Special Section on Transporters in Toxicity and Disease—Minireview

The Major Facilitative Folate Transporters Solute Carrier 19A1 and Solute Carrier 46A1: Biology and Role in Antifolate Chemotherapy of Cancer

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ABSTRACT

This review summarizes the biology of the major facilitative membrane transporters, the reduced folate carrier (RFC) (Solute Carrier 19A1) and the proton-coupled folate transporter (PCFT) (Solute Carrier 46A1). Folates are essential vitamins, and folate deficiency contributes to a variety of health disorders. RFC is ubiquitously expressed and is the major folate transporter in mammalian cells and tissues. PCFT mediates the intestinal absorption of dietary folates and appears to be important for transport of folates into the central nervous system. Clinically relevant antifolates for cancer, such as methotrexate and pralatrexate, are transported by RFC, and loss of RFC transport is an important mechanism of methotrexate resistance in cancer cell lines and in patients. PCFT is expressed in human tumors, and is active at pH conditions associated with the tumor microenvironment. Pemetrexed is an excellent substrate for both RFC and PCFT. Novel tumor-targeted antifolates related to pemetrexed with selective membrane transport by PCFT over RFC are being developed. In recent years, there have been major advances in understanding the structural and functional properties and the regulation of RFC and PCFT. The molecular bases for methotrexate resistance associated with loss of RFC transport and for hereditary folate malabsorption, attributable to mutant PCFT, were determined. Future studies should continue to translate molecular insights from basic studies of RFC and PCFT biology into new therapeutic strategies for cancer and other diseases.

Introduction

Folates are B9 vitamins that are required for synthesis of thymidylate, purine nucleotides, serine, and methionine (Stokstad, 1990). Folates are essential for cell growth and tissue development and must be obtained from exogenous sources since mammals cannot support their own folate synthesis. RFC and PCFT play key roles in folate and antifolate transport across cell membranes. RFC mediates the intestinal absorption of dietary folates and appears to be important for transport of folates into the central nervous system. PCFT is expressed in human tumors, and is active at pH conditions associated with the tumor microenvironment. Pemetrexed is an excellent substrate for both RFC and PCFT. Novel tumor-targeted antifolates related to pemetrexed with selective membrane transport by PCFT over RFC are being developed. In recent years, there have been major advances in understanding the structural and functional properties and the regulation of RFC and PCFT. The molecular bases for methotrexate resistance associated with loss of RFC transport and for hereditary folate malabsorption, attributable to mutant PCFT, were determined. Future studies should continue to translate molecular insights from basic studies of RFC and PCFT biology into new therapeutic strategies for cancer and other diseases.

ABBREVIATIONS: ABCG2, ATP-binding cassette subfamily G member 2; AG2034, N-[5-{2-[(6S)-2-amino-4-oxo-4,6,7,8-tetrahydro-1H-pyrimido[5,4-b][1,4]thiazin-9-yl]ethyl}thiophen-2-yl]carbonyl]-L-glutamic acid; AG2037, N-[5-{2-[(6S)-2-amino-4-oxo-4,6,7,8-tetrahydro-1H-pyrimido[5,4-b][1,4]thiazin-9-yl]ethyl}thiophen-2-yl]carbonyl]-L-glutamic acid; AICA, 5-aminoimidazole-4-carboxamide; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; AICARFTase, 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase; ALL, acute lymphoblastic leukemia; AMPK, AMP-activated protein kinase; AMT, aminopterin; AP2, activating protein 2; bp, base pairs; CB3717, N10-propargyl-5,8-dideazafolic acid, N-[4-[N-[(2-amino-4-hydroxy-6-quinazolinyl)methyl]prop-2-ynylamino]benzoyl]-L-glutamic acid; C/EBP, CCAAT/enhancer-binding protein α; CNS, central nervous system; cRNAs, complementary RNAs; CSF, cerebral spinal fluid; DHFR, dihydrofolate reductase; ECFP, enhanced cyan fluorescent protein; FDA, Food and Drug Administration; FPGS, folylpolyglutamate synthase; FR, folate receptor; FRET, fluorescence resonance energy transfer; GAR, β-glycinamidine ribonucleotide; GARFase, glycaminamide ribonucleotide formyltransferase; GI, gastrointestinal; GW184388, (S)-2-[4-[(2S)-4-amino-4-oxo-1,4,5,6,7,8-hexahydropyrido[2,3-d]pyrimidin-6-yl]thiophene-2-carboxamido]pentanedioic acid; HFM, hereditary folate malabsorption; hPCFT, human proton-coupled folate transporter; hRFC, human reduced folate carrier; IC198583, N-{4-[[[(1,4-dihydro-2-methyl-4-oxo-6-quinazolinyl)methyl]prop-2-ynylamino]benzoyl]-L-glutamic acid; K, inhibition constant; K, Michaelis constant; LMX, lometrexol, 6R,5,10-dideazatetrahydrofolate; (S)-2-(4-[(2R)-2-amino-4-oxo-1,4,5,6,7,8-hexahydropyrido[2,3-d]pyrimidin-6-yl]thiophene-2-carboxamido]pentanedioic acid; LY309887, 6R,5,10-dideazatetrahydrofolate; MRS, major facilitator superfamily; MRP, multidrug resistance-associated protein; MTS, methanethiosulfonate; MTSES, 2-sulfanatoethyl methanethiosulfonate; PT523, N(alpha)-(4-amino-4-deoxypteroyl)-N(delta)-hemiphthaloyl-L-ornithine; RFC, reduced folate carrier; RTX, raltitrexed, ZD1694, (S)-2-(5-[(1S)-2-[4-(((2,7-dimethyl-4-oxo-3,4-dihydroquinazolin-6-yl)methyl)prop-2-ynylamino]benzoyl]-L-glutamic acid; SLC, Solute Carrier; SP1, specificity protein 1; THF, tetrahydrofolate; TMD, transmembrane domain; TS, thymidylate synthase; UTR, untranslated region; USF1, upstream transcription factor 1; VDR, vitamin D receptor; Ypet, FRET-optimized yellow fluorescent protein; ZD9331, Plevitrexed, BGC9331, (S)-2-(4-[(2,7-dimethyl-4-oxo-3,4-dihydroquinazolin-6-yl)methyl]prop-2-yn-1-y]amino)-2-fluorobenzamido)-4-(1H-tetrazol-5-yl)butanoic acid; ZMP, 5-aminomimidazole-4-carboxamide ribonucleotide.
synthesize these derivatives de novo. Folates are also hydrophilic molecules that are anions at physiologic pH and do not cross biologic membranes by diffusion alone.

Genetically distinct systems have evolved in mammalian cells to facilitate membrane transport of folates (Matherly and Goldman, 2003; Zhao et al., 2011a; Desmoulin et al., 2012a; Zhao and Goldman, 2013). The best characterized folate transporter is the ubiquitously expressed reduced folate carrier [RFC; Solute Carrier (SLC) 19A1] (Matherly et al., 2007; Matherly and Hou, 2008). RFC was initially characterized over 35 years ago in relation to its kinetics and thermodynamics (Goldman et al., 1968; Goldman, 1969, 1971). Following its cloning in the mid-1990s (Dixon et al., 1994; Williams et al., 1994; Moscow et al., 1995; Prasad et al., 1995; Williams and Flintoff, 1995; Wong et al., 1995), RFC was recognized as the major cellular and tissue folate transporter in mammals. In 2006, the proton-coupled folate transporter (PCFT; SLC46A1) was identified with characteristics distinctly different from those of RFC, including its acidic pH optimum and substrate specificity (Qiu et al., 2006). Although PCFT turned out to be identical to a carrier previously reported to transport heme (Shayeghi et al., 2005), this activity was later recognized to be at most a minor component, as it soon became clear that the primary role for PCFT involved transport of dietary folates across the apical brush border of the small intestine (Zhao et al., 2009a). PCFT is also important to the transport of folates into the central nervous system (CNS) (Wollack et al., 2008; Zhao et al., 2009b). PCFT is expressed in other tissues, although given its modest transport activity at neutral pH, its broader physiologic role remains uncertain.

In addition to its established role in the membrane transport of physiologic folates, RFC is a major systemic transport system for antifolate drugs used for cancer chemotherapy, including methotrexate (MTX), pemetrexed (PMX; Alimta), raltitrexed (RTX), and pralatrexate (PDX) (Folotyn, 10-propargyl-10-deazaaminopterin, N-4-{1-[(2,4-diamino-6-pteridinyl)methyl]-3-butynyl}benzoyl-L-glutamic acid) (Matherly et al., 2007; Desmoulin et al., 2012a) (Fig. 1). These drugs are also substrates for PCFT, albeit to different extents (Zhao and Goldman, 2007; Desmoulin et al., 2012a). However, it is the RFC transport component which predominates; i.e., although the PCFT transport flux for these clinically relevant antifolates can be significant, particularly at the acidic pH characterizing the tumor microenvironment, there would be no net therapeutic gain since membrane transport by RFC in normal tissues would continue. These considerations provided impetus for developing a new therapeutic paradigm for antifolate drug development, namely, the rational development of tumor-targeted therapies based on tumor-specific high-level expression and/or function of PCFT (Desmoulin et al., 2012a).

In this review, we focus on the molecular, regulatory, and functional characteristics of the major facilitative folate transporters, RFC and PCFT. This includes their basic biology, along with their physiology and roles in cancer therapy.

