The human proton-coupled folate transporter
Biology and therapeutic applications to cancer

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Abbreviations: AICA, 5-amino-4-imidazole carboxamide; AICAR, 5-amino-4-imidazole carboxamide ribonucleotide; AICARFase, 5-amino-4-imidazole carboxamide ribonucleotide formyltransferase; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; AMPK, AMP-activated protein kinase; AMT, aminopterin; APRT, adenine phosphoribosyl transferase; ATIC, 5-amino-4-imidazole carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase; CAIRS, carboxyaminimidazole ribonucleotide synthase; CNS, central nervous system; DHF, dihydrofolate; DHFR, dihydrofolate reductase; EL, extracellular loop; FGAM, formylglycinamidine ribonucleotide synthase; FPGS, folylpolyglutamate synthase; FR, folate receptor; GAR, β-glycinamidine ribonucleotide; GARGase, glycinamine ribonucleotide formyl transferase; GAT, glutamine phosphoribosyltransferase; GPI, glycosylphosphatidylinositol; HA, hemagglutinin; HFM, hereditary folate malabsorption; hPCFT, human proton-coupled folate transporter; HPRT, hypoxanthine phosphoribosyl transferase; hRFC, human reduced folate carrier; IL, intracellular loop; LMX, lometrexol; MFS, major facilitator superfamily; MTAP, methylthioadenosine phosphorylase; MTX, methotrexate; NRF-1, nuclear respiratory factor 1; NSCLC, non-small cell lung cancer; PAICS, phosphoribosylaminimidazole carboxylase/phosphoribosylaminimidazole succinocarboxamidase synthetase; PCFT, proton-coupled folate transporter; PDX, pralatrexate; PMX, pemetrexed; PPRP, phosphoribosylpyrophosphate; RFC, reduced folate carrier; RTX, raltitrexed; SAM, S-adenosylmethionine; THF, tetrahydrofolate; TMD, transmembrane domain; TS, thymidylate synthase; VDR, vitamin D receptor

This review summarizes the biology of the proton-coupled folate transporter (PCFT). PCFT was identified in 2006 as the primary transporter for intestinal absorption of dietary folates, as mutations in PCFT are causal in hereditary folate malabsorption (HFM) syndrome. Since 2006, there have been major advances in understanding the mechanistic roles of critical amino acids and/or domains in the PCFT protein, many of which were identified as mutated in HFM patients, and in characterizing transcriptional control of the human PCFT gene. With the recognition that PCFT is abundantly expressed in human tumors and is active at pHs characterizing the tumor microenvironment, attention turned to exploiting PCFT for delivering novel cytotoxic antifolates for solid tumors. The finding that pemetrexed is an excellent PCFT substrate explains its demonstrated clinical efficacy for mesothelioma and non-small cell lung cancer, and prompted development of more PCFT-selective tumor-targeted 6-substituted pyrrolo[2,3-d]pyrimidine antifolates that derive their cytotoxic effects by targeting de novo purine nucleotide biosynthesis.

Introduction

Proliferating tumor cells have unique metabolic requirements characterized by enhanced cell-autonomous nutrient uptake and reorganization of metabolic pathways to support the biosynthesis of macromolecules needed for cell growth and division. This includes the folate-dependent de novo synthesis of purine nucleotides and thymidylate.

Early observations by Farber and colleagues established the importance of folates to cancer progression.1 It was hypothesized that folic acid antagonists may inhibit or arrest the proliferation of cancer cells. In the late 1940s, a series of folic acid analogs including aminopterin (AMT) (Fig. 1) was synthesized. When administered to children with acute lymphoblastic leukemia (ALL), AMT became the first drug to induce remissions in this malignancy.2 This was followed by the synthesis of additional antifolates, including methotrexate (MTX).3 Remarkably, MTX continues to achieve widespread clinical use as an essential component of multidrug regimens for treating ALL, lymphomas, and solid tumors worldwide.4

While targeting folate metabolism and nucleotide biosynthesis is a well established therapeutic strategy for cancer, for MTX, clinical efficacy is limited by a lack of tumor selectivity and the presence of de novo and acquired drug resistance.5 These challenges led to decades of drug discovery efforts to identify more effective antifolates with improved pharmacology over MTX. Prominent examples include pemetrexed (PMX; Alimta), FDA-approved for treating malignant pleural mesothelioma in 20046 and non-small cell lung cancer (NSCLC) in 2008,7 and pralatrexed (PDX; Folotyn), FDA-approved in 2009 for treating refractory peripheral T-cell lymphoma8 (Fig. 1). Additional efforts were...
The reduced folate carrier (RFC) is a member of the major facilitator superfamily (MFS) of solute carriers. RFC is a secondary active anionic exchanger which transports reduced folates via counter-transport with organic anions. RFC has a much lower (~50- to 100-fold) affinity for folate than for organic anions such as glucose. This is important because it allows RFC to selectively transport folates into cells while minimizing the uptake of competing organic anions. RFC is the major transport system for reduced folates in mammalian cells and tissues and its physiologic substrate is 5-methyl THF, the major circulating folate form. RFC has a much lower (~50- to 100-fold) affinity for folate than for organic anions such as glucose. This is important because it allows RFC to selectively transport folates into cells while minimizing the uptake of competing organic anions. RFC is the major transport system for reduced folates in mammalian cells and tissues and its physiologic substrate is 5-methyl THF, the major circulating folate form.
membrane protein with 12 transmembrane domains (TMDs) and cytoplasmic-oriented amino and carboxyl termini. hRFC is N-glycosylated at Asn58 in the first extracellular loop (EL) between TMDs 1 and 2. While hRFC, like other MFS transporters, exists as a homo-oligomer, each hRFC monomer functions independently. However, homo-oligomeric forms of hRFC are critical to intracellular trafficking and surface expression of the functional transporter. hRFC is ubiquitously expressed in tissues and tumors. RFC is the major folate transporter in mammals and transports folates from blood into cells of peripheral tissues.

