exists in two compartmentalized isoforms, i.e. cytosolic (cFPGS) and mitochondrial (mFPGS) (Fig. 3). Polyglutamylation of folates by the mFPGS results in mitochondrial accumulation of folate polyglutamates, a process crucial for glycine biosynthesis which occurs exclusively in mitochondria (Lin et al., 1993). (c) The third function of FPGS concerns the bioactivity of polyglutamates of THF cofactors and antifolates; it is well established that most polyglutamate THF cofactors are much better substrates than their parent monoglutamate forms for various folate-dependent enzymes. This can result from either increased affinity and/or enhanced Vmax (Schirch and Strong, 1989). This is also true for antifolates where it has been shown that polyglutamate conjugates of antifolates display a markedly enhanced inhibitory capacity of their target enzymes (Allegra et al., 1985, 1987; Baggott et al., 1986; Jackman et al., 1991; Shih et al., 1997).

From a physiological point of view, the process of polyglutamylation is reversed by the counteracting activity of the lysosomal enzyme γ-glutamyl hydrolase (GGH). GGH is a secreted glycoprotein enzyme located in the lysosome. GGH catalyzes the hydrolysis of the γ-glutamyl tail of folate and antifolate polyglutamates (Fig. 3). GGH exhibits a Km value in the micromolar range and is a high-turnover enzyme that possesses a catalytic cysteine residue at the active site (Reviewed in Galivan et al., 2000; Schneider and Ryan, 2006).

2. Antifolates and their mode of action

Due to the major role that folates play in the *de novo* biosynthesis of purines and thymidylate, it was realized in the 1940s that antifolates are effective in the treatment of childhood acute leukemia (Farber et al., 1948). Decades afterwards, this discovery led to the development of a large group of rationally designed, new generation antifolates (Walling, 2006) (Fig. 4 and Table 1). These antifolates were designed to target and inhibit key folate-dependent enzymes, thereby leading to inhibition of nucleotide biosynthesis and consequent cell death (Walling, 2006). The current section describes the different antifolates that are used in the clinic and those that are currently developed or that are under different stages of clinical evaluation. This will include their uptake and efflux routes as well as their cellular target enzymes (Fig. 4 and Table 1). Moreover, special emphasis will be put on the molecular mechanisms of antifolate resistance (Section 3).

Antifolates can be generally divided into two main categories: polyglutamatable and non-polyglutamatable antifolates. Like naturally occurring folates, polyglutamatable antifolates also contain a glutamic acid residue and therefore undergo intracellular polyglutamylation. In contrast, non-polyglutamatable antifolates are devoid of a glutamate residue and thus cannot undergo polyglutamylation (Fig. 4 and Table 1).

2.1. Polyglutamatable antifolates

2.1.1. Methotrexate (MTX)

Although first introduced into the clinic in 1948, it took another two to three decades to completely understand the kinetics of the inhibitory interaction between 4-amino antifolates including aminopterin (AMT) and MTX and their target enzyme, DHFR. Animal studies published in 1956 showed that the therapeutic index of MTX was better than that of AMT, and the clinical use of AMT was thus abandoned in favor of MTX. MTX is currently used as an integral component of chemotherapeutic regimens that display substantial efficacy in the treatment of a number of malignancies including: breast cancer, head and neck cancer, non-Hodgkin lymphoma, osteosarcoma, bladder cancer, and choriocarcinoma (Assaraf, 2007), Moreover, MTX is also used for the treatment of autoimmune disorders including rheumatoid arthritis, psoriasis, psoriatic arthritis, systemic lupus erythematosus and Crohn's disease (Assaraf, 2007).

When studied in a cell-free system, it was demonstrated that MTX is a potent inhibitor of DHFR with a Ki of 5 pM (Appleman et al., 1988; Domin et al., 1982). However, it was soon realized that in order to completely block DHFR activity in living cells, a six orders of magnitude higher MTX concentration above the Ki value is necessary, i.e. ≥1 μM, (Goldman, 1974; Sirotnak and Donsbach, 1974; White et al., 1975). The basis for this difference was both experimentally elucidated as well as examined by computational models (Assaraf et al., 2006; Jackson and Harrap, 1973; Jackson et al., 1977; White, 1979; White and Goldman, 1981). Unlike in a cell-free system where there is a stoichiometric inhibition of DHFR by MTX, the relationship between MTX and DHFR is more complex in living cells as it is being influenced by multiple parameters including the levels of DHF and THF cofactors, as well as the ability to retain MTX within the cell. DHFR activity in mammalian cells is very high when compared to the activity of TS. As a result, DHF, which is the byproduct of TS activity, is retained at very low levels in the cell (~10 nM) (Moran et al., 1976) and is being efficiently reduced to THF by DHFR. As the Km of DHF for DHFR is in the range of 0.1–1 μM (Domin et al., 1982), this enzyme operates at a very low level of saturation, hence, only a very small fraction of DHFR activity is sufficient to maintain the cellular need for THF cofactors (~5% of total enzyme). Once MTX is taken up into cells via a carrier-mediated process (typically RFC and PCFT), it tightly binds DHFR with a very high affinity, thereby resulting in accumulation of DHF. In turn, DHF binds the MTX-unbound DHFR, still allowing appropriate synthesis of THF. When DHF accumulates to a concentration of ~10 μM (i.e. 1000-fold increase), it starts to compete with MTX for the residual amount of free DHFR critical for the maintenance of THF pool. As such, in order to achieve a complete inhibition of this small, but yet crucial fraction of DHFR, relatively high levels of intracellular MTX is needed (Jackson et al., 1977; White, 1979; White and Goldman, 1981).

Another parameter that has a major impact on the activity of MTX is polyglutamylation. The formation and relatively slow accumulation of MTX polyglutamates can markedly alter antifolate retention and cytotoxic activity since long-chain MTX

polyglutamate congeners can no longer be exported out of the cell (Assaraf, 2007, 2006). Unlike with some antifolates where polyglutamylation can lead to a markedly increased affinity to key folate-dependent target enzymes (as detailed below), MTX inhibition of DHFR is not affected by its polyglutamylation. Instead, polyglutamylation of MTX alters its target specificity by converting its polyglutamates into potent inhibitors of TS and AICARFT in addition to the potent inhibition of DHFR (Allegra et al., 1985, 1987; Baggott et al., 1986).

With the cloning of the mouse (Dixon et al., 1994), hamster (Williams et al., 1994) and human RFC (Moscow et al., 1995; Prasad et al., 1995; Wong et al., 1995), the ultimate proof that impaired RFC transport activity results in MTX resistance was revealed, establishing RFC as a major uptake route for this antifolate (Km = 2–26 μM) (Prasad et al., 1995; Wong et al., 1995). MTX is also transported by PCFT with comparable affinities (Km = 2 μM at pH 5.5) (Qiu et al., 2006) and to a markedly lesser extent by FRs; the MTX Ki values for FRα and FRβ were 1900 nM and 114 nM, respectively, determined by inhibition of [^{3}H]folic acid binding when compared to the folic acid Ki values in the subnanomolar range (Wang et al., 1992).

MTX is also a substrate of MRP1-5 (ABCC1 through ABCC5) and BCRP (ABCG2) (Bakos et al., 2000; Chen et al., 2002; van Aubel et al., 2002; Volk and Schneider, 2003; Wielinga et al., 2005; Zeng et al., 2001; for a comprehensive review see Assaraf, 2006).

2.1.2. Raltitrexed

Raltitrexed (Tomudex®; ZD1694) is a water-soluble, quinazoline-based antifolate and a potent inhibitor of TS (Ki = 62 nM) (Jackman et al., 1991) (Figs. 1 and 4). Raltitrexed is actively transported into cells by RFC and FRα (Westerhof et al., 1995b). In contrast, transfection of the human PCFT into transporter-null HeLa cells did not result in any increased cytotoxicity of raltitrexed, suggesting that PCFT does not play a significant role in ralitrexed uptake at physiological pH (Zhao et al., 2008). Upon entry into the cell, raltitrexed is rapidly polyglutamylated, a process that dramatically increases its inhibitory potency towards TS by a factor of 100-fold when comparing the tetraglutamate form to the parent monoglutamate form of ralitrexed. In support of its inhibitory activity of TS, the cytotoxic effect of raltitrexed can be reversed by the addition of thymidine or by a simultaneous administration of leucovorin (5-CHO-THF) (Jackman et al., 1991). Raltitrexed was approved in Europe, Canada and Australia for the treatment of advanced colorectal cancer; however, it is usually given only to patients with intolerance to 5-flurouracil (5-FU), a widely used fluoropyrimidine analogue (Cunningham et al., 2002; Thomas et al., 2003). In addition, following a successful phase II trial exploring the activity of raltitrexed either as a single agent or in combination with a platinum compound, a phase III trial was conducted, which demonstrated that a combination of raltitrexed and cisplatin improved the overall survival in malignant pleural mesothelioma (MPM), a solid cancer with poor prognosis. Hence, raltitrexed was registered for the treatment of MPM in several European countries (Surmont and van Meerbeeck, 2011).

2.1.3. Lometrexol

Lometrexol (5,10-dideazatetrahydrofolate, DDATHF) was the first class of GARFT inhibitors to be synthesized (Beardsley et al., 1989). It is a specific and potent inhibitor of GARFT with no inhibitory effect on either DHFR or TS, hence its cytotoxicity solely relies on inhibition of the *de novo* purine synthesis (Beardsley et al., 1989). As a result of GARFT inhibition, a rapid drop in intracellular ATP and GTP pools occurs which can be completely reversed by the addition of a preformed purine such as hypoxanthine (Pizzorno et al., 1991a). Lometrexol uptake into the cell is mediated via RFC and FR (Pizzorno et al., 1993; Westerhof et al., 1995a). Lometrexol is highly dependent on polyglutamylation for its cytotoxic activity and displays very slow elimination of polyglutamates (Pizzorno et al., 1991b). As such, impaired polyglutamylation due to decreased FPGS activity is considered a major mechanism of resistance to this antifolate (Pizzorno et al., 1995). One of the major obstacles in using lometrexol in the clinic is its high toxicity to healthy tissues and as such, severe side effects (Ray et al., 1993). Although lometrexol was administered with folate supplementation it was not further developed for cancer treatment (Roberts et al., 2000).

2.1.4. AG2034

Following pioneering studies with lometrexol, the first GARFT inhibitor, AG2034 (4-[2-(2-amino-4-oxo-4,6,7,8-tetrahydro-3H-pyrimidino[5,4-6] [1,4]thiazin-6-yl)-(S)-ethyl]-2,5-thienoyl-L-glutamic acid), a second generation, potent GARFT inhibitor was designed using knowledge of the X-ray crystal structure of GARFT from *E. coli* and of the GARFT domain of the human trifunctional enzyme (Boritzki et al., 1996). Human GARTF harbors three catalytic activities: phosphoribosylglycinamide formyltransferase, phosphoribosylglycinamide synthetase, and phosphoribosylaminoimidazole synthetase, all of which are essential for *de novo* purine biosynthesis. Computational analysis of the GARFT active site suggested that sulfur atoms should have particular affinity with two regions of the folate cofactor binding site. Thus, AG2034 was designed to fulfill this requirement, while retaining substrate activity for RFC and FPGS. Indeed, in preclinical studies, AG2034 was similar to lometrexol, in terms of GARFT inhibition (Ki = 28 nM) and FPGS substrate specificity (Km = 6.4 μM), but displayed higher binding affinity for FR (Kd = 0.0042 nM) (Boritzki et al., 1996). A recent study aimed to evaluate the membrane transport properties of GARFT inhibitors including AG2034 demonstrated that the latter displayed 4–5-fold better RFC transport efficiency than MTX. Moreover, AG2034 displayed a higher affinity towards FR than folic acid (Jansen et al., 2010). AG2034 demonstrated a broad spectrum antitumor activity in *in vitro* and *in vivo* model systems, with greater potency than lometrexol (Boritzki et al., 1996). AG2034 retained its pharmacological activity regardless of the intracellular folate pool size (Jansen et al., 2010). Interestingly, AG2034 exhibited preferential cytotoxicity against tumor cells lacking a functional G1 checkpoint (Zhang et al., 1998a). Recently, the anti-proliferative effect of AG2034 was shown to involve increasing phosphorylation of AMP-activated protein kinase, (AMPK); it was specifically shown that AG2034 activates p53 and AMPK, hence mediating the induction of signaling pathways leading to senescence in prostate cancer cells (Obajimi et al., 2009). However, dose-limiting toxicities in the form of mucositis, diarrhea and vomiting were observed at doses of ≥6 mg/m^2; significant levels of thrombocytopenia, neutropenia and anemia were also recorded (Bissett et al., 2001). Unfortunately, no objective antitumor responses were observed in this study. Based on these findings the development of AG2034 was terminated.

2.1.5. Pemetrexed

Pemetrexed (Alimta®, MTA, LY231514) is a rationally designed antifolate which is primarily a potent inhibitor of TS (Figs. 1 and 4). Pemetrexed was synthesized by Eli Lilly on the basis of lometrexol. Substitution of the 5-deazapteridine ring of lometrexol by a pyrrolopyrimidine ring yielded pemetrexed, which surprisingly turned out to be a potent inhibitor of TS, while exhibiting far less inhibitory capacity towards GARFT (Shih et al., 1997; Taylor et al., 1992). Subsequent studies with pemetrexed revealed that pemetrexed is quickly and efficiently taken up into tumor cells by RFC and especially PCFT (Qiu et al., 2006); pemetrexed was found to undergo a rapid and efficient polyglutamylation (Habeck et al., 1995; Taylor et al., 1992).

A key parameter that sets pemetrexed superior over MTX appears to be the very rapid polyglutamylation that begins when pemetrexed is present at very low intracellular concentrations. This effect is mediated by the superior kinetic characteristics of pemetrexed as an excellent FPGS substrate, i.e. pemetrexed exhibits 100-fold lower Km towards FPGS than does MTX (Habeck et al., 1995). This high affinity of pemetrexed for mammalian FPGS when compared to MTX was further demonstrated in murine L1210 leukemia cells (Zhao et al., 2000a). The cellular accumulation of both MTX and pemetrexed was examined; while the initial uptake rates of both antifolates were comparable, a steady-state was rapidly attained with MTX, whereas a continuous increase, albeit at a lower rate than initial, was documented for pemetrexed. This difference in cellular accumulation could be reversed by the use of an FPGS-deficient subline of L1210 (Chattopadhyay et al., 2007a; Zhao et al., 2000a). The polyglutamylation of pemetrexed also affects its inhibitory potency towards target enzymes such as TS. In this respect, it was demonstrated that the pentaglutamate form of pemetrexed displayed an 84-fold increase in the inhibitory potential towards TS, when compared to the parent monoglutamate form (Ki = 1.3 nM vs 109 nM, respectively) (Shih et al., 1997; Taylor et al., 1992). Apart from the increased inhibitory potency towards TS, pemetrexed polyglutamates also exhibit a significantly increased inhibitory activity towards GARFT (Ki = 65 nM for the pentaglutamate congener vs 9.3 μM for the monoglutamate form) and AICARFT (Ki = 265 nM for the pentaglutamate vs 3.6 μM for the monoglutamate form) (Shih et al., 1997). Pemetrexed also displayed an inhibitory activity against DHFR, however, no significant changes in the affinity of the mono- and pentaglutamate derivatives were observed (Ki = 7.2 nM for the pentaglutamate vs 7 nM for the monoglutamate) (Shih et al., 1997). Due to the superior ability of pemetrexed to inhibit several key folate-dependent enzymes, pemetrexed was termed "multitargeted antifolate" (MTA). However, one should note that on a molar basis, the inhibitory capacity of pemetrexed towards TS is 50-fold more potent than that towards GARFT, further confirming that TS is the primary target enzyme of pemetrexed, whereas GARFT is a secondary target.

Interestingly, a recent study discovered that pemetrexed has an additional secondary target enzyme AICARFT, (Racanelli et al., 2009). As a result of AICARFT inhibition by pemetrexed, its substrate, ZMP, markedly accumulated. ZMP is also a known activator of AMPK, which is a key component in the mammalian target of rapamycin (mTOR) pathway. Racanelli et al. (2009) demonstrated that ZMP accumulation induced an activation of AMPK with subsequent inhibition of mTOR and hypophosphorylation of the downstream targets of mTOR that control the initiation of protein synthesis and cell growth. These novel results suggest that the activity of pemetrexed in cancer cells comprises of both direct inhibition of folate-dependent enzymes and a more prolonged inhibition of the mTOR pathway.

Pemetrexed is transported into cells via RFC with a transport Km of 1.1 μM (Wang et al., 2004). Interestingly, pemetrexed is currently the antifolate with the highest documented transport affinity towards PCFT with a remarkable transport Km as low as 50 nM at the optimal pH of 5.5 (Chattopadhyay et al., 2007a; Wang et al., 2004). The major role that PCFT (initially termed the low pH folate transporter; (Sierra et al., 1997; Sierra and Goldman, 1998)) plays in the transport of pemetrexed was uncovered by the isolation of a HeLa subline with a genomic deletion of the RFC locus, termed HeLa-R5 (Zhao et al., 2004b). These HeLa-R5 cells exhibited a marked resistance towards MTX, raltitrexed and talotrexin, while remaining highly sensitive to pemetrexed (Zhao et al., 2004c). This sensitivity was due to the preservation of an efficient pemetrexed transport via PCFT, which retained a good transport affinity towards pemetrexed at physiological pH (Km = 12 μM at pH 7.4) but low transport affinity towards raltitrexed, MTX and talotrexin (Ki = 90, 100 and 250 μM, respectively). Furthermore, following MTX selection, a HeLa-R5 subline termed HeLa-R1 was further established which displayed a markedly decreased transport activity via PCFT (Zhao et al., 2004a). As expected, this subline exhibited markedly decreased influx of pemetrexed, further supporting the major role of PCFT as a transport route of pemetrexed, both in the presence or absence of RFC activity (Zhao et al., 2004a). This HeLa-R1 subline was subsequently found to harbor a silenced PCFT gene via promoter methylation (Diop-Bove et al., 2009); this finding was consistent with the original observation that the PCFT gene undergoes silencing in certain tumor cells via dense promoter methylation (Gonen et al., 2008).

Another important determinant that markedly affects the cytotoxic activity of pemetrexed is the intracellular folate pool size (Taylor et al., 1992; Zhao et al., 2001a). An expanded intracellular folate pool markedly decreases the cytotoxic activity of pemetrexed; this is due to competition between pemetrexed and the abundant cellular folates both for binding to target enzymes as well as for polyglutamylation by FPGS. It was shown that under conditions of expanded cellular 5-CHO-THF pools, there is a marked decrease in the polyglutamylation of pemetrexed, thereby leading to reduced inhibitory capacity of this antifolate (Zhao et al., 2001a). Consistently, another study in HeLa cells with loss of RFC transport activity, showed a major decrease in 5-CHO-THF levels; this resulted in a marked increase in the inhibitory capacity of pemetrexed on GARFT, an inhibition that highly relies on intact polyglutamylation (Zhao et al., 2005). Interestingly, a phase III clinical trial with pemetrexed and cisplatin in MPM showed different results as to folate supplementation (Vogelzang et al., 2003). This study examined whether or not treatment with pemetrexed and cisplatin results in a superior survival time to that achieved with cisplatin monotherapy in MPM patients. Whereas there was a clear improvement in survival time, time to progression, and response rates in the combination arm of pemetrexed + cisplatin, the toxicity was high. During this study, Eli Lilly amended the treatment protocol by adding folates and B_{12} to both arms. This resulted in significantly reduced toxicity without negatively affecting survival time (Vogelzang et al., 2003). Hence, during pemetrexed treatment of human malignancies including MPM, it is important to clinically evaluate folate vitamin supplementation, as it might have a significant effect on the cytotoxic activity of pemetrexed both in tumor cells and normal tissues.

In 2004, pemetrexed (Alimta®) was approved by the FDA and European Medicines Agency (EMA) for the treatment of MPM, in combination with cisplatin (Hazarika et al., 2004; Manegold, 2003). In 2008, pemetrexed was further approved by the FDA and EMA as the first line treatment for non-squamous, non-small cell lung cancer (NSCLC) in combination with cisplatin (Cohen et al., 2010, 2009). Furthermore, on 2009, the FDA approved pemetrexed for maintenance treatment of patients with locally advanced or metastatic non-squamous NSCLC whose disease has not progressed after four cycles of platinum-based first-line chemotherapy. The therapeutic activity of pemetrexed is currently being assessed in more than 132 ongoing clinical trials for the treatment of various solid tumors including breast cancer, gastric cancer, head and neck cancer, CNS lymphoma, ovarian cancer, advanced solid tumors and more (ClinicalTrials.gov) (Reviewed in Jarmula, 2010; Martin, 2006).

2.1.6. Pralatrexate

Pralatrexate (10-propargyl-10-deazaaminopterin; Folotyn®) was introduced as part of a rationally designed synthesis aimed at improving the cytotoxic activity of antifolates such as MTX. Sirotnak and colleagues discovered that structural modifications of the N^{10} position of 4-amino folates increased membrane transport of antifolates in mammalian cells but had little effect on the inhibition of the target enzyme DHFR (Schmid et al., 1985; Sirotnak

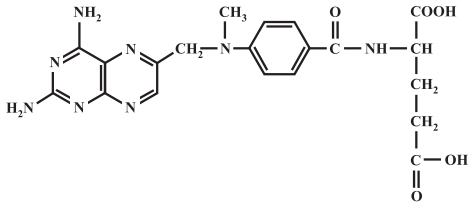
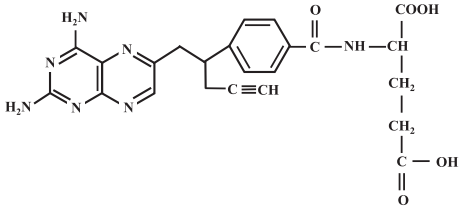
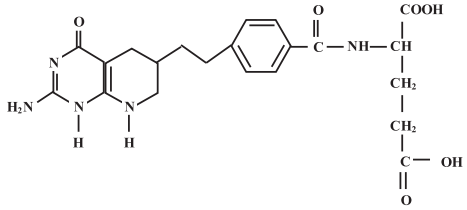
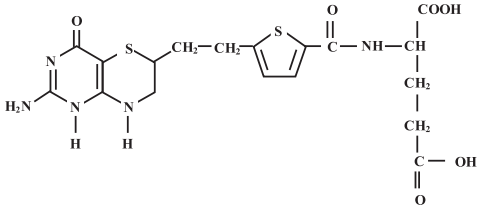
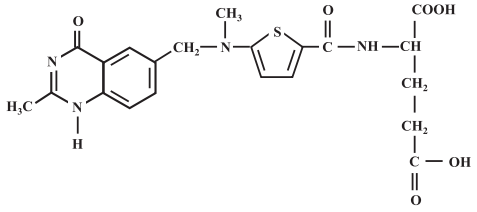
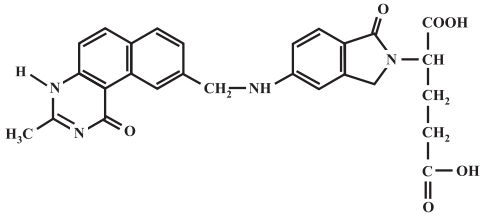
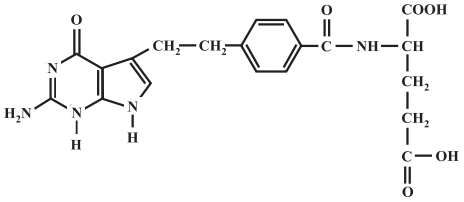
Polyglutamatable Antifolate		
Antifolate	**Chemical Structure**	**Target Enzyme**
Methotrexate		**DHFR**
Pralatrexate		**DHFR**
Lometrexol		**GARFT**
AG2034		**GARFT**
Raltitrexed		**TS**
GW1843		**TS**
Pemetrexed		**TS** **DHFR** **GARFT**

Fig. 4. Chemical structures of various polyglutamatable and non-polyglutamatable antifolates and the folate-dependent enzymes that they target.