The Role of Membrane Transport in In Vivo Folate Homeostasis

Folic acid is the synthetic form of the folate derivatives found in cells. Folates differ in oxidation of the pteridine ring, and for reduced...
or tetrahydrofolate (THF) cofactor forms, in the nature of their associated one-carbon unit (methyl, formyl, methylene, methenyl) and its position of substitution (N10 or N5 positions) (Stokstad, 1990). Within cells, folate cofactors exist primarily as poly-γ-glutamates which include 2–8 glutamate moieties, conjugated to the parent molecule in an ATP-dependent step catalyzed by folyl-γ-glutamate synthetase (Shane, 1989). Polyglutamylation is an essential metabolic function as folate polyglutamates are retained intracellularly due to their polyanionic character and their poor substrate activities for the major folate efflux pumps (discussed later). Further, polyglutamyl folates are preferred substrates for folate-dependent enzymes, although substrate activity varies for different polyglutamyl forms (Shane, 1989; Moran, 1999). Within cells, one-carbon-substituted THF polyglutamate cofactors participate in the carbon-transfer reactions leading to purine nucleotides, thymidylate, and the amino acids serine and methionine (Fig. 2). Methionine is converted to S-adenosyl methionine, required for biologic methylation reactions, including those involving DNA, RNA, neurotransmitters, and proteins such as histones (Lu, 2000).

10-Formyl THF is the one carbon donor in reactions catalyzed by β-glycinamide ribonucleotide (GAR) formyltransferase (GARFTase) and 5-aminoimidazole-4-carboxamide (AICA) ribonucleotide (AICAR) formyltransferase (AICARFTase), leading to the purine nucleotides (Fig. 2). Thymidylate synthase (TS) catalyzes synthesis of dTMP from dUMP using 5,10-methylene THF as a one-carbon donor, generating dihydrofolate. Dihydrofolate is reduced back to THF by dihydrofolate reductase (DHFR). 5,10-Methylene THF is also a source of one-carbon units for the synthesis of serine from glycine by serine hydroxymethyltransferase (both mitochondrial and cytosolic). Further, 5,10-methylene THF is converted by 5,10-methylene THF reductase to 5-methyl THF, which provides a one-carbon unit for methylation of homocysteine to methionine by methionine synthetase.

Excellent sources of folates include dark green leafy vegetables, orange juice, liver, and strawberries (Gregory, 1995). Cooking decreases folate levels in food. Dietary folates are absorbed in the proximal gastrointestinal (GI) tract via their transport across the enterocyte brush-border apical membrane by PCFT (Zhao et al., 2009a, 2011a). Although RFC is expressed throughout the intestine (Qiu et al., 2006, 2007; Inoue et al., 2008), the acidic pH optimum of the upper GI (pH ~5.8–6.0) is highly conducive to PCFT membrane transport, but not to RFC transport (Yun et al., 1995). Whether RFC contributes to intestinal folate absorption in the lower GI where the pH is less acidic is unclear. After entering the enterocytes by PCFT, folates are transported across the basolateral membrane surface [most likely by multidrug resistance-associated protein (MRP) 3] into the bloodstream, and are delivered to the liver by the hepatic portal vein (Zhao et al., 2009a, 2011a). Folates are released from the liver into the blood primarily as 5-methyl THF, which is the major circulating form of folate.

5-Methyl THF, like all folates, is hydrophilic and incapable of permeating plasma membranes by diffusion alone. RFC is expressed ubiquitously in tissues and tumors and is the major folate transporter which transports folate cofactors from the blood into cells of peripheral tissues (Matherly et al., 2007). In human tissues, highly elevated human RFC (hRFC) transcripts are detected in the placenta and liver, with significant levels in other tissues including leukocytes, kidneys, lungs, bone marrow, intestines, and portions of the CNS and brain (Whetsel et al., 2002a). By immunohistochemistry of mouse tissues, RFC was detected at the apical brush-border membrane of the small intestine and colon, hepatocyte membranes, the apical membrane of the choroid plexus, the basolateral membrane of the renal tubule epithelium, and the apical membrane of the cells lining the spinal canal (Wang et al., 2001).

Folate deficiency results from insufficient dietary folate or impaired intestinal folate absorption (discussed later). This can result in pathologic conditions such as cardiovascular disease, neural tube defects, neurologic disorders, and cancer (Lucocq, 2000). RFC is essential for development, since in mice, inactivating both RFC alleles by targeted homologous recombination is embryonic lethal (Zhao et al., 2001c). Whereas approximately 10% of RFC-null mice could be brought to live birth by folic acid supplementation, these animals went on to die within 1–2 weeks due to failure of hematopoietic organs such as bone marrow, the thymus, or the spleen (Zhao et al., 2001c).

PCFT is also expressed in many normal tissues, although levels are generally modest (Desmoulin et al., 2012a). Major sites of PCFT expression include the apical brush-border surface of the jejunum and duodenum, as well as kidney, the sinusoidal membrane of the liver, and the basolateral membrane of the choroid plexus and retinal pigment epithelium (Qiu et al., 2006; Umaphathy et al., 2007; Inoue et al., 2008).
et al., 2008; Zhao et al., 2009a,b). PCFT is also expressed in the placenta and spleen. Whereas PCFT is highly active in the acidic conditions which characterize the upper GI, given its acidic pH optimum, PCFT seems unlikely to represent a generalized mechanism for folate uptake into tissues where it is expressed, although it appears to be essential for transport across the choroid plexus (discussed later). Human PCFT (hPCFT) is abundantly expressed in human tumor cell lines (e.g., breast, prostate, ovarian, lung) and at very low-undetectable levels in leukemias (Zhao et al., 2004a; Gonen et al., 2008; Kugel Desmoulin et al., 2011).

Loss of hPCFT is associated with hereditary folate malabsorption (HFM) syndrome, a rare autosomal recessive disorder characterized by the onset of macrocytic folate deficiency, anemia, and failure to thrive within the first few months of life (Geller et al., 2002; Qiu et al., 2006; Zhao et al., 2007; Lasry et al., 2008; Min et al., 2008; Atabay et al., 2010; Mahadeo et al., 2010, 2011; Meyer et al., 2010; Shin et al., 2010, 2011; Diop-Bove et al., 2013). Other manifestations of HFM include hyponatremoglobulinemia, developmental delays, gait disorders, peripheral neuropathies, and seizures. HFM derives from homozgyous mutations in the hPCFT gene including base insertions, deletions, or substitutions, manifesting as exon skipping, frame shifts, premature translation terminations, and amino acid substitutions. Loss of hPCFT function leads to impaired intestinal folate absorption, resulting in severe systemic folate deficiency and impaired transport of folates across the choroid plexus into the CNS (Wollack et al., 2008; Zhao et al., 2009b). PCFT knockout mice provide an excellent in vivo model of HFM that largely recapitulates the HFM syndrome seen in humans with mutated hPCFT, including undetectable serum folate and elevated plasma homocysteine. The phenotype can be rescued by oral supplementation with high levels of 5-methyl THF or parenteral administration of 5-methyl THF or leucovorin [(6R,S)-5-formyl THF] (Salojin et al., 2011).

Other folate uptake systems in mammalian cells and tissues include folate receptors (FRs) α and β, glycosyl phosphatidylinositol-tethered proteins (Elnakat and Ratnam, 2004; Zhao et al., 2011a). FRs mediate folate internalization by endocytosis (Sabharanjak and Mayor, 2004). FRα is expressed in the choroid plexus, proximal renal tubules, retinal pigment epithelium, uterus, and placenta (Elnakat and Ratnam, 2004). In polarized epithelial cells, FRETα is expressed on the apical membrane whereas it is not in contact with the circulation (Chancy et al., 2000). FRβ is expressed in placenta and hematopoietic cells, as well as in activated macrophages (Elnakat and Ratnam, 2004). In normal bone marrow and peripheral blood cells, FRβ is nonfunctional (Reddy et al., 1999). In malignant tissues such as nonmucinous adenocarcinomas of the ovary, uterus, and cervix, FRα is expressed in high levels and is exposed to the circulation (Elnakat and Ratnam, 2004). FRβ is expressed in chronic myelogenous leukemia and acute myelogenous leukemia cells (Ross et al., 1994; Pan et al., 2002). The expression of FRα in the plasma membranes of solid tumors and FRβ in leukemias has prompted the development of folate-based therapeutics as targeting and cytotoxic agents for therapeutic applications including cancer and inflammatory diseases (Salazar and Ratnam, 2007; Wang et al., 2010, 2011; Xia and Low, 2010; Yang et al., 2012).

The organic anion transporters (OATs) and organic anion–transporting polypeptides (OATPs) transport a diverse spectrum of organic ions such as bromosulphthalein, taurocholate, and probenecid, as well as folates, into epithelial tissues such as kidney and intestine (Burckhard, 2012; Konig et al., 2013). Finally, efflux pumps including MRPs (MRPs 1-5 and 8) and ABCG2 (ATP-binding cassette subfamily G member 2) also transport folates (Kruh et al., 2007; Gonen and Assaraf, 2012; Natarajan et al., 2012), thereby opposing the cellular uptake mediated by the other major transporters.

In proximal renal tubules, both PCFT and OATP1 are expressed at the apical brush-border membrane, along with FRα, whereas RFC and OAT1/OAT3 are present at the basolateral membrane (Zhao et al., 2009a, 2011a). Fulates are filtered via the glomerulus and are reabsorbed from the urine primarily by an FRα-mediated process, although OATP1 may contribute to folate reabsorption. Whether PCFT might contribute to folate reabsorption is not clear. Fulates are transported into the circulation by folate transporters at the basolateral membrane. Although FRs, RFC, and PCFT are all expressed in the placenta, their contributions to transplacental transport of folates are not entirely clear. FRα- and PCFT-null mice are fertile. A woman with HFM due to a homozgyous stop codon in the hPCFT coding sequence was recently reported to experience a normal term pregnancy and delivery (Zhao et al., 2011a).