Figure 2. Folate transporters, folate metabolic pathways and intracellular enzyme targets of antifolates. Folate and antifolate transport across biological membranes is mediated by the reduced folate carrier (RFC), the proton-coupled folate transporter (PCFT) and folate receptors (FR). While RFC and PCFT are facilitative transporters, FRs mediate transport by a non-classical endocytosis involving formation of endosomes that migrate to the cytoplasm. Exit of the folate ligand from the endosome involves diffusion or a mediated process. PCFT has been proposed to facilitate endosomal exit, however this does not appear to be obligatory. Intracelualar folates including tetrahydrofolate (THF), dihydrofolate (DHF), 10-formyl tetrahydrofolate (10-CHO-THF), 5, 10-methylene tetrahydrofolate (5,10-CH2-THF), 5,10-methyl tetrahydrofolate (5,10-CH+-THF), and 5-methyl tetrahydrofolate (5-CH3-THF) participate in folate interconverting and biosynthetic reactions. Reactions 1–4 are in both the cytoplasmic and mitochondrial compartments. Reactions 1 and 9 are also present in the nucleus. Reaction 1 designates serine hydroxymethyltransferase. In the mitochondria, reactions 2 and 3 are catalyzed by bifunctional 5,10-CH2-THF dehydrogenase (MTHFD) 2 or MTHFD2L and 4 is catalyzed by monofunctional MTHFD1L. In the cytoplasm, reactions 4, 3 and 2 are catalyzed by the 10-CHO-THF synthetase, 5,10-CH2-THF cyclohydrolase and MTFD activities, respectively, of the trifunctional C1-THF synthase (MTHFD1). Other steps shown are catalyzed by β-glycinamide ribonucleotide formyltransferase (GARFTase; reaction 5), 5-amino-4-imidazolecarboxamide ribonucleotide formyltransferase (AICARFTase; reaction 6), thymidylate synthase (TS; reaction 7), dihydrofolate reductase (DHFR; reaction 8), 5,10-methyleneTHF reductase (MTHFR; reaction 9), and methionine synthase (MS; reaction 10). Antifolates inhibit folate biosynthetic reactions as shown and include aminopterin (AMT), methotrexate (MTX), pralatrexate (PDX), raltitrexed (RTX), lometrexol (LMX), pemetrexed (PMX), ONX-0801 (ONX), and compounds 3, 16 and 17.
the highest hRFC transcript levels were recorded in placenta and liver, with significant hRFC levels in leukocytes, kidney, lung, bone marrow, intestine, and portions of the central nervous system (CNS) and brain.31 By immunohistochemistry of mouse tissues probed with antibody to mouse RFC, RFC was identified at the apical brush border membrane of small intestine and colon, hepatocyte membranes, the apical surface of the choroid plexus, the basolateral membrane of renal tubular epithelium, and the apical membrane of cells lining the spinal canal.32 RFC is essential for development since targeting both RFC alleles is embryonic lethal.33 In at least some tissues (e.g., small intestine), mouse RFC is responsive to dietary folates such that increased RFC transcripts and proteins were detected under conditions of dietary folate deficiency.34 However, the significance of this result in intestine is unclear since RFC is unlikely to be active at the acid pH of the gut and PCFT is the major intestinal transporter for absorption of dietary folates (see below).

Folate receptor. FRs bind folic acid, reduced folates, many antifolates and folate conjugates with high (low nanomolar) affinities. The three major isoforms of human FR, α, β and γ, are encoded by distinct genes localized to chromosome 11q13.3-q13.5.23 Human FR isoforms are homologous, with 68–79% identical amino acid sequences and two (α) N-glycosylation sites. FRα and β are cell surface glycosyl phosphatidylinositol (GPI)-anchored glycoproteins, while FRγ lacks a signal for GPI-anchor attachment and is a secretory protein of unknown function.25

Membrane-bound FRs mediate cellular uptake of folates via a non-classical endocytic mechanism whereby folate ligands bind FRs at the cell membrane, followed by invagination and the formation of cytoplasmic vesicles (endosomes)35,36 (Fig. 2). Release of bound ligands occurs upon endosomal acidification which facilitates dissociation of the ligand-FR complex, and exit of the folate ligand from the endosome to the cytoplasm by diffusion or a transport-mediated process that operates at acidic pH.37 PCFT has been implicated in endosomal efflux of folates,38,39 FRα is predominantly expressed on the apical (luminal) surface of polarized epithelial cells where it is not in contact with circulating folate.40 Among normal tissues, FRα is expressed in the choroid plexus, retina pigment epithelium, proximal tubules in kidney, fallopian tubes, uterus and placenta.25 The polarized expression of FRα appears to protect normal tissues from FR-targeted cytotoxic agents in the circulation.41 FRβ is detected in placenta and hematopoietic cells.25 In normal bone marrow and peripheral blood cells, expression of FRβ is restricted to the myelomonocytic lineage such as mature neutrophils and was reported to be non-functional.42

Overexpression of FRα has been reported in malignant tissues, such as non-mucinous adenocarcinomas of ovary, uterus and cervix, and ependymal brain tumors.24 FRα levels positively correlate with tumor grades and stages.43-46 FRβ has been reported to involve a substantial fraction of chronic myelogenous leukemia and acute myelogenous leukemia (AML) cells, but not ALL.39,47 Both FRα and FRβ in malignant tissues seem to be functional,41,47 prompting use of folic acid and pteroyl moieties for tumor targeting of toxins, liposomes, imaging and cytotoxic agents.30

Proton-coupled folate transporter. A low pH folate transporter in mammalian cells was reported more than three decades ago,48 however, the responsible carrier system remained elusive. In 2006, a protein previously reported to be a low-affinity heme transporter49 was identified as the proton-coupled folate transporter or PCFT (SLC46A1).49 PCFT is a proton-folate symporter that functions optimally at acidic pH by coupling the flow of protons down an electrochemical concentration gradient to the uptake of folates into cells.50,51,52 Like RFC, PCFT is a MFS protein. Human PCFT (hPCFT) shares only ~14% amino acid identity with hRFC.24,25 Although PCFT can transport heme, its primary role involves intestinal absorption of dietary folates and as such it plays a major role in in vivo folate homeostasis.24,53,54

Biology of the Proton-Coupled Folate Transporter

PCFT structure. The hPCFT gene is localized to chromosome 17q11.2 and consists of five exons. hPCFT is comprised of 459 amino acids with a molecular mass of 49.8 kDa and is predicted to include 12 TMDs with N- and C-termini oriented to the cytoplasm (Fig. 3). This structure has been validated by immunofluorescence analysis of hemagglutin (HA)-tagged hPCFT molecules53 and scanning cysteine-accessibility methods.56 The EL domain between TMDs 1 and 2 contains two N-glycosylation sites (Asn58, Asn68). On SDS gels, hPCFT migrates broadly with a molecular mass centered at ~50–55 kDa. Digestion with N-glycosidase F, pretreatment of cells with tunicamycin, or mutagenesis of Asn58 and Asn68 to Gln converts this diffuse migrating species to a sharply banding protein at ~35–45 kDa.35,57 While expression or transport function was not appreciably impacted by loss of N-glycosylation in individual Gln58 and Gln68 mutants, activity decreased to ~40% for the double Gln58/Gln68 mutant.59 In MDCK and Caco2 cells, C-terminal-tagged yellow fluorescent protein hPCFT was expressed at the apical membrane, distinct from the basolateral localization for hRFC.58 Truncation of the hPCFT C-terminus (to position 449) does not affect apical membrane targeting or transport activity.58 Although the detailed tertiary structure has not been determined for hPCFT, Cys66 in the first EL forms a disulphide bond with Cys298 in the fourth EL.56

Transport characteristics. A distinguishing characteristic of PCFT involves its acidic pH optimum.24,25,50,53 For PCFT, transport is maximal at pH 5–5.5. As the pH increases from pH 5.5, transport decreases dramatically; above pH 7, activity is scarcely detectable. Transport conforms to Michaelis-Menten kinetics such that decreased transport activity with increasing pH is due to prominent effects on both Kt and Vmax, although the impact on these parameters varies for different substrates.12,15,25,59 PCFT has similar Ks for reduced folates (5-methyl THF, 5-formyl THF) and folic acid and is stereospecific for 5-formyl THF.35 Direct measurements confirmed transport of [3H]MTX by PCFT.16,54,59 Further, cytotoxicity and transport inhibition experiments indicate that RTX and lometrexol (LMX) are all transported by PCFT.14,16,59 PDX also appears to be transported by PCFT (C. Cherian and L.H. Matherly, unpublished). PMX has been widely considered to be the best PCFT substrate.25,50 However, a series of
could interfere with intestinal absorption of dietary folates or oral medications (e.g., MTX) when co-administered.