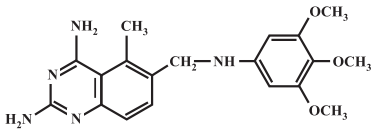
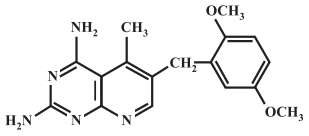
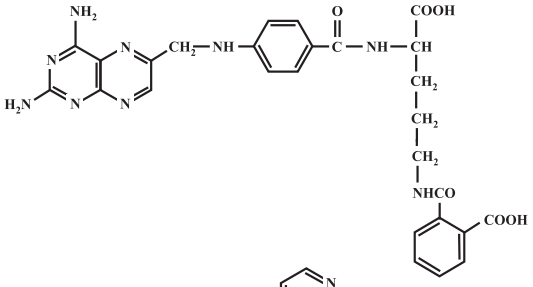
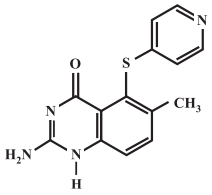
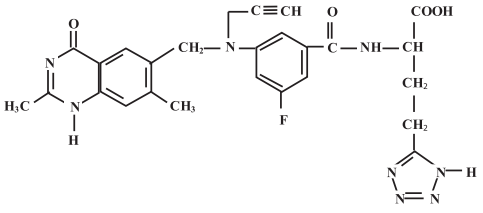
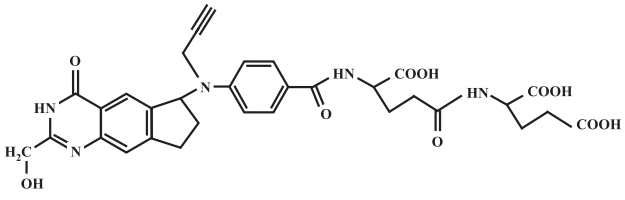
Non-Polyglutamatable Antifolate		
Antifolate	**Chemical Structure**	**Target Enzyme**
Trimetrexate		**DHFR**
Piritrexim		**DHFR**
Talotrexin		**DHFR**
Nolatrexed		**TS**
Plevitrexed		**TS**
BGC 945		**TS**

Fig. 4. *Continued*.

et al., 1984a,b). The improved activity of these antifolates was originally attributed to enhanced transport via RFC (Sirotnak et al., 1984a, 1987). However, it is possible that PCFT also contributed to membrane transport of pralatrexate. The addition of a propargyl moiety at the N^{10} position yielded the most favorable antifolate in this group with regards to uptake characteristics via RFC as well as cellular retention mediated by polyglutamylation (Sirotnak et al., 1998) (Fig. 4 and Table 1). Pralatrexate displayed greater *in vitro* and *in vivo* antitumor efficacy than MTX and edatrexate in murine tumor models and human tumor xenografts (Sirotnak et al., 1998). Pralatrexate has successfully passed phase I and II clinical trials in NSCLC (Krug et al., 2003, 2000). The efficacy and safety of pralatrexate have also been demonstrated in a prospective phase II study called PROPEL, in patients with relapsed or

Table 1
Summary of transport, polyglutamylation and target enzyme properties of various antifolates.

Antifolate	Synonyms	Target enzyme	Polyglutamylation	Transport system	Approved for treatment
Polyglutamatable					
Methotrexate	MTX	DHFR	+	RFC	+
Pralatrexate	Folotyn®	DHFR	+	RFC	+
Lometrexol	DDATHF	GARFT	+	RFC/FRα	–
AG2034		GARFT	+	RFC/FRα	–
Pemetrexed	Alimta®/PMX/MTA/LY231514	TS/DHFR/GARFT	+	PCFT/RFC	+
Raltitrexed	Tomudex®/ZD1694	TS	+	RFC/FRα	+
GW1843	GSL7904L/BW1843/1843U89/OSI-7904	TS	+	RFC	–
Non-polyglutamatable					
Trimetrexate	TMQ/Neutrexin®	DHFR	–	PD*	–
Piritrexim	PTX/BW3014	DHFR	–	PD	–
Talotrexin	PT523	DHFR	–	RFC	+
Nolatrexed	AG337/Thymitaq®	TS	–	PD	+
Plevitrexed	ZD9331/BGC9331	TS	–	RFC/FRα	–
BGC 945	ONX-0801	TS	–	FRα	–

*PD—Passive diffusion.

refractory peripheral T-cell lymphoma (PTCL) (O'Connor et al., 2009). On this basis, pralatrexate was granted an accelerated approval by the FDA for the treatment of patients with PTCL. Remarkably, this was the first chemotherapeutic drug approved for this specific hematological malignancy (Foss, 2011; O'Connor et al., 2011; Thompson, 2009). Building on the cytotoxic activity of pralatrexate with PTCL, a recent phase I study was completed in patients with previously treated, advanced NSCLC (Azzoli et al., 2011). Pralatrexate with folic acid and B_{12} supplementation was safely administered in previously treated NSCLC patients, and durable responses were observed. However, mucositis remained the dose-limiting toxicity of pralatrexate, and this study failed to demonstrate that vitamin supplementation prevents mucositis. There are several ongoing clinical trials with pralatrexate for the treatment of head and neck squamous carcinoma, non-Hodgkin's lymphoma, multiple myeloma and breast cancer (ClinicalTrials.gov).

2.1.7. GW1843

GW1843 (BW1843, 1843U89, OSI-7904, GSL7904L) is a very potent antifolate inhibitor of TS (Dev et al., 1994; Duch et al., 1993; Jackman and Calvert, 1995) (Fig. 4 and Table 1). It was developed using a series of benzoquinazoline folate analogues aimed at inhibiting TS and was confirmed to be a very potent inhibitor of human TS with a remarkable Ki of 0.09 nM (Duch et al., 1993). GW1843 is efficiently transported into human leukemia MOLT-4 cells via RFC with a remarkable transport Km of 0.33 μM (Duch et al., 1993). In addition, GW1843 exhibited potent cytotoxic activity in various tumor cell lines with IC_{50} values lower than 1 nM. Growth inhibition was reversed by provision of thymidine, indicating that TS was the sole site of inhibitory activity of GW1843 (Duch et al., 1993). Interestingly, although it is an excellent substrate for FPGS, the parent compound is as potent TS inhibitor as its polyglutamate conjugates (Hanlon and Ferone, 1996; Jackman and Calvert, 1995). Although FPGS displays high affinity towards the parent GW1843 compound, GW1843 mostly undergoes diglutamylation with approximately 80% of intracellular GW1843 present in the diglutamate form (Hanlon and Ferone, 1996). This was found to be due to a major fall in the affinity of the diglutamate form for FPGS when compared to the parent compound; i.e. the diglutamate congener of GW1843 was only 1/100 as an efficient substrate for further polyglutamylation as was the parent drug itself. In this respect, it was demonstrated, in two independent studies, that unlike raltitrexed and MTX, GW1843 retained high cytotoxic activity in FPGS-deficient human and mouse soft tissue sarcoma, leukemia and ovarian cancer cell lines (Jackman et al., 1995; Li et al., 1995). The diglutamate form of GW1843 can be retained in the cell for a longer period than the parent compound; however, it persisted for shorter time than did long-chain polyglutamates of other antifolates. As such, a more frequent administration protocol would be necessary in order to achieve a continuous inhibition of TS activity (Hanlon and Ferone, 1996).

Encouraging pre-clinical results with human tumor xenografts implanted in mice (Banks et al., 1994; Smith et al., 1999, 1995) led to phase I clinical trials. A phase I clinical trial was conducted with the aim to evaluate the pharmacokinetics of GW1843 and to seek preliminary evidence for anticancer activity in patients with advanced solid tumors before this drug was reformulated (Schwartz et al., 2001). In 2000, GW1843 was reformulated by encapsulation into liposomes in order to enhance its therapeutic index and for dose schedule convenience; the drug was then renamed OSI-7904L. Subsequent studies with OSI-7904L in a human colon adenocarcinoma xenograft model in mice demonstrated superior antitumor effects compared to the non-encapsulated drug. Due to better bioavailability of the liposomal-encapsulated drug, it could be administered less frequently than GW1843, while achieving greater tumor growth inhibition. Phase I clinical trials were conducted with OSI-7904L alone or in combination with other antitumor agents including cisplatin or oxaliplatin in patients with solid tumors (Beutel et al., 2005; Ricart et al., 2008) and advanced colorectal cancer (Clamp et al., 2008). OSI Pharmaceuticals conducted phase II clinical trials in patients with advanced gastric or gastro-esophageal junction adenocarcinoma (Falk et al., 2006), advanced biliary cancer (Ciuleanu et al., 2007) and metastatic or recurrent squamous cell carcinoma of the head and neck (SCCHN) (Hough et al., 2009).

2.2. Non-polyglutamatable antifolates

2.2.1. Trimetrexate

Trimetrexate (TMQ, Neutrexin®) is a nonclassical, lipophilic quinazoline derivative which was developed in order to overcome MTX resistance mediated by impaired drug transport (Fig. 4 and Table 1). Hence, although the glutamate residue was truncated and a trimethoxy substitution was introduced, TMQ still retained potent inhibition of DHFR (Bertino et al., 1979; Jackson et al., 1984; Marshall and DeLap, 1994). Being a lipid-soluble antifolate, TMQ is not a substrate for the dominant influx transporters RFC and PCFT; this lipophilic antifol efficiently enters cells by passive diffusion (Assaraf et al., 1989a; Fry and Besserer, 1988). As such, tumor cell lines displaying MTX resistance due to impaired transport, retain their sensitivity towards TMQ and frequently display a remarkable hypersensitivity to this antifolate due to diminished intracellular folate pools (Rothem et al., 2002). Moreover, although

leucovorin (5-formyl-THF) efficiently protects MTX-sensitive cells and healthy tissues from TMQ toxicity, MTX-transport defective tumor cells are poorly rescued by leucovorin as they cannot take up this reduced folate via their impaired RFC. In this respect, the Bertino group showed that SCID mice bearing MTX-transport defective human ALL cells can be successfully treated by a combination of TMQ and leucovorin with no toxic side effects to healthy tissues (Lacerda et al., 1995). As mentioned above, TMQ is devoid of a glutamate residue and hence is not a substrate for FPGS; this latter property renders it a superior antifolate when antifolate resistance emerges due to loss of FPGS activity (Liani et al., 2003). Liani et al. (2003) reported up to 14-fold hypersensitivity to TMQ in multiple human leukemia cell lines that acquired resistance to polyglutamatable antifolates due to loss of FPGS function and hence markedly diminished intracellular folate pool. However, due to lack of polyglutamylation and despite its lipophilicity, TMQ is not retained within the cell for prolonged periods; therefore, a longer exposure is required in order to obtain optimal DHFR inhibitory activity (Fleming and Schilsky, 1992).

In early studies it was shown that acquisition of TMQ resistance in cultured mammalian cells following multiple step selection to TMQ, occurs due to DHFR and ABCB1 gene amplification and consequent overexpression of both DHFR and P-glycoprotein (ABCB1) (Assaraf et al., 1989b). This and subsequent studies provided the first evidence that TMQ is a *bona fide* transport substrate of the MDR efflux transporter, ABCB1 (Arkin et al., 1989; Assaraf et al., 1989a,b). Additionally, overexpression of a mutant G482 and T482 but not the WT R482 of another MDR efflux transporter called ABCG2 (BCRP), conferred high level resistance to TMQ, along with other lipophilic antifolates, hence demonstrating that TMQ is a transport substrate of ABCG2 as well (Bram et al., 2006).

On 1993 TMQ co-administered with 5-formyl-THF, was approved by the FDA for the treatment of *Pneumocystis carinii* pneumonia in AIDS patients. In the oncology setting, TMQ had undergone broad phase II clinical testing, mainly for the treatment of solid tumors; the overall activity in most tumors was not favorable when compared to existing treatments (Reviewed in Takimoto, 1996). A recent Southwest Oncology Group phase II clinical trial explored the combination of TMQ, 5-FU, and leucovorin in gastric adenocarcinoma; it was found that this regimen achieved response rates that were comparable to other 5-FU-based regimens, when used in treatment of incurable gastric cancer; the untoward toxicity appeared manageable (Blanke et al., 2010).

2.2.2. Piritrexim

Similar to TMQ, piritrexim (PTX/BW301U) is a lipophilic antifolate which also inhibits DHFR (Fig. 4 and Table 1). PTX rapidly enters cells by diffusion, does not undergo polyglutamylation and has proven effective against cancer cells displaying MTX resistance due to impaired transport (Assaraf and Schimke, 1987; Taylor et al., 1985). Interestingly, this effect was not seen in other tumor cell lines including L5178Y/MTX or L1210/MTX R, where resistance to MTX was correlated with either overproduction of DHFR or with combined MTX transport defect and increased DHFR levels, respectively (Taylor et al., 1985). Resistance to PTX can be acquired due to increased DHFR expression (Assaraf et al., 1989b), mutations in DHFR that decrease the binding capacity of the drug (Lewis et al., 1995) and increased efflux due to increased expression of MDR efflux pumps (Klohs et al., 1986).

The antitumor effect of PTX was evaluated in a variety of Phase II clinical trials for the treatment of carcinoma of the urothelium (Khorsand et al., 1997; Lassiter et al., 2008; Roth et al., 2002), metastatic melanoma (Feun et al., 1991, 1995), glioma (Bleehen et al., 1995), head and neck carcinoma (Degardin et al., 1994; Uen et al., 1992; Vokes et al., 1991), metastatic breast cancer (de Vries et al., 1993) as well as soft tissue sarcoma (Schiesel et al., 1992). In summary, although PTX underwent many phase I/II clinical trials for the treatment of a variety of solid cancers, it failed to exhibit any therapeutic superiority and is therefore no longer developed for clinical use.

2.2.3. Talotrexin

Talotrexin (PT523) and its close homologue PT632 (5,8-dideaza PT523) are extremely potent non-polyglutamatable DHFR inhibitors (Fig. 4 and Table 1). Talotrexin is similar in structure to aminopterin; however, it contains a side chain that contains a hydrophobic aromatic ring harboring an acid group (Rosowsky et al., 1991). Talotrexin is a very potent inhibitor of DHFR (Ki = 0.35 pM), with 11-fold and 15-fold lower Ki values than those obtained with aminopterin and MTX, respectively (Rosowsky et al., 1998).

Talotrexin and its close homologues utilize the RFC for their cellular entry and the transport Km values of these compounds for RFC are approximately 10-fold lower than that of MTX (Ki = 0.7 μM for talotrexin, determined by inhibition of [^{3}H]MTX uptake) (Wright et al., 2000, 2003). Consistently, resistance to talotrexin in human leukemia cells occurs primarily due to impaired transport via RFC; this was mediated by several alterations in RFC including transcriptional silencing, inactivating mutations, and allele loss (Kaufman et al., 2006; Rothem et al., 2002). In contrast, PCFT displays a very poor transport affinity for talotrexin and its close homologue PT632 (Zhao et al., 2004c, 2008).

Talotrexin is devoid of a glutamic acid side chain and therefore cannot undergo polyglutamylation. In addition, due to its very high affinity for DHFR, talotrexin is less affected by the intracellular THF pool size, when compared to MTX and TMQ (Rosowsky et al., 1998; Wright et al., 2000; Zhao and Goldman, 2003). A phase I study of talotrexin was conducted in patients with relapsed or refractory NSCLC, where talotrexin demonstrated acceptable tolerability with encouraging cytotoxic activity after multiple cycles of chemotherapy (Rocha Lima et al., 2006). The FDA has granted orphan drug status for talotrexin in patients with ALL to Hana Biosciences which subsequently became Talon Therapeutics. Two phase I clinical trials with talotrexin in solid tumors, leukemia and NSCLC were recently withdrawn.

2.2.4. Nolatrexed

Drug discovery and development is an interdisciplinary, expensive and time-consuming process. Scientific advancements have revolutionized the way pharmaceutical research generates novel bioactive molecules. Advances in computational techniques and in parallel hardware support have enabled *in silico* methods, and in particular structure-based drug design techniques, to accelerate new target selection through the identification of hits to the optimization of lead compounds in the drug discovery process. In this respect, nolatrexed (AG337/Thymitaq®) is a nonclassical lipophilic inhibitor of TS that was rationally designed using such advanced X-ray structure-based methodologies in order to block the folate cofactor-binding site of TS (Fig. 4 and Table 1). Nolatrexed inhibited purified recombinant human TS with a Ki of 11 nM and displayed non-competitive inhibitory kinetics (Webber et al., 1996). Like other antifolate inhibitors of TS including raltitrexed, pemetrexed and plevitrexed, nolatrexed binds TS at the folate-binding site and not at the nucleotide-binding domain like 5-fluoro-dUMP (the bioactive form of 5-FU). A severe depletion of dTMP and a marked accumulation of dUMP occurs following treatment with nolatrexed, thereby resulting in cell death (Reviewed in Chu et al., 2003; Jarmula, 2010).

Due to its lipophilicity, nolatrexed enters cells by passive diffusion and since it is devoid of a glutamate residue it cannot undergo polyglutamylation (Webber et al., 1996). Nolatrexed was found to be a transport substrate of ABCB1 (P-gp) but not of MRP1 (Hu and

Chen, 2004; van Triest et al., 1997). Resistance to nolatrexed can be acquired via TS overexpression due to TS gene amplification as well as point mutations in TS that decrease nolatrexed binding to TS (Tong et al., 1998).

Two phase II clinical studies and a randomized phase III study comparing nolatrexed with doxorubicin for the treatment of advanced hepatocellular carcinoma (HCC) were conducted (Gish et al., 2007; Jhawer et al., 2007; Mok et al., 1999; Stuart et al., 1999). On that basis, nolatrexed was designated a fast track product for the treatment of unresectable HCC by the FDA in 2001, and by the EMA on 2007 (Reviewed in Niculescu-Duvaz, 2001). Enrolment for pivotal Phase III trial of nolatrexed compared to doxorubicin, for the treatment of patients with HCC was completed on 2005. Unfortunately, this trial failed to show survival benefit of nolatrexed over doxorubicin (Gish et al., 2007). Hence, the development of nolatrexed was discontinued in the US and Europe for such antitumor indications.

2.2.5. Plevitrexed

Plevitrexed (ZD9331, BGC9331) is a rationally designed, orally bioavailable, non-polyglutamatable quinazoline antifolate that is a selective inhibitor of TS with potent antineoplastic activity (Fig. 4 and Table 1). It was developed to have increased efficiency owing to its ability to overcome antifolate resistance that is due to decreased FPGS activity, in addition to having reduced toxicity presumably due to a lesser cellular retention than polyglutamatable TS inhibitors. Plevitrexed selectively binds to the folate binding site of TS with high affinity and blocks thymidylate biosynthesis (Ki = 0.4 nM, Jackman et al., 1997), resulting in inhibition of DNA synthesis and consequent apoptosis.

Plevitrexed is primarily transported into cells via RFC; consistently, it exhibited reduced cytotoxic activity in two leukemia cell lines (L1210:1565 and CEM/MTX) with low transport activity via RFC (Jackman et al., 1997). Plevitrexed can also be transported by FRα, albeit, to a lesser extent than via the RFC (Reviewed in Benepal and Judson, 2005). Consistent with its inability to undergo polyglutamylation, it was demonstrated that a mouse leukemia cell line (L1210:RD1694), with acquired resistance to raltitrexed due to impaired FPGS activity, retained sensitivity to plevitrexed (Jackman et al., 1997). Resistance to plevitrexed can result from both TS gene amplification and from impaired transport via RFC (Kobayashi et al., 1998).

Plevitrexed was assessed in several phase II clinical studies for the treatment of various solid cancers including metastatic colorectal cancer (Louvet et al., 2004), advanced colorectal cancer (Schulz et al., 2004), ovarian cancer (Rader et al., 2003), pancreatic cancer (Smith and Gallagher, 2003) as well as relapsed or refractory solid tumors (Hainsworth et al., 2003; Niculescu-Duvaz, 2001).

2.2.6. BGC 945

As FRα is highly expressed in 90% of ovarian cancers and in many other epithelial tumors (Reviewed in Antony, 2004; Elnakat and Ratnam, 2004; Jackman et al., 2004; Xia and Low, 2010), BGC 945 (ONX 0801) is considered an excellent selective agent for cancer treatment through the development of novel FRα-targeted antifolates. Indeed, a novel class of FRα-targeted TS inhibitors was synthesized all of which are cyclopenta[g]quinazoline-based compounds (Bavetsias et al., 2000), among which are BGC 638 (Theti et al., 2003) and BGC 945 (Gibbs et al., 2005). BGC 638 and BGC 945 are non-polyglutamatable antifolates with some structural similarity to plevitrexed (Theti et al., 2003) (Fig. 4 and Table 1). Both BGC 638 and BGC 945 have a modified glutamate moiety, in the form of L-Glu-γ-D-Glu dipeptide, with the D-enantiomer stabilizing them against enzymatic hydrolysis *in vivo* (Gibbs et al., 2005; Theti et al., 2003). Although displaying 5-fold lower inhibition of TS when compared to BGC 638 (BGC 945 Ki = 1.2 nM; BGC 638 Ki = 0.24 nM), BGC 945 was found to be superior to BGC 638 in *in vitro* studies in terms of its ability to target FRα-overexpressing tumor cells (Gibbs et al., 2005). Remarkably, the affinity of FRα for BGC 945 was 70% that of folic acid (Kd = 0.1–1 nM). Growth inhibition experiments in mouse leukemia L1210 cells demonstrated that the IC_{50} of BGC 945 (7.6 μM) was 30-fold higher than that of BGC 638. Additionally, RFC-negative L1210-FBP cells which overexpress FRα showed a marked sensitivity to BGC 945 (IC_{50} = 0.02 nM), which could be abrogated by addition of 1 μM folic acid, due to competition on transport via FRα. While the IC_{50} value of BGC 945 in the FRα-negative A431 cell line was as high as 7 μM, this value decreased significantly in FRα-overexpressing human tumor cell lines ($IC_{50} \sim$ 1–300 nM), demonstrating that BGC 945 is a potent TS inhibitor.

In vivo experiments in mice bearing human KB tumor xenografts demonstrated that due to the restricted pattern of FRα expression in the various tissues, the tumor was the only site of incorporation of 5-[^{125}I]-iodo-2′-deoxyuridine (Gibbs et al., 2005). These encouraging results indicated that BGC 945 is a selective inhibitor of TS in FRα-overexpressing tumors, and that the use of BGC 945 may result in minimal toxicity to healthy tissues (Gibbs et al., 2005). Onyx Pharmaceuticals initiated a phase I clinical study in the UK in 2009 in order to evaluate the safety and pharmacokinetics of BGC 945 in advanced solid tumors. This study may lead to further clinical trials with BGC 945, where it is expected to be mostly efficient in epithelial tumors overexpressing FRα, including ovarian cancer, lung cancer, breast cancer, and colorectal cancer.

3. Molecular mechanisms of antifolate resistance in cancer

Mechanisms of resistance to antifolates frequently emerge that hinder their clinical efficacy. Intensive molecular research provided deep mechanistic insights into the various mechanisms underlying resistance to various antifolates (Fig. 5). Notably, many of the mechanisms that were characterized *in vitro* were further identified *in vivo*, in patients who either presented with (i.e. inherent drug resistance) or developed resistance (acquired drug resistance) to antifolate-containing chemotherapy. The in-depth understanding of the molecular mechanisms underlying drug resistance enhanced the rational design of new generation antifolates, some of which were aimed at overcoming drug resistance (Section 2). The documented mechanisms of antifolate resistance are as follows (Fig. 5).

The following section describes and discusses the preexisting mechanisms of resistance or those that were acquired following antifolate-containing treatment.