Fulates are concentrated in the cerebral spinal fluid via active transport at the choroid plexus (Spector and Lorenzo, 1975; Gonen et al., 2002). FRα is localized to the basal and apical membranes (Spector and Lorenzo, 1975; Zhao et al., 2011a), and RFC is present on the apical membrane of the choroid plexus (Wang et al., 2001). PCFT is also localized to the basolateral membrane of the choroid plexus (Zhao et al., 2009b). Although the neutral pH at both interfaces is inconsistent with PCFT transport, a localized low pH conducive to PCFT transport may occur at the basolateral membrane of ependymal cells, reflecting the presence of sodium-hydrogen exchangers (Zhao et al., 2009a, 2011a). HFM is accompanied by low levels of CNS folates, even in patients who take folate supplements (Diop-Bove et al., 1993; Geller et al., 2002), establishing the role of hPCFT in folate uptake into the CNS. Moreover, loss of function mutations in FRα were described in children with cerebral folate deficiency that appears several years after birth (Cario et al., 2009; Steinfeld et al., 2009). Although RFC is positioned to extract folates from the CSF (cerebral spinal fluid), its role in CNS transport is not clear.

**Biology of RFC**

**Transport Characteristics and Structure/Function.** RFC is the major membrane transporter of circulating folate cofactors (Matherly et al., 2007). The transport kinetics and thermodynamics for RFC were first characterized in the mid- to late1960s (Goldman, 1968, 1969, 1971). RFC transport is temperature-dependent and characterized by a neutral pH optimum such that transport activity decreases dramatically below pH 7 (Sierra et al., 1997). RFC substrates are structurally diverse and include ring systems differing in aromaticities and in the presence or absence of heteroatoms or substituents, the length and character of the linker domain connecting the aromatic rings, and the identity and charge character of the terminal amino acid (Fig. 1).

(6S)5-Formyl THF and (6S)5-methyl THF are excellent substrates for RFC (Goldman et al., 1968). Transport of (6S)5-formyl THF is preferred over the (6R) stereoisomer (Sirotnak et al., 1979), although transport is not stereospecific for 5-methyl THF (White et al., 1978; Chello et al., 1982). Whereas 5-methyl and 5-formyl THF both show low micromolar affinities for RFC, folic acid is a poor RFC substrate with binding affinities one to two orders of magnitude less than those for the reduced folate forms (Goldman et al., 1968; Westerhof et al., 1995). The clinically used antifolates MTX, PMX, RTX, and PDX are all excellent RFC substrates, with Kᵦ (Michaels constant) values in the low micromolar range (Sirotnak et al., 1998; Jansen, 1999; Matherly et al., 2007; Matherly and Hou, 2008; Visentin et al., 2013). The hemiphthaloylornithine antifolate PT523 [(Nalpha)-(4-amino-4-deoxypenteryl)-N(delta)-hemiphthaloyl-L-ornithine] (Rhee et al., 1994; Rosowsky et al., 1994; Jansen, 1999; Wright et al., 2000) and the
whereby the transmembrane gradient (inside 1971). The net result is the transport of (anti)folates into cells by RFC, (anti)folates seems to involve gradients of organic phosphates across the membrane (Goldman, 1971). Further, thiamine pyrophosphate and ZMP (AICA ribonucleotide) are bona fide RFC substrates which, when present within cells, trans-stimulate folate influx by RFC while inhibiting (anti)folate export via this mechanism (Zhao et al., 2001b, 2002; Visentin et al., 2001). RFC belongs to the major facilitator superfamily (MFS) of transporters including the membrane surface and carrier function, deletion of larger segments (positions 302–591) abolished surface targeting (Marchant et al., 2002). Deletion of major segments (49 or 60 amino acids; positions 215–263 and 204–263, respectively) from the loop domain connecting TMDs 6 and 7 of hRFC (Fig. 3) also abolished transport (Liu et al., 2003). Interestingly, when these deleted loop segments in hRFC were replaced by the corresponding segment from the MFS homolog SLC19A2, which transports thiamine, transport was restored (Liu et al., 2003). Further, when hRFC was expressed in cells as individual TMD1–6 and TMD7–12 half molecules, transport was restored (Witt et al., 2004). Collectively, these results establish that neither the N nor C termini, nor the intracellular loop domain connecting TMDs 6 and 7, participate in binding and translocation of folate substrates. The primary role for the TMD6–7 loop domain is to provide appropriate spacing between the TMD1–6 and TMD7–12 segments for optimal membrane transport.

A functional “Cys-less” hRFC was generated by replacement of the 11 cysteine residues in hRFC with serine (Cao and Matherly, 2003). Cys-less hRFC was used for exhaustive Cys-scanning insertional mutagenesis and substituted-cysteine accessibility methods. The 282 cysteine mutants were individually expressed in an hRFC-null (R5) HeLa subline treated with 2-sulfonatothiole methanethiosulfonate (MTSES) to identify aqueous accessible TMD residues involved in substrate binding and translocation (Hou et al., 2005, 2006). Based on patterns of MTSES inhibition of transport and protection with excess substrate (leucovorin), TMDs 4, 5, 7, 8, 10, and 11 were identified as comprising the membrane translocation pathway for anionic folate substrates. Interestingly, of the 282 hRFC Cys mutants, only 10 Cys mutants were inactive for transport. These included 10 positions in a stretch of TMD4 (Arg133, Ile134, Ala135, Tyr136, Ser138), Tyr281 in TMD7, Ser313 in TMD8, and Arg373 in TMD10. Several of these amino acids were previously implicated as functionally or structurally important by site-directed mutagenesis (Arg133, Arg373) (Liu and Matherly, 2001; Sharina et al., 2001; Sadlish et al., 2002) or from studies of nonfunctional hRFC in cells selected for MTX resistance (Ser313) (Zhao et al., 1999).

From studies in mouse and human RFCS, other residues were implicated as functionally important, including (numbers are based on hRFC) Val29, Gly44, Glu45, Ser46, Ile48, Val106, Trp107, Ser127, and Ala132 (Brigle et al., 1995; Jansen et al., 1998; Roy et al., 1998; Zhao et al., 1998, 1999, 2000; Wong et al., 1999; Drori et al., 2000a; ). Arg133 in TMD4 forms a charge pair with Asp88 in TMD2 of hRFC (Liu and Matherly, 2001). A charge-pair association was also suggested for Glu45 and Lys404 (equivalent to Lys411 in hRFC) in mouse RFC (Zhao et al., 2003). Lys411 is in TMD11 of hRFC, and was labeled by a radioaffinity ligand for RFC (N-hydroxysuccinimide
N-hydroxysuccinimide esters of diamino furo[2,3-d]pyrimidine antifolates with modified amino acids including a substituted α or γ group were used for affinity labeling hRFC. Labeling was increased for analogs with unmodified γ- over α-carboxylates, establishing that the γ-carboxylate forms an ionic association with Lys411. From the solved structures for the bacterial MFS homologs, the lactose/proton symporter (Abramson et al., 2003) and glycerol-2-phosphate/inorganic phosphate antiporter (Huang et al., 2003), a 3-dimensional homology model for hRFC was generated with a membrane translocation pathway composed of TMDs 1, 2, 4, 5, 7, 8, 10, and 11, and mechanistically important roles for Tyr281, Ser313, and Arg373 (Hou et al., 2006).

Recent studies suggest that, like many MFS proteins, hRFC exists as a homo-oligomer (Hou and Matherly, 2009). Each hRFC monomer functions independently, i.e., each hRFC monomer comprises a separate translocation pathway for folate substrates (Hou et al., 2010). However, cofolding of hRFC monomers to form oligomeric hRFC appears to be necessary for intracellular trafficking and surface expression of the functional transporter (Hou and Matherly, 2009). Indeed, coexpression of wild-type and inactive mutant S138C hRFCs, combined with surface biotinylation and confocal microscopy, a dominant-negative phenotype was demonstrated, involving markedly decreased cell surface expression of both mutant and wild-type hRFCs caused by impaired intracellular trafficking.

**Regulation of RFC Expression and Function.** The hRFC gene maps to chromosome 21q22.2 (Moscow et al., 1995) and includes five coding exons with conserved intron-exon boundaries and as many as 6 noncoding regions and promoters (Matherly et al., 2007). Five of these (designated A, B, C, D, and E) are separate noncoding exons, whereas the A1/A2 noncoding sequence is fused to the first hRFC coding exon (Whetstine et al., 2002a; Flatley et al., 2004). Promoter activity was localized to the 5′ regions proximal to the A1/A2, A, B, C, and D noncoding regions, and for four of these promoters, ubiquitously expressed [e.g., SP1 (specificity protein 1), USF1 (upstream transcription factor 1)] and tissue-specific [e.g., AP2 (activating protein 2), CCAAT/enhancer-binding protein (C/EBP), Ikaros] transcription factors and cis elements were identified as important for hRFC transcription (Matherly et al., 2007). Thus, hRFC levels in various cells and tissues are likely to reflect differential promoter usage, combined with differing levels of critical transcription factors. Other likely determinants of hRFC transcriptional activity include additional up- and downstream cis elements, polymorphisms in the hRFC promoters (discussed later), and general promoter architecture and chromatin structure. A downstream region proximal to hRFC exon B was reported to be methylated in MDA-MB-231 human breast cancer cells (Worm et al., 2001) and primary lymphomas (Ferreri et al., 2004), resulting in loss of hRFC transcripts. However, methylation was not detected in other cell culture models with reduced hRFC levels (Rothem et al., 2004), nor in primary acute lymphoblastic leukemia (ALL) specimens (Liu et al., 2006).