High levels of PCFT are expressed in apical brush-border membranes in the proximal jejunum and duodenum; however, PCFT levels decrease markedly in other segments of the intestine and colon. PCFT is also detected in the choroid plexus. In normal human tissues, elevated hPCFT transcripts were measured in kidney and liver with modest levels in most tissues and undetectable levels in bone marrow and colon (Fig. 4). For a number of human tissues, including small intestine, hPCFT proteins were detected by immunohistochemistry with hPCFT-specific antibody (not shown). These results substantiate those of Qui et al. and demonstrate that while hPCFT is expressed in normal human tissues, its levels are more limited than for hRFC. While PCFT in the upper gastrointestinal tract is involved in absorption of dietary folates, the physiologic role of PCFT in tissues not normally associated with low pH microenvironments is less obvious. PCFT may conceivably still contribute to folate internalization in such tissues by virtue of localized acidification or at sufficiently elevated levels to transport 5-methyl THF and related folates. However, at comparatively neutral pHs characterizing most tissues, RFC is far more efficient at delivering reduced folates than PCFT.

Regulation of PCFT gene expression. Studies have begun to explore the transcriptional regulation of hPCFT. The hPCFT minimal transcriptional regulatory region is localized between

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**Figure 3.** Schematic structure of PCFT topology. Structurally or functionally important amino acids, as determined from published mutagenesis studies, are shown as purple circles. Amino acids mutated in patients with hereditary folate malabsorption (HFM) are shown as red and blue circles. GxxxG putative oligomerization motifs are shown as yellow circles and glycosylated residues Asn58 and Asn68 are shown as green circles.
Induction of PCFT is accompanied by enhanced transport at pH 5.5. The vitamin D receptor (VDR) heterodimerizes with retinoid X receptor-α in response to vitamin D3 and binds a VDR response element in the hPCFT promoter (positions -1694 to -1680), resulting in transactivation. These results suggest that vitamin D3 supplementation can affect bioavailability of dietary folates and toxicity of PCFT-targeted therapeutics for cancer (see below).

Finally, PCFT expression may be regulated in response to clinically used proton pump inhibitors (omeprazole, pantoprazole, lansoprazole). For instance, PCFT transcript levels were decreased by nearly 50% in duodenal biopsies from patients treated with proton-pump inhibitors in comparison with untreated controls. Although it is unclear whether this is a direct or indirect response to these widely used medications, these results raise the possibility that changes in the tissue microenvironmental pHs may regulate PCFT levels.

Hereditary folate malabsorption. Direct evidence that PCFT is responsible for low pH intestinal transport activity and absorption of dietary folates involved the discovery that homozygous mutations in the hPCFT gene were associated with a rare autosomal recessive disorder, hereditary folate malabsorption (HFM). Clinically relevant mutations include base insertions, deletions, or substitutions, manifesting as exon skipping, frame shifts, premature translation terminations and amino acid substitutions.

Expression of PCFT is increased in a dose-dependent fashion in Caco-2 cells in vitro and in duodenal rat biopsies ex vivo treated with 1,25-dihydroxyvitamin D3 (vitamin D3). Induction of PCFT is accompanied by enhanced transport at pH 5.5. The vitamin D receptor (VDR) heterodimerizes with retinoid X receptor-α in response to vitamin D3 and binds a VDR response element in the hPCFT promoter (positions -1694 to -1680), resulting in transactivation. These results suggest that vitamin D3 supplementation can affect bioavailability of dietary folates and toxicity of PCFT-targeted therapeutics for cancer (see below).

Figure 4. PCFT and RFC transcript expression in human normal tissues. PCFT transcripts were measured by real-time RT-PCR using an Origene cDNA array prepared from 48 pathologist-verified human normal tissues as previously described. Transcript levels were normalized to GAPDH transcripts.
Pneumocystis jiroveci pneumonia. HFM is characterized by developmental delays, gait disorders, peripheral neuropathies, and seizures. 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. These findings establish the important role of PCFT in folate transport across the gastrointestinal epithelium and into the CNS, and indicate that RFC does not significantly contribute to intestinal folate absorption.

Functionally important residues in hPCFT. Structural insights into PCFT transport function have resulted from characterization of clinically relevant loss-of-function hPCFT mutations in HFM cases, and mutagenesis of conserved amino acids implicated as functionally important from considerations of PCFT homologies, charge properties and TMD localization (Fig. 3).

Functionally important residues include Glu185 (TMD5) (required for proton coupling), His281 (TMD7) (important for substrate binding) and Arg376 (TMD10) (impacts proton and substrate binding). Amino acids mapping to a highly conserved stretch between TMDs 2 and 3 (DXXGRR; positions 109–114) including a β-turn were also implicated as important for hPCFT transport. Arg113 is essential for transport since regardless of charge or polarity, amino acid replacement abolishes substrate binding and membrane translocation. From the loss of transport activity for Arg113Cys mutant hPCFT, a molecular model (based on the GlpT template) was proposed in which Arg113 is buried in a hydrophobic cavity made up of TMDs 1, 3, 4 and 6. However, this has not been experimentally confirmed. Arg113 may directly participate in substrate binding and/or membrane translocation of negatively charged transport substrates.

For His247, mutation (Ala, Arg, Glu, Gln) resulted in markedly decreased rates of transport (decreased V max) and increased substrate affinities (decreased K) for folate substrates compared with wild-type hPCFT. By homology modeling, His247 was localized in a highly electropositive region at the cytoplasmic opening to the water-filled translocation pathway and interacted with Ser172, limiting substrate access to the putative folate-binding pocket (thus determining substrate selectivity). As expected, the Ser172Ala mutant hPCFT showed a similar transport phenotype to that for His247Ala hPCFT and enhanced proton transport in the absence of folate substrate (“slippage”).

Other residues implicated as functionally important include Glu232 (TMD6), Leu161 (TMD4), Ile304 (TMD8) and Pro425 (EL6, flanking TMD12). Loss of transport was associated with a decreased rate of carrier translocation (Glu232Gly mutant) or decreased substrate affinities (Ile304Phe and Leu161Arg mutants). For Pro425, mutation to Arg resulted in loss of binding for MTX and other substrates, but substantial preservation of PMX binding, presumably reflecting a conformation change induced by the Arg substitution.