3.1. Impaired antifolate uptake

One of the first documented mechanisms of antifolate resistance was impaired MTX transport (Assaraf and Schimke, 1987; Hakala, 1965; Hill et al., 1979; Niethammer and Jackson, 1975; Sirotnak et al., 1968, 1981). Since RFC is a major transporter mediating antifolate uptake into cells, it was not surprising to find that loss of RFC function was a frequent mechanism of antifolate resistance. This was associated with allele loss, inactivating RFC mutations, decreased expression or silencing due to loss of function of transcription factors. Consistently, RFC expression was found to be decreased in cancer patient specimens, hence resulting in antifolate resistance in several malignancies including ALL (Belkov et al., 1999; Gorlick et al., 1997; Kastrup et al., 2008; Levy et al., 2003; Rots et al., 2000; Zhang et al., 1998b), colorectal carcinoma (Wettergren et al., 2005), osteosarcoma (Guo et al., 1999; Ifergan et al., 2003; Yang et al., 2008b), diffuse large B-cell lymphomas

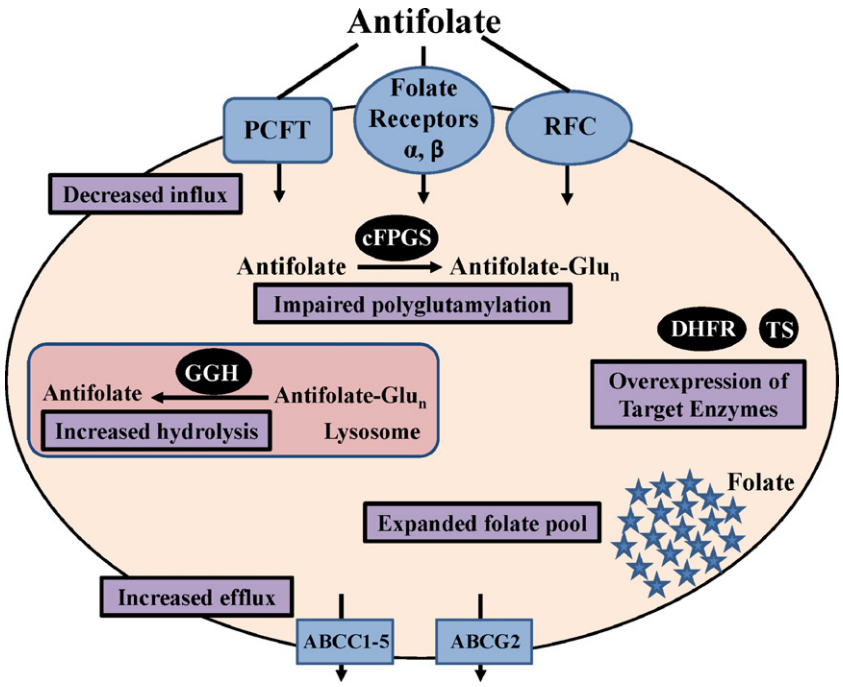


Fig. 5. Molecular mechanisms underlying antifolate resistance in cancer.

(DLBCL) (Kastrup et al., 2008) and primary central nervous system lymphoma (PCNSL) (Ferreri et al., 2004).

To date, no resistance mechanisms associated with the primary loss of function of PCFT were documented in antifolate-resistant cells. However, it is possible that under conditions where RFC is less active, PCFT may remain the primary route of entry, and thus resistance mechanisms that involve down-regulation of PCFT may evolve. Supportive evidence for this hypothesis stems from antifolate selection augmented by chemical mutagenesis in HeLa cells (Diop-Bove et al., 2009); in a HeLa cell line (HeLa R5), in which the RFC locus was genomically deleted following chemical mutagenesis and MTX selective pressure, growth inhibition by pemetrexed was fully preserved due to transport mediated by PCFT (Zhao et al., 2004b). However, these HeLa R5 cells displayed resistance to MTX and were highly cross-resistant to raltitrexed and talotrexin. When, in addition, PCFT was silenced due to a second round of MTX selection, the resultant HeLa R1 cell line now exhibited resistance to all of these antifolates (Zhao et al., 2004a).

As to the third route of entry, FRα and FRβ, there is paucity of data to suggest that alterations in FR expression could result in antifolate resistance. This can be attributed to the fact that under conditions where RFC and PCFT are expressed, the net impact of the relatively slow, yet high affinity FR would be small. However, in tumors with high level FR expression as in nasopharyngeal epidermoid carcinoma (KB) cells, it was demonstrated that selection with MTX can result in decreased level of the FRα protein, leading to decreased transport, increased IC_{50} of MTX and drug resistance (Saikawa et al., 1993). Furthermore, transfection of FRα into human breast carcinoma MCF-7 cells resulted in increased uptake and sensitivity to MTX (Chung et al., 1993). Another report demonstrated that a MTX-resistant KB subline (KB1BT) which exhibited 70–80% decrease in FRα mRNA was associated with DNA methylation; FRα expression could be restored by treatment with 5-aza-2′-deoxycytidine (Hsueh and Dolnick, 1994). While these reports suggest the involvement of other transport routes in mediating antifolate resistance, the vast majority of mechanisms of impaired antifolate transport involve quantitative and/or qualitative alterations at the RFC level as follows:

(i) Mutations in RFC that alter its transport activity.

The first inactivating mutation in the RFC gene was identified in MTX-resistant murine L1210 leukemia cells (Brigle et al., 1995). This tumor cell line exhibited a point mutation which resulted in the substitution of a proline for an alanine (Ala130Pro) in the highly conserved TMD3. This mutation resulted in a markedly decreased MTX transport due to decreased Vmax, associated with impaired carrier mobility. Interestingly, despite the presence of two RFC alleles, only the mutant allele was expressed, indicating that this cell line has acquired yet another alteration in RFC expression that presumably silenced the wild type allele; however, this putative alteration was not confirmed experimentally.

Among the frequent mechanisms of impaired transport were inactivating mutations in the RFC coding region, the majority of which mapped to TMDs (Drori et al., 2000; Jansen et al., 1998; Kaufman et al., 2006; Mauritz et al., 2002; Rothem et al., 2002; Roy et al., 1998; Zhao et al., 1998a,b, 1999). Some of these RFC mutations formed a mutation cluster region (i.e. "hot spot") in TMD1, supporting its key role in antifolate binding and/or substrate translocation (Drori et al., 2000; Jansen et al., 1998; Rothem et al., 2002; Zhao et al., 1998a,b, 1999). Several mutations exhibited a unique phenotype of reduced entry of antifolate either due to decreased transport affinity (higher Km) or lower transport Vmax, resulting in antifolate resistance. Remarkably however, they retained sufficient cellular folate transport to meet their folate requirement necessary to sustain DNA replication and cellular proliferation. The latter involved markedly increased affinity towards folic acid or reduced folates (Drori et al., 2000; Jansen et al., 1998; Zhao et al., 1998a,b). In one case it was shown that a 100-fold resistance towards GW1843 and MTX was acquired in human CCRF-CEM leukemia cells, through decreased RFC-dependent antifolate transport. Specifically, these cells exhibited 6-fold increase in the transport Km of GW1843 (decreased affinity), while conversely displaying 11-fold decrease in the Km for folic acid (increased affinity), including a slight increase in the Vmax for folic acid. By combining these opposite transport kinetic properties towards the antifolate and folic acid, cells were able to acquire resistance

to GW1843 and MTX, while allowing sufficient folic acid uptake to sustain cell growth and DNA replication. These cells adopted this unique antifolate resistance phenotype by acquiring three point mutations in amino acids located in TMD1 of RFC (Val29Leu, Glu45Lys and Ser46Ile) (Drori et al., 2000). Consistently, the same amino acid residues located at positions 45 and 46 were identified as mediating antifolate resistance in murine leukemia cells (Zhao et al., 1998a,b). Notably, substitution of amino acid 45 from Glu to Lys (Glu45Lys) was identified in both murine and human RFC, following antifolate selection (Drori et al., 2000; Jansen et al., 1998; Zhao et al., 1998a). MTX-resistant human leukemia cells harboring this mutation, showed markedly increased affinity to both folic acid and 5-CHO-THF (transport Km values decreased by 9- and 31-fold, respectively) as well as decreased transport Vmax. Hence, the unique Glu45Lys mutation increased the transport affinity towards folates, thereby selectively compensating for the decreased transport Vmax, resulting in the retention of sufficient cellular folate cofactor pools. In support of the above findings, upon gradual folate deprivation from the culture medium, this unique Glu45Lys mutant cell line exhibited up to 24-fold elevation in the folate pool compared with parental cells, owing to RFC overexpression due to 10-fold RFC gene amplification of this altered Glu45Lys RFC allele (Jansen et al., 1998). Consequently, the markedly expanded intracellular folate pool conferred a novel mechanism of resistance to polyglutamatable (e.g. ralitrexed, lometrexol, and AG2034) and to lipophilic antifolates (e.g. trimetrexate and pyrimethamine) by abolishing their polyglutamylation and circumventing target enzyme inhibition. Substitution of serine to asparagine in the adjacent amino acid residue 46 (Ser46Asn) also conferred MTX-resistance in mouse leukemia cells (Zhao et al., 1998b). L1210 cells were grown with 5-CHO-THF as the sole source of folates and upon chemical mutagenesis and MTX selection, a MTX-resistant clone was isolated; this clone displayed a markedly decreased MTX transport Vmax, with far less decrease in 5-CHO-THF transport, further confirming the differential preservation of folate influx while losing antifolate uptake (Zhao et al., 1998b).

Exploration of mechanisms of antifolate resistance mediated by RFC alterations in cancer patients undergoing MTX-containing chemotherapy, revealed that RFC mutations can also occur *in vivo* (Flintoff et al., 2004; Kastrup et al., 2008; Kaufman et al., 2004; Whetstine et al., 2001; Yang et al., 2003). Two large studies that examined the potential presence of RFC mutations as a mechanism of MTX resistance in ALL patients failed to identity frequent RFC mutations (Kaufman et al., 2004; Whetstine et al., 2001). In contrast, in a study with 162 osteosarcoma specimens obtained after treatment with high dose MTX, it was found that various heterozygous mutations in RFC are relatively common (Yang et al., 2003). Not only were missense mutations identified but also frameshift mutations were present. Interestingly, among these mutations was also the Ser46Asn, which was initially described *in vitro* with MTX-resistant mouse and human leukemia cells (Zhao et al., 1998b). A follow up study assessed the functionality of the altered RFC proteins encoded by these RFC mutant genes and found that some mutations may contribute to MTX-resistance in osteosarcoma (Flintoff et al., 2004). These alterations in the RFC gene in osteosarcoma specimens are consistent with previous findings that decreased RFC transcript is evident in half of osteosarcoma specimens at the time of diagnosis (Guo et al., 1999). Consistently, a significant correlation between low RFC levels at osteosarcoma diagnosis and poor histological response to preoperative chemotherapy was identified, hence suggesting that RFC levels at diagnosis may be a useful predictor of chemosensitivity (Ifergan et al., 2003). Another study that examined genetic alterations in RFC in a relatively small number of ALL as well as in large B-cell lymphoma (DLBCL) patients found a nonsense RFC mutation (Gln466X) in DLBCL, as well as a missense mutation (Ile417Thr) mapping to TMD11 in ALL specimens (Kastrup et al., 2008).

(ii) RFC Silencing via promoter methylation and 3′-UTR alterations.

The first indication for promoter methylation as a mechanism of antifolate resistance was documented in a human breast cancer cell line MDA-MB-231 which is known for its high DNA methylation status (Worm et al., 2001). This study demonstrated that this inherently MTX-resistant tumor cell line, which is completely devoid of RFC expression, harbors CpG island methylation in the RFC promoter region. Transfection of MDA-MB-231 cells with RFC cDNA restored MTX uptake and markedly increased MTX sensitivity by approximately 50-fold (Worm et al., 2001).

In support of these pre-clinical *in vitro* findings, some clinical studies with tumor specimens derived from primary central nervous system lymphoma, osteosarcoma and large B-cell lymphoma identified RFC silencing, mediated by promoter methylation, as a possible mechanism of MTX-resistance (Ferreri et al., 2004; Kastrup et al., 2008; Yang et al., 2008a,b). In a study with 66 osteosarcoma specimens, RFC promoter methylation was observed in 84% of the tumor samples (Yang et al., 2008b). Additionally, this study also characterized several heterozygous polymorphisms in the 3′-UTR of RFC that were associated with reduced RFC expression; these results provide some novel aspects as to the possible mechanisms of MTX resistance that involve RFC transcript alterations in osteosarcoma (Yang et al., 2008b). In contrast, other studies with antifolate-resistant tumor cell lines failed to detect RFC promoter methylation (Ding et al., 2001; Rothem et al., 2003).

(iii) RFC silencing via loss of function of transcriptional regulators.

The molecular characterization of the basal and the inducible promoters driving RFC expression (Whetstine and Matherly, 2001) paved the way for further investigation of the possibility that alterations in transcription factors may be possibly involved in decreased RFC expression and thereby contribute to antifolate resistance. Several studies with an assortment of transport-defective human leukemia cell lines displaying resistance to different antifolates, demonstrated multiple alterations in the activity of transcription factors known to regulate RFC expression; this resulted in down-regulation of RFC and consequent antifolate resistance (Kaufman et al., 2006; Rothem et al., 2003, 2004a,b; Stark and Assaraf, 2006). In this respect, it was shown that alterations in post-translational modifications, i.e. hyperphosphorylation of Sp1 and/or loss of CREB-1 phosphorylation resulted in a marked decrease in RFC mRNA levels, thereby leading to antifolate resistance. Consistently, treatment with inhibitors of Sp1 phosphorylation or with agents that enhance CREB-1 phosphorylation restored RFC expression (Rothem et al., 2004a; Stark and Assaraf, 2006).

(iv) Alterations in gene copy number of RFC in antifolate-resistant cells.

RFC is located in a telomeric region of chromosome 21 (21q22.2–q22.3) (Moscow et al., 1995; Yang-Feng et al., 1995). This telomeric localization of RFC along with the genomic instability, that is a hallmark of many cancers, may lead to alterations in both RFC copy number and in its genomic localization. Selection with various antifolates leading to antifolate resistance was due to genomic deletion, allele loss and translocation of the RFC genomic locus (Ding et al., 2001; Kaufman et al., 2006; Zhao et al., 2004c). Ding et al. (2001) studied MTX-resistant K562 cells with marked decrease in RFC mRNA and protein levels. It was found that decreased RFC expression was due to a combination of RFC

allele loss along with translocation of the remaining RFC allele with the formation of a 21/22 fusion chromosome (Ding et al., 2001). Whether or not RFC translocation or allele loss mediates antifolate-resistance in the clinic remains to be determined.

3.2. Loss of FPGS function as a mechanism of defective antifolate polyglutamylation and decreased cellular retention

Since polyglutamylation enhances cellular retention of antifolates, it is not surprising that loss of FPGS activity is an important determinant of resistance to polyglutamylation-dependent antifolates. A large number of studies demonstrated that MTX-resistant tumor cells, from various cell lineages, exhibit markedly reduced activity of FPGS, leading to reduced intracellular levels of MTX polyglutamates (Barakat et al., 1993; Li et al., 1992; Liani et al., 2003; Mauritz et al., 2002; McCloskey et al., 1991; McGuire et al., 1993; Pizzorno et al., 1989, 1988). Similarly, loss of FPGS activity was also documented in drug-resistant cancer cells following treatment with other polyglutamatable-dependent antifolates such as aminopterin, edatrexate, raltitrexed, pemetrexed, GW1843 and lometrexol (Cheradame et al., 1999; Drake et al., 1996; Liani et al., 2003; Lu et al., 1995; Mauritz et al., 2002; Pizzorno et al., 1995; Rumberger et al., 1990; Takemura et al., 1999). For some polyglutamatable-antifolates like MTX, polyglutamylation is mostly crucial for cellular retention but has little effect on the affinity of MTX towards its primary target enzyme DHFR; therefore, upon continuous exposure to MTX, a significant cytotoxic effect may be retained even in the presence of diminished FPGS activity (Barnes et al., 1999). However, in certain polyglutamatable-antifolates like raltitrexed and pemetrexed where polyglutamylation is required to enhance the binding affinity to the primary target enzymes, even a continuous exposure to the antifolate cannot restore antifolate sensitivity.

In a large study with multiple antifolates, fourteen antifolate-resistant sublines of the parental CCRF-CEM human leukemia cell line were isolated following a clinically relevant selection protocol of exposure to high dose intermittent pulses to polyglutamatable antifolates. Among these antifolates were the DHFR inhibitors MTX, aminopterin and edatrexate, the TS inhibitors raltitrexed and GW1843, the GARFT inhibitor lometrexol as well as the multitargeted antifolate pemetrexed which inhibits primarily TS but also targets DHFR and GARFT (Liani et al., 2003). Eleven out of the fourteen sublines exhibited antifolate resistance due to impaired FPGS activity with more than 90% loss of FPGS activity. Interestingly, one of these antifolate-resistant sublines harbored a Cys346Phe inactivating mutation in FPGS, which upon 3D modeling, mapped to the active site, therefore suggesting an interference with its catalytic activity. Indeed, this mutant Cys346Phe FPGS displayed a 23-fold decrease in the affinity towards its substrate L-glutamate. Four other sublines exhibited decreased FPGS mRNA levels, while the mechanism underlying loss of FPGS activity in the remaining sublines was not identified (Liani et al., 2003), but is currently under investigation.

Consistent with these findings, other studies have also demonstrated loss of FPGS activity, albeit in the absence of decreased FPGS mRNA levels (Drake et al., 1996; McGuire et al., 1995; McGuire and Russell, 1998; Pizzorno et al., 1995; Roy et al., 1997). This implies the presence of additional, yet unidentified post-transcriptional mechanisms or mutations involved in the regulation of FPGS transcript levels and catalytic activity. An example for such an impaired post-transcriptional modification emerged with the discovery that aberrant splicing of FPGS and hence marked loss of FPGS activity can emerge in MTX-resistant human leukemia cells *in vitro* and in leukemia patients (Stark et al., 2009b). In this study, MTX-resistant leukemia cell lines with markedly impaired FPGS activity were shown to have defective splicing of FPGS mRNA; the latter was based on intron retention and/or exon skipping, leading to premature translation termination. These *in vitro* results were further corroborated in childhood ALL patients, where exon 12 skipping was identified both at diagnosis and at relapse, the latter of which occurred following high-dose MTX-containing chemotherapy (Stark et al., 2009b). In this respect, it is well established that under high-dose, intermittent exposure to polyglutamatable antifolates including MTX, raltitrexed, and pemetrexed, antifolate resistance that is dependent on loss of FPGS function frequently occurs. This is in contrast to the continuous, low dose antifolate exposure, where mechanisms of antifolate resistance frequently arise due to impaired drug transport (Assaraf and Schimke, 1987; Drake et al., 1996; Liani et al., 2003; Lu et al., 1995; McCloskey et al., 1991; McGuire et al., 1995, 1993; Pizzorno et al., 1989, 1988). It is interesting to note that exon 12 skipping pre-existed in some ALL patients at diagnosis, albeit at low levels; this propensity may explain why FPGS-dependent mechanisms of drug resistance would frequently emerge following intermittent, high dose exposure to polyglutamatable-antifolates. Apparently, the severe selection imposed by intermittent, pulse exposure to high doses of antifolates does not allow for the *de novo* acquisition of novel mutations, thereby favoring the positive selection of pre-existent mechanisms. Owing to the genomic instability of leukemic cells, the pre-existence of such a modality of antifolate resistance in cancer cells but not in normal healthy cells, can be easily enhanced under high dose pulse exposure to antifolates. Therefore, the enhancement of a pre-existing mechanism would be superior to the *de novo* generation of a new mechanism, probably involving quantitative (decrease) or qualitative alterations (inactivating mutations) in RFC that would frequently lead to impaired antifolate transport.

Epigenetic regulation of the FPGS gene was recently shown to be involved in FPGS activity in ALL cell lines. Treatment with histone deacetylase (HDAC) inhibitors resulted in increased FPGS expression and enhanced intracellular accumulation of long-chain MTX polyglutamates (i.e. $Glu_{3\text{-}7}$) in ALL cells (Leclerc et al., 2010). This effect proved to be mediated via epigenetic mechanisms involving chromatin remodeling through a NFY-B- and Sp1-dependent recruitment of HDAC1 to the FPGS promoter (Leclerc et al., 2010). Whether or not this mechanism mediates MTX resistance in ALL patients warrants further investigation.

Inactivating mutations in FPGS could also constitute a mechanism mediating resistance towards polyglutamatable antifolates. However, as previously shown (Liani et al., 2003), only upon enhancement with a chemical mutagen, such mutations occurred in the FPGS gene; specifically, four murine leukemia L1210 cell lines resistant to lometrexol were isolated after pre-exposure to the mutagen *N*-methyl-*N*-nitrosourea (Zhao et al., 2000b). These variants exhibited >98% decrease in FPGS activity, where each cell line harbored distinct single point mutations in both FPGS alleles (Zhao et al., 2000b). Although inactivating mutations in FPGS do exist, they do not constitute a frequent mechanism of antifolate-resistance. In summary, loss of FPGS function infrequently mediated by inactivating mutations, decreased mRNA levels and/or post-transcriptional modifications such as aberrant splicing, constitutes an important determinant of resistance to polyglutamatable antifolates.

3.3. Increased expression of GGH

GGH cleaves the γ-glutamyl chain of both folates and antifolates, hence converting them into the monoglutamate form which renders them transport substrates of the MDR efflux pumps (Reviewed in Coward and McGuire, 2008; Galivan et al., 2000; Schneider and Ryan, 2006). Thus, under increased GGH activity, antifolates can be readily extruded out of the cell, thereby abolishing their cytotoxic activity. Notably, such increased GGH activity would result

in the same outcome for folates, i.e. diminished intracellular folate pool. Several early reports demonstrated that increased GGH activity can lead to decreased accumulation of long-chain antifolate polyglutamates, hence facilitating antifolate resistance; however, this was not a sole mechanism rendering antifolate resistance and these resistant cells also displayed the coexistence of both reduced FPGS activity and impaired RFC-dependent transport (Li et al., 1992, 1993; Rhee et al., 1993).

In an attempt to assess the contribution of increased GGH activity to antifolate resistance, GGH was ectopically overexpressed. Consequently, these transfectant cells overexpressed GGH transcript by 15–30-fold and displayed 15–90-fold increased GGH activity. Expectedly, these tumor cells exhibited a shift in the intracellular MTX pool towards the unmodified MTX (MTX-Glu_1) and MTX-Glu_2, whereas parental cells contained mostly long-chain MTX polyglutamates (MTX-Glu_{3-6}). The same was true for 5-CH_3-THF but to a lesser extent than for MTX. Surprisingly however, GGH overexpression did not confer resistance to short exposure to MTX (Cole et al., 2001). This failure to confer MTX-resistance can be mediated by the shrinkage in the folate pool and, therefore, enhancement of the cytotoxic effect of MTX. Therefore, it remains unclear whether increased GGH expression can be a primary determinant mediating MTX-resistance (Cole et al., 2001). However, recent studies provided ample evidence that epigenetic regulation and single nucleotide polymorphism (SNP) in the promoter and coding region of GGH can affect GGH expression and hence responsiveness to antifolate-containing therapy (Adjei et al., 2010; Chave et al., 2003; Cheng et al., 2006, 2004; DeVos et al., 2008; Hayashi et al., 2007; Organista-Nava et al., 2010). Collectively, alterations in the levels and activity of GGH can contribute to the outcome of the treatment with polyglutamatable antifolates. While reduced GGH activity would favor sensitivity to antifolates, increased GGH activity could lead to antifolate resistance. Further studies are warranted to conclusively determine whether or not increased GGH activity can mediate antifolate resistance as a primary mechanism of antifolate resistance in the clinical oncology setting.