The noncoding exons for the hRFC gene are alternately spliced to generate multiple hRFC transcripts with unique untranslated regions (UTRs) (as many as 15 have been reported) linked to a common hRFC coding sequence (Whetstine et al., 2002a; Flatley et al., 2004; Payton et al., 2007). hRFC 5′UTR transcript heterogeneity was reported to impact the efficiency of 5′CAP-dependent translation and result in...
differences in hRFC transcript stabilities (Payton et al., 2007). For the A1/A2 and A 5’UTRs, upstream AUGs occur in frame with the hRFC coding sequence and result in modified hRFC proteins with 62 and 22 additional amino acids linked to the N terminus of the 591 amino acid hRFC protein form encoded from hRFC transcripts, including the B 5’UTR (Flatley et al., 2004; Payton et al., 2007). Although the physiologic significance of these alternate hRFC forms remains uncertain, the hRFC A1/A2 carrier isoform including 62 additional N-terminal residues was reported to exhibit slightly decreased transport activity (Flatley et al., 2004).

Reflecting the importance of RFC to in vivo folate homeostasis and the impact of folate deficiency on human health and disease, interest in mechanisms of RFC regulation in relation to exogenous folate levels is high. For instance, elevated RFC levels were reported in cell lines (CCRF-CEM, L1210, K562) following prolonged in vitro culture with subphysiologic concentrations of reduced folates (Sirotnak et al., 1984b; Jansen et al., 1990; Matherly et al., 1991). In mice fed folate-deficient diets, RFC transcripts and proteins increased in the small intestine (Liu et al., 2005). However, the physiologic significance of these changes in intestinal RFC is unclear given the acidic pH of the GI, which favors intestinal transport by PCFT over RFC. In Caco-2 and HuTu-80 cells, hRFC transcripts and proteins were induced in response to folate deficiency in vitro, and a transcriptionally active putative folate-responsive region was identified upstream of the hRFC-B minimal promoter (Subramanian et al., 2003). However, in another study using transport-upregulated CEM/7A T-cell leukemia cells and MCF7/MR breast cancer cells, hRFC levels decreased in response to folate deficiency (Ifergan et al., 2008). This result was suggested to represent an adaptive-protective response to folate deficiency which counteracts the detrimental effects of high-affinity folate extrusion via the hRFC. However, it is unclear how this can be reconciled with the formation of polyglutamyl folates within cells, which themselves are poor substrates for efflux, and the small net efflux of folates via RFC relative to MRP-mediated export.

Most recently, post-transcriptional regulatory effects on hRFC transcripts, protein, and transport were examined in hRFC-null HeLa cells stably transfected with hRFC and cultured with increasing subphysiologic to physiologic concentrations of extracellular folate (leucovorin) (Hou et al., 2013). The results suggested a novel regulation of hRFC in response to increasing extracellular folates involving increased hRFC transcripts and hRFC protein, reflecting differences in hRFC transcript stabilities. At higher folate concentrations, there was impaired intracellular trafficking and plasma membrane targeting with increased endoplasmic reticulum–trapped hRFC (Hou et al., 2013).

High-frequency polymorphisms have been identified in the hRFC gene and include nucleotide substitutions, deletions, and insertions in the hRFC coding region (G80A, resulting in replacement of Arg27 by His), the 3’ noncoding region (T2582G, C2617G), the A1/A2 promoter and 5’ noncoding region, and promoter A (Matherly et al., 2007). Although the functional impact and broader clinical significance of these alterations are still uncertain or remain controversial, increased hRFC transcriptional activity was associated with the 61 bp (base pairs) repeat polymorphism identified in hRFC promoter A (Whetstine et al., 2002b). When transport function of Arg27-hRFC was compared with His27-hRFC, there was no significant difference (Whetstine et al., 2001).

hRFC transcript variants have been described. These include 1) a CATG insertion at position 191 in an MTX-resistant ALL cell line and in primary ALL specimens that generates a frame shift and an early translational termination at position 1176 (Wong et al., 1999; Whetstine et al., 2001); 2) a 625 bp deletion in exon 7 (positions 1569–2193) that preserves a functional hRFC protein (Wong et al., 1995); and 3) a 988 bp deletion (positions 1294–2281), including all of TMD12, that generates an inactive transportor (Drori et al., 2000b).

A regulation of hRFC by its phosphorylation was implied (Kumar et al., 1997), although this has not been further studied. The original finding that AICA ribonucleotide regulates hRFC transport (McGuire et al., 2006) now appears to be unrelated to the activating effect of AICA ribonucleotide (ZMP) on AMP-activated protein kinase (AMPK), but rather reflects trans-stimulation of hRFC by intracellular ZMP (Visentin et al., 2012b) (described previously).

Thus, multiple regulatory mechanisms operate to ensure that there are sufficient levels of RFC protein and folate cofactor transport to meet needs for cell proliferation and tissue regeneration under diverse tissue environments. Further, alterations involving these mechanisms may significantly impact RFC levels and function, including specialized tissue functions, thus contributing to the pathophysiology of folate deficiency.

**Biology of PCFT**

**Transport Characteristics and Structure/Function Considerations.** hPCFT is composed of 459 amino acids (Fig. 4). The predicted molecular mass is 49.8 kDa. PCFT, like RFC, is a member of the MFS of secondary transporters, although hPCFT and hRFC share only 14% amino acid identity. hPCFT includes 12 TMDs with cytosolic N and C termini, as established by immunofluorescence studies of N- and C-terminal hemagglutinin-tagged hPCFT and by scanning cysteine accessibility methods with 2-aminoethyl methanethiosulfonate–bixin (Unal et al., 2008; Zhao et al., 2010). There are two N-glycosylation sites (Asn58 and Asn68) in the extracellular loop domain connecting TMDs 1 and 2 in hPCFT (Unal et al., 2008). When Asn58 and Asn68 were individually mutated to Gln, hPCFT expression and function were unaffected; however, transport activity decreased to ~40% for the Asn58/Asn68 double mutant. Expression of C-terminal yellow fluorescent protein-tagged hPCFT localized to the apical membranes of Madin-Darby canine kidney and Caco-2 cells (Subramanian et al., 2008). Deletion of carboxyl-terminal amino acids (to position 449) had no effect on apical membrane targeting or transport activity. Whereas Cys66 in the first extracellular loop forms a disulfide bond with Cys298 in the fourth extracellular loop (connects TMDs 7 and 8), this is not essential for transport activity (Zhao et al., 2010).

The transport properties of PCFT have been characterized in transfected cell lines and in oocytes microinjected with PCFT cRNAs (complementary RNAs) (Qiu et al., 2006; Zhao and Goldman, 2007; Deng et al., 2009). In human embryonic kidney 293 cells, transport activity was maximal at pH 4.5 (Nakai et al., 2007), although it was appreciable up to pH 6.5 (Zhao and Goldman, 2007). With further increased pH, there is a dramatic loss of transport activity such that, above pH 7, transport is very low. Decreased transport reflects both increased Kt and decreased Vmax values, although this varies for different transport substrates (Zhao and Goldman, 2007; Zhao et al., 2008; Desmoulin et al., 2010; Wang et al., 2010, 2011; Kugel Desmoulin et al., 2011). RFC substrates including 5-methyl THF and 5-formyl THF, MTX, aminopterin (AMT), PMX, and PDX are also transported by PCFT, particularly at low pH, although with increasing pH there are substantial differences in KtS (Qiu et al., 2007; Zhao and Goldman, 2007; Zhao et al., 2008; Deng et al., 2009; Desmoulin et al., 2012a; Menter et al., 2012). PCFT shows similar KtS for reduced (5-methyl and 5-formyl THF) and oxidized (folic acid) folate forms and is stereospecific for (6S)-5-formyl THF (Zhao and Goldman, 2007). PCFT also is stereospecific for L- over D-AMT (Menter et al., 2012). This was attributable almost entirely to differences in KtS. From
growth inhibition patterns in Chinese hamster ovary or HeLa cell lines engineered to express hPCFT without RFC, both RTX and Iometrexol (LMX) are transported by hPCFT (Zhao et al., 2008; Deng et al., 2009; Desmoulin et al., 2010); however, PT523 and GW1843U89 are not PCFT substrates (Zhao and Goldman, 2007; Deng et al., 2009). The 5-substituted pyrrolo[2,3-d]pyrimidine antifolate PMX is among the best PCFT substrates reported (Zhao and Goldman, 2007). More recently, a series of novel 6-substituted pyrrolo[2,3-d]pyrimidine antifolates were described as excellent PCFT substrates, with Ks comparable to that for PMX (Wang et al., 2010, 2011, 2012; Kugel Desmoulin et al., 2011; Desmoulin et al., 2010, 2012a; Cherian et al., 2013). The apparent affinities of these 5- and 6-substituted pyrrolo [2,3-d]pyrimidine analogs were less impacted by pH than for other PCFT transport substrates.