Oligomerization of hPCFT. MFS proteins including hRFC often exist as oligomers (e.g., dimers, tetramers, etc.). By protein cross-linking and blue native gel electrophoresis of ectopically-expressed hPCFT, hPCFT species were identified with molecular masses approximating those of oligomeric hPCFT. Physical associations between HA- and His 6-tagged hPCFT monomers were established by co-expression in hPCFT-null HeLa cells and co-binding to nickel affinity columns, and by fluorescence resonance energy transfer between co-expressed YPet- and ECFP*-tagged hPCFT monomers in transfected cells. Wild-type and inactive mutant Pro425Arg hPCFTs were co-expressed and exhibited a “dominant-positive” functional phenotype, consistent with positive cooperativity between monomers and suggesting a functional “rescue” of mutant hPCFT by wild-type carrier. Interestingly, hPCFT primary sequence includes GXXG motifs in TMD 2 (amino acids 93–97) and TMD 4 (amino acids 155–159), analogous to “dimerization motifs” in other amphipathic proteins. While mutation of Gly93 and Gly97 to Ala preserved hPCFT oligomerization, as assessed by thiol-reactive (MTS-1-MTS) protein cross-linking, when the 7 native Cys residues in wild-type hPCFT were invidually replaced with Ser, only Cys229Ser abolished cross-linking. This suggests that TMD6 represents an interface between individual hPCFT monomers.

An “alternate access” model for hPCFT, analogous to that suggested for LacY and adapted from that for monomeric hRFC, was proposed which includes the notion of a functional impact for hPCFT oligomerization (Fig. 5). The model assumes that hPCFT monomers occur as hPCFT homo-dimers which undergo the transport cycle in tandem and a functional cooperativity between hPCFT monomers which permits ordered loading and release of both substrates and protons.

The Role of Antifolates in Cancer Biology

Antifolates continue to occupy an important niche in the chemotherapy of a number of cancers, including pediatric ALL, osteogenic sarcoma, lymphoma, breast cancer, non-small cell lung cancer and malignant pleural mesothelioma. While MTX had origins during the late 1940s, this agent remains an extraordinary important drug for both cancer and non-malignant diseases. In recent years, newer antifolates have been introduced that were subsequently approved for patients with cancer, including PDX, RTX, PMX. Although numerous other antifolates have been synthesized and tested pre-clinically or even advanced to clinical trials, only to later fail, these were nonetheless invaluable prototypes, fostering development of clinically important agents or tumor-targeted antifolates still in development, typified by FR-selective ONXY 08107 and PCFT-selective 6-substituted pyrrolo[2,3-d]pyrimidine antifolates (see below).

As classical antifolates, like their folate cofactor counterparts, are all anions at physiologic pH, membrane transport is essential to drug efficacies. RFC has long been considered the primary transport route for antifolate drugs into both tumors and normal tissues, even though their cellular uptake by other routes (i.e., FRs and/or PCFT) can occur. For MTX, membrane transport is requisite to generating adequate intracellular drug concentrations to maximize inhibition of DHFR and for synthesis of polyglutamyl forms required for drug retention and sustained antitumor effects. For non-DHFR inhibitors such as PMX,
Antifolate inhibitors of dihydrofolate reductase and the de novo thymidylate biosynthesis pathway. Antifolates that inhibit DHFR include AMT, MTX, and PDX (Fig. 1). Inhibition of DHFR blocks synthesis of THF from DHF, generated during synthesis of thymidylate by TS (**Fig. 1**). The effect is a buildup of DHF, a “depletion” of unsubstituted and C1-substituted THF pools, resulting in suppression of biosynthesis of purines, thymidylate, serine, and methionine. The extent of THF depletion varies for different cofactor forms and cell types, 101-103 likely because the 2-amino group is replaced with a 2-desamino substituent in these cell types.

**Fig. 1.** Proposed reaction scheme for hPCFT-mediated cellular uptake involving cooperative interactions between hPCFT monomers. Based on the "alternate access model" for secondary transporters such as Lac Y,87 adapted from that of Unal et al. for monomeric PCFT,82 an analogous reaction scheme is depicted for hPCFT-mediated transport which incorporates the functional impact of hPCFT oligomerization. The model starts from the outward-facing unloaded dimer, followed by the ordered binding of the co-transported protons (step 1) and (anti)folate substrates (step 2), which triggers a conformational change resulting in simultaneous transition of the two hPCFT monomers to an inward-facing state (step 3). This is followed by an ordered release of substrates (step 4) and then protons (step 5) into the cytoplasm. The unloaded homo-oligomeric unit then returns to the outward-facing state (step 6) to complete the transport cycle. In this model, the two hPCFT monomers are suggested to function cooperatively in facilitating substrate and proton binding, conformational changes, and substrate and proton release. From Hou et al.87

AMT preceded MTX in use for pediatric ALL.3 AMT is a better substrate than MTX for both hRFC and FPGS and is therefore transported into cells and metabolized to polyglutamates much more rapidly.105-106 This increases its antitumor potency but may also contribute to its increased toxicity over MTX. While there has been renewed clinical interest of AMT for cancer and inflammatory diseases,108,109 MTX remains the most extensively used and widely-studied antifolate and is the standard by which newer agents of this class are compared.

PDX (Fig. 1) was discovered through collaboration between F.M. Sirotkak (Memorial Sloan-Kettering Cancer Center) and J.I. Degraw (Southern Research Institute) to identify antifolates with improved cellular pharmacology over MTX. 10-Deaza-AMT was more potent than MTX110 toward preclinical models and its 10-ethyl derivative (edatrexate) was even more potent.111,112 A 3rd generation analog, 10-propargyl-10-deaza-AMT (PDX), was a less potent DHFR inhibitor than AMT, MTX, or edatrexate but was more active for RFC transport and polyglutamylation than these analogs.113 This resulted in substantially increased cytotoxicity toward leukemia, breast cancer and NSCLC cell lines in vitro and in vivo. PDX showed efficacy and safety in phase I and in phase II trials, including patients with NSCLC114 and peripheral T-cell lymphoma.107,115 In September 2009, the FDA approved use of PDX for the treating relapsed, refractory peripheral T-cell lymphoma.9

The quinazoline antifolate RTX (Tomudex, ZD1694) (**Fig. 1**) is a potent TS inhibitor that grew from rational drug design efforts of scientists at the Institute for Cancer Research and Astra Zeneca.98,116 RTX was an outgrowth of N10-propargyl-5,8-dideazafolic acid (CB3717) which in phase I/II clinical trials showed activity against ovarian, liver, and breast cancers along with hepatic toxicity and dose-limiting nephrotoxicity. To ameliorate toxicity, the 2-amino group was replaced with a 2-desamino-2-methyl, a thiophene was substituted for p-amino-benzoate, and the N10-propargyl was replaced with a methyl, producing RTX.117 RTX is a less potent TS inhibitor than CB3717 but exhibits substantially greater RFC uptake and metabolism to polyglutamates, leading to more potent anti-tumor effects in vitro and in vivo. RTX was approved for advanced colorectal cancer outside the US96 and shows efficacy toward malignant pleural mesothelioma combined with cisplatin.118

Jackman and colleagues extended their search for a new generation of TS-targeted therapeutics to include the FRα-targeted agents BGC638 and BGC945, both cyclopenta[g]quinazoline analogs, neither of which are RFC or FPGS substrates.9,119 BGC945 showed superior in vitro efficacy over BGC638 with FRα-expressing tumors. BGC945 was tested in vivo in mice bearing human KB tumor xenografts and treated with 5-[125I]-iodo-2'-deoxyuridine.9,119 The results established that BGC945 was a selective TS inhibitor toward FRα-expressing tumors. BGC945 was licensed by Onyx Pharmaceuticals and renamed ONYX0801, and in 2009 a phase I clinical trial was initiated in the UK.