3.4. Overexpression of DHFR and mutations that decrease its affinity towards antifolates

Many antifolates target DHFR, a key enzyme in the folate metabolic pathway, among which are aminopterin, MTX, pralatrexate, trimetrexate, piritrexim and talotrexin (see Section 2). One could postulate that increased DHFR levels would result in further elevation in the already high level of DHFR, leading to a markedly increased concentration of antifolate necessary to fully block DHFR activity. Indeed, overexpression of DHFR, sometimes mediated by DHFR gene amplification, was discovered both in cancer cell lines and in specimens from cancer patients as an underlying mechanism of MTX-resistance (Alt et al., 1978; Assaraf et al., 1992; Dolnick et al., 1979; Hamkalo et al., 1985; Horns et al., 1984; Kaufman et al., 1979; Matherly et al., 1997; Mini et al., 1985; Morales et al., 2009; Nunberg et al., 1978; Singer et al., 2000; Trent et al., 1984).

Two different genomic mechanisms account for DHFR gene amplification: the more stable one in which the DHFR amplicon resides on a chromosome is known as homogeneously staining region (HSR). This mechanism involves gene amplification that is typically located at the same locus of the original gene or translocated to another chromosomal region, hence being retained upon cell division and is therefore considered stable. DHFR gene amplification involving the latter mechanism was well documented following gradually increasing (stepwise selection) concentrations of MTX using a multitude of tumor cell lines (Dolnick et al., 1979; Mini et al., 1985; Morales et al., 2009; Nunberg et al., 1978). The second mechanism for DHFR gene amplification is an unstable form, involving DHFR amplicons residing on acentromeric circular DNA termed double minute chromosomes (DMs). DMs are small, acentric and autonomously replicating extrachromosomal DNA structures, which are therefore frequently lost upon cell division. Examples for DHFR gene amplification through the DMs mechanism are well documented (Haber and Schimke, 1981; Hamkalo et al., 1985; Kaufman et al., 1979; Morales et al., 2009; Singer et al., 2000).

The dynamics of acquisition of MTX resistance mediated by DHFR gene amplification in human colon adenocarcinoma HT29 cells was recently determined (Morales et al., 2009). The authors re-sensitized these MTX-resistant cells to MTX by leaving out drug selection, followed by re-exposure to MTX. They demonstrated passive loss of the DHFR amplicon upon withdrawal of MTX selection, which resulted in re-sensitization of these tumor cells to MTX. When subjected to a second cycle of MTX treatment, these tumor cells exhibited a reduced capacity to mount antifolate resistance. Cells which regained MTX resistance displayed a different structure of the amplicon, suggesting that the formation of the DHFR amplicon, as in the first cycle of treatment, is not feasible. These interesting findings suggest that MTX-resistant tumor cells may become responsive to a second round of treatment if left untreated during a sufficient period of time (Morales et al., 2009). Whether or not this mechanism of acquired sensitivity exists in the clinic requires further investigation. It is important to note that DHFR gene amplification was clinically observed in several cancers, including leukemia (Horns et al., 1984) and ovarian carcinoma (Trent et al., 1984) following MTX-containing chemotherapy.

Another level of regulation at the DHFR enzyme level emerged with the original discovery that apart from being a DHF reductase, human DHFR is also an RNA-binding protein (Chu et al., 1993a; Ercikan et al., 1993). Several early *in vitro* and *in vivo* studies found an increase in DHFR protein levels in response to MTX treatment (Bertino et al., 1965, 1970, 1963, 1962; Hillcoat et al., 1967). Interestingly, this increase in DHFR protein level, upon exposure to MTX, was unaffected by the transcriptional inhibitor actinomycin D but was blocked by the eukaryotic translation inhibitor cycloheximide, indicating the presence of a transcriptional-independent autoregulatory mechanism (Hillcoat et al., 1967). The ultimate support to this hypothesis emerged from *in vitro* translation assays which demonstrated that addition of exogenous DHFR protein to its own mRNA blocked its translation. Additionally, UV cross-linking assays showed that DHFR can bind its own mRNA (Ercikan-Abali et al., 1997). Based on the above, it was proposed that human DHFR protein can bind its own mRNA, resulting in translation inhibition. Addition of MTX which binds the DHFR protein with high affinity resulted in conformational changes which prevented the formation of the binary DHFR protein-mRNA complex. This enabled mRNA translation and resulted in increased DHFR protein levels (Appleman et al., 1988; Bystroff and Kraut, 1991). Further studies have identified the specific amino acids in the DHFR protein that mediate its binding to the mRNA (Tai et al., 2002), as well as amino acids that are essential for the upregulation of DHFR protein in response to antifolate treatment (Skacel et al., 2005). Furthermore, the *cis*-acting autoregulatory element in the human DHFR gene that allows the binding of the DHFR protein was also identified (Tai et al., 2004a, 2008). A 27-nt sequence (DHFR27, corresponding to nucleotides 407–433) was isolated using enzymatic footprinting assays and RNA-binding experiments, which was found to bind the DHFR protein with high affinity and specificity, forming a ribonucleoprotein complex. Transient transfection experiments using a luciferase reporter system revealed that DHFR27 RNA could repress luciferase gene expression in a DHFR-dependent manner when placed upstream of luciferase mRNA. This study provided

new insights into the essential molecular elements that mediate RNA-protein interactions and the autoregulatory loop. Collectively, these studies establish the presence of a translational autoregulation of DHFR that can be abolished by the presence of an antifolate, thereby leading to increased DHFR protein levels (Reviewed in Abali et al., 2008; Banerjee et al., 2002; Tai et al., 2004b). The association between the translational autoregulatory loop of DHFR and antifolate resistance needs to be further explored.

Decreased affinity of DHFR towards antifolates was also identified as a determinant of antifolate-resistance; however, this mechanism appears to be far less frequent than DHFR gene amplification (Albrecht et al., 1972; Flintoff and Essani, 1980; Goldie et al., 1980; Jackson et al., 1976). The kinetic properties of such an altered mouse DHFR isolated from MTX-resistant cells were previously described (Haber and Schimke, 1981). This altered DHFR displayed a 270-fold fall in the binding affinity for MTX, with only a 3-fold increase in the Km for its substrate DHF (Haber and Schimke, 1981), therefore, allowing sufficient THF synthesis while blocking the inhibitory activity of MTX. It was soon realized that this altered DHFR harbors a Leu22Arg mutation (Simonsen and Levinson, 1983). Subsequent studies described additional mutations in DHFR including Leu22Arg or Leu22Phe, Gly15Trp and Phe31Trp or Phe31Ser, all of which resulted in substantial MTX resistance (Dicker et al., 1990; McIvor and Simonsen, 1990; Melera et al., 1984; Melera et al., 1988; Miyachi et al., 1995; Srimatkandada et al., 1989). It is important to note that in some cases, the mutation in DHFR was accompanied by allele-specific gene amplification (Dicker et al., 1990; Haber and Schimke, 1981; Miyachi et al., 1995; Srimatkandada et al., 1989). As most mutations decreased the affinity of DHFR towards DHF, it is likely that gene amplification occurred in order to produce sufficient amounts of altered DHFR to allow for adequate levels of THF to be formed that can sustain cellular growth, DNA replication and cellular proliferation. Although mutations in DHFR were identified in MTX-resistant tumor cell lines, it is important to note that no such mutations were found in patients exposed to MTX-containing chemotherapy (Spencer et al., 1996).

3.5. Overexpression of TS and mutations that decrease its affinity towards antifolates

TS catalyzes the conversion of deoxyuridylate (dUMP) and 5,10-CH_2-THF to thymidylate (dTMP) and DHF. This reaction constitutes a crucial step in the *de novo* pyrimidine biosynthetic pathway resulting in the formation of dTMP. Due to its pivotal role in DNA replication, TS has proven a prime target for chemotherapy. Two distinct types of TS inhibitors exist: dUMP analogues such as 5-FU (Heidelberger et al., 1957) and antifolates. The later is composed of rationally-designed THF antagonists that potently inhibit TS activity, including raltitrexed, nolatrexed, GW1843, pemetrexed, plevitrexed and BGC 945 (see Section 2; (Jarmula, 2010)).

The molecular mechanisms underlying resistance to these antifolates include TS overexpression, sometimes due to gene amplification and/or polymorphism, and TS mutations that decrease its affinity towards the antifolate. In this respect, it was recently shown that increased expression of TS confers resistance to pemetrexed in NSCLC cell lines (Takezawa et al., 2011). Similarly to DHFR, TS gene amplification underlies the acquisition of resistance to different antifolates including ICI198583 (C2-desamino-C2-methyl-N10-propargyl-5,8-dideazafolic acid) (O'Connor et al., 1992), raltitrexed (Drake et al., 1996; Freemantle et al., 1995), and pemetrexed (Schultz et al., 1999; Sigmond et al., 2003; Wang et al., 2001).

Another mechanism involved in regulation of TS expression and sensitivity to fluoropyrimidines relates to polymorphisms in both the 5′-UTR and 3′-UTR of TS (Reviewed in Lurje et al., 2009; Zhou et al., 2012). The first polymorphism is a tandem repeat in the TS promoter enhancer region (TSER); this 28-bp sequence region can appear either as a double (2R) or triple (3R) tandem repeat (Horie et al., 1995). Moreover, a single-nucleotide polymorphism (G > C) within the second repeat of the 3R alleles also exists which might influence TS expression/activity and response to fluoropyrimidines. The second common polymorphism affecting TS levels is a 6-bp deletion in the 3′-UTR of the TS gene (TS1494del6) (Ulrich et al., 2000). It was proposed that alterations in the 3′-UTR may influence TS mRNA stability, thereby altering TS mRNA and protein levels (Ulrich et al., 2000). Several recent clinical studies in patients with colorectal cancer failed to find a consistent relationship between TSER polymorphisms and protein levels as well as with clinical outcome (Mauritz et al., 2009). Combined analysis of these studies revealed that while some correlation exists between TSER genotype and TS mRNA and protein levels in normal tissues, no such correlation is observed in malignant tissues (Galvani et al., 2011; Mauritz et al., 2009). Furthermore, a recent pooled analysis performed a quantitative study of the association between these two polymorphisms and cancer risk, including 63 studies for TSER polymorphism and 39 studies for the TS1494del6 polymorphism. Overall, these two polymorphisms failed to exhibit an association with cancer risk when pooled together; however, upon division to different authentic groups, such an association was found (Zhou et al., 2012). Gene amplification of these TS polymorphisms was also documented (Brody et al., 2006).

Recent studies explored the role of TS and excision repair cross-complementing group-1 (ERCC1) protein as predictors of chemotherapy responsiveness in MPM patients treated with pemetrexed/carboplatin (Righi et al., 2010; Zucali et al., 2011). This pemetrexed/platinum combination represents the standard of care in first-line treatment for MPM. Currently however, there are no established indicators of responsiveness that can be used to optimize the treatment. Thus, the primary goal of the first retrospective study was to assess the role of ERCC1 and TS in MPM tumors, and to correlate the expression levels of these key determinants of drug activity with the outcome of MPM patients treated with carboplatin/pemetrexed as first-line chemotherapy. Sixty histologically confirmed MPM patients previously treated with pemetrexed and platinum (45 of 60) or as pemetrexed monotherapy (15 of 60) were retrospectively considered. Eighty-one control patients with MPM which were not treated with pemetrexed were also evaluated. TS and ERCC1 gene expression levels were evaluated by real-time PCR and by immunohistochemistry using the H-score (histologic score). A significant correlation between low TS protein expression and longer time to progression (TTP; 17.9 versus 7.9 months; hazard ratio [HR], 2.05, 95% CI, 1.19–3.77; $P = 0.02$) or overall survival (OS; 30 versus 16.7 months; HR, 2.38; 95% CI, 1.15–4.91; $P = 0.019$) was found when patients were divided according to median H-score. Conversely, TS mRNA levels were not significantly correlated with outcome. In platinum-treated patients ($n = 45$), no correlation was found with survival according to ERCC1 median H-score, but patients in the lower tertile had a significantly shorter survival (HR, 3.06; 95% CI, 1.08–8.69; $P = 0.035$). In untreated MPMs, TS had no prognostic role. At multivariate analysis, TS protein levels were the only independent prognostic factor for both TTP (HR, 2.71; 95% CI, 1.13–6.49; $P = 0.02$) and OS (HR, 6.91; 95% CI, 1.90–25.07; $P = 0.003$). These findings suggest that in MPM patients treated with pemetrexed-based chemotherapy, low TS protein levels are predictive of improved TTP and OS.

Consistent results were obtained in a follow up study (Zucali et al., 2011). Here, the aim of the study was to assess the role of ERCC1 and TS in MPM tumors, and to correlate the expression levels and polymorphisms of these determinants of drug activity with the outcome of MPM patients treated with carboplatin/pemetrexed as first-line chemotherapy. Towards this end,

analysis of TS and ERCC1 polymorphisms, mRNA and protein expression was performed using PCR and immunohistochemistry in tumor specimens from 126 MPM patients, including 99 carboplatin/pemetrexed-treated patients. A significant correlation between low TS protein expression and MPM disease control (DC) to carboplatin/pemetrexed therapy ($P = 0.027$), longer progression-free survival (PFS; $P = 0.017$), and longer overall survival (OS; $P = 0.022$) was found when patients were categorized according to median H-score. In contrast, patients with the higher tertile of TS mRNA expression correlated with higher risk of developing progressive disease ($P = 0.022$), shorter PFS ($P < 0.001$), and shorter OS ($P < 0.001$). At multivariate analysis, the higher tertile of TS mRNA level and TS H-score confirmed their independent prognostic role for DC, PFS, and OS. No significant associations were found among ERCC1 protein expression, TS and ERCC1 polymorphisms, and clinical outcome. Thus, in this important study with carboplatin-/pemetrexed-treated MPM patients, low TS protein and mRNA levels were significantly associated with DC, improved PFS, and OS. Prospective trials for the validation of the prognostic/predictive role of TS in MPM patients treated with pemetrexed-based regimens are thus warranted.

As with the autoregulation of DHFR protein levels, TS was also shown to function as an RNA-binding protein. TS protein was found to bind its own mRNA, resulting in translational inhibition (Reviewed in Liu and Matherly, 2002; Tai et al., 2004b). The first indication for this autoregulatory loop was achieved through cell-free studies with a rabbit reticulocyte lysate system. It was shown that exogenous addition of purified human recombinant TS protein to an *in vitro* translation reaction, inhibited translation of TS mRNA. Additionally, electrophoretic mobility shift assays (EMSA) confirmed a specific interaction between TS protein and its corresponding mRNA (Chu et al., 1991). This protein-mRNA complex was also identified in cultured human colon cancer cells (Chu et al., 1994). Consequent studies pin-pointed the sites crucial for this specific interaction both in the mRNA (Chu et al., 1993b; Lin et al., 2000) and in the TS protein (Lin et al., 2003). Interestingly, addition of dUMP, 5-F-dUMP or 5,10-CH_2-THF to the *in vitro* translation assays, completely released the inhibitory effect on the mRNA translation, due to competitive binding to the TS protein (Chu and Allegra, 1996; Chu et al., 1994).

Although relatively rare, mutations occurring in the TS coding region can also confer resistance to both TS-inhibitory antifolates as well as to fluoropyrimidines including 5-FU. Two mutations that confer resistance to both nolatrexed and fluorodeoxyuridines were isolated after mutagenesis with the chemical mutagen ethyl methanesulfonate followed by nolatrexed selection in the human fibrosarcoma HT-1080 cells (Tong et al., 1998). Both TS gene amplification and point mutations contributed to nolatrexed resistance. Interestingly, two mutations, located in a conserved Arg50 loop, Asp49Gly and Gly52Ser displayed resistance to nolatrexed in cells transfected with the mutant TS cDNAs, with IC_{50} values 40- and 12-fold higher than their wild type TS, respectively. Consistently, these mutations also conferred resistance to fluorodeoxyuridine (26- and 97-fold increase in the IC_{50} values, respectively). Surprisingly, no resistance was observed towards other antifolates including raltitrexed and GW1843, indicating that these amino acids contribute to the binding of nolatrexed and fluorodeoxyuridines, but not to other antifolates such as raltitrexed and GW1843 (Tong et al., 1998). Additional studies have also identified amino acid 33 as a highly conserved residue that is crucial for the binding of 5-fluoro-2′-deoxyuridine and 5,10-CH_2-THF to TS (Barbour et al., 1990; Hughey et al., 1993; Reilly et al., 1997). In summary, it appears that TS mutations can confer resistance to both antifolates and 5-FU; however, they are relatively rare. The possible emergence of such TS mutations in colorectal cancers undergoing TS targeted chemotherapy has not been identified to date.

3.6. Expansion of intracellular THF cofactor pool

Since antifolates target specific enzymes in the folate metabolic pathway, a constant competition exists between folates and antifolates at the levels of uptake, target enzyme binding, polyglutamylation, and efflux. As such, it is expected that alterations at the level of the intracellular THF-cofactor pool size will have major impact on the pharmacological activity of antifolates. Expansion of the cellular folate pool will result in enhanced competition both on polyglutamylation as well as on target enzyme binding, leading to decreased cytotoxic effects and consequent antifolate resistance. In contrast, shrinkage in the cellular folate pool will result is enhanced cytotoxic effects. Indeed, expanded cellular folate pool due to multiple mechanisms that lead to antifolate resistance is a well recognized determinant of antifolate resistance (Assaraf and Goldman, 1997; Chattopadhyay et al., 2006; Drori et al., 2000; Jansen et al., 1999, 1998, 1990; Tse and Moran, 1998; van der Wilt et al., 2001; Zhao et al., 2001a).

Several studies indicated that increased RFC transport activity could contribute to the expansion of cellular folate pool (Drori et al., 2000; Jansen et al., 1998; Tse and Moran, 1998). However, the understanding that increased RFC activity cannot serve as a standalone mechanism for intracellular folate expansion was first demonstrated in a study where RFC was overexpressed in murine L1210 leukemia cells (Zhao et al., 1997). The impact of the bidirectional transport activity of RFC on the folate pool size was further explored in a study which demonstrated that increased RFC expression can be lethal to cells cultured under conditions of severe folate deprivation, causing major export of folates from the cell, hence bringing about a decrease in the folate pool (Ifergan and Assaraf, 2008; Ifergan et al., 2008).

A different mechanism that was shown to mediate an increase in the cellular folate pool is loss of folate exporter activity, typically via ATP-driven MDR efflux transporters (Assaraf and Goldman, 1997). In this respect, studies with a pyrimethamine-resistant Chinese hamster ovary cell line (Pyr^{R100}) shed light on the involvement of folate exporters in facilitating the expansion of intracellular folate pool (Assaraf and Slotky, 1993; Jansen et al., 1999). This cell line was established using a gradual deprivation of folates by a stepwise selection to the lipophilic antifolate pyrimethamine. Consequently, this cell line exhibited 1000-fold resistance to pyrimethamine. These cells displayed 17-fold increase in the intracellular pool of folic acid (under conditions of DHFR blockade with trimetrexate which prevents folic acid reduction), and the underlying mechanism was complete loss of folate efflux pump activity (Assaraf and Goldman, 1997). Subsequent studies revealed the complete loss of MRP1 (ABCC1) expression as well as a marked decrease in MRP5 (ABCC5) levels, thereby leading to 5-fold decrease in the ATP-dependent efflux of folic acid (Stark et al., 2003). Additional studies with different antifolate-resistant tumor cell lines further confirmed that decreased activity of ATP-driven folate efflux pumps plays a pivotal role in the expansion of cellular folate pools (Assaraf et al., 2003).

Once expansion in the intracellular folate pool is accomplished, it can mediate antifolate resistance through several mechanisms, mainly competition at the level of either (a) FPGS activity and/or (b) folate-dependent target enzymes, i.e., DHFR, TS or GARFT. The first indications that elevated intracellular folate pools will inhibit MTX polyglutamylation stemmed from early studies in 1983; studies with H35-rat hepatoma cells showed that the maximal rate of MTX polyglutamylation occurred in folate-depleted cells (Johnson et al., 1988; Nimec and Galivan, 1983). Furthermore, supplementation of these folate-depleted cells with THF-coenzymes resulted in 90% decrease in MTX polyglutamylation (Johnson et al., 1988; Nimec and Galivan, 1983). Consequently, several other studies consistently showed that elevated cellular folate pools result in

antifolate resistance mediated by diminished polyglutamylation (Assaraf and Goldman, 1997; Assaraf et al., 2003; Drori et al., 2000; Jansen et al., 1998; Tse and Moran, 1998). In this respect, a dedicated study was conducted, aimed at evaluating the impact of the intracellular THF-cofactor pool size on the activity of various polyglutamatable and non-polyglutamatable antifolates (Zhao et al., 2001a). Murine leukemia L1210 cells were grown in different concentrations of leucovorin and it was demonstrated that the intracellular folate pool increased in proportion to the elevation in the extracellular leucovorin concentration. This increase in the intracellular folate pool led to a significant increase in the IC_{50} values of polyglutamatable antifolates including lometrexol, pemetrexed and raltitrexed. The lower the affinity of the monoglutamate antifolate to the target enzyme (and FPGS), the more compromised was its cytotoxic effect by the expanding cellular folate pool. Consistently, no effect was observed with the IC_{50} value of plevitrexed, a non-polyglutamatable TS-inhibitor (Zhao et al., 2001a).

Increased folate pool can also mediate resistance to antifolates that do not undergo polyglutamylation mainly by competition at the level of the target enzyme. The cytotoxic activity of trimetrexate, a less potent DHFR inhibitor that cannot undergo polyglutamylation, was also found to be highly compromised by increased cellular folate pool (Assaraf and Goldman, 1997; Assaraf and Slotky, 1993; Zhao et al., 2001a). This is in contrast to non-polyglutamatable, potent DHFR inhibitors such as PT523 and PT632 that retain their cytotoxic activity even when the intracellular folate pool was expanded (Zhao et al., 2001a). The above results demonstrate that the higher affinity the antifolate has for its target enzyme, the less it will be affected by expanded folate pool.

The significance of expanded cellular folate pool was not assessed in the clinical oncology setting, although it is expected to have a significant impact on the cytotoxic activity of several antifolates that are currently used in cancer chemotherapy. In addition, many antifolates are administered with folate supplementation in order to decrease toxic side effects to normal tissues. In this respect, it was recently suggested following pre-clinical experiments with human solid tumor cell lines, that the addition of folic acid to cancer patients undergoing pemetrexed-based chemotherapy, should be limited to the lowest dose possible (Chattopadhyay et al., 2007b). This should be done in order to provide optimal protection from pemetrexed toxicity, while allowing potent cytotoxic effect of pemetrexed in tumor cells.

3.7. Enhanced efflux of antifolates due to increased expression of the ATP-driven MDR efflux transporters of the ABC superfamily

Members of the MDR efflux transporters including MRP1 (ABCC1) through MRP5 (ABCC5) and ABCG2 (BCRP) mediate the ATP-dependent efflux of mono- di- and tri-glutamate of folates and antifolates (Assaraf, 2006). Early experiments with hyperbilirubinemic rats lacking ABCC2 (MRP2) were the first to show the role of this ABC transporter in MTX efflux; these hyperbilirubinemic rats exhibited impaired clearance and excretion of MTX (Masuda et al., 1997). Subsequent studies demonstrated that other members of the family, i.e. MRP1-4 (ABCC1-4) are all involved in MTX efflux when overexpressed and can therefore mediate MTX-resistance following short exposure to the drug (Chen et al., 2002; Hooijberg et al., 1999, 2003; Lee et al., 2000; Zeng et al., 2001). The same principle was found to be true for BCRP (ABCG2) where overexpression of this efflux pump was shown to mediate resistance to MTX (Shafran et al., 2005; Volk et al., 2000). Like MTX, other antifolates were found to be transport substrates for the above ABC-transporters including raltitrexed and GW1843. Trimetrexate, however, was shown to be a substrate of P-gp (ABCB1) (Arkin et al., 1989; Assaraf et al., 1989a; Bram et al., 2006; Hooijberg et al., 1999; Schlemmer and Sirotnak, 1995; Shafran et al., 2005).