PCFT transport activity was not affected by removal of Na+, K+, Ca2+, Mg2+, or Cl- (Qiu et al., 2006). Treatment with carbonyl cyanide p-trifluoromethoxyphenylhydrazone (a proton ionophore) (Qiu et al., 2006) or nigericin (a K+/H+-exchanging ionophore) (Inoue et al., 2006) reduced transport by PCFT. Similarly, treatment of HeLa cells with nitrate or bisulfite abolished the pH gradient and inhibited PCFT transport (Zhao et al., 2013). From studies in Xenopus oocytes, PCFT transport of folates is electrogenic such that there is a net translocation of positive charges for each negatively charged folate molecule (Qiu et al., 2006), although the coupling ratio is not known. Further, in Xenopus oocytes, PCFT transport was accompanied by intracellular acidification (Unal et al., 2009a). In the absence of a transmembrane pH gradient, PCFT can still function. In this case, transport is driven by the membrane potential (Qiu et al., 2006; Umaphathy et al., 2007). At acidic pH, PCFT was reported to exhibit channel-like activities, i.e., the proton flux was uncoupled from transport of folate substrates (Unal et al., 2009a; Mahadeo et al., 2010).

Structural determinants of hPCFT transport have been deduced from studies of loss-of-function hPCFT mutations identified in HFM patients, and by mutagenesis of amino acids implicated as potentially functionally important from considerations of species homologies, amino acid charge or polarity, and TMD localization. Residues implicated as functionally important include Glu185 (TMD5) (important for proton coupling) (Unal et al., 2009b), His281 (TMD7) (important for PCFT protonation which augments substrate binding) (Unal et al., 2009a), and Arg376 (TMD10) (impacts proton and substrate binding) (Mahadeo et al., 2010) (Fig. 4). A conserved stretch of amino acids linking TMDs 2 and 3 (DXXGRR; positions 109–114) including a β-turn was implicated as functionally important for transport (Zhao et al., 2007; Lasry et al., 2008; Subramanian et al., 2008; Shin et al., 2010). Both Asp109 and Arg113 are essential for hPCFT transport since amino acid replacements at these positions abolished transport regardless of charge or polarity. From the loss of hPCFT transport for the R113C mutant, homology modeling based on the glycerol-2-phosphate/inorganic phosphate antiporter template was used and predicted that Arg113 protrudes into a hydrophobic cavity composed of TMDs 1, 3, 4, and 6 (Lasry et al., 2008). However, this was not experimentally confirmed. Both Asp109 and Arg113 may directly participate in binding and translocation of (anti)folate substrates.

Mutations at His247 (Ala, Arg, Gln, Glu) resulted in substantially decreased rates of transport (decreased Vmax) and increased affinities (decreased Kd) for folate substrates compared with wild-type hPCFT (Unal et al., 2009a). In an hPCFT homology model, His247 was predicted to reside in a highly electropositive region at the cytoplasmic opening to the water-filled translocation pathway where it interacted with Ser172, thus limiting substrate access to the putative folate binding pocket. Consistent with this interpretation, the S172A hPCFT mutant showed a transport phenotype similar to that for H247A hPCFT and enhanced proton transport in the absence of substrate.

Other residues implicated as functionally important to hPCFT transport include Leu161 (TMD4), Glu232 (TMD6), Ile304 (TMD8), and Pro425 (flanks TMD12) (Zhao et al., 2011b) (Fig. 4). Mutation of Glu232 (Gly) decreased the rate of carrier translocation, whereas mutations at Ile304 (Phe) and Leu161 (Arg) decreased substrate affinities (Zhao et al., 2011b). Mutation of Pro425 to Arg resulted in decreased binding of MTX and other (anti)folate substrates; however, PMX binding was preserved (Shin et al., 2012). From mutant studies,
Cysteine-scanning mutagenesis, combined with reactivity with membrane-impermeable sulfhydryl-reactive methanethiosulfonate (MTS) reagents [MTSES, (2-trimethylammonium)ethyl methanethiosulfonate, 2-aminoethyl methanethiosulfonate-biotin], was used to localize residues in hPCFT to the substrate binding region. Thus, Phe157, Gly158, and Leu161 in TMD4 and Ile188 in TMD5 were reactive with MTS reagents and could be protected by PMX, placing these residues within or near the folate binding site in hPCFT (Zhao et al., 2012; Shin et al., 2013).

Like other MFS proteins, including hRFC, hPCFT exists as a homo-oligomer (Hou et al., 2012). In ectopically expressed hPCFT, oligomerization was demonstrated by protein cross-linking with 1,1-methanediyl bismethanethiosulfonate (MTS-1-MTS), blue native gel electrophoresis, coeling of coexpressed epitope-tagged (hemagglutinin and His[9]) hPCFT monomers to nickel affinity columns, and fluorescence resonance energy transfer (FRET) between coexpressed YPet (FRET-optimized yellow fluorescent protein)- and ECFP* (enhanced cyan fluorescent protein)-tagged hPCFT monomers. Oligomerization was functionally significant, as coexpression of wild-type and mutant P425R hPCFTs exhibited a “dominant-positive” functional phenotype, establishing positive cooperativity between monomers, and a functional rescue of the inactive mutant hPCFT by wild-type hPCFT. Based on these results, an “alternate access” model for hPCFT, analogous to that suggested for monomeric hMCT1, was proposed which incorporates a functionally important role for hPCFT oligomerization (Hou et al., 2012).

The hPCFT primary sequence includes GXXXG motifs in TMD2 (amino acids 93–97) and TMD4 (amino acids 155–159), analogous to dimerization motifs reported for other anfipathic proteins (Polgar et al., 2010; Duan et al., 2011). Although mutation of Gly93 and Gly97 to Ala did not inhibit transport activity or oligomer formation, as determined with thiol-reactive (MTS-1-MTS) protein cross-linking (Zhao et al., 2012), analogous studies with the GXXXG motif in TMD4 were not performed. Using cross-linking with MTS-1-MTS as a metric for hPCFT oligomerization, Zhao et al. (2012) individually mutated each of the seven cysteine residues in wild-type hPCFT to assess the impact on PCFT oligomerization. Whereas serine replacement of Cys21, -66, -151, -298, -328, and -397 had no impact on cross-linking with MTS-1-MTS, C229C in TMD6 abolished cross-linking (Zhao et al., 2012). This implies that TMD6 provides a structural interface between individual hPCFT monomers.

In contrast to the aforementioned evidence that oligomeric hPCFT is structurally and functionally important, a study by Duddemudi et al. (2013) suggested that hPCFT may not be oligomeric when expressed in Chinese hamster ovary cells or Xenopus oocytes and isolated from plasma membranes prepared by polymerization with colloidal silica and polyacrylic acid. As this latter report used entirely different methods and metrics than the earlier study by Hou et al. (2012), it is not possible to reconcile their disparate conclusions.

**Regulation of PCFT Expression.** The hPCFT gene consists of five exons and is localized to chromosome 17q11.2. The hPCFT promoter includes a minimal transcriptional unit localized between positions −42 and +96 (Diop-Bove et al., 2009; Stark et al., 2009). The promoter is G/C rich and includes a 1085 bp CpG island spanning the transcriptional start site which is hypermethylated, accompanying low-level hPCFT expression in MTX-resistant HeLa and T-cell ALL (Jurkat, CCFL-CEM) cells (Gonen et al., 2008; Diop-Bove et al., 2009). Treatment with 5-aza-2′-deoxycytidine resulted in restoration of hPCFT mRNA expression and transport. In mice fed a folate-deficient diet, PCFT transcript levels increased (∼13-fold) in the proximal small intestine compared with levels in mice fed a folate-replete diet (Qiu et al., 2007).

Studies have begun to identify transcriptional regulatory factors and cis elements which regulate the hPCFT gene (Gonen et al., 2008; Eloranta et al., 2009; Stark et al., 2009; Gonen and Assaraf, 2010; Furumiya et al., 2013). Three nuclear respiratory factor-1 sites (positions −108 to −97, −93 to −82, and −10 to +1) were identified in the hPCFT minimal promoter, and nuclear respiratory factor-1 binds and transactivates the hPCFT gene, leading to increased hPCFT transcripts (Gonen and Assaraf, 2010). 1,25-Dihydroxyvitamin D3 (vitamin D3) induced hPCFT levels in Caco-2 cells in vitro and in rat duodenal biopsies ex vivo (Eloranta et al., 2009). Induction of hPCFT by vitamin D3 resulted in enhanced transport at pH 5.5. In the presence of vitamin D3, vitamin D receptor (VDR) heterodimerized with retinoid X receptor-α and bound a VDR response element in the hPCFT promoter (positions −1694 to −1680). Although these results suggested that vitamin D3 could affect bioavailability of dietary folates via PCFT transactivation, in VDR homozygous knockout mice, hepatic and plasma folates, as well as intestinal PCFT transcripts, were unchanged from wild-type mice (Brandsch et al., 2013). Moreover, in rat dams and their offspring, there were no changes in levels of plasma folates in response to dietary vitamin D (Brandsch et al., 2013).

Recent studies explored the transcriptional basis for tissue-specific expression patterns for PCFT in the small intestine, including the localization of PCFT primarily to the proximal GI, with lower levels in other regions of the GI tract (Furumiya et al., 2013). The focus was on the effect of individual transcription factors which are specifically or abundantly expressed in the small intestine. Using a reporter construct including hPCFT upstream sequence from positions −1695 to +96 in luciferase reporter assays, transactivation was seen with Krüppel-like factor 4, and this was further enhanced by hepatocyte nuclear factor 4α. Conversely, caudal-type homeobox transcription factor 2 and CCAAT/enhancer-binding protein α (C/EBPα) suppressed hPCFT promoter activity. Western blots of rat small intestine proteins demonstrated uniform expression of Krüppel-like factor 4 along the entire length of the intestinal tract, proximally expressed hepatocyte nuclear factor 4α, and distal expression of caudal-type homeobox transcription factor 2 and C/EBPα, consistent with the observed proximal-to-distal expression of PCFT in the GI tract.