**Antifolate inhibitors of de novo purine nucleotide biosynthesis**. Purines serve as building blocks of RNA and DNA, and as components of ATP, cyclic AMP, NADH and coenzyme A. Differentiated adult cells often satisfy their purine requirements through purine salvage.120,121 Conversely, proliferating cells require de novo synthesis to meet their greater demands for purine nucleotides for DNA and RNA synthesis.

Both purine salvage and de novo biosynthetic pathways use phosphoribosyl pyrophosphate (PRPP). In purine salvage, hypoxanthine phosphoribosyl transferase (HPRT) converts guanine and hypoxanthine to GMP and IMP, respectively, and adenine...
phosphoribosyl transferase (APRT) converts adenine to AMP. The de novo purine nucleotide biosynthetic pathway consists of 10 reactions catalyzed by 6 distinct enzymes including multifunctional proteins, eventually generating IMP (Fig. 6). The two folate-dependent steps are catalyzed by GARFTase and AICARFTase.

In an effort to develop new inhibitors of folate metabolism at targets other than DHFR, E.C. Taylor at Princeton and Chuan (Joe) Shih at Eli Lilly collaborated to synthesize the (6R) diastereomer of 5,10-dideazatetrahydrofolate or LMX (Fig. 7).121,123 LMX is structurally analogous to THF except that the 5 and 10 nitrogens are replaced by carbons. While LMX is an excellent substrate for FRs,124 it is primarily transported into tumors by hRFC, whereupon it is extensively polyglutamylated by FPGS and inhibits GARFTase, leading to ATP and GTP depletion and potent antitumor activity with preclinical models in vitro and in vivo.99,121,123 LMX is so extensive as to effectively negate the impact of loss of RFC and the resistant phenotype.126 In a phase I study without folic acid co-administration, LMX caused severe cumulative toxicity, with dose-limiting myelosuppression (anemia, thrombocytopenia and neutopenia) and mucositis.127 When LMX was administered with folic acid, there was a reduction in clinical toxicity, permitting a 10-fold dose escalation over that without folate supplementation.128

To reduce toxicity, second generation GARFTase inhibitors were synthesized (Fig. 7). LY309887 had a lower affinity for FRs, reduced polyglutamylation and a 9-fold increased affinity for GARFTase compared with LMX.99 AG2034 (Fig. 7) and AG2037 were based on X-ray crystal structures of E. coli GARFTase and the GARFTase domain of the bifunctional human enzyme.129 AG2034 differed from AG2037 in its lower substrate activity toward FRs, although AG2034 and AG2037 were both transported by RFC and were potent GARFTase inhibitors.129 Clinical evaluation of AG2034 and LY309887 showed similar cumulative toxicities to that for LMX.130,131

Development of a multitargeted antifolate. To meet FDA requirements of purity and to eliminate the chirality at the 6-position of LMX, the 5-deazapteridine ring was replaced with a pyrrolo[2,3-d]pyrimidine, resulting in LY231514 or PMX (Alimta) (Fig. 1).132 Cell culture experiments with nucleoside (e.g., thymidine, hypoxanthine) additions confirmed that PMX was primarily a TS inhibitor, although secondary targets were suggested, including folate-dependent enzymes in de novo purine biosynthesis.132,133 Enzymology studies confirmed potent inhibition of TS and weaker inhibition of DHFR, GARFTase and AICARFTase.132,133 Inhibitions for PMX polyglutamates were especially potent, with a Kᵢ for PMX pentaglutamate (PMX plus 4 glutamates) of 1.3 nM at TS, compared with 109 nM for the parent drug. For GARFTase and AICARFTase, Kᵢ were substantially increased, suggesting decreased inhibitory potencies. Reflecting this multi-targeted enzyme inhibition, PMX was originally designated “multi-targeted antifolate” or “MTA.”

PMX is an excellent transport substrate for both RFC and PCFT,131 and is among the best substrates for FPGS.131,134 Polyglutamylation of PMX is highly sensitive to cellular folate status, such that its in vitro efficacy is enhanced in RFC deficient cells in the presence of physiologic concentrations of THF cofactors, as long as PCFT is present.134-137 For antifolates such as MTX or RTX that are poorer substrates for PCFT and are primarily transported by RFC, loss of RFC results in drug resistance. It is its PCFT transport which is a defining characteristic of PMX, as PMX is among the best known substrates for PCFT and shows reduced pH sensitivity for transport compared with other (anti)folate substrates.135,139

Although GARFTase was originally suggested to be an important secondary target of PMX, R.G. Moran and colleagues suggested that the second folate-dependent step in de novo purine nucleotide biosynthesis, AICARFTase, was likely a more important target for PMX than originally envisaged. They showed that treatment of CCRF-CEM ALL and a number of solid tumor cell lines with PMX resulted in marked accumulations of the AICARFTase substrate, ZMP,138 even though ATP pools were not depleted.138 ZMP is an AMP mimetic and activator of AMP-activated protein kinase (AMPK). AMPK activation causes phosphorylation of AMPK target proteins involved in initiation of cap-dependent translation, lipid synthesis, and energy metabolism. AMPK phosphorylates tuberous sclerosis complex 2 and raptor (component of mTORc1 complex) proteins, leading to inhibition of mTOR signaling. Interestingly, PMX synergizes with sorafenib, enhancing tumor killing via a toxic form of autophagy and activation of the intrinsic apoptosis pathway.140

PMX was FDA approved in 2004 for use (with cisplatin) in treating malignant pleural mesothelioma4 and in 2008 as a first-line treatment for non-squamous NSCLC in combination with cisplatin.131 In 2009, PMX was approved for maintenance therapy of patients with locally advanced or metastatic non-squamous NSCLC.141