These efflux transporters can also expel reduced folates out of the cell including THF, 5-CH_3-THF, 5,10-CH_2-THF and 10-CHO-THF (Assaraf, 2006; Kusuhara et al., 1998). It is important to note that enhanced efflux of THF cofactors decreases the cellular folate pool, hence enhancing the cytotoxic effect exerted by different antifolates. Therefore, increased expression of various MRPs and BCRP (ABCG2) can mediate two distinct opposing effects on antifolate cytotoxicity. The net effect of such overexpression will result in a different outcome; this depends on the specific antifolate, in terms of affinity for the ABC transporters, and to what extent this antifolate is affected by the cellular folate pool that may affect its cytotoxic activity.

Despite the major role that overexpression of these MDR efflux transporters has on antifolate-resistance, it is important to note that no such primary overexpression of an ABC transporter was ever provoked in tumor cell lines selected for resistance to antifolates. It is only when cells are treated with other MDR type cytotoxic agents such as anthracyclines, epipodophyllotoxins, camptothecins, taxenes and *Vinca* alkaloids, that overexpression of ABC transporters can be observed as a primary mechanism of drug resistance and cross-resistance to antifolates. Hence, this efflux pump-dependent mechanism of antifolate resistance is mostly critical in cancers where a combined chemotherapeutic regimen is administered, where other cytotoxic agents that are *bona fide* transport substrates of ABC transporters can provoke increased expression of ABC transporters, thereby leading to combined resistance both towards antifolates and other cytotoxic agents.

4. Future perspectives: emerging antifolates and small molecule folate-drug conjugates for personalized medicine

For some antifolates, tumor selectivity may not be optimal since many antifolates are taken up by RFC which is ubiquitously expressed in normal cells from healthy tissues, while poorly expressed or mutated in drug resistant tumor cells (Assaraf, 2007, 2006). This may consequently result in untoward toxicity to various proliferating healthy tissues. For example, clinical studies with lometrexol and AG2034 were discontinued due to extensive accumulation and long-term retention of lometrexol-polyglutamates in normal tissues leading to undesired toxicities to healthy tissues. Specifically, this explains the severe myelosuppression previously encountered in phase I studies with lometrexol (Ray et al., 1993) as well as the untoward toxicity observed with AG2034 (Bissett et al., 2001). Hence, one could postulate that if an FR-targeted antifolate were itself cytotoxic without being recognized as an RFC transport substrate, selective tumor targeting would ensue. Moreover, if an antifolate could be rationally designed to be recognized as a transport substrate of PCFT and FR but not of RFC, this could result in enhanced tumor selectivity, owing to the acidic microenvironment of tumors where PCFT is supposed to display its optimal influx activity. In support of this notion, antifolates that selectively target FRs over RFC have been originally described (Jansen, 1999). In this respect, cyclopenta[g]quinazoline antifolates that target FRs for their cellular entry including BGC 945 were described, that potently inhibit TS in tumor cells (Jackman et al., 2004). Moreover, when tested in mice bearing human tumor xenografts, BGC 945 was found to be active and in the same time had no toxicity to normal tissues, as reflected in the lack of weight loss, or any macroscopic signs of toxicity to major organs. This is consistent with the premise that FR-targeting without the involvement of RFC is highly selective, since various human carcinomas overexpress FRs (Gibbs et al., 2005).

The Gangjee group initially reported the synthesis and discovery of novel series of 6-substituted classical pyrrolo[2,3-*d*]pyrimidine antifolates with a 3–6-carbon bridge between the heterocycle and

the benzoyl-L-glutamate; some of these compounds were transported via FRs, displayed high affinity inhibition of GARFT, thereby resulting in potent antitumor activity in tumor cell lines with high FR expression (Deng et al., 2008). The potent inhibition of *de novo* purine biosynthesis via blockade of GARFT was further confirmed by the fact that these antifolates markedly decreased the GTP and ATP pools and that 5-amino-4-imidazolecarboxamide (AICAR) abolished their cytotoxic effect. Subsequent studies have shown that these novel 6-substituted classical pyrrolo[2,3-*d*]pyrimidine antifolates were taken up via FRs and PCFT, but not by RFC; these compounds which were potent inhibitors of GARFT, displayed antitumor activity in SCID mice harboring advanced human KB carcinoma tumors with FR-overexpression (Wang et al., 2010). Under *in vitro* low pH conditions that mimic the acidic microenvironment of solid tumors, these radiolabeled antifolates were shown to be efficiently transported via PCFT but not RFC and subsequently underwent polyglutamylation (Kugel Desmoulin et al., 2011). Moreover, an *in vivo* efficacy trial with nude mice implanted with PCFT-overexpressing human hepatoma HepG2 xenografts confirmed that this 6-substituted pyrrolo[2,3-d]pyrimidine thienoyl antifolates were active (Kugel Desmoulin et al., 2011). Collectively, these studies offer some new therapeutic possibilities in the delivery of rationally-designed novel antifolates via PCFT- and/or FR-dependent transport (but not via RFC) in the acidic microenvironment of solid tumors that frequently express substantial levels of these antifolate transport systems (Wang et al., 2012). Clinical evaluation of these rationally designed novel targeted antifolates is warranted.

Cancer therapies using targeting ligands including folic acid aimed at selectively delivering cytotoxic drug conjugates to malignant cells which frequently overexpress FRs, are currently receiving a great deal of attention (Leamon et al., 2008; Low and Kularatne, 2009; Xia and Low, 2010). Over the past three decades, the FR has emerged as an attractive tumor biomarker with the potential to be exploited for therapeutic and imaging purposes (Muller, 2012). Increasing evidence suggests that these endocytosing FRs can functionally mediate the cellular uptake and retention of natural folates, certain antifolates, and folate-small molecule drug conjugates. FR has a restricted tissue expression profile which appears to be limited to either tissues responsible for whole body retention of folates (e.g., kidney and placenta), or certain pathologic tissues including malignant tumors or activated macrophages. Hence, the FR is increasingly proving to be a useful biological target for disease management and personalized medicine as well as for imaging and diagnostic purposes. In this respect, it was recently demonstrated that FRα can be efficiently harnessed for intraoperative tumor-specific fluorescence imaging in patients harboring ovarian cancer (van Dam et al., 2011). In this study, folic acid conjugated to fluorescein isothiocyanate (folate-FITC) for FRα targeting was used together with a real-time multispectral intraoperative fluorescence imaging system. Hence, in ovarian cancer patients, intraoperative tumor-specific fluorescence imaging with a FRα-targeted fluorescent agent showcased the potential applications in ovarian cancer patients for improved intraoperative staging and more radical cytoreductive surgery. Additionally, the recent years have been peppered with reports of novel FR-targeted therapies, and many have demonstrated impressive *in vivo* potency, particularly against tumor xenografts, without the undesirable toxicity that often accompanies non-targeted drug regimens (Leamon et al., 2008; Low and Kularatne, 2009; Naumann and Coleman, 2011; Xia and Low, 2010). Thus, folate conjugates bind to FRs that are overexpressed on approximately 40% of human cancers and mediate internalization of their attached drugs via receptor-mediated endocytosis. With the rational use of proper linkers, folate-conjugated small molecule drugs can be released inside their target tumor cells where they can elicit their desired cytotoxic activity. Based on this strategy, multiple FR-targeted folic acid-drug conjugates have been synthesized and developed by Endocye Inc., some of which are currently undergoing advanced stages of clinical trials for cancer therapeutics. In this respect, a randomized phase II trial termed PRECEDENT used pegylated liposomal doxorubicin (PLD; Doxil®, which is known outside the US as Caelyx®) with or without Vintafolide (EC145) against recurrent platinum-resistant ovarian cancer. Vintafolide is a folic acid-linked desacetylvinblastine hydrazide, i.e. a *Vinca* alkaloid conjugate which disrupts microtubule polymerization (Naumann and Coleman, 2011; Naumann et al., 2011). This novel drug combination regimen of Vintafolide and platinum compounds has shown a significant progression-free survival advantage over standard therapy. Moreover, the most significant progression-free survival was observed in ovarian cancer patients harboring tumors and metastases that were FR-positive. Consequently, Endocyte Inc. who developed these folic acid small molecule drug conjugates for personalized medicine has launched a new phase III clinical study, called PROCEED; this large study is currently evaluating the effectiveness and safety of Vintafolide in combination with Doxil® for the treatment of women with ovarian cancer whose tumors developed resistance to standard platinum-based chemotherapy. This clinical study is being conducted in 160 locations in the US, Canada, and Europe and will include 640 women harboring platinum-resistant ovarian cancer. This novel FR-targeted therapeutic strategy offers a new hope for personalized medicine of drug-resistant human cancers while minimizing untoward side effects.

References

Abali, E.E., et al., 2008. Regulation of human dihydrofolate reductase activity and expression. Vitamins & Hormones 79, 267–292.

Adjei, A.A., et al., 2010. Phase II trial of pemetrexed plus bevacizumab for second-line therapy of patients with advanced non-small-cell lung cancer: NCCTG and SWOG study N0426. Journal of Clinical Oncology 28, 614–619.

Albrecht, A.M., Biedler, J.L., Hutchison, D.J., 1972. Two different species of dihydrofolate reductase in mammalian cells differentially resistant to amethopterin and methasquin. Cancer Research 32, 1539–1546.

Allegra, C.J., et al., 1985. Enhanced inhibition of thymidylate synthase by methotrexate polyglutamates. Journal of Biological Chemistry 260, 9720–9726.

Allegra, C.J., et al., 1987. Evidence for direct inhibition of de novo purine synthesis in human MCF-7 breast cells as a principal mode of metabolic inhibition by methotrexate. Journal of Biological Chemistry 262, 13520–13526.

Alt, F.W., et al., 1978. Selective multiplication of dihydrofolate reductase genes in methotrexate-resistant variants of cultured murine cells. Journal of Biological Chemistry 253, 1357–1370.

Antony, A., et al., 2004. Translational upregulation of folate receptors is mediated by homocysteine via RNA-heterogeneous nuclear ribonucleoprotein E1 interactions. Journal of Clinical Investigation 113, 285–301.

Antony, A.C., 2004. Folate receptors: reflections on a personal odyssey and a perspective on unfolding truth. Advance Drug Delivery Reviews 56, 1059–1066.

Appleman, J.R., et al., 1988. Kinetics of the formation and isomerization of methotrexate complexes of recombinant human dihydrofolate reductase. Journal of Biological Chemistry 263, 10304–10313.

Arkin, H., et al., 1989. Multidrug resistance in a human leukemic cell line selected for resistance to trimetrexate. Cancer Research 49, 6556–6561.

Assaraf, Y.G., 2007. Molecular basis of antifolate resistance. Cancer and Metastasis Reviews 26, 153–181.

Assaraf, Y.G., 2006. The role of multidrug resistance efflux transporters in antifolate resistance and folate homeostasis. Drug Resistance Updates: Reviews and Commentaries in Antimicrobial and Anticancer Chemotherapy 9, 227–246.

Assaraf, Y.G., et al., 1992. Characterization of the coexisting multiple mechanisms of methotrexate resistance in mouse 3T6 R50 fibroblasts. Journal of Biological Chemistry 267, 5776–5784.

Assaraf, Y.G., Goldman, I.D., 1997. Loss of folic acid exporter function with markedly augmented folate accumulation in lipophilic antifolate-resistant mammalian cells. Journal of Biological Chemistry 272, 17460–17466.

Assaraf, Y.G., et al., 2006. Computer modelling of antifolate inhibition of folate metabolism using hybrid functional petri nets. Journal of Theoretical Biology 240, 637–647.

Assaraf, Y.G., Molina, A., Schimke, R.T., 1989a. Cross-resistance to the lipid-soluble antifolate trimetrexate in human carcinoma cells with the multidrug-resistant phenotype. Journal of the National Cancer Institute 81, 290–294.

Assaraf, Y.G., Molina, A., Schimke, R.T., 1989b. Sequential amplification of dihydrofolate reductase and multidrug resistance genes in Chinese hamster ovary

cells selected for stepwise resistance to the lipid-soluble antifolate trimetrexate. Journal of Biological Chemistry 264, 18326–18334.
Assaraf, Y.G., et al., 2003. Loss of multidrug resistance protein 1 expression and folate efflux activity results in a highly concentrative folate transport in human leukemia cells. Journal of Biological Chemistry 278, 6680–6686.
Assaraf, Y.G., Schimke, R.T., 1987. Identification of methotrexate transport deficiency in mammalian cells using fluoresceinated methotrexate and flow cytometry. Proceedings of the National Academy of Sciences United States of America 84, 7154–7158.
Assaraf, Y.G., Slotky, J.I., 1993. Characterization of a lipophilic antifolate resistance provoked by treatment of mammalian cells with the antiparasitic agent pyrimethamine. Journal of Biological Chemistry 268, 4556–4566.
Atabay, B., et al., 2010. Mutation of the proton-coupled folate transporter gene (PCFT-SLC46A1) in Turkish siblings with hereditary folate malabsorption. Pediatric Hematology Oncology 27, 614–619.
Azzoli, C.G., et al., 2011. Pralatrexate with vitamin supplementation in patients with previously treated, advanced non-small cell lung cancer: safety and efficacy in a phase 1 trial. Journal of Thoracic Oncology 6, 1915–1922.
Baggott, J.E., Vaughn, W.H., Hudson, B.B., 1986. Inhibition of 5-aminoimidazole-4-carboxamide ribotide transformylase, adenosine deaminase and 5'-adenylate deaminase by polyglutamates of methotrexate and oxidized folates and by 5-aminoimidazole-4-carboxamide riboside and ribotide. Biochemical Journal 236, 193–200.
Bakos, E., et al., 2000. Interactions of the human multidrug resistance proteins MRP1 and MRP2 with organic anions. Molecular Pharmacology 57, 760–768.
Banerjee, D., et al., 2002. Novel aspects of resistance to drugs targeted to dihydrofolate reductase and thymidylate synthase. Biochimica et Biophysica Acta 1587, 164–173.
Banks, S.D., et al., 1994. Destruction of WiDr multicellular tumor spheroids with the novel thymidylate synthase inhibitor 1843U89 at physiological thymidine concentrations. Cancer Chemotherapy and Pharmacology 33, 455–459.
Barakat, R.R., et al., 1993. Intrinsic resistance of cervical squamous cell carcinoma cell lines to methotrexate (MTX) as a result of decreased accumulation of intracellular MTX polyglutamates. Gynecologic Oncology 51, 54–60.
Barbour, K.W., Berger, S.H., Berger, F.G., 1990. Single amino acid substitution defines a naturally occurring genetic variant of human thymidylate synthase. Molecular Pharmacology 37, 515–518.
Barnes, M.J., et al., 1999. Impact of polyglutamation on sensitivity to raltitrexed and methotrexate in relation to drug-induced inhibition of de novo thymidylate and purine biosynthesis in CCRF-CEM cell lines. Clinical Cancer Research 5, 2548–2558.
Baugh, C.M., Krumdieck, C.L., Nair, M.G., 1973. Polygammaglutamyl metabolites of methotrexate. Biochemical and Biophysical Research Communications 52, 27–34.
Bavetsias, V., et al., 2000. Design and synthesis of Cyclopenta[g]quinazoline-based antifolates as inhibitors of thymidylate synthase and potential antitumor agents. Journal of Medicinal Chemistry 43, 1910–1926.
Beardsley, G.P., et al., 1989. A new folate antimetabolite, 5,10-dideaza-5,6,7,8-tetrahydrofolate is a potent inhibitor of de novo purine synthesis. Journal of Biological Chemistry 264, 328–333.
Belkov, V.M., et al., 1999. Reduced folate carrier expression in acute lymphoblastic leukemia: a mechanism for ploidy but not lineage differences in methotrexate accumulation. Blood 93, 1643–1650.
Benepal, T.S., Judson, I., 2005. ZD9331: discovery to clinical development. Anticancer Drugs 16, 1–9.
Bertino, J.R., et al., 1965. The "induction" of leukocyte and erythrocyte dihydrofolate reductase by methotrexate. II. Clinical and pharmacologic studies. Clinical Pharmacology & Therapeutics 6, 763–770.
Bertino, J.R., Cashmore, A.R., Hillcoat, B.L., 1970. Induction" of dihydrofolate reductase: purification and properties of the "induced" human erythrocyte and leukocyte enzyme and normal bone marrow enzyme. Cancer Research 30, 2372–2378.
Bertino, J.R., et al., 1963. The "Induction" of dihydrofolic reductase activity in leukocytes and erythrocytes of patients treated with amethopterin. Journal of Clinical Investigation 42, 466–475.
Bertino, J.R., et al., 1962. Increased level of dihydrofolic reductase in leucocytes of patients treated with amethopterin. Nature 193, 140–142.
Bertino, J.R., et al., 1979. 2,4-diamino-5-methyl-6-[(3,4,5-trimethoxyanilino) methyl]quinazoline (tmq), a potent non-classical folate antagonist inhibitor–I effect on dihydrofolate reductase and growth of rodent tumors in vitro and in vivo. Biochemical Pharmacology 28, 1983–1987.
Beutel, G., et al., 2005. Phase I study of OSI-7904L, a novel liposomal thymidylate synthase inhibitor in patients with refractory solid tumors. Clinical Cancer Research 11, 5487–5495.
Bissett, D., et al., 2001. Phase I dose-escalation and pharmacokinetic study of a novel folate analogue AG2034. British Journal of Cancer 84, 308–312.
Blanke, C.D., et al., 2010. S9511: a Southwest Oncology Group phase II study of trimetrexate, 5-fluorouracil, and leucovorin in unresectable or metastatic adenocarcinoma of the stomach. American Journal of Clinical Oncology 33, 117–120.
Bleehen, N.M., et al., 1995. A phase II study of oral piritrexim in recurrent high-grade (III, IV) glioma. British Journal of Cancer 72, 766–768.
Bolton, J.A., et al., 1999. Retinoic acid-dependent upregulation of mouse folate receptor-alpha expression in embryonic stem cells, and conservation of alternative splicing patterns. Gene 230, 215–224.
Boritzki, T.J., et al., 1996. AG2034: a novel inhibitor of glycinamide ribonucleotide formyltransferase. Investigational New Drugs 14, 295–303.
Borst, P., Elferink, R.O., 2002. Mammalian ABC transporters in health and disease. Annual Review of Biochemistry 71, 537–592.
Bram, E., et al., 2006. Mutant Gly482 and Thr482 ABCG2 mediate high-level resistance to lipophilic antifolates. Cancer Chemotherapy and Pharmacology 58, 826–834.
Brigle, K.E., et al., 1995. Characterization of a mutation in the reduced folate carrier in a transport defective L1210 murine leukemia cell line. Journal of Biological Chemistry 270, 22974–22979.
Brody, J.R., et al., 2006. Genomic copy number changes affecting the thymidylate synthase (TYMS) gene in cancer: a model for patient classification to aid fluoropyrimidine therapy. Cancer Research 66, 9369–9373.
Bystroff, C., Kraut, J., 1991. Crystal structure of unliganded Escherichia coli dihydrofolate reductase. Ligand-induced conformational changes and cooperativity in binding. Biochemistry 30, 2227–2239.
Cao, W., Matherly, L.H., 2004. Analysis of the membrane topology for transmembrane domains 7-12 of the human reduced folate carrier by scanning cysteine accessibility methods. Biochemical Journal 378, 201–206.
Chattopadhyay, S., Moran, R.G., Goldman, I.D., 2007a. Pemetrexed: biochemical and cellular pharmacology, mechanisms, and clinical applications. Molecular Cancer Therapeutics 6, 404–417.
Chattopadhyay, S., et al., 2007b. Commentary: a case for minimizing folate supplementation in clinical regimens with pemetrexed based on the marked sensitivity of the drug to folate availability. Oncologist 12, 808–815.
Chattopadhyay, S., et al., 2006. The inverse relationship between reduced folate carrier function and pemetrexed activity in a human colon cancer cell line. Molecular Cancer Therapeutics 5, 438–449.
Chave, K.J., et al., 2003. Identification of single nucleotide polymorphisms in the human gamma-glutamyl hydrolase gene and characterization of promoter polymorphisms. Gene 319, 167–175.
Chen, Z.S., et al., 2002. Analysis of methotrexate and folate transport by multidrug resistance protein 4 (ABCC4): MRP4 is a component of the methotrexate efflux system. Cancer Research 62, 3144–3150.
Cheng, Q., et al., 2006. Epigenetic regulation of human gamma-glutamyl hydrolase activity in acute lymphoblastic leukemia cells. American Journal of Human Genetics 79, 264–274.
Cheng, Q., et al., 2004. A substrate specific functional polymorphism of human gamma-glutamyl hydrolase alters catalytic activity and methotrexate polyglutamate accumulation in acute lymphoblastic leukaemia cells. Pharmacogenetics 14, 557–567.
Cheradame, S., et al., 1999. Variable intrinsic sensitivity of human tumor cell lines to raltitrexed (Tomudex) and folylpolyglutamate synthetase activity. Anticancer Drugs 10, 505–510.
Chu, E., Allegra, C.J., 1996. The role of thymidylate synthase as an RNA binding protein. BioEssays: News and Reviews in Molecular, Cellular and Developmental Biology 18, 191–198.
Chu, E., et al., 2003. Thymidylate synthase inhibitors as anticancer agents: from bench to bedside. Cancer Chemotherapy and Pharmacology Suppl. 1 (52), S80–S89.
Chu, E., et al., 1991. Autoregulation of human thymidylate synthase messenger RNA translation by thymidylate synthase. Proceedings of the National Academy of Sciences United States of America 88, 8977–8981.
Chu, E., et al., 1993a. Specific binding of human dihydrofolate reductase protein to dihydrofolate reductase messenger RNA in vitro. Biochemistry 32, 4756–4760.
Chu, E., et al., 1993b. Identification of an RNA binding site for human thymidylate synthase. Proceedings of the National Academy of Sciences United States of America 90, 517–521.
Chu, E., et al., 1994. Identification of a thymidylate synthase ribonucleoprotein complex in human colon cancer cells. Molecular and Cellular Biology 14, 207–213.
Chung, K.N., et al., 1993. Stable transfectants of human MCF-7 breast cancer cells with increased levels of the human folate receptor exhibit an increased sensitivity to antifolates. Journal of Clinical Investigation 91, 1289–1294.
Ciuleanu, T., et al., 2007. A randomised phase II study of OSI-7904L versus 5-fluorouracil (FU)/leucovorin (LV) as first-line treatment in patients with advanced biliary cancers. Investigational New Drugs 25, 385–390.
Clamp, A.R., et al., 2008. A phase I and pharmacokinetic study of OSI-7904L, a liposomal thymidylate synthase inhibitor in combination with oxaliplatin in patients with advanced colorectal cancer. Cancer Chemotherapy and Pharmacology 61, 579–585.
Cohen, M.H., et al., 2010. Approval summary: pemetrexed maintenance therapy of advanced/metastatic nonsquamous, non-small cell lung cancer (NSCLC). Oncologist 15, 1352–1358.
Cohen, M.H., Justice, R., Pazdur, R., 2009. Approval summary: pemetrexed in the initial treatment of advanced/metastatic non-small cell lung cancer. Oncologist 14, 930–935.
Cole, P.D., et al., 2001. Effects of overexpression of gamma-Glutamyl hydrolase on methotrexate metabolism and resistance. Cancer Research 61, 4599–4604.
Coward, J.K., McGuire, J.J., 2008. Mechanism-based inhibitors of folylpoly-gamma-glutamate synthetase and gamma-glutamyl hydrolase: control of folylpoly-gamma-glutamate homeostasis as a drug target. Vitamins & Hormones 79, 347–373.
Cunningham, D., et al., 2002. Efficacy, tolerability and management of raltitrexed (Tomudex) monotherapy in patients with advanced colorectal cancer. a review of phase II/III trials. European Journal of Cancer 38, 478–486.