**Role of Antifolates in Cancer Therapy**

The antifolates remain an important class of drugs for the treatment of numerous cancers, notably pediatric ALL, osteogenic sarcoma, lymphoma, breast cancer, non–small-cell lung cancer, and malignant pleural mesothelioma (Monahan and Allegra, 2011; Desmoulin et al., 2012a; Gonen and Assaraf, 2012; Visentin et al., 2012a). MTX was introduced more than 60 years ago (Farber and Diamond, 1948; Farber, 1949) yet remains a vital drug for both cancer (Monahan and Allegra, 2011; Gonen and Assaraf, 2012; Visentin et al., 2012a) and nonmalignant diseases such as rheumatoid arthritis and psoriasis (Chladek et al., 1998; Wessels et al., 2008). Numerous other antifolates have since been synthesized and tested preclinically, in many cases drawing from the enhanced understanding of the pharmacology and biology of MTX or AMT, including their membrane transport, polyglutamylation, and binding to intracellular targets. In recent years, a new generation of clinically relevant antifolates has emerged including PDX (Sirotnak et al., 1998; Thompson, 2009; Marchi et al., 2013), RTX (Wilson and Malfair Taylor, 2009), and PMX (Hazarika et al., 2005; Cohen et al., 2009) (Fig. 1). Other agents are still in the pipeline and are in various stages of development, including, most recently, a series of novel
PCFT-selective 6-substituted 2,3(3H)[1,2,4]triazolo[4,3-
A]pyridine antifolates designed to selectively target solid tumors by virtue of their substantial PCFT expression and their acid microenvironments which favor membrane transport by PCFT (Desmoulin et al., 2012a) (Fig. 5).

Classic antifolates, like folate cofactors, are anions at physiologic pH, such that facilitative membrane transport is critical to their cellular uptake and drug efficacy (Goldman and Matherly, 1985; Zhao and Goldman, 2003; Matherly et al., 2007; Desmoulin et al., 2012a; Gonen and Assaraf, 2012). The ubiquitously expressed RFC is the major transport route for antifolate drugs such as MTX, RTX, and PDX into both normal tissues and tumors, even though cellular uptake by FRs and/or PCFT can also occur (Matherly et al., 2007; Desmoulin et al., 2012a; Gonen and Assaraf, 2012). The relative contributions of these route reflect levels of these uptake systems in different tissues and tumors, the pH of the tissue/tumor microenvironment, and substrate specificities for the individual uptake systems. Transport of antifolates by RFC into normal tissues contributes to the toxicities associated with these agents.

The role of membrane transport in MTX antitumor activity has been extensively documented (Goldman and Matherly, 1985; Zhao and Goldman, 2003; Matherly et al., 2007; Monahan and Allegra, 2011; Desmoulin et al., 2012a; Gonen and Assaraf, 2012). For MTX, transport is essential to generate sufficient intracellular drug to maximally inhibit DHFR and to provide substrate for synthesis of polyglutamyl derivatives required for cellular drug retention and to sustain antitumor effects despite decreasing extracellular drug (Goldman and Matherly, 1985; Zhao and Goldman, 2003). Polyglutamylation of MTX is critical to drug efficacy, as tumors with elevated capacity to synthesize MTX polyglutamates are generally more responsive to drug (Goldman and Matherly, 1985; Zhao and Goldman, 2003; Monahan and Allegra, 2011; Gonen and Assaraf, 2012). Further, the extent of MTX polyglutamylation is likely a contributing factor to tumor selectivity over normal tissues, and to the selectivity of leucovorin rescue from MTX toxicity (Zhao and Goldman, 2003). Similar considerations would apply to other DHFR inhibitors, such as PDX, that are metabolized to polyglutamates (Visentin et al., 2013), but not to antifolates, such as PT523, that are not metabolized to polyglutamates (discussed later). For PMX, RTX, and LMX, all of which inhibit enzymes other than DHFR as their primary cellular targets, polyglutamylation is especially important since polyglutamate forms of these drugs are more potent enzyme inhibitors than the nonpolyglutamyl drug forms (Hughes et al., 1999; Mendelsohn et al., 1999; Shih and Thornton, 1999; Chattopadhyay et al., 2007).

Impaired membrane transport results in MTX resistance with in vitro and in vivo preclinical models, and has been implicated in clinical resistance to MTX in ALL and osteogenic sarcomas (Zhao and Goldman, 2003; Matherly et al., 2007; Gonen and Assaraf, 2012). Impaired RFC transport has also been described for other antifolate inhibitors (Gonen and Assaraf, 2012). In non–small-cell lung cancer and malignant pleural mesothelioma, expression of hRFC was associated with responses to treatment with PMX (Alvarez-Fernandez et al., 2013; Mairinger et al., 2013). In cell lines, transport resistance reflects loss of RFC due to decreased levels or point mutations and synthesis of inactive transporters (Zhao and Goldman, 2003; Matherly et al., 2007; Gonen and Assaraf, 2012). Loss of transport frequently accompanies other cellular alterations, including decreased polyglutamate synthesis and/or increased levels of intracellular target enzymes (Zhao and Goldman, 2003; Gonen and Assaraf, 2012). For LMX, which is an especially good substrate for folypoly-γ-glutamyl synthetase (FGPS) and is extensively converted to polyglutamates (far exceeding levels for MTX; discussed later), sensitivity can be preserved toward MTX-resistant cells despite substantial losses of hRFC, as long as FGPS activity is preserved (Matherly et al., 1993).

In the following sections, we describe the biologic and pharmacologic principles behind the major antifolate drugs, including clinically relevant agents and experimental prototypes in various stages of clinical and preclinical development, for which drug efficacy can be attributed to their membrane transport by the major facilitative folate transporters, RFC and PCFT.

DHFR Inhibitors. Based on observations establishing the importance of folate cofactors to cancer progression, Farber and colleagues (1947) hypothesized that folate antagonists could inhibit the proliferation of cancer cells. A series of folate analogs were synthesized, one of which (AMT) (Fig. 1) was administered to children with ALL and induced clinical remissions (Farber and Diamond, 1948). Thus, AMT was the first drug to induce remissions in this devastating disease. MTX (Fig. 1) was subsequently tested and found to induce remissions with less toxicity than was encountered with AMT (Farber, 1949). Today, MTX continues to be used throughout the world as an essential component of multidrug regimens for treating ALL, lymphomas, and solid tumors (Monahan and Allegra, 2011; Gonen and Assaraf, 2012; Visentin et al., 2012a). MTX is also used for treating other conditions ranging from rheumatoid arthritis and psoriasis to Crohn’s disease (Feagan et al., 1995; Cllake et al., 1998; Wessels et al., 2008).

Both AMT and MTX are potent inhibitors of DHFR (Zhao and Goldman, 2003; Monahan and Allegra, 2011; Gonen and Assaraf, 2012; Visentin et al., 2012a). Inhibition of DHFR results in accumulation of dihydrofolate from 5,10-methylene THF, generated during synthesis of thymidylate by TS (Fig. 2). Dihydrofolate is reduced to THF by DHFR such that, in the absence of DHFR, the buildup of dihydrofolate results in “depletion” of unsubstituted THF and C1-substituted THF pools, and cessation of THF-dependent biosynthesis of thymidylate, purine nucleotides, serine, and methionine. The magnitude of this net loss of THF cofactors varies for different THF forms and for different cell types (Allegra et al., 1986; Matherly et al., 1987; Trent et al., 1991b) and is attributable to binding of folates to cellular proteins and sequestration of folate cofactors in cellular organelles (e.g., mitochondria) (Trent et al., 1991a; Matherly and Muench, 1990; Tibbett and Appling, 2010).

AMT is a better substrate than MTX for RFC transport and polyglutamylation by FGPS (Matherly et al., 1985). In tumor cells, high levels of AMT polyglutamates accumulate, far exceeding levels of MTX polyglutamates. Reflecting its high levels of transport and polyglutamylation, AMT also exhibits more potent antitumor activity than MTX (Goldin et al., 1955; Moccio et al., 1984). This may also...
explain increased toxicity of AMT over MTX seen clinically. In recent years, there has been renewed clinical interest in AMT for treating cancer and inflammatory diseases (Cole et al., 2008; Menter et al., 2012).

PDX (Fig. 1) or 10-propargyl-10-deaza-AMT was a result of the collaboration between F. M. Sirotnak (Memorial Sloan-Kettering Cancer Center) and J. I. Degraw (Southern Research Institute) to identify novel antifolates with improved cellular pharmacology over MTX. In preclinical studies, 10-deaza-AMT was more potent than MTX (Sirotnak et al., 1984a) and 10-ethyl-10-deaza-AMT (edatrexate) was even more potent (Schmid et al., 1985; Sirotnak et al., 1993). PDX, a third generation analog of this series, was a less potent DHFR inhibitor than AMT, MTX, or edatrexate, but exhibited better RFC-mediated transport and polyglutamation than these compounds (Sirotnak et al., 1998; Visentin et al., 2013). The net result was increased drug efficacy toward leukemia, breast cancer, and non–small-cell lung cancer cell lines in vitro and in vivo. In phase I and phase II trials, including patients with non–small-cell lung cancer (Krug et al., 2003) and peripheral T-cell lymphoma (O’Connor et al., 2009; Marchi et al., 2013), PDX showed efficacy and safety. The Food and Drug Administration (FDA) approved the use of PDX in 2009 for the treatment of relapsed, refractory peripheral T-cell lymphoma (Thompson, 2009).