**Hijacking the Acidic Tumor Microenvironment for Solid Tumor Targeting**

Cancer cells often have a greater need for energy and metabolic precursors (e.g., nucleotides) than normal differentiated cells. This increased biosynthetic demand can be, at least in part, met by an altered metabolic program known as the “Warburg effect” or aerobic glycolysis in which cancer cells become highly glycolytic even in the presence of normal oxygen tension.142 To avoid intracellular acidification, glycolytically-produced acid must be extruded. This is achieved by increased expression and/or activity of plasma membrane ion pumps and transporters, including H⁺-ATPases or vacuolar ATPases,143 the Na⁺/H⁺ exchanger (NHE1) of the SLA9A family,144 monocarboxylate-H⁺ efflux symporters (MCT1 and MCT4) of the SLC16A family,145,146 carbonic anhydrases (CAIX and CAXII),147 the Cl⁻/HCO₃⁻ exchanger (CBE),148 and the Na⁺/HCO₃⁻ co-transporter (NBC).149 The net result is reversal of intra-to-extracellular pH gradients, such that tumors generate significant acidification of their extracellular environments. The extracellular pH (pHₑ) in the vicinity of tumor cells can be as low as pH ~6.7 to ~7.1, while tumors maintain a normal to slightly alkaline intracellular pH (pHᵢ) of ≥ 7.4. This compares to pHᵢ of ~7.2 and pHₑ of ~7.3 in normal
Figure 6. De novo purine nucleotide biosynthesis pathway. The de novo purine nucleotide biosynthetic pathway from phosphoribosyl pyrophosphate (PRPP) to IMP is shown. The numbered reactions are catalyzed by the following monofunctional enzymes: 1, glutamine phosphoribosylpyrophosphate amidotransferase (GPAT); 4, formylglycinamide ribonucleotide synthase (FGAM synthetase); and 8, adenylosuccinate lyase (ASL). Reactions 2, 3 and 5 are catalyzed by the trifunctional glycinamide ribonucleotide (GAR) formyltransferase (GARFTase) which contains GAR synthase (GARS; reaction 2), GAR formyltransferase (GARFTase; reaction 3) and 5-amino-4-imidazole ribonucleotide synthase (AIRS; reaction 5) activities. Reactions 6 and 7 are catalyzed by the bifunctional phosphoribosylaminoimidazole carboxylase/phosphoribosylaminoimidazole succinocarboxamide synthetase (PAICS) enzyme, which contains carboxyaminoimidazole ribonucleotide synthase (CAIRS; reaction 6) and 5-aminoimidazole-4-(N-succinylcarboxamide) ribonucleotide synthase (SAICARS; reaction 7) activities. Reactions 9 and 10 are catalyzed by a bifunctional enzyme, 5-amino-4-imidazolecarboxamide ribonucleotide (AICAR) formyltransferase (AICARFTase)/IMP cyclohydrolase (ATIC) that sequentially catalyzes the last two steps in the pathway for de novo synthesis of IMP. Folate-dependent reactions (reactions 3 and 9) in which 10-CHO-THF serves as the one-carbon donor are catalyzed by GARFTase and AICARFTase. 5-Aminouracil (AICAR) and AICAR can be metabolized to AICAR monophosphate (ZMP) by adenine phosphoribosyl transferase (APRT) and adenosine kinase (AK), respectively, thus circumventing the reaction catalyzed by GARFTase.
differentiated cells. Acidification of the tumor extracellular environment is exacerbated by limited removal of glycolytic waste products due to poor perfusion, which can be affected by tumor size and abnormal vascularization. The resulting H+ electrochemical gradient favors passive weak acid uptake at the tumor plasma membrane by pH partition and also acts as a driving force for H+-coupled membrane transport of solutes including chemotherapy drugs used for cancer.

A targeted drug strategy for cancer founded on the selective uptake of therapeutics into tumors by H+-coupled transporters is novel but not unprecedented. Aberrant H+-coupled di- / tripeptide (PepT1 and PepT2) transport can be used for targeting experimental and clinical anticancer substrates to tumor cells, including the photodynamic therapy and imaging agent 5-aminolevulinic acid and the aminopeptidase inhibitor bestatin. Prodrugs of flouxuridine and cytarabine can also be transported by PepT1. The H+-coupled amino acid transporter PAT1 and the pH-dependent OATPs also facilitate uptake of anticancer drugs. PAT1 can transport 5-aminolevulinic acid and L-cycloserine and OATP1A2 shows MTX transport activity at low pH. Recently, OATP2B1 was identified as a low affinity/low pH transporter for classic antifolates.

**Therapeutic Targeting of Solid Tumors with Pcft-Selective Antifolates**

Expression of the major folate transporters in human tumors. Following reports of a low pH transport activity in solid tumor cells and PCFT expression in a small cohort of human tumors, studies were performed to establish a comprehensive expression profile for hPCFT compared with hRFC and FRs in 80 established cell lines derived from human solid tumors (n = 53) and leukemias (n = 27). Transcript levels for hPCFT, hRFC, and FRs α and β were measured by real-time RT-PCR. The results confirmed substantial levels of hPCFT transcripts in the majority of tumor cell lines of different origins (e.g., breast, prostate, ovarian, etc.) and very low-to-undetectable hPCFT levels in leukemias. hPCFT levels were highest in Caco-2 (colorectal adenocarcinoma), SKOV3 (ovarian carcinoma), HepG2 (hepatoma), and H69 (small cell lung cancer) cells, with substantial hPCFT in numerous other tumor sublines. hRFC transcripts were detected in all tumor and leukemia cell lines with the exception of MDA-MB-231 breast cancer cells. FRα was detected in a small cohort of ovarian, cervical, and breast cancer cell lines and FRβ was detected in a few AML and T-cell ALL cell lines. In ten tumor cell lines, hPCFT protein was measured on western blots and [H]MTX transport was measured at pH 5.5, thus corroborating the original findings of Zhao et al. and identifying hPCFT as the transporter responsible for low pH transport activity.

Development of hPCFT-selective antifolates. The clinical successes with PMX, combined with evidence that hPCFT may offer a tumor-selective mode of antifolate delivery, led to an intensive effort to develop novel cytotoxic antifolates with transport specificity for PCFT over RFC. Analogs were initially tested in engineered CHO sublines derived from RFC-, FR- and PCFT-null cells (RIMTX⁸⁸Oua2–4) to express individually hRFC (PC43–10) or hPCFT (R2/hPCFT4). While tissue culture media is at pH 7.2–7.4, during cell outgrowth the pH of the culture media decreases to pH ~6.7. PMX is a 5-substituted 2-amino-4-oxo-pyrrolo[2,3-d]pyrimidine antifolate with a 2 carbon bridge attached to a p-aminobenzoyl glutamate (Fig. 1). While the 6-regiosomer of PMX was inert toward tumor cells in culture, when the bridge region between the heterocycle and p-aminobenzoate was elongated to 3 (compound 3) or 4 (compound 4) carbons to provide greater conformational flexibility, the resulting analogs were modest inhibitors of proliferation of CCRF-CEM ALL cells at elevated (micromolar) drug and folic acid concentrations. Later studies with the engineered PC43–10 and R2/hPCFT4 CHO sublines convincingly showed that compounds 3 and 4 were highly selective inhibitors of proliferation of hPCFT-expressing cells (but not hRFC-expressing cells) at nanomolar concentrations. Compound 3 was 9-fold more active than compound 4 (IC₅₀ of 23 and 213 nM) toward hPCFT-expressing R2/hPCFT4 cells. These results contrast with those for anti-folates such as MTX, PMX, or RTX which showed no selectivity for hPCFT over hRFC. Compounds 3 and 4 were also inhibitors of FR-expressing CHO cells.