de Vries, E.G., et al., 1993. A phase II and pharmacokinetic study with oral piritrexim for metastatic breast cancer. British Journal of Cancer 68, 641–644.
Deeley, R.G., Westlake, C., Cole, S.P., 2006. Transmembrane transport of endo- and xenobiotics by mammalian ATP-binding cassette multidrug resistance proteins. Physiological Review 86, 849–899.
Degardin, M., et al., 1994. A phase II study of piritrexim in patients with advanced squamous cell carcinoma of the head and neck. European Journal of Cancer 30A, 1044–1045.
Deng, Y., et al., 2008. Synthesis and discovery of high affinity folate receptor-specific glycinamide ribonucleotide formyltransferase inhibitors with antitumor activity. Journal of Medicinal Chemistry 51, 5052–5063.
Dev, I.K., et al., 1994. Mode of binding of folate analogs to thymidylate synthase. Evidence for two asymmetric but interactive substrate binding sites. Journal of Biological Chemistry 269, 1873–1882.
DeVos, L., et al., 2008. Associations between single nucleotide polymorphisms in folate uptake and metabolizing genes with blood folate, homocysteine, and DNA uracil concentrations. American Journal of Clinical Nutrition 88, 1149–1158.
Diaz, G.A., et al., 1999. Mutations in a new gene encoding a thiamine transporter cause thiamine-responsive megaloblastic anaemia syndrome. Nature Genetics 22, 309–312.
Dicker, A.P., et al., 1990. Identification and characterization of a mutation in the dihydrofolate reductase gene from the methotrexate-resistant Chinese hamster ovary cell line Pro-3 MtxRIII. Journal of Biological Chemistry 265, 8317–8321.
Ding, B.C., et al., 2001. Association of deletions and translocation of the reduced folate carrier gene with profound loss of gene expression in methotrexate-resistant K562 human erythroleukemia cells. Biochemical Pharmacology 61, 665–675.
Diop-Bove, N.K., et al., 2009. Hypermethylation of the human proton-coupled folate transporter (SLC46A1) minimal transcriptional regulatory region in an antifolate-resistant HeLa cell line. Molecular Cancer Therapeutics 8, 2424–2431.
Dixon, K.H., et al., 1994. A novel cDNA restores reduced folate carrier activity and methotrexate sensitivity to transport deficient cells. Journal of Biological Chemistry 269, 17–20.
Dolnick, B.J., et al., 1979. Correlation of dihydrofolate reductase elevation with gene amplification in a homogeneously staining chromosomal region in L5178Y cells. Journal of Cell Biology 83, 394–402.
Domin, B.A., Cheng, Y.C., Hakala, M.T., 1982. Properties of dihydrofolate reductase from a methotrexate-resistant subline of human KB cells and comparison with enzyme from KB parent cells and mouse S180 AT/3000 cells. Molecular Pharmacology 21, 231–238.
Drake, J.C., et al., 1996. Resistance to tomudex (ZD1694): multifactorial in human breast and colon carcinoma cell lines. Biochemical Pharmacology 51, 1349–1355.
Drori, S., et al., 2000. Clustering of mutations in the first transmembrane domain of the human reduced folate carrier in GW1843U89-resistant leukemia cells with impaired antifolate transport and augmented folate uptake. Journal of Biological Chemistry 275, 30855–30863.
Duch, D.S., et al., 1993. Biochemical and cellular pharmacology of 1843U89, a novel benzoquinazoline inhibitor of thymidylate synthase. Cancer Research 53, 810–818.
Dutta, B., et al., 1999. Cloning of the human thiamine transporter, a member of the folate transporter family. Journal of Biological Chemistry 274, 31925–31929.
Elnakat, H., Ratnam, M., 2004. Distribution, functionality and gene regulation of folate receptor isoforms: implications in targeted therapy. Advance Drug Delivery Reviews 56, 1067–1084.
Elnakat, H., Ratnam, M., 2006. Role of folate receptor genes in reproduction and related cancers. Frontiers in Bioscience 11, 506–519.
Eloranta, J.J., et al., 2009. Vitamin D3 and its nuclear receptor increase the expression and activity of the human proton-coupled folate transporter. Molecular Pharmacology 76, 1062–1071.
Elwood, P.C., et al., 1997. The divergent 5' termini of the alpha human folate receptor (hFR) mRNAs originate from two tissue-specific promoters and alternative splicing: characterization of the alpha hFR gene structure. Biochemistry 36, 1467–1478.
Ercikan-Abali, E.A., et al., 1997. Dihydrofolate reductase protein inhibits its own translation by binding to dihydrofolate reductase mRNA sequences within the coding region. Biochemistry 36, 12317–12322.
Ercikan, E., et al., 1993. Translational regulation of the synthesis of dihydrofolate reductase. Advances in Experimental Medicine and Biology 338, 537–540.
Falk, S., et al., 2006. Multicentre phase II pharmacokinetic and pharmacodynamic study of OSI-7904L in previously untreated patients with advanced gastric or gastroesophageal junction adenocarcinoma. British Journal of Cancer 95, 450–456.
Farber, S., et al., 1948. Temporary remissions in acute leukemia produced by folic acid antagonist, 4-aminopteroylglutamic acid (aminopterin). New England Journal of Medicine 238, 787–793.
Ferguson, P.L., Flintoff, W.F., 1999. Topological and functional analysis of the human reduced folate carrier by hemagglutinin epitope insertion. Journal of Biological Chemistry 274, 16269–16278.
Ferreri, A.J., et al., 2004. Aberrant methylation in the promoter region of the reduced folate carrier gene is a potential mechanism of resistance to methotrexate in primary central nervous system lymphomas. British Journal of Haematology 126, 657–664.
Feun, L.G., et al., 1991. Phase II trial of piritrexim in metastatic melanoma using intermittent, low-dose administration. Journal of Clinical Oncology 9, 464–467.
Feun, L.G., et al., 1995. Phase II trial of piritrexim and DTIC using an alternating dose schedule in metastatic melanoma. American Journal of Clinical Oncology 18, 488–490.
Fleming, G.F., Schilsky, R.L., 1992. Antifolates: the next generation. Seminars in Oncology 19, 707–719.
Fleming, J.C., et al., 1999. The gene mutated in thiamine-responsive anaemia with diabetes and deafness (TRMA) encodes a functional thiamine transporter. Nature Genetics 22, 305–308.
Flintoff, W.F., Essani, K., 1980. Methotrexate-resistant Chinese hamster ovary cells contain a dihydrofolate reductase with an altered affinity for methotrexate. Biochemistry 19, 4321–4327.
Flintoff, W.F., et al., 2004. Functional analysis of altered reduced folate carrier sequence changes identified in osteosarcomas. Biochimica et Biophysica Acta 1690, 110–117.
Foss, F.M., 2011. Evaluation of the pharmacokinetics, preclinical and clinical efficacy of pralatrexate for the treatment of T-cell lymphoma. Expert Opinion on Drug Metabolism & Toxicology 7, 1141–1152.
Fox, J.T., Stover, P.J., 2008. Folate-mediated one-carbon metabolism. Vitamins & Hormones 79, 1–44.
Freemantle, S.J., et al., 1995. Molecular characterisation of two cell lines selected for resistance to the folate-based thymidylate synthase inhibitor, ZD1694. British Journal of Cancer 71, 925–930.
Fry, D.W., Besserer, J.A., 1988. Characterization of trimetrexate transport in human lymphoblastoid cells and development of impaired influx as a mechanism of resistance to lipophilic antifolates. Cancer Research 48, 6986–6991.
Galivan, J., et al., 2000. Glutamyl hydrolase. pharmacological role and enzymatic characterization. Pharmacology & Therapeutics 85, 207–215.
Galvani, E., Peters, G.J., Giovannetti, E., 2011. Thymidylate synthase inhibitors for non-small cell lung cancer. Expert Opinion on Investigational Drugs 20, 1343–1356.
Gibbs, D.D., et al., 2005. BGC 945, a novel tumor-selective thymidylate synthase inhibitor targeted to alpha-folate receptor-overexpressing tumors. Cancer Research 65, 11721–11728.
Gish, R.G., et al., 2007. Phase III randomized controlled trial comparing the survival of patients with unresectable hepatocellular carcinoma treated with nolatrexed or doxorubicin. Journal of Clinical Oncology 25, 3069–3075.
Goldie, J.H., et al., 1980. A methotrexate insensitive variant of folate reductase present in two lines of methotrexate-resistant L5178Y cells. European Journal of Cancer 16, 1539–1546.
Goldman, I.D., 1971. The characteristics of the membrane transport of amethopterin and the naturally occurring folates. Annals of the New York Academy of Sciences 186, 400–422.
Goldman, I.D., 1974. The mechanism of action of methotrexate. I. Interaction with a low-affinity intracellular site required for maximum inhibition of deoxyribonucleic acid synthesis in L-cell mouse fibroblasts. Molecular Pharmacology 10, 257–274.
Goldman, I.D., Lichtenstein, N.S., Oliverio, V.T., 1968. Carrier-mediated transport of the folic acid analogue, methotrexate, in the L1210 leukemia cell. Journal of Biological Chemistry 243, 5007–5017.
Gonen, N., Assaraf, Y.G., 2010. The obligatory intestinal folate transporter PCFT (SLC46A1) is regulated by nuclear respiratory factor 1. Journal of Biological Chemistry 285, 33602–33613.
Gonen, N., Bram, E.E., Assaraf, Y.G., 2008. PCFT/SLC46A1 promoter methylation and restoration of gene expression in human leukemia cells. Biochemical and Biophysical Research Communications 376, 787–792.
Gong, M., et al., 1999. Isolation and characterization of genomic sequences involved in the regulation of the human reduced folate carrier gene (RFC1). Gene 233, 21–31.
Gorlick, R., et al., 1997. Defective transport is a common mechanism of acquired methotrexate resistance in acute lymphocytic leukemia and is associated with decreased reduced folate carrier expression. Blood 89, 1013–1018.
Gottesman, M.M., Fojo, T., Bates, S.E., 2002. Multidrug resistance in cancer: role of ATP-dependent transporters. Nature Reviews Cancer 2, 48–58.
Guo, W., et al., 1999. Mechanisms of methotrexate resistance in osteosarcoma. Clinical Cancer Research 5, 621–627.
Habeck, L.L., et al., 1995. Substrate specificity of mammalian folylpolyglutamate synthetase for 5, 10-dideazatetrahydrofolate analogs. Molecular Pharmacology 48, 326–333.
Haber, D.A., Schimke, R.T., 1981. Unstable amplification of an altered dihydrofolate reductase gene associated with double-minute chromosomes. Cell 26, 355–362.
Hainsworth, J., Vergote, I., Janssens, J., 2003. A review of phase II studies of ZD9331 treatment for relapsed or refractory solid tumours. Anticancer Drugs 1 (14 Suppl), S13–S19.
Hakala, M.T., 1965. On the role of drug penetration in amethopterin resistance of Sarcoma-180 cells in vitro. Biochimica et Biophysica Acta 102, 198–209.
Hamkalo, B.A., et al., 1985. Ultrastructural features of minute chromosomes in a methotrexate-resistant mouse 3T3 cell line. Proceedings of the National Academy of Sciences United States of America 82, 1126–1130.
Hanlon, M.H., Ferone, R., 1996. In vitro uptake, anabolism, and cellular retention of 1843U89 and other benzoquinazoline inhibitors of thymidylate synthase. Cancer Research 56, 3301–3306.
Hao, H., Qi, H., Ratnam, M., 2003. Modulation of the folate receptor type beta gene by coordinate actions of retinoic acid receptors at activator Sp1/ets and repressor AP-1 sites. Blood 101, 4551–4560.

Hayashi, H., et al., 2007. Genetic polymorphism of C452T (T127I) in human gamma-glutamyl hydrolase in a Japanese population. Biological and Pharmaceutical Bulletin 30, 839–841.
Hazarika, M., et al., 2004. FDA drug approval summaries: pemetrexed (Alimta). Oncologist 9, 482–488.
Heidelberger, C., et al., 1957. Fluorinated pyrimidines, a new class of tumour-inhibitory compounds. Nature 179, 663–666.
Henderson, G.B., Zevely, E.M., 1983. Structural requirements for anion substrates of the methotrexate transport system in L1210 cells. Archives of Biochemistry and Biophysics 221, 438–446.
Hilgenbrink, A.R., Low, P.S., 2005. Folate receptor-mediated drug targeting: from therapeutics to diagnostics. Journal of Pharmaceutical Sciences 94, 2135–2146.
Hill, B.T., et al., 1979. Characteristics of transport of 4-amino antifolates and folate compounds by two lines of L5178Y lymphoblasts, one with impaired transport of methotrexate. Cancer Research 39, 2440–2446.
Hillcoat, B.L., Swett, V., Bertino, J.R., 1967. Increase of dihydrofolate reductase activity in cultured mammalian cells after exposure to methotrexate. Proceedings of the National Academy of Sciences United States of America 58, 1632–1637.
Hooijberg, J.H., et al., 1999. Antifolate resistance mediated by the multidrug resistance proteins MRP1 and MRP2. Cancer Research 59, 2532–2535.
Hooijberg, J.H., et al., 2003. The role of multidrug resistance proteins MRP1, MRP2 and MRP3 in cellular folate homeostasis. Biochemical Pharmacology 65, 765–771.
Horie, N., et al., 1995. Functional analysis and DNA polymorphism of the tandemly repeated sequences in the 5'-terminal regulatory region of the human gene for thymidylate synthase. Cell Structure and Function 20, 191–197.
Horns Jr., R.C., Dower, W.J., Schimke, R.T., 1984. Gene amplification in a leukemic patient treated with methotrexate. Journal of Clinical Oncology 2, 2–7.
Hou, Z., et al., 2011. Identification and functional impact of homo-oligomers of the human proton-coupled folate transporter. Journal of Biological Chemistry 287, 4982–4995.
Hough, B., et al., 2009. A phase II study of single agent OSI-7904L in patients with metastatic or recurrent squamous cell carcinoma of the head and neck (SCCHN). Journal of Clinical Oncology 27 (suppl; abstr e17005).
Hsueh, C.T., Dolnick, B.J., 1994. Regulation of folate-binding protein gene expression by DNA methylation in methotrexate-resistant KB cells. Biochemical Pharmacology 47, 1019–1027.
Hu, M., Chen, J., 2004. Nucleobase- and p-glycoprotein-mediated transport of AG337 in a Caco-2 cell culture model. Molecular Pharmacology 1, 194–200.
Hughey, C.T., et al., 1993. Genetic variation in thymidylate synthase confers resistance to 5-fluorodeoxyuridine. Advances in Experimental Medicine and Biology 339, 67–76.
Ifergan, I., Assaraf, Y.G., 2008. Molecular mechanisms of adaptation to folate deficiency. Vitamins & Hormones 79, 99–143.
Ifergan, I., Jansen, G., Assaraf, Y.G., 2008. The reduced folate carrier (RFC) is cytotoxic to cells under conditions of severe folate deprivation. RFC as a double edged sword in folate homeostasis, Journal of Biological Chemistry 283, 20687–20695.
Ifergan, I., et al., 2003. Reduced folate carrier protein expression in osteosarcoma: implications for the prediction of tumor chemosensitivity. Cancer 98, 1958–1966.
Jackman, A.L., Calvert, A.H., 1995. Folate-based thymidylate synthase inhibitors as anticancer drugs. Annals of Oncology 6, 871–881.
Jackman, A.L., et al., 1995. Mechanisms of acquired resistance to the quinazoline thymidylate synthase inhibitor ZD1694 (Tomudex) in one mouse and three human cell lines. British Journal of Cancer 71, 914–924.
Jackman, A.L., et al., 1997. Cellular pharmacology and in vivo activity of a new anticancer agent, ZD9331: a water-soluble, nonpolyglutamatable, quinazoline-based inhibitor of thymidylate synthase. Clinical Cancer Research 3, 911–921.
Jackman, A.L., et al., 1991. ICI D1694, a quinazoline antifolate thymidylate synthase inhibitor that is a potent inhibitor of L1210 tumor cell growth in vitro and in vivo: a new agent for clinical study. Cancer Research 51, 5579–5586.
Jackman, A.L., Theti, D.S., Gibbs, D.D., 2004. Antifolates targeted specifically to the folate receptor. Advance Drug Delivery Reviews 56, 1111–1125.
Jackson, R.C., et al., 1984. Biochemical pharmacology of the lipophilic antifolate, trimetrexate. Advances in Enzyme Regulation 22, 187–206.
Jackson, R.C., Harrap, K.R., 1973. Studies with a mathematical model of folate metabolism. Archives of Biochemistry and Biophysics 158, 827–841.
Jackson, R.C., Hart, L.I., Harrap, K.R., 1976. Intrinsic resistance to methotrexate of cultured mammalian cells in relation to the inhibition kinetics of their dihydrololate reductases. Cancer Research 36, 1991–1997.
Jackson, R.C., Niethammer, D., Hart, L.I., 1977. Reactivation of dihydrofolate reductase inhibted by methotrexate or aminopterin. Archives of Biochemistry and Biophysics 182, 646–656.
Jansen, G., 1999. Receptor- and Carrier-Mediated Transport Systems for Folates and Antifolates. Exploitation for Folate Chemotherapy and Immunotherapy. Humana Press Inc., Totowa, NJ.
Jansen, G., et al., 1999. Multiple mechanisms of resistance to polyglutamatable and lipophilic antifolates in mammalian cells: role of increased folylpolyglutamylation, expanded folate pools, and intralysosomal drug sequestration. Molecular Pharmacology 55, 761–769.
Jansen, G., et al., 1998. A structurally altered human reduced folate carrier with increased folic acid transport mediates a novel mechanism of antifolate resistance. Journal of Biological Chemistry 273, 30189–30198.
Jansen, G., et al., 2010. Membrane transport properties and biological activity of stereo-isomers of glycinamide ribonucleotide formyltransferase (GARFT) inhibitors AG2032 and AG2034. Pteridines 20, 109–114.
Jansen, G., et al., 1990. Methotrexate transport in variant human CCRF-CEM leukemia cells with elevated levels of the reduced folate carrier. Selective effect on carrier-mediated transport of physiological concentrations of reduced folates. Journal of Biological Chemistry 265, 18272–18277.
Jarmula, A., 2010. Antifolate inhibitors of thymidylate synthase as anticancer drugs. Mini-Reviews in Medicinal Chemistry 10, 1211–1222.
Jhawer, M., et al., 2007. Phase II trial of nolatrexed dihydrochloride [Thymitaq, AG 337] in patients with advanced hepatocellular carcinoma. Investigational New Drugs 25, 85–94.
Johnson, T.B., Nair, M.G., Galivan, J., 1988. Role of folylpolyglutamate synthetase in the regulation of methotrexate polyglutamate formation in H35 hepatoma cells. Cancer Research 48, 2426–2431.
Kamen, B.A., et al., 1988. Delivery of folates to the cytoplasm of MA104 cells is mediated by a surface membrane receptor that recycles. Journal of Biological Chemistry 263, 13602–13609.
Kane, M.A., et al., 1988. Influence on immunoreactive folate-binding proteins of extracellular folate concentration in cultured human cells. Journal of Clinical Investigation 81, 1398–1406.
Kastrup, I.B., et al., 2008. Genetic and epigenetic alterations of the reduced folate carrier in untreated diffuse large B-cell lymphoma. European Journal of Haematology 80, 61–66.
Kaufman, R.J., Brown, P.C., Schimke, R.T., 1979. Amplified dihydrofolate reductase genes in unstably methotrexate-resistant cells are associated with double minute chromosomes. Proceedings of the National Academy of Sciences United States of America 76, 5669–5673.
Kaufman, Y., et al., 2004. Reduced folate carrier mutations are not the mechanism underlying methotrexate resistance in childhood acute lymphoblastic leukemia. Cancer 100, 773–782.
Kaufman, Y., et al., 2006. Coexistence of multiple mechanisms of PT523 resistance in human leukemia cells harboring 3 reduced folate carrier alleles: transcriptional silencing, inactivating mutations, and allele loss. Blood 107, 3288–3294.
Kelemen, L.E., 2006. The role of folate receptor alpha in cancer development, progression and treatment: cause, consequence or innocent bystander? International Journal of Cancer 119, 243–250.
Kelley, K.M., Rowan, B.G., Ratnam, M., 2003. Modulation of the folate receptor alpha gene by the estrogen receptor: mechanism and implications in tumor targeting. Cancer Research 63, 2820–2828.
Khorsand, M., et al., 1997. Phase II trial of oral piritrexim in advanced, previously treated transitional cell cancer of bladder. Investigational New Drugs 15, 157–163.
Klohs, W.D., et al., 1986. Cross resistance of pleiotropically drug resistant P338 leukemia cells to the lipophilic antifolates trimetrexate and BW 301U. Cancer Letters 31, 253–260.
Kobayashi, H., Takemura, Y., Miyachi, H., 1998. Molecular characterization of human acute leukemia cell line resistant to ZD9331, a non-polyglutamatable thymidylate synthase inhibitor. Cancer Chemotherapy and Pharmacology 42, 105–110.
Krug, L.M., et al., 2003. 10-propargyl-10-deazaaminopterin: an antifolate with activity in patients with previously treated non-small cell lung cancer. Clinical Cancer Research 9, 2072–2078.
Krug, L.M., et al., 2000. Phase I and pharmacokinetic study of 10-propargyl-10-deazaaminopterin, a new antifolate. Clinical Cancer Research 6, 3493–3498.
Kugel Desmoulin, S., et al., 2011. Therapeutic targeting of a novel 6-substituted pyrrolo [2,3-d]pyrimidine thienoyl antifolate to human solid tumors based on selective uptake by the proton-coupled folate transporter. Molecular Pharmacology 80, 1096–1107.
Kusuhara, H., et al., 1998. Reduced folate derivatives are endogenous substrates for cMOAT in rats. American Journal of Physiology 275, G789–G796.
Labay, V., et al., 1999. Mutations in SLC19A2 cause thiamine-responsive megaloblastic anaemia associated with diabetes mellitus and deafness. Nature Genetics 22, 300–304.
Lacerda, J.F., et al., 1995. Selective treatment of SCID mice bearing methotrexate-transport-resistant human acute lymphoblastic leukemia tumors with trimetrexate and leucovorin protection. Blood 85, 2675–2679.
Laftah, A.H., et al., 2009. Haem and folate transport by proton-coupled folate transporter/haem carrier protein 1 (SLC46A1). British Journal of Nutrition 101, 1150–1156.
Lasry, I., et al., 2008. A novel loss-of-function mutation in the proton-coupled folate transporter from a patient with hereditary folate malabsorption reveals that Arg 113 is crucial for function. Blood 112, 2055–2061.
Lassiter, L.K., et al., 2008. Phase II open-label study of oral piritrexim in patients with advanced carcinoma of the urothelium who have experienced failure with standard chemotherapy. Clinical Genitourinary Cancer 6, 31–35.
Lawrence, S.A., Hackett, J.C., Moran, R.G., 2011. Tetrahydrofolate recognition by the mitochondrial folate transporter. Journal of Biological Chemistry 286, 31480–31489.
Leamon, C.P., et al., 2008. Folate targeting enables durable and specific antitumor responses from a therapeutically null tubulysin B analogue. Cancer Research 68, 9839–9844.
Leclerc, G.J., et al., 2010. Histone deacetylase inhibitors induce FPGS mRNA expression and intracellular accumulation of long-chain methotrexate polyglutamates in childhood acute lymphoblastic leukemia: implications for combination therapy. Leukemia 24, 552–562.
Lee, K., Klein-Szanto, A.J., Kruh, G.D., 2000. Analysis of the MRP4 drug resistance profile in transfected NIH3T3 cells. Journal of the National Cancer Institute 92, 1934–1940.