PT523 (talotrexin) is a hemithalylolornithine antifolate (Fig. 1) synthesized by A. Rosowsky and colleagues (Dana Farber) (Rosowsky et al., 1988). PT523 is a potent DHFR inhibitor (Rosowsky et al., 1988; Rhee et al., 1994) and is among the best substrates for RFC with a submicromolar Ki for the human carrier (Rhee et al., 1994; Rosowsky et al., 1994; Wright et al., 2000). PT523 is a very poor substrate for PCFT (Zhao and Goldman, 2007; Desmoulin et al., 2010; Wang et al., 2010). Reflecting the absence of a terminal glutamate, PT523 is not a substrate for polyglutamation and is less impacted by levels of intracellular THF cofactors than is MTX. PT523 was tested in a phase I study in 18 patients with relapsed or refractory non–small-cell lung cancer where it showed acceptable toxicity and efficacy (2 partial responses, 9 stable disease) after multiple (median 3–4) chemotherapy cycles (Roca Lima et al., 2006).

Thymidylate Synthase Inhibitors. RTX [Tomudex, ZD1694(S)-2-(5-(methyl)((2-methyl-4-oxo-1,4-dihydroquinazolin-6-yl)methyl)amino)thiophene-2-carboxamido)pentanedioic acid] (Fig. 1) is a quinazoline antifolate inhibitor of TS that was the result of rational drug design by scientists at the Institute for Cancer Research and Astra Zeneca (Jackman and Calvert, 1995; Hughes et al., 1999). Early efforts to develop a TS-targeted antifolate resulted in N10-propargyl-5,8-dideazafolic acid (CB3717, N-[4-[N-([2-Amino-4-hydroxy-6-quinazolyl)methyl]prop-2-ylamino]benzoyl]-L-glutamic acid). In phase I/II clinical trials, CB3717 showed efficacy against ovarian, liver, and breast cancers, but also resulted in hepatic toxicity and dose-limiting nephrotoxicity (Jackman and Calvert, 1995). To reduce toxicity, modifications were introduced, including substitution at the 2-amino group by a 2-desamino-2-methyl, replacement of the benzoyl ring by a thiophene, and the replacement of the N10-propargyl by a methyl (Jackman et al., 1991). The resulting compound, RTX, is less potent than CB3717 as a TS inhibitor, but is a far better substrate for RFC transport and polyglutamation by FPGS. These properties resulted in more potent antitumor efficacy in vitro and in vivo (Jackman et al., 1991; Hughes et al., 1999). RTX can also be transported by FRs and PCFT, although RFC is the major transport route. RTX was approved for treatment of advanced colorectal cancer in Europe, Canada, and Australia (Chu et al., 2003). Based on evidence of efficacy of combined RTX and cisplatin toward malignant pleural mesothelioma, RTX was approved for treatment of this disease in a number of European countries (Surmont and van Meerbeeck, 2011).

ZD9331 (Plevitrexed, BGC9331) (Fig. 1) is a water-soluble quinazoline antifolate with a γ tetrazole that lacks FPGS substrate activity but retains high affinity for RFC. ZD9331 is a potent inhibitor of TS (Jackman and Calvert, 1995; Jackman et al., 1997). Although its principal mode of transport is by RFC, ZD9331 is also a substrate for FRs (Jansen, 1999) and PCFT (Matherly and Gangjee, 2011). The rationale for developing nonpolyglutamylated antifolates was that such compounds would be active against tumors expressing low FPGS or high γ-glutamyl hydrodase activities, both of which can confer resistance to polyglutamylated antifolates (Zhao and Goldman, 2003). In addition, this property was believed to result in reduced toxicity due to decreased drug retention. ZD9331 inhibitory activity was preserved in murine L1210 leukemia cells resistant to RTX due to reduced FPGS activity (Jackman et al., 1997). Based on promising preclinical results, phase II clinical trials were conducted with ZD9331 with advanced and metastatic colorectal cancer, ovarian cancer, pancreatic cancer, as well as other solid tumors (Hainsworth et al., 2003; Rader et al., 2003; Smith and Gallagher, 2003; Louvet et al., 2004; Schulz et al., 2004). ZD9331 showed a manageable toxicity profile and some evidence of activity in patients with relapsed or refractory disease.

GW1843U89 (Fig. 1) was an outgrowth of a program at the Burroughs Wellcome Company to discover folate inhibitors as antimicrobial agents (Smith et al., 1999). This resulted in a series of benzof[quinazolin-1(2H)-ones including GW1843U89. GW1843U89 is an extremely potent noncompetitive inhibitor of human TS with a Ki (inhibitory constant) of 0.09 nM (Duch et al., 1993). TS binds GW1843U89 in a binary complex which is further stabilized upon binding of its deoxyuridylate substrate. GW1843U89 was reported to be an excellent substrate for hRFC with a Ki of 0.33 μM, whereas RFC transport of GW1843U89, unlike other RFC substrates, by rodent cells is poor (Duch et al., 1993). GW1843U89 is a good substrate for FPGS with the major cellular metabolite being the diglutamate (Duch et al., 1993; Hanlon and Ferone, 1996). This reflects the poor FPGS substrate activity of diglutamyl GW1843U89 (Hanlon and Ferone, 1996). TS inhibition was unaffected by polyglutamylation such that antitumor activity was preserved in tumors with defective polyglutamation. GW1843U89 showed potent inhibitory activity against a number of human tumor cell lines, including human tumor xenografts engrafted into mice (Smith et al., 1995, 1999). A phase I clinical trial was performed in patients with advanced solid tumors (Schwartz et al., 2001). GW1843U89 was reformulated by encapsulation into liposomes and renamed OSI-794. OSI-794 showed better bioavailability and superior antimetabolic effects than free GW1843U89, prompting phase I trials conducted in patients with advanced cancers (Beutel et al., 2005; Clamp et al., 2008; Ricart et al., 2008). In phase II trials, OSI-794 was well tolerated. Clinical responses were seen in patients with advanced gastric or gastroesophageal cancers (Falk et al., 2006) but not in patients with advanced biliary cancer (Ciauleanu et al., 2007).

De Novo Purine Nucleotide Biosynthesis Inhibitors. Purines are critical for the synthesis of DNA and RNA, and as components of ATP, cyclic AMP, NAD/NADP, and coenzyme A. Differentiated cells frequently obtain purines through purine salvage reactions, whereas proliferating cells achieve their requirements for purine nucleotides primarily by de novo synthesis (Howell et al., 1981; Jackson and Harkrader, 1981). Both salvage and de novo pathways use phosphoribosyl pyrophosphate, or PRPP. For purine salvage, hypoxanthine phosphoribosyl transferase converts hypoxanthine and guanine to IMP and GMP, respectively; adenine is incorporated into AMP by adenosine phosphoribosyl transferase. In de novo purine
biosynthesis, PRPP is converted in 10 steps to IMP, a precursor of GMP and AMP. There are two folate-dependent reactions, catalyzed by the multifunctional proteins GARFTase and AICARFTase (Fig. 6).

In a collaboration between academic and pharmaceutical sectors designed to develop antifolates which inhibit enzyme targets other than DHFR, E. C. Taylor (Princeton University) and Chuan (Joe) Shih (Eli Lilly) collaborated to synthesize the (6R) diastereomer of 5,10-dideaza THF known as LMX (Taylor et al., 1985; Moran et al., 1989; Mendelsohn et al., 1999) (Fig. 1). LMX is a substrate for RFC (Matherly et al., 1989; Jansen, 1999), although it can also be transported by both FRs and PCFT (Jansen, 1999; Desmoulin et al., 2012a). Following internalization, LMX is extensively polyglutamylated (Moran et al., 1989; Matherly et al., 1993). Polyglutamyl forms of LMX are potent inhibitors of GARFTase, and result in ATP and GTP depletion (Beardsley et al., 1989; Moran et al., 1989; Mendelsohn et al., 1999). Interestingly, loss of ATP renders p53 transcriptionally inert such that LMX showed cytotoxic activity independent of p53 status (Bronder and Moran, 2003). LMX showed promising preclinical antitumor activity in vitro and in vivo with assorted tumor models (Taylor et al., 1985; Beardsley et al., 1989; Moran et al., 1989; Mendelsohn et al., 1999). In a phase I clinical trial, LMX caused severe cumulative toxicity, including dose-limiting myelosuppression and mucositis (Ray et al., 1993). Toxicity was reduced if LMX was administered with folic acid, permitting a 10-fold dose escalation over the dose administered without folic acid supplementation (Roberts et al., 2000).