Additional studies established structure-activity profiles for PCFT transport, using the analogous 6-substituted pyrrolo[2,3-d]pyrimidine antifolates with one (compound 1), two (compound 2), five (compound 5), or six (compound 6) bridge region methylenes (Fig. 8). Further, replacement of the pyrrolo ring of compounds 3 and 4 with an isosteric thieno ring (compounds 8 and 9, respectively), resulted in a larger ring more closely approximating the 6-6 fused ring system of the THF cofactor, and replaced the hydrogen bond (NH) donor with an S. None of these analogs were active for hPCFT- or hRFC-expressing CHO cells, although compounds 8 and 9 preserved substantial anti-proliferative activities toward FR-expressing cells.

The synthesis of 6-substituted pyrrolo[2,3-d]pyrimidines based on compounds 3 and 4 with a thienoyl-for-benzoyl
and 4, compounds 16, 17, 23 and 24 were also active toward cell lines expressing FRs.\textsuperscript{11-13,15}

Tumor-targeting by PCFT-mediated uptake of novel antifolates. The anti-proliferative effects of 6-substituted pyrrolo[2,3-d] pyrimidine antifolates 3, 16, 17, 23 and 24 toward hPCFT-expressing CHO cells provided strong impetus for additional studies to explore the therapeutic potential of hPCFT for the chemotherapy of human tumors.\textsuperscript{11-13,15} With compounds 16 and 17, selective inhibition of proliferation was confirmed in hPCFT-expressing HeLa cells (R1-11-PCFT4), derived from a hRFC- and hPCFT-null HeLa subline (R1-11), whereas activity was nominal in the isoegenic R1-11-RFC6 subline which expresses only hRFC (Fig. 9 shows results for compound 17). Compound 17 was a potent inhibitor of R1-11-PCFT4 colony formation during intermittent drug exposures and showed dose- and time-dependence.\textsuperscript{12} hPCFT transport of compounds 16 and 17 was inferred from competition assays in R1-11-PCFT4 HeLa and R2/hPCFT4 CHO cells incubated with [3H]MTX in the presence of 16 and 17, and from induced currents in Xenopus oocytes microinjected with hPCFT cRNA in the presence of PCFT-substrates.\textsuperscript{12,13,15} Direct transport measurements of [3H] compounds 16 and 17 established kinetics and pH dependencies consistent with the known properties of hPCFT.\textsuperscript{12,60} Substrate activities with compounds 16 and 17 were at least equivalent to those of PMX. In HeLa cells treated at pH 6.8 with [3H] compounds 16 and 17, polyglutamates were detected, with ~6-fold higher levels for 16 over 17.\textsuperscript{60} Polyglutamyltion of compound 17 was also reported in HepG2 hepatoma cells.\textsuperscript{12}

In assorted cell lines treated with compounds 3, 16, 17, 23 and 24, growth inhibition protection assays were performed with adenosine and thymidine, and de novo purine biosynthesis was implicated as the targeted pathway.\textsuperscript{11-13,15} Since de novo purine biosynthesis involves folate-dependent reactions catalyzed by GARFTase and AICARFTase (Fig. 6), additional protection experiments used AICA, a precursor of ZMP, which circumvents the GARFTase step.\textsuperscript{11-15} For compounds 3, 16 and 17, GARFTase inhibition was accompanied by a dramatic drop in ATP levels, to an extent approximating that seen with LMX but far exceeding the modest impact of PMX on ATP pools.\textsuperscript{11-15} For compound 17, treatment of R1-11-PCFT4 HeLa cells resulted in replacement afforded the most potent PCFT-selective antifolates (compounds 16 and 17; Figure 8).\textsuperscript{12,13,15} This modification was partly based on the previous GARFTase inhibitors LY309887\textsuperscript{99} and AG2034\textsuperscript{129} (Fig. 7). Compounds 16 and 17 with 3 and 4 bridge carbons, respectively, showed substantial PCFT-targeted activity for R2/hPCFT4 CHO cells, with IC\textsubscript{50} values of 3.34 and 43 nM, respectively.\textsuperscript{13} Activity for this series declined dramatically for analogs with carbon bridge lengths shorter than 3 or greater than 4.

A series of pyrrolo[2,3-d]pyrimidine thienoyle regioisomers of compound 17 with a 4 carbon bridge and thienoyl ring substitutions, 4',5' (compound 20), 2',3' (compound 21), 3',4' (compound 22), 2',4' (compound 23), and 3',5' (compound 24) (Fig. 8), were synthesized and tested, as a means of forcing the bicyclic scaffold and l-glutamate closer together than in the parent 3-atom bridge compound 16.\textsuperscript{11} While the analogs were all inactive toward hRFC-expressing CHO cells, 23 and 24 were quite inhibitory toward hPCFT-expressing R2/hPCFT4 CHO cells, essentially equivalent to compound 17. This establishes the selective cellular uptake of compounds 23 and 24 by hPCFT. Like compounds 3 and 17 was also reported in HepG2 hepatoma cells.\textsuperscript{12}
panied by cell death, apparently by a non-apoptotic mechanism. These compelling in vitro results with compounds 16 and 17 were extended in vivo in SCID mice bearing human tumor xenografts (HepG2, HeLa), and provide proof-of-concept of in vivo tumor targeting of these novel analogs via hPCFT.

Considerations for selectivity and efficacy of current generation PCFT-targeted therapeutics. The 6-substituted pyrrolo[2,3-d]pyrimidine antifolates 16 and 17, with 3- or 4-carbon bridge lengths, respectively, are lead compounds for hPCFT-targeted chemotherapy of human solid tumors. While thienoyl-for-benzoyl ring replacement was comparatively neutral within the context of the 4-carbon bridge platform, the pyrrolo[2,3-d]pyrimidine thienoyl 3-carbon bridge analog 16 is the most potent of the hPCFT-selective antifolates thus far identified. At both pH 5.5 and pH 6.8, compound 16 is at least comparable to PMX as a PCFT transport substrate, yet it shows greater tumor selectivity than does PMX due to its near complete absence of RFC transport. Compound 16 is efficiently converted to polyglutamates, to levels exceeding those for compound 17, resulting in substantially increased GARFTase inhibition and ATP depletion at lower drug concentrations, thus explaining differences in drug sensitivities.

Previous generation GARFTase-targeted drugs include LMX, LY309887 and AG2034, all of which entered into clinical trials and resulted in significant toxicity. In retrospect, toxicities of these agents were most likely due, at least in part, to their membrane transport by hRFC in normal tissues. Indeed, GARFTase inhibitors with hPCFT selectivity such as compound 16 would seem to afford substantial advantages over not only classic antifolates such as MTX and PMX, but also previous iterations of GARFTase-targeted drugs which are primarily transported by hRFC.