Levy, A.S., et al., 2003. Reduced folate carrier and dihydrofolate reductase expression in acute lymphocytic leukemia may predict outcome: a Children's Cancer Group Study. Journal of Pediatric Hematology Oncology 25, 688–695.
Lewis, W.S., et al., 1995. Methotrexate-resistant variants of human dihydrofolate reductase with substitutions of leucine 22. Kinetics, crystallography, and potential as selectable markers. Journal of Biological Chemistry 270, 5057–5064.
Li, J., et al., 2011. Xenopus reduced folate carrier regulates neural crest development epigenetically. PLoS One 6, pe27198.
Li, W.W., et al., 1992. Intrinsic resistance to methotrexate in human soft tissue sarcoma cell lines. Cancer Research 52, 3908–3913.
Li, W.W., Tong, W.P., Bertino, J.R., 1995. Antitumor activity of antifolate inhibitors of thymidylate and purine synthesis in human soft tissue sarcoma cell lines with intrinsic resistance to methotrexate. Clinical Cancer Research 1, 631–636.
Li, W.W., et al., 1993. Increased activity of gamma-glutamyl hydrolase in human sarcoma cell lines: a novel mechanism of intrinsic resistance to methotrexate (MTX). Advances in Experimental Medicine and Biology 338, 635–638.
Liani, E., et al., 2003. Loss of folylpoly-gamma-glutamate synthetase activity is a dominant mechanism of resistance to polyglutamylation-dependent novel antifolates in multiple human leukemia sublines. International Journal of Cancer 103, 587–599.
Lin, B.F., Huang, R.F., Shane, B., 1993. Regulation of folate and one-carbon metabolism in mammalian cells. III. Role of mitochondrial folylpoly-gamma-glutamate synthetase. Journal of Biological Chemistry 268, 21674–21679.
Lin, X., et al., 2003. Role of cysteine amino acid residues on the RNA binding activity of human thymidylate synthase. Nucleic Acids Research 31, 4882–4887.
Lin, X., et al., 2000. Characterization of a cis-acting regulatory element in the protein coding region of thymidylate synthase mRNA. Nucleic Acids Research 28, 1381–1389.
Liu, M., et al., 2005. Structure and regulation of the murine reduced folate carrier gene: identification of four noncoding exons and promoters and regulation by dietary folates. Journal of Biological Chemistry 280, 5588–5597.
Liu, X.Y., Matherly, L.H., 2002. Analysis of membrane topology of the human reduced folate carrier protein by hemagglutinin epitope insertion and scanning glycosylation insertion mutagenesis. Biochimica et Biophysica Acta 1564, 333–342.
Louvet, C., et al., 2004. A phase I-II, dose-escalating trial of ZD9331 in combination with irinotecan (CPT11) in previously pretreated metastatic colorectal cancer patients. Bull Cancer 91, 279–284.
Low, P.S., Kularatne, S.A., 2009. Folate-targeted therapeutic and imaging agents for cancer. Current Opinion in Chemical Biology 13, 256–262.
Lu, K., et al., 1995. Mechanisms of resistance to N-[5-[N-(3, 4-dihydro-2-methyl-4- oxoquinazolin-6-ylmethyl)-N-methylamino]-2-thenoyl]-L-glutamic acid (ZD1694), a folate-based thymidylate synthase inhibitor, in the HCT-8 human ileocecal adenocarcinoma cell line. Biochemical Pharmacology 50, 391–398.
Lu, Y., Low, P.S., 2002. Folate-mediated delivery of macromolecular anticancer therapeutic agents. Advance Drug Delivery Reviews 54, 675–693.
Lurje, G., et al., 2009. Thymidylate synthase gene variations: predictive and prognostic markers. Molecular Cancer Therapeutics 8, 1000–1007.
Manegold, C., 2003. Pemetrexed (Alimta, MTA, multitargeted antifolate, LY231514) for malignant pleural mesothelioma. Seminars in Oncology 30, 32–36.
Marshall, J.L., DeLap, R.J., 1994. Clinical pharmacokinetics and pharmacology of trimetrexate. Clinical Pharmacokinetics 26, 190–200.
Martin, M., 2006. Clinical experience with pemetrexed in breast cancer. Seminars in Oncology 33, S15–S18.
Masuda, M., et al., 1997. Methotrexate is excreted into the bile by canalicular multispecific organic anion transporter in rats. Cancer Research 57, 3506–3510.
Matherly, L.H., Angeles, S.M., 1994. Role of N-glycosylation in the structure and function of the methotrexate membrane transporter from CCRF-CEM human lymphoblastic leukemia cells. Biochemical Pharmacology 47, 1094–1098.
Matherly, L.H., Angeles, S.M., Czajkowski, C.A., 1992. Characterization of transport-mediated methotrexate resistance in human tumor cells with antibodies to the membrane carrier for methotrexate and tetrahydrofolate cofactors. Journal of Biological Chemistry 267, 23253–23260.
Matherly, L.H., Goldman, D.I., 2003. Membrane transport of folates. Vitamins & Hormones 66, 403–456.
Matherly, L.H., et al., 1997. Increased frequency of expression of elevated dihydrofolate reductase in T-cell versus B-precursor acute lymphoblastic leukemia in children. Blood 90, 578–589.
Mauritz, R., et al., 2009. Polymorphisms in the enhancer region of the thymidylate synthase gene are associated with thymidylate synthase levels in normal tissues but not in malignant tissues of patients with colorectal cancer. Clinical Colorectal Cancer 8, 146–154.
Mauritz, R., et al., 2002. Multiple mechanisms of resistance to methotrexate and novel antifolates in human CCRF-CEM leukemia cells and their implications for folate homeostasis. Biochemical Pharmacology 63, 105–115.
McBurney, M.W., Whitmore, G.F., 1974. Characterization of a Chinese hamster cell with a temperature-sensitive mutation in folate metabolism. Cell 2, 183–188.
McCloskey, D.E., et al., 1991. Decreased folylpolyglutamate synthetase activity as a mechanism of methotrexate resistance in CCRF-CEM human leukemia sublines. Journal of Biological Chemistry 266, 6181–6187.
McGuire, J., et al., 1995. Evolution of drug resistance in CCRF-CEM human leukemia cells selected by intermittent methotrexate exposure. Oncology Research 7, 535–543.
McGuire, J.J., et al., 1993. Cross-resistance studies of folylpolyglutamate synthetase-deficient, methotrexate-resistant CCRF-CEM human leukemia sublines. Leukemia 7, 1996–2003.
McGuire, J.J., et al., 1980. Enzymatic synthesis of folylpolyglutamates. Characterization of the reaction and its products. Journal of Biological Chemistry 255, 5776–5788.
McGuire, J.J., Russell, C.A., 1998. Folylpolyglutamate synthetase expression in antifolate-sensitive and -resistant human cell lines. Oncology Research 10, 193–200.
McIvor, R.S., Simonsen, C.C., 1990. Isolation and characterization of a variant dihydrofolate reductase cDNA from methotrexate-resistant murine L5178Y cells. Nucleic Acids Research 18, 7025–7032.
Melera, P.W., et al., 1984. Phenotypic expression in Escherichia coli and nucleotide sequence of two Chinese hamster lung cell cDNAs encoding different dihydrofolate reductases. Molecular and Cellular Biology 4, 38–48.
Melera, P.W., Davide, J.P., Oen, H., 1988. Antifolate-resistant Chinese hamster cells. Molecular basis for the biochemical and structural heterogeneity among dihydrofolate reductases produced by drug-sensitive and drug-resistant cell lines. Journal of Biological Chemistry 263, 1978–1990.
Meyer, E., et al., 2010. A novel PCFT gene mutation (p.Cys66LeufsX99) causing hereditary folate malabsorption. Molecular Genetics and Metabolism 99, 325–328.
Mini, E., et al., 1985. Molecular and karyological analysis of methotrexate-resistant and -sensitive human leukemic CCRF-CEM cells. Cancer Research 45, 317–324.
Miyachi, H., et al., 1995. Expression of variant dihydrofolate reductase with decreased binding affinity to antifolates in MOLT-3 human leukemia cell lines resistant to trimetrexate. Cancer Letters 88, 93–99.
Mok, T.S., et al., 1999. A multi-centre randomized phase II study of nolatrexed versus doxorubicin in treatment of Chinese patients with advanced hepatocellular carcinoma. Cancer Chemotherapy and Pharmacology 44, 307–311.
Morales, C., et al., 2009. Dihydrofolate reductase amplification and sensitization to methotrexate of methotrexate-resistant colon cancer cells. Molecular Cancer Therapeutics 8, 424–432.
Moran, R.G., 1999. Roles of folylpoly-gamma-glutamate synthetase in therapeutics with tetrahydrofolate antimetabolites: an overview. Seminars in Oncology 26, 24–32.
Moran, R.G., Werkheiser, W.C., Zakrzewski, S.F., 1976. Folate metabolism in mammalian cells in culture. I Partial characterization of the folate derivatives present in L1210 mouse leukemia cells. Journal of Biological Chemistry 251, 3569–3575.
Moscow, J.A., et al., 1995. Isolation of a gene encoding a human reduced folate carrier (RFC1) and analysis of its expression in transport-deficient, methotrexate-resistant human breast cancer cells. Cancer Research 55, 3790–3794.
Muller, C., 2012. Folate based radiopharmaceuticals for imaging and therapy of cancer and inflammation. Current Pharmaceutical Design 18, 1058–1083.
Naumann, R.W., Coleman, R.L., 2011. Management strategies for recurrent platinum-resistant ovarian cancer. Drugs 71, 1397–1412.
Naumann, R.W., et al., 2011. PRECEDENT: A randomized phase II trial comparing EC145 and pegylated liposomal doxorubicin (PLD) in combination, versus PLD alone, in subjects with platinum-resistant ovarian cancer. Journal of Clinical Oncology, 29 (suppl; abstr 5045).
Niculescu-Duvaz, I., 2001. Thymitaq (Zarix). Current Opinion in Investigational Drugs 2, 693–705.
Niethammer, D., Jackson, R.C., 1975. Changes of molecular properties associated with the development of resistance against methotrexate in human lymphoblastoid cells. European Journal of Cancer 11, 845–854.
Nimec, Z., Galivan, J., 1983. Regulatory aspects of the glutamylation of methotrexate in cultured hepatoma cells. Archives of Biochemistry and Biophysics 226, 671–680.
Nunberg, J.H., et al., 1978. Amplified dihydrofolate reductase genes are localized to a homogeneously staining region of a single chromosome in a methotrexate-resistant Chinese hamster ovary cell line. Proceedings of the National Academy of Sciences United States of America 75, 5553–5556.
O'Connor, B.M., et al., 1992. Human lymphoblastoid cells with acquired resistance to C2-desamino-C2-methyl-N10-propargyl-5, 8-dideazafolic acid: a novel folate-based thymidylate synthase inhibitor. Cancer Research 52, 1137–1143.
O'Connor, O.A., et al., 2009. Phase II-I-II study of two different doses and schedules of pralatrexate, a high-affinity substrate for the reduced folate carrier, in patients with relapsed or refractory lymphoma reveals marked activity in T-cell malignancies. Journal of Clinical Oncology 27, 4357–4364.
O'Connor, O.A., et al., 2011. Pralatrexate in patients with relapsed or refractory peripheral T-cell lymphoma: results from the pivotal PROPEL study. Journal of Clinical Oncology 29, 1182–1189.
Obajimi, O., Keen, J.C., Melera, P.W., 2009. Inhibition of de novo purine synthesis in human prostate cells results in ATP depletion, AMPK activation and induces senescence. The Prostate 69, 1206–1221.
Organista-Nava, J., et al., 2010. Polymorphisms of the gamma-glutamyl hydrolase gene and risk of relapse to acute lymphoblastic leukemia in Mexico. Leukemia Research 34, 728–732.
Pao, S.S., Paulsen, I.T., Saier Jr., M.H., 1998. Major facilitator superfamily. Microbiology and Molecular Biology Reviews 62, 1–34.
Parker, N., et al., 2005. Folate receptor expression in carcinomas and normal tissues determined by a quantitative radioligand binding assay. Analytical Biochemistry 338, 284–293.
Paulos, C.M., et al., 2004. Folate receptor-mediated targeting of therapeutic and imaging agents to activated macrophages in rheumatoid arthritis. Advance Drug Delivery Reviews 56, 1205–1217.

Pizzorno, G., et al., 1993. 5,10-Dideazatetrahydrofolic acid (DDATHF) transport in CCRF-CEM and MA104 cell lines. Journal of Biological Chemistry 268, 1017–1023.
Pizzorno, G., et al., 1989. Inherent resistance of human squamous carcinoma cell lines to methotrexate as a result of decreased polyglutamylation of this drug. Cancer Research 49, 5275–5280.
Pizzorno, G., et al., 1988. Impaired polyglutamylation of methotrexate as a cause of resistance in CCRF-CEM cells after short-term, high-dose treatment with this drug. Cancer Research 48, 2149–2155.
Pizzorno, G., et al., 1991a. (6R)-5,10-Dideaza-5,6,7,8-tetrahydrofolic acid effects on nucleotide metabolism in CCRF-CEM human T-lymphoblast leukemia cells. Cancer Research 51, 2291–2295.
Pizzorno, G., et al., 1995. Multifactorial resistance to 5, 10-dideazatetrahydrofolic acid in cell lines derived from human lymphoblastic leukemia CCRF-CEM. Cancer Research 55, 566–573.
Pizzorno, G., et al., 1991b. Intracellular metabolism of 5, 10-dideazatetrahydrofolic acid in human leukemia cell lines. Molecular Pharmacology 39, 85–89.
Polgar, O., Robey, R.W., Bates, S.E., 2008. ABCG2: structure, function and role in drug response. Expert Opinion on Drug Metabolism & Toxicology 4, 1–15.
Prasad, P.D., et al., 1995. Molecular cloning of the human placental folate transporter. Biochemical and Biophysical Research Communications 206, 681–687.
Qiu, A., et al., 2006. Identification of an intestinal folate transporter and the molecular basis for hereditary folate malabsorption. Cell 127, 917–928.
Qiu, A., et al., 2007. Rodent intestinal folate transporters (SLC46A1): secondary structure, functional properties, and response to dietary folate restriction. American Journal of Physiology Cell Physiology 293, C1669–C1678.
Racanelli, A.C., et al., 2009. Therapeutics by cytotoxic metabolite accumulation: pemetrexed causes ZMP accumulation, AMPK activation, and mammalian target of rapamycin inhibition. Cancer Research 69, 5467–5474.
Rader, J.S., et al., 2003. A phase II study to determine the efficacy and tolerability of intravenous ZD9331 in heavily pretreated patients with ovarian cancer. Gynecologic Oncology 91, 318–325.
Rajgopal, A., et al., 2001. SLC19A3 encodes a second thiamine transporter ThTr2. Biochimica et Biophysica Acta 1537, 175–178.
Ratnam, M., et al., 1989. Homologous membrane folate binding proteins in human placenta: cloning and sequence of a cDNA. Biochemistry 28, 8249–8254.
Ray, M.S., et al., 1993. Phase I study of (6R)-5, 10-dideazatetrahydrofolate: a folate antimetabolite inhibitory to de novo purine synthesis. Journal of the National Cancer Institute 85, 1154–1159.
Reilly, R.T., Forsthoefel, A.M., Berger, F.G., 1997. Functional effects of amino acid substitutions at residue 33 of human thymidylate synthase. Archives of Biochemistry and Biophysics 342, 338–343.
Rhee, M.S., et al., 1993. Acquisition of resistance to antifolates caused by enhanced gamma-glutamyl hydrolase activity. Cancer Research 53, 2227–2230.
Ricart, A.D., et al., 2008. Phase I, pharmacokinetic and biological correlative study of OSI-7904L, a novel liposomal thymidylate synthase inhibitor, and cisplatin in patients with solid tumors. Clinical Cancer Research 14, 7947–7955.
Righi, L., et al., 2010. Thymidylate synthase but not excision repair cross-complementation group 1 tumor expression predicts outcome in patients with malignant pleural mesothelioma treated with pemetrexed-based chemotherapy. Journal of Clinical Oncology 28, 1534–1539.
Roberts, J.D., et al., 2000. Weekly lometrexol with daily oral folic acid is appropriate for phase II evaluation. Cancer Chemotherapy and Pharmacology 45, 103–110.
Rocha Lima, C., et al., 2006. A phase I study of talotrexin (PT-523) in patients with relapsed or refractory non-small cell lung cancer (NSCLC). Journal of Clinical Oncology 24, 7142, No. 18S (June 20 Supplement).
Rochman, H., Selhub, J., Karrison, T., 1985. Folate binding protein and the estrogen receptor in breast cancer. Cancer Detection and Prevention 8, 71–75.
Rosowsky, A., Bader, H., Freisheim, J.H., 1991. Synthesis and biological activity of methotrexate analogues with two acid groups and a hydrophobic aromatic ring in the side chain. Journal of Medicinal Chemistry 34, 574–579.
Rosowsky, A., et al., 1998. Synthesis and potent antifolate activity and cytotoxicity of B-ring deaza analogues of the nonpolyglutamatable dihydrofolate reductase inhibitor Nalpha-(4-amino-4-deoxypteroyl)-Ndelta-hemiphthaloyl-L-ornithine (PT523). Journal of Medicinal Chemistry 41, 5310–5319.
Ross, J.F., Chaudhuri, P.K., Ratnam, M., 1994. Differential regulation of folate receptor isoforms in normal and malignant tissues in vivo and in established cell lines. Physiologic and clinical implications. Cancer 73, 2432–2443.
Ross, J.F., et al., 1999. Folate receptor type beta is a neutrophilic lineage marker and is differentially expressed in myeloid leukemia. Cancer 85, 348–357.
Roth, B.J., et al., 2002. Piritrexim in advanced, refractory carcinoma of the urothelium (E3896): a phase II trial of the Eastern Cooperative Oncology Group. Investigational New Drugs 20, 425–429.
Rothberg, K.G., et al., 1990. The glycophospholipid-linked folate receptor internalizes folate without entering the clathrin-coated pit endocytic pathway. Journal of Cell Biology 110, 637–649.
Rothem, L., Aronheim, A., Assaraf, Y.G., 2003. Alterations in the expression of transcription factors and the reduced folate carrier as a novel mechanism of antifolate resistance in human leukemia cells. Journal of Biological Chemistry 278, 8935–8941.
Rothem, L., et al., 2002. Resistance to multiple novel antifolates is mediated via defective drug transport resulting from clustered mutations in the reduced folate carrier gene in human leukaemia cell lines. Biochemical Journal 367, 741–750.
Rothem, L., Stark, M., Assaraf, Y.G., 2004a. Impaired CREB-1 phosphorylation in antifolate-resistant cell lines with down-regulation of the reduced folate carrier gene. Molecular Pharmacology 66, 1536–1543.
Rothem, L., et al., 2004b. Reduced folate carrier gene silencing in multiple antifolate-resistant tumor cell lines is due to a simultaneous loss of function of multiple transcription factors but not promoter methylation. Journal of Biological Chemistry 279, 374–384.
Rots, M.G., et al., 2000. mRNA expression levels of methotrexate resistance-related proteins in childhood leukemia as determined by a standardized competitive template-based RT-PCR method. Leukemia 14, 2166–2175.
Roy, K., et al., 1998. Chromosomal localization of the murine RFC-1 gene encoding a folate transporter and its amplification in an antifolate resistant variant overproducing the transporter. Cancer Genetics and Cytogenetics 105, 29–38.
Roy, K., et al., 1997. Posttranscriptionally mediated decreases in folylpolyglutamate synthetase gene expression in some folate analogue-resistant variants of the L1210 cell. Evidence for an altered cognate mRNA in the variants affecting the rate of de novo synthesis of the enzyme. Journal of Biological Chemistry 272, 6903–6908.
Rumberger, B.G., et al., 1990. Preferential selection during therapy in vivo by edatrexate compared to methotrexate of resistant L1210 cell variants with decreased folylpolyglutamate synthetase activity. Cancer Communication 2, 305–310.
Sadasivan, E., Cedeno, M.M., Rothenberg, S.P., 1994. Characterization of the gene encoding a folate-binding protein expressed in human placenta. Identification of promoter activity in a G-rich SP1 site linked with the tandemly repeated GGAAG motif for the ets encoded GA-binding protein. Journal of Biological Chemistry 269, 4725–4735.
Saier Jr., M.H., 1999. A functional-phylogenetic system for the classification of transport proteins. Journal of Cellular Biochemistry Supplement 32–33, 84–94.
Saikawa, Y., et al., 1993. Decreased expression of the human folate receptor mediates transport-defective methotrexate resistance in KB cells. Journal of Biological Chemistry 268, 5293–5301.
Saikawa, Y., et al., 1995. Structural and functional analysis of the human KB cell folate receptor gene P4 promoter: cooperation of three clustered Sp1-binding sites with initiator region for basal promoter activity. Biochemistry 34, 9951–9961.
Salazar, M.D., Ratnam, M., 2007. The folate receptor: what does it promise in tissue-targeted therapeutics? Cancer and Metastasis Reviews 26, 141–152.
Schiesel, J.D., et al., 1992. Oral piritrexim—a phase II study in patients with advanced soft tissue sarcoma. Investigational New Drugs 10, 97–98.
Schirch, V., Strong, W.B., 1989. Interaction of folylpolyglutamates with enzymes in one-carbon metabolism. Archives of Biochemistry and Biophysics 269, 371–380.
Schlemmer, S.R., Sirotnak, F.M., 1995. Structural preferences among folate compounds and their analogues for ATPase-mediated efflux by inside-out plasma membrane vesicles derived from L1210 cells. Biochemical Pharmacology 49, 1427–1433.
Schmid, F.A., et al., 1985. New folate analogs of the 10-deaza-aminopterin series: markedly increased antitumor activity of the 10-ethyl analog compared to the parent compound and methotrexate against some human tumor xenografts in nude mice. Cancer Treatment Reports 69, 551–553.
Schneider, E., Ryan, T.J., 2006. Gamma-glutamyl hydrolase and drug resistance. Clinica Chimica Acta 374, 25–32.
Schultz, R.M., et al., 1999. Biological activity of the multitargeted antifolate, MTA (LY231514), in human cell lines with different resistance mechanisms to antifolate drugs. Seminars in Oncology 26, 68–73.
Schulz, J., et al., 2004. ZD9331 as second- or third-line therapy in patients with advanced colorectal cancer: a phase II multicenter trial. American Journal of Clinical Oncology 27, 337–342.
Schwartz, G., et al., 2001. A phase I and pharmacokinetic study of 1843U89, a non-competitive inhibitor of thymidylate synthase, in patients with advanced solid malignancies. Clinical Cancer Research 7, 1901–1911.
Shafran, A., et al., 2005. ABCG2 harboring the Gly482 mutation confers high-level resistance to various hydrophilic antifolates. Cancer Research 65, 8414–8422.
Shayeghi, M., et al., 2005. Identification of an intestinal heme transporter. Cell 122, 789–801.
Shen, F., et al., 1994. Identification of a novel folate receptor, a truncated receptor, and receptor type beta in hematopoietic cells: cDNA cloning, expression, immunoreactivity, and tissue specificity. Biochemistry 33, 1209–1215.
Shen, F., et al., 1995. Folate receptor type gamma is primarily a secretory protein due to lack of an efficient signal for glycosylphosphatidylinositol modification: protein characterization and cell type specificity. Biochemistry 34, 5660–5665.
Shih, C., et al., 1997. LY231514, a pyrrolo[2,3-d]pyrimidine-based antifolate that inhibits multiple folate-requiring enzymes. Cancer Research 57, 1116–1123.
Shin, D.S., et al., 2011. Identification of novel mutations in the proton-coupled folate transporter (PCFT-SLC46A1) associated with hereditary folate malabsorption. Molecular Genetics and Metabolism 103, 33–37.
Shin, D.S., et al., 2012. A P425R mutation of the proton-coupled folate transporter causing hereditary folate malabsorption produces a highly selective alteration in folate binding. American Journal of Physiology Cell Physiology.
Sierra, E.E., et al., 1997. pH dependence of methotrexate transport by the reduced folate carrier and the folate receptor in L1210 leukemia cells. Further evidence for a third route mediated at low pH. Biochemical Pharmacology 53, 223–231.
Sierra, E.E., Goldman, I.D., 1998. Characterization of folate transport mediated by a low pH route in mouse L1210 leukemia cells with defective reduced folate carrier function. Biochemical Pharmacology 55, 1505–1512.
Sigmond, J., et al., 2003. Induction of resistance to the multitargeted antifolate Pemetrexed (ALIMTA) in WiDr human colon cancer cells is associated with thymidylate synthase overexpression. Biochemical Pharmacology 66, 431–438.