Second generation GARFTase inhibitors were synthesized and tested, including LY309887 [(2S)-2-[5-[2-[6(R)-2-amino-4-oxo-5,6,7,8-tetrahydro-1H-pyrido[2,3-d]pyrimidin-6-yl]ethyl]thiophene-2-carbonyl]amino]pentanedioic acid], AG2034 [N-[5-[2-[6S]-2-amino-4-oxo-4,6,7,8-tetrahydro-1H-pyrimidin-5,4-b][1,4]thiazin-6-yl]ethyl]thiophen-2-yl]carbonyl]-L-glutamic acid], and AG2037 [N-[5-[2-[6S]-2-amino-1,4,5,6,7,8-hexahydro-4-oxopyrido[2,3-d]pyrimidin-6-yl]ethyl]-4-methyl-2-thienyl]carbonyl]-L-glutamic acid] (Fig. 1 shows structures of LY309887 and AG2034), as a step toward reducing the toxicity encountered with LMX (Boritzki et al., 1996; Mendelsohn et al., 1999). All these newer compounds were substrates for RFC and were potent inhibitors of GARFTase. LY309887 had a lower affinity for FRs than LMX (Mendelsohn et al., 1999). AG2034 differed from AG2037 in its lower affinity toward FRs (Boritzki et al., 1996). In phase I studies, AG2034 and LY309887 showed similar cumulative toxicities to those encountered with LMX (Bissett et al., 2001; Budman et al., 2001).

Pemetrexed, a Multitargeted Antifolate. PMX (LY231514, Alimta, N-[4-[2-(2-amino-4,7-dihydro-4-oxo1H-pyrrolo[2,3-d]pyrimidin-5-yl)ethyl]benzoyl]-L-glutamic acid) (Fig. 1) was synthesized by Eli Lilly and Company in an attempt to meet FDA requirements for purity and to eliminate chirality at the 6 position of the 5-deazapteridine ring of LMX (Taylor et al., 1992). PMX is an excellent transport substrate for RFC and PCFT (Chattopadhyay et al., 2007; Matherly et al., 2007; Zhao and Goldman, 2007; Desmoulin et al., 2012a). For PCFT, PMX is among the best substrates, and its transport is much less sensitive to pH

Fig. 6. The de novo purine nucleotide biosynthesis pathway. The 10 steps from PRPP to inosine monophosphate (IMP) are shown. Antifolate drugs that inhibit the folate-dependent enzymes, GARFTase and AICARFTase, are marked in red, as described in the text. AIR, aminoimidazole ribonucleotide; CAIR, carboxyaminoimidazole ribonucleotide; FAICAR, formyl 5-aminimidazole-4-carboxamide ribonucleotide; FAICAR, formyl 5-aminimidazole-4-carboxamide ribonucleotide; FGM, N-formylglycinamidine ribonucleotide; GFA, formyl glycaminamide ribonucleotide; GFA, formyl glycaminamide ribonucleotide; SAICAR, 5-aminimidazole-4-(N-succinyl)carboxamide).
than other (anti)folate substrates. Within cells, PMX is extensively polyglylutamylated, and its polyglutamylation is negatively impacted by cellular folate status (Shih et al., 1997; Zhao et al., 2001a, 2004b; Kugel Desmoulin et al., 2011). In contrast to antifolates such as MTX or RTX, antitumor effects of PMX are maintained or even enhanced in RFC-deficient cells, as long as PCFT is present (Zhao et al., 2008). In initial cell culture experiments, TS appeared to be the primary cellular target, although secondary targets were implied, including the folate-dependent enzymes in de novo purine nucleotide biosynthesis, GARFTase and AICARFTase (Taylor et al., 1992; Shih et al., 1997). PMX has a very low affinity for DHFR. Further, the impact of DHFR inhibition would be nominal since primary inhibition of TS would obviate DHFR as a secondary target, as dihydrofolate would not be generated. These results were confirmed by studies with isolated enzyme preparations. PMX polyglutamates were especially potent inhibitors of TS, with a Ki for PMX pentaglutamate of 1.3 nM, compared with a Ki of 109 nM for unmethabolized PMX (Shih et al., 1997). Inhibitions of GARFTase, AICARFTase, and DHFR were all confirmed, albeit less than for TS. PMX was originally termed a “multitargeted antifolate” to reflect its inhibition of multiple folate-dependent enzyme targets. In 2004, PMX was approved by the FDA for use (with cisplatin) in treating malignant glioblastomas. PMX inhibition of multiple folate-dependent enzyme targets. In 2004, PMX was approved for maintenance therapy of patients with locally advanced or metastatic non–small-cell lung cancer (Cohen et al., 2010).

R. G. Moran (Virginia Commonwealth University) presented interesting evidence that AICARFTase may be a more important secondary therapeutic target for PMX than previously realized (Racanelli et al., 2009; Rothbart et al., 2010). Thus, treatment of CCRF-CEM T cell ALL cells and several solid tumor cell lines with PMX resulted in accumulations of ZMP, the substrate of the AICARFTase reaction. In contrast to results with the GARFTase inhibitor LMX, PMX treatment did not deplete cellular ATP pools. ZMP acts as an AMP mimic that activates AMPK which, in turn, phosphorylates target proteins involved in initiation of cap-dependent translation, lipid synthesis, and energy metabolism. Tuberculosis complex 2 and raptor (component of mTORC1 or mammalian target of rapamycin complex 1) proteins are AMPK targets, such that AMPK activation results in inhibition of mTOR signaling (Iino et al., 2003; Gwinn et al., 2008). Although this could contribute to the antitumor efficacy of PMX, particularly in the absence of a primary inhibition of TS, in KB tumor cells, AMPK activation in response to PMX or direct polyglutamates for compound 9 and 10 were extended in vivo in severe combined immunodeficient mice bearing human tumor xenografts (HepG2, HeLa, H2452) (Kugel Desmoulin et al., 2011). In hRFC-null HeLa cells expressing hPCFT, the antiproliferative effects of 9 and 10 were greater compared with wild-type HeLa cells expressing hPCFT with intact hRFC, due to the depletion of intracellular folate cofactors (Desmoulin et al., 2012b).

Additional studies confirmed that compounds 3, 4, 9, and 10 all targeted de novo purine nucleotide biosynthesis with potent inhibition of GARFTase and a dramatic fall in ATP levels (Desmoulin et al., 2010; Wang et al., 2010, 2011; Kugel Desmoulin et al., 2011; Cherian et al., 2013). Compounds 9 and 10 were cytotoxic. Further, for compound 10, treatment of HeLa cells resulted in time- and dose-dependent accumulation in late S-phase, accompanied by cell death, in part by an apoptotic mechanism (Kugel Desmoulin et al., 2011). These compelling in vitro results with compounds 9 and 10 were extended in vivo in severe combined immunodeficient mice bearing human tumor xenografts (HepG2, HeLa, H2452) (Kugel Desmoulin et al., 2011; Desmoulin et al., 2012b; Cherian et al., 2013). These results provide definitive proof of concept of in vivo tumor targeting via PCFT.

Future Directions

This review summarizes the biology and therapy of the major facilitative folate transporters, RFC and PCFT. Although the advances in the understanding of the biology of the major facilitative folate transporters have been substantial, there remain important unresolved issues.

For instance, further characterization of the transcriptional and post-transcriptional regulation of hPCFT is certainly warranted to clarify the basis for differential hPCFT expression levels between many tumors and normal tissues, or among tumors with vastly differing hPCFT levels. Likewise, the role of hPCFT promoter methylation to differential expression of hPCFT among tumors must be established. Better understanding of critical determinants of hPCFT transcriptional
regulation may spur development of strategies for modulating hPCFT levels in tumors, including combined therapies that include hPCFT-targeted antifolates and demethylating agents. For hRFC, the physiologic significance of differential S^UTR usage on hRFC translational efficiency or transcript stabilities, or the role of N-terminally modified hRFC proteins needs further clarification. For both hRFC and hPCFT, this should extend to characterizing post-translational mechanisms that regulate carrier levels and function.

The finding that both hRFC and hPCFT can form homo-oligomers implies yet another level of regulation, namely, the possibility that heterozygous hPCFT mutants from HFM patients or heterozygous mutant hRFC in MTX-resistant tumors may impact trafficking and function of the wild-type transporter secondary to formation of mutant/wild-type oligomers. In future studies, it will be important to further identify the functional impact and structural determinants of transporter oligomerization, as this may foster development of approaches for biochemically modulating this process with small-molecule “pharmacologic chaperones” or peptidomimetics that will enhance transporter levels and activity.

Of additional importance will be better understanding the impact of exogenous factors that could regulate transporter levels and function in vivo, including dietary components (e.g., folates) and the tissue/tumor microenvironment. Despite extensive research, the functional or clinical significance of high-frequency hRFC polymorphisms remains largely unresolved and at best controversial. Although novel 6-substituted pyrrolo[2,3-d]pyrimidine antifolates with selectivity for hPCFT over hRFC and potent antitumor efficacies have been developed, it will be essential to better understand the structure-activity relationships for binding and translocation of (anti) folate substrates by these transporters. Given the challenges in the structural biology of membrane transporters, the focus will continue to be on multiparameter optimization of novel analogs based on medicinal chemistry and experimentally tested molecular homology models. As the most promising of these agents are GARFTase inhibitors, it will be especially important to definitively establish the therapeutic potential of targeting GARFTase by these non-RFC PCFT-specific antifolates. This extends to other determinants likely to impact drug efficacy, including purine salvage and substrate activities for the major folate efflux pumps such as MRPI and ABCG2, since these could significantly affect their in vivo pharmacology and antitumor efficacies. Finally, it will be important to better understand resistance to these novel hPCFT-selective antifolates that will invariably arise, the extent to which resistance involves molecular alterations to hPCFT, and the possibility that hPCFT transport resistance can be circumvented by structurally distinct cytotoxic hPCFT substrates or the presence of other non-RFC uptake mechanisms, such as FRs, for which these agents also have high affinity.

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