Thus, in normal tissues, the tissue milieu is generally at neutral pH which would greatly favor (anti)folate membrane transport by hRFC. While hPCFT expression is more limited in normal tissues than hRFC, even if hPCFT is present, the decreased electrochemical proton gradient would result in only modest accumulations of hPCFT substrates such as compound 16. Further, the increased capacity of hRFC to transport reduced folates compared with hRFC should result in elevated intracellular folate pools in normal tissues which compete at FPGS for polyglutamylation (with consequent effects on drug retention and inhibition of folate-dependent enzyme targets), or at intracellular drug targets such as GARFTase, protecting normal cells from drug-induced cytotoxicity. Other compensatory effects in response to elevated intracellular folate pools are also possible including decreased FPGS activity or increased levels and/or altered cellular distributions of ABC transporters. In solid tumors, if sufficient hRFC is present, this may transport reduced folates even at slightly acidic pHs to decrease efficacy of hPCFT-targeted drugs. Consistent with this are studies with hRFC-null HeLa cells in which the inhibitory effects of compounds 16 and 17 were exacerbated compared with wild-type HeLa with intact hRFC, as long as hPCFT was present. Thus, tumor selectivity of cytotoxic hPCFT-targeted antifolates such as compound 16 is not only determined by differences in hPCFT levels between normal tissues and tumors, but also by the interstitial pH and the activity of hRFC.

GARFTase inhibitors such as compounds 16 and 17 would seem to have other features that render them uniquely useful for therapy of solid tumors. For instance, based on studies with LMX, GARFTase inhibition resulting in cytotoxicity appears to be independent of p53 status. This reflects depletion of ATP/ GTP pools, resulting in p53 hypophosphorylation and hypoa- cetylation which, while not impeding nuclear retention and p21 promoter binding, renders p53 transcriptionally inert. Differences in purine salvage between normal and tumor cells can confer selectivity for antipurine antifolates. While low rates of de novo purine synthesis in bone marrow and suggests that inhibitors of de novo purine synthesis should exhibit minimal marrow toxicity. Further, methylthioadenosine phosphorylase (MTAP), another salvage enzyme that releases adenine from methylthioadenosine formed during polyamine biosynthesis is abundantly expressed in normal tissues yet is co-deleted with CDKN2A in many tumors. This...
would potentially render tumors deficient in purine salvage especially sensitive to GARFTase inhibitors, while functional purine salvage via MTAP in normal tissues should provide selective protection.168

Conclusions

This review summarizes the biology of PCFT. PCFT was originally identified as a heme transporter49 and in 2006, it was reclassified as the primary folate transporter involved in intestinal absorption of dietary folates.50 Loss of PCFT function, secondary to hypermutation of functionally or structurally important amino acids, was recognized as causal in the rare autosomal recessive condition, HFM.51-62,75-84 Characterization of the causal basis for HFM identified mechanistically important residues and regions involved in substrate binding or proton coupling, prompting further mechanistic studies. While formation of hPCFT homo-oligomers was confirmed, establishing their functional significance relied on studies of HFM mutant/wild-type hetero-oligomers.85 Further studies documented mechanisms of hPCFT transcriptional control including promoter methylation,65-67 regulation by NRF-1,68 and effects of exogenous vitamin D3 or possibly changes in microenvironmental pH65 on PCFT expression.

With the recognition that hPCFT was abundantly expressed in many human solid tumors,12,13,16 attention soon turned to considerations of how PCFT could be therapeutically exploited for cancer. The demonstration that PMX is an excellent hPCFT transport substrate provided validation of its established clinical efficacy for malignant mesothelioma and NSCLC, and studies of cross-resistance patterns provided a rational explanation for collateral sensitivities to PMX of tumors that are deficient in hRFC.135-137 These successes prompted development of more selective PCFT-targeted 6-substituted pyrrolo[2,3-d]pyrimidine antifolates such as compounds 16 and 17 that, unlike PMX, derive their cytotoxic effects by targeting de novo purine nucleotide biosynthesis and GARFTase.12,13,15 Selectivity for these novel analogs is enhanced by virtue of the acidic pHs of solid tumors which favor hPCFT membrane transport over hRFC, and the preferential membrane transport of THF cofactors into normal tissues at neutral pH that favors hRFC over hPCFT transport.18

The progress in our understanding of the biology and clinical applications of PCFT since 2006 has been nothing short of staggering. With this said, there are significant questions that must be answered should basic science studies of PCFT be further extended to patients with HFM and potentially cancer. For instance, further understanding of hPCFT regulation is essential including both transcriptional and posttranscriptional controls that account for elevated levels of hPCFT in many solid tumors and comparatively modest hPCFT levels in many normal tissues. Of particular interest will be the possible regulatory impact of exogenous folates or microenvironmental factors. The demonstration that hPCFT can form functionally important homo-oligomers87 points to another level of regulation, namely formation and intracellular trafficking of these higher-order hPCFT complexes. Evidence that wild-type/mutant hPCFT mixtures exhibit a dominant-positive phenotype provides an explanation for exclusive homozygosity in HFM but is also relevant to potential resistant phenotypes involving mutant hPCFT in tumors treated with hPCFT-targeted antifolates. It will be especially important to identify the structural determinants of hPCFT oligomerization, as this may eventually lead to approaches for modulating this critical process, i.e., through the use of small molecule chaperones or peptidomimetics based on the peptide interface that can impact membrane trafficking.

Although the 6-substituted pyrrolo[2,3-d]pyrimidine antifolates exhibit near exclusive selectivity for hPCFT over hRFC, it will be important to better identify structure-activity relationships for substrate binding and transport for both these carriers, and also for FRs. Indeed, while it has been possible to identify cytotoxic folate analogs with selective membrane transport by hRFC or FRs over other transporters, to date no compounds have been identified with hPCFT transport selectivity without substantial FR uptake, as well. Indeed, it is not yet certain whether it might be beneficial to develop exclusive hPCFT-selective substrates without FR transport, as long as hRFC transport is nominal. These studies will be undoubtedly facilitated by continued structural and molecular modeling studies of these transporters. It will be especially important to study resistance to novel hPCFT-selective antifolates including alterations involving the hPCFT protein and the potential impact of alterations in hRFC and/or FRs on the development of resistance. Finally, it will be important to identify the activities of hPCFT-specific antifolates for the major efflux pumps, MRPs and ABCG2, since these could also impact the antitumor efficacies for these novel agents.

The intracellular impact of tumor-targeted hPCFT-selective inhibitors must be further defined, including specificities and binding determinants for FPGS and folate-dependent enzymes such as GARFTase, potential effects of altered purine nucleotide pools on AMPK and mTOR signaling pathways, and effects on the formation and catalytic function of the multienzyme de novo purine biosynthetic complex termed the “purinosome.”169 It will be important to determine the mechanisms by which tumor-targeted hPCFT-selective antifolates that inhibit de novo purine enzymes actually kill tumor cells. This may further validate the therapeutic value of targeting de novo purine biosynthesis and/or imply that developing hPCFT-selective antifolates with different intracellular targets (e.g., TS) may be warranted. Clearly, better understanding of these pathways and the perturbations induced by novel hPCFT-selective antifolates will be essential for optimizing their potential clinical utility for targeting solid tumors.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed

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