Simonsen, C.C., Levinson, A.D., 1983. Isolation and expression of an altered mouse dihydrofolate reductase cDNA. Proceedings of the National Academy of Sciences United States of America 80, 2495–2499.
Singer, M.J., et al., 2000. Amplification of the human dihydrofolate reductase gene via double minutes is initiated by chromosome breaks. Proceedings of the National Academy of Sciences United States of America 97, 7921–7926.
Sirotnak, F.M., 1985. Obligate genetic expression in tumor cells of a fetal membrane property mediating "folate" transport: biological significance and implications for improved therapy of human cancer. Cancer Research 45, 3992–4000.
Sirotnak, F.M., et al., 1998. A new analogue of 10-deazaaminopterin with markedly enhanced curative effects against human tumor xenografts in mice. Cancer Chemotherapy and Pharmacology 42, 313–318.
Sirotnak, F.M., et al., 1984a. New folate analogs of the 10-deaza-aminopterin series. Basis for structural design and biochemical and pharmacologic properties. Cancer Chemotherapy and Pharmacology 12, 18–25.
Sirotnak, F.M., et al., 1984b. New folate analogs of the 10-deaza-aminopterin series. Further evidence for markedly increased antitumor efficacy compared with methotrexate in ascitic and solid murine tumor models. Cancer Chemotherapy and Pharmacology 12, 26–30.
Sirotnak, F.M., Donsbach, R.C., 1974. The intracellular concentration dependence of antifolate inhibition of DNA synthesis in L1210 leukemia cells. Cancer Research 34, 332–340.
Sirotnak, F.M., Kurita, S., Hutchison, D.J., 1968. On the nature of a transport alteration determining resistance to amethopterin in the L1210 leukemia. Cancer Research 28, 75–80.
Sirotnak, F.M., et al., 1981. Relative frequency and kinetic properties of transport-defective phenotypes among methotrexate-resistant L1210 clonal cell lines derived in vivo. Cancer Research 41, 4447–4452.
Sirotnak, F.M., et al., 1987. 10-Ethyl-10-deaza-aminopterin: structural design and biochemical, pharmacologic, and antitumor properties. NCI Monographs, 127–131.
Skacel, N., et al., 2005. Identification of amino acids required for the functional up-regulation of human dihydrofolate reductase protein in response to antifolate Treatment. Journal of Biological Chemistry 280, 22721–22731.
Smith, D., Gallagher, N., 2003. A phase II/III study comparing intravenous ZD9331 with gemcitabine in patients with pancreatic cancer. European Journal of Cancer 39, 1377–1383.
Smith, G., et al., 1999. GW1843, a Potent, Noncompetitive Thymidylate Synthase Inhibitor—Preclinical and Preliminary Clinical Studies. Humana Press, Inc.
Smith, G.K., et al., 1995. Enhanced antitumor activity for the thymidylate synthase inhibitor 1843U89 through decreased host toxicity with oral folic acid. Cancer Research 55, 6117–6125.
Spencer, H.T., et al., 1996. Mutations in the gene for human dihydrofolate reductase: an unlikely cause of clinical relapse in pediatric leukemia after therapy with methotrexate. Leukemia 10, 439–446.
Srimatkandada, S., et al., 1989. Amplification of a polymorphic dihydrofolate reductase gene expressing an enzyme with decreased binding to methotrexate in a human colon carcinoma cell line, HCT-8R4, resistant to this drug. Journal of Biological Chemistry 264, 3524–3528.
Stark, M., Assaraf, Y.G., 2006. Loss of Sp1 function via inhibitory phosphorylation in antifolate-resistant human leukemia cells with down-regulation of the reduced folate carrier. Blood 107, 708–715.
Stark, M., Gonen, N., Assaraf, Y.G., 2009a. Functional elements in the minimal promoter of the human proton-coupled folate transporter. Biochemical and Biophysical Research Communications 388, 79–85.
Stark, M., et al., 2003. Antifolate resistance associated with loss of MRP1 expression and function in Chinese hamster ovary cells with markedly impaired export of folate and cholate. Molecular Pharmacology 64, 220–227.
Stark, M., et al., 2009b. Aberrant splicing of folylpolyglutamate synthetase as a novel mechanism of antifolate resistance in leukemia. Blood 113, 4362–4369.
Stockstad, E.L.R., 1990. Historical perspective on key advances in the biochemistry and physiology of folates. In: Folic Acid Metabolism in Health and Disease. Wiley-Liss, New York, pp. 1–21.
Stuart, K., et al., 1999. A Phase II trial of nolatrexed dihydrochloride in patients with advanced hepatocellular carcinoma. Cancer 86, 410–414.
Surmont, V.F., van Meerbeeck, J.P., 2011. Raltitrexed in mesothelioma. Expert Review of Anticancer Therapy 11, 1481–1490.
Szakacs, G., et al., 2006. Targeting multidrug resistance in cancer. Nature Reviews Drug Discovery 5, 219–234.
Tai, N., et al., 2002. Identification of critical amino acid residues on human dihydrofolate reductase protein that mediate RNA recognition. Nucleic Acids Research 30, 4481–4488.
Tai, N., et al., 2004a. Characterization of a cis-acting regulatory element in the protein-coding region of human dihydrofolate reductase mRNA. Biochemical Journal 378, 999–1006.
Tai, N., et al., 2008. Identification of a cis-acting element of human dihydrofolate reductase mRNA. Biochemical and Biophysical Research Communications 369, 795–800.
Tai, N., et al., 2004b. Translational autoregulation of thymidylate synthase and dihydrofolate reductase. Frontiers in Bioscience 9, 2521–2526.
Takemura, Y., Kobayashi, H., Miyachi, H., 1999. Variable expression of the folylpolyglutamate synthetase gene at the level of mRNA transcription in human leukemia cell lines sensitive, or made resistant, to various antifolate drugs. Anticancer Drugs 10, 677–683.
Takezawa, K., et al., 2011. Thymidylate synthase as a determinant of pemetrexed sensitivity in non-small cell lung cancer. British Journal of Cancer 104, 1594–1601.
Takimoto, C.H., 1996. New antifolates: pharmacology and clinical applications. Oncologist 1, 68–81.
Taylor, E.C., et al., 1992. A dideazatetrahydrofolate analogue lacking a chiral center at C-6, N-[4-[2-(2-amino-3, 4-dihydro-4-oxo-7H-pyrrolo[2,3-d]pyrimidin-5-yl)ethyl]benzoyl]-L-glutamic acid, is an inhibitor of thymidylate synthase,. Journal of Medicinal Chemistry 35, 4450–4454.
Taylor, I.W., et al., 1985. Selective toxicity of a new lipophilic antifolate, BW301U, for methotrexate-resistant cells with reduced drug uptake. Cancer Research 45, 978–982.
Theti, D.S., et al., 2003. Selective delivery of CB300638, a cyclopenta[g]quinazoline-based thymidylate synthase inhibitor into human tumor cell lines overexpressing the alpha-isoform of the folate receptor. Cancer Research 63, 3612–3618.
Thomas, R.J., Williams, M., Garcia-Vargas, J., 2003. Lessons learned from raltitrexed–quality assurance, patient education and intensive supportive drugs to optimise tolerability. Clinical Oncology (R Coll Radiol) 15, 227–232.
Thompson, C.A., 2009. FDA approves pralatrexate for treatment of rare lymphoma. American Journal of Health-System Pharmacy 66, p1890.
Tibbetts, A.S., Appling, D.R., 2010. Compartmentalization of Mammalian folate-mediated one-carbon metabolism. Annual Review of Nutrition 30, 57–81.
Titus, S.A., Moran, R.G., 2000. Retrovirally mediated complementation of the glyB phenotype, Cloning of a human gene encoding the carrier for entry of folates into mitochondria. Journal of Biological Chemistry 275, 36811–36817.
Tolner, B., Roy, K., Sirotnak, F.M., 1998. Structural analysis of the human RFC-1 gene encoding a folate transporter reveals multiple promoters and alternatively spliced transcripts with 5' end heterogeneity. Gene 211, 331–341.
Tong, Y., et al., 1998. Isolation and characterization of thymitaq (AG337) and 5-fluoro-2-deoxyuridylate-resistant mutants of human thymidylate synthase from ethyl methanesulfonate-exposed human sarcoma HT1080 cells. Journal of Biological Chemistry 273, 11611–11618.
Tran, T., et al., 2005. Enhancement of folate receptor alpha expression in tumor cells through the glucocorticoid receptor: a promising means to improved tumor detection and targeting. Cancer Research 65, 4431–4441.
Trent, J.M., et al., 1984. Cytologic evidence for gene amplification in methotrexate-resistant cells obtained from a patient with ovarian adenocarcinoma. Journal of Clinical Oncology 2, 8–15.
Tse, A., Moran, R.G., 1998. Cellular folates prevent polyglutamation of 5, 10-dideazatetrahydrofolate. A novel mechanism of resistance to folate antimetabolites. Journal of Biological Chemistry 273, 25944–25952.
Uen, W.C., et al., 1992. A phase II study of piritrexim in patients with advanced squamous head and neck cancer. Cancer 69, 1008–1011.
Ulrich, C.M., et al., 2000. Searching expressed sequence tag databases: discovery and confirmation of a common polymorphism in the thymidylate synthase gene. Cancer Epidemiology, Biomarkers & Prevention 9, 1381–1385.
Unal, E.S., et al., 2008. N-linked glycosylation and its impact on the electrophoretic mobility and function of the human proton-coupled folate transporter (HsPCFT). Biochimica et Biophysica Acta 1778, 1407–1414.
van Aubel, R.A., et al., 2002. The MRP4/ABCC4 gene encodes a novel apical organic anion transporter in human kidney proximal tubules: putative efflux pump for urinary cAMP and cGMP. Journal of the American Society of Nephrology 13, 595–603.
van Dam, G.M., et al., 2011. Intraoperative tumor-specific fluorescence imaging in ovarian cancer by folate receptor-alpha targeting: first in-human results. Nature Medicine 17, 1315–1319.
van der Wilt, C.L., et al., 2001. Modulation of both endogenous folates and thymidine enhance the therapeutic efficacy of thymidylate synthase inhibitors. Cancer Research 61, 3675–3681.
van Triest, B., et al., 1997. Cross-resistance to antifolates in multidrug resistant cell lines with P-glycoprotein or multidrug resistance protein expression. Biochemical Pharmacology 53, 1855–1866.
Vogelzang, N.J., et al., 2003. Phase III study of pemetrexed in combination with cisplatin versus cisplatin alone in patients with malignant pleural mesothelioma. Journal of Clinical Oncology 21, 2636–2644.
Vokes, E.E., et al., 1991. A phase II study of piritrexim in combination with methotrexate in recurrent and metastatic head and neck cancer. Cancer 67, 2253–2257.
Volk, E.L., et al., 2000. Methotrexate cross-resistance in a mitoxantrone-selected multidrug-resistant MCF7 breast cancer cell line is attributable to enhanced energy-dependent drug efflux. Cancer Research 60, 3514–3521.
Volk, E.L., Schneider, E., 2003. Wild-type breast cancer resistance protein (BCRP/ABCG2) is a methotrexate polyglutamate transporter. Cancer Research 63, 5538–5543.
Walling, J., 2006. From methotrexate to pemetrexed and beyond. A review of the pharmacodynamic and clinical properties of antifolates. Investigational New Drugs 24, 37–77.
Wang, H., et al., 2000. Differentiation-independent retinoid induction of folate receptor type beta, a potential tumor target in myeloid leukemia. Blood 96, 3529–3536.
Wang, L., et al., 2010. Synthesis and antitumor activity of a novel series of 6-substituted pyrrolo[2,3-d]pyrimidine thienoyl antifolate inhibitors of purine biosynthesis with selectivity for high affinity folate receptors and the proton-coupled folate transporter over the reduced folate carrier for cellular entry. Journal of Medicinal Chemistry 53, 1306–1318.
Wang, L., et al., 2012. Synthesis and Biological Activity of 6-Substituted Pyrrolo[2, 3-d]pyrimidine Thienoyl Regioisomers as Inhibitors of de Novo Purine Biosynthesis with Selectivity for Cellular Uptake by High Affinity Folate Receptors and the Proton-Coupled Folate Transporter over the Reduced Folate Carrier. Journal of Medicinal Chemistry 55, 1758–1770.

Wang, W., et al., 2001. Pharmacogenomic dissection of resistance to thymidylate synthase inhibitors. Cancer Research 61, 5505–5510.
Wang, X., et al., 1992. Differential stereospecificities and affinities of folate receptor isoforms for folate compounds and antifolates. Biochemical Pharmacology 44, 1898–1901.
Wang, Y., Zhao, R., Goldman, I.D., 2004. Characterization of a folate transporter in HeLa cells with a low pH optimum and high affinity for pemetrexed distinct from the reduced folate carrier. Clinical Cancer Research 10, 6256–6264.
Webber, S., et al., 1996. AG337, a novel lipophilic thymidylate synthase inhibitor: in vitro and in vivo preclinical studies. Cancer Chemotherapy and Pharmacology 37, 509–517.
Westerhof, G.R., et al., 1995a. Functional activity of the reduced folate carrier in KB, MA104, and IGROV-I cells expressing folate-binding protein. Cancer Research 55, 3795–3802.
Westerhof, G.R., et al., 1995b. Carrier- and receptor-mediated transport of folate antagonists targeting folate-dependent enzymes: correlates of molecular-structure and biological activity. Molecular Pharmacology 48, 459–471.
Wettergren, Y., et al., 2005. Low expression of reduced folate carrier-1 and folylpolyglutamate synthase correlates with lack of a deleted in colorectal carcinoma mRNA splice variant in normal-appearing mucosa of colorectal carcinoma patients. Cancer Detection and Prevention 29, 348–355.
Whetstine, J.R., Flatley, R.M., Matherly, L.H., 2002a. The human reduced folate carrier gene is ubiquitously and differentially expressed in normal human tissues: identification of seven non-coding exons and characterization of a novel promoter. Biochemical Journal 367, 629–640.
Whetstine, J.R., et al., 2001. Single nucleotide polymorphisms in the human reduced folate carrier: characterization of a high-frequency G/A variant at position 80 and transport properties of the His(27) and Arg(27) carriers. Clinical Cancer Research 7, 3416–3422.
Whetstine, J.R., Matherly, L.H., 2001. The basal promoters for the human reduced folate carrier gene are regulated by a GC-box and a cAMP-response element/AP-1-like element. Basis for tissue-specific gene expression. Journal of Biological Chemistry 276, 6350–6358.
Whetstine, J.R., Witt, T.L., Matherly, L.H., 2002b. The human reduced folate carrier gene is regulated by the AP2 and sp1 transcription factor families and a functional 61-base pair polymorphism. Journal of Biological Chemistry 277, 43873–43880.
White, J.C., 1979. Reversal of methotrexate binding to dihydrofolate reductase by dihydrofolate. Studies with pure enzyme and computer modeling using network thermodynamics. Journal of Biological Chemistry 254, 10889–10895.
White, J.C., Goldman, I.D., 1981. Methotrexate resistance in al L1210 cell line resulting from increased dihydrofolate reductase, decreased thymidylate synthetase activity, and normal membrane transport, Computer simulations based on network thermodynamics. Journal of Biological Chemistry 256, 5722–5727.
White, J.C., Loftfield, S., Goldman, I.D., 1975. The mechanism of action of methotrexate. III. Requirement of free intracellular methotrexate for maximal suppression of (14C)formate incorporation into nucleic acids and protein. Molecular Pharmacology 11, 287–297.
Wielinga, P., et al., 2005. The human multidrug resistance protein MRP5 transports folates and can mediate cellular resistance against antifolates. Cancer Research 65, 4425–4430.
Williams, F.M., et al., 1994. Isolation of a hamster cDNA clone coding for a function involved in methotrexate uptake. Journal of Biological Chemistry 269, 5810–5816.
Wong, S.C., et al., 1995. Isolation of human cDNAs that restore methotrexate sensitivity and reduced folate carrier activity in methotrexate transport-defective Chinese hamster ovary cells. Journal of Biological Chemistry 270, 17468–17475.
Wong, S.C., et al., 1998. Effects of the loss of capacity for N-glycosylation on the transport activity and cellular localization of the human reduced folate carrier. Biochimica et Biophysica Acta 1375, 6–12.
Worm, J., et al., 2001. Methylation-dependent silencing of the reduced folate carrier gene in inherently methotrexate-resistant human breast cancer cells. Journal of Biological Chemistry 276, 39990–40000.
Wright, J.E., et al., 2000. Efficient utilization of the reduced folate carrier in CCRF-CEM human leukemic lymphoblasts by the potent antifolate N(alpha)-(4-amino-4-deoxypteroyl)-N(delta)-hemiphthaloyl-L- ornithine (PT523) and its B-ring analogues. Biochemical Pharmacology 60, 41–46.
Wright, J.E., et al., 2003. Further studies on the interaction of nonpolyglutamatable aminopterin analogs with dihydrofolate reductase and the reduced folate carrier as determinants of in vitro antitumor activity. Biochemical Pharmacology 65, 1427–1433.
Xia, W., Low, P.S., 2010. Folate-targeted therapies for cancer. Journal of Medicinal Chemistry 53, 6811–6824.
Yang-Feng, T.L., et al., 1995. Assignment of the human folate transporter gene to chromosome 21q22.3 by somatic cell hybrid analysis and in situ hybridization. Biochemical and Biophysical Research Communications 210, 874–879.
Yang, R., et al., 2008a. Quantitative correlation between promoter methylation and messenger RNA levels of the reduced folate carrier. BMC Cancer 8, p124.
Yang, R., et al., 2008b. Polymorphisms and methylation of the reduced folate carrier in osteosarcoma. Clinical Orthopaedics and Related Research 466, 2046–2051.
Yang, R., et al., 2003. Sequence alterations in the reduced folate carrier are observed in osteosarcoma tumor samples. Clinical Cancer Research 9, 837–844.
Zeng, H., et al., 2001. Transport of methotrexate (MTX) and folates by multidrug resistance protein (MRP) 3 and MRP1: effect of polyglutamylation on MTX transport. Cancer Research 61, 7225–7232.
Zeng, H., et al., 2000. Transport of amphipathic anions by human multidrug resistance protein 3. Cancer Research 60, 4779–4784.
Zhang, C.C., Boritzki, T.J., Jackson, R.C., 1998a. An inhibitor of glycinamide ribonucleotide formyltransferase is selectively cytotoxic to cells that lack a functional G1 checkpoint. Cancer Chemotherapy and Pharmacology 41, 223–228.
Zhang, L., et al., 1998b. Reduced folate carrier gene expression in childhood acute lymphoblastic leukemia: relationship to immunophenotype and ploidy. Clinical Cancer Research 4, 2169–2177.
Zhao, R., Assaraf, Y.G., Goldman, I.D., 1998a. A mutated murine reduced folate carrier (RFC1) with increased affinity for folic acid, decreased affinity for methotrexate, and an obligatory anion requirement for transport function. Journal of Biological Chemistry 273, 19065–19071.
Zhao, R., Assaraf, Y.G., Goldman, I.D., 1998b. A reduced folate carrier mutation produces substrate-dependent alterations in carrier mobility in murine leukemia cells and methotrexate resistance with conservation of growth in 5-formyltetrahydrofolate. Journal of Biological Chemistry 273, 7873–7879.
Zhao, R., et al., 2000a. The mechanism of transport of the multitargeted antifolate (MTA) and its cross-resistance pattern in cells with markedly impaired transport of methotrexate. Clin Cancer Research 6, 3687–3695.
Zhao, R., et al., 2004a. Antifolate resistance in a HeLa cell line associated with impaired transport independent of the reduced folate carrier. Clinical Cancer Research 10, 8735–8742.
Zhao, R., Gao, F., Goldman, I.D., 2001a. Marked suppression of the activity of some, but not all, antifolate compounds by augmentation of folate cofactor pools within tumor cells. Biochemical Pharmacology 61, 857–865.
Zhao, R., Gao, F., Goldman, I.D., 2002. Reduced folate carrier transports thiamine monophosphate: an alternative route for thiamine delivery into mammalian cells. American Journal of Physiology Cell Physiology 282, C1512–C1517.
Zhao, R., et al., 2004b. A prominent low-pH methotrexate transport activity in human solid tumors: contribution to the preservation of methotrexate pharmacologic activity in HeLa cells lacking the reduced folate carrier. Clin Cancer Research 10, 718–727.
Zhao, R., et al., 2001b. Impact of the reduced folate carrier on the accumulation of active thiamin metabolites in murine leukemia cells. Journal of Biological Chemistry 276, 1114–1118.
Zhao, R., Goldman, I.D., 2003. Resistance to antifolates. Oncogene 22, 7431–7457.
Zhao, R., et al., 2004c. Selective preservation of pemetrexed pharmacological activity in HeLa cells lacking the reduced folate carrier: association with the presence of a secondary transport pathway. Cancer Research 64, 3313–3319.
Zhao, R., Matherly, L.H., Goldman, I.D., 2009a. Membrane transporters and folate homeostasis: intestinal absorption and transport into systemic compartments and tissues. Expert Reviews in Molecular Medicine 11.
Zhao, R., et al., 2007. The spectrum of mutations in the PCFT gene, coding for an intestinal folate transporter, that are the basis for hereditary folate malabsorption. Blood 110, 1147–1152.
Zhao, R., et al., 2009b. A role for the proton-coupled folate transporter (PCFT-SLC46A1) in folate receptor-mediated endocytosis. Journal of Biological Chemistry 284, 4267–4274.
Zhao, R., et al., 2008. The proton-coupled folate transporter: impact on pemetrexed transport and on antifolates activities compared with the reduced folate carrier. Molecular Pharmacology 74, 854–862.
Zhao, R., et al., 1997. Impact of overexpression of the reduced folate carrier (RFC1), an anion exchanger, on concentrative transport in murine L1210 leukemia cells. Journal of Biological Chemistry 272, 21207–21212.
Zhao, R., Sharina, I.G., Goldman, I.D., 1999. Pattern of mutations that results in loss of reduced folate carrier function under antifolate selective pressure augmented by chemical mutagenesis. Molecular Pharmacology 56, 68–76.
Zhao, R., et al., 2000b. Molecular analysis of murine leukemia cell lines resistant to 5, 10-dideazatetrahydrofolate identifies several amino acids critical to the function of folylpolyglutamate synthetase,. Journal of Biological Chemistry 275, 26599–26606.
Zhao, R., et al., 2005. Loss of reduced folate carrier function and folate depletion result in enhanced pemetrexed inhibition of purine synthesis. Clinical Cancer Research 11, 1294–1301.
Zhou, J.Y., et al., 2012. The association between two polymorphisms in the TS gene and risk of cancer: a systematic review and pooled analysis. International Journal of Cancer.
Zucali, P.A., et al., 2011. Thymidylate synthase and excision repair cross-complementing group-1 as predictors of responsiveness in mesothelioma patients treated with pemetrexed/carboplatin. Clinical Cancer Research 17, 2581–2590.