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The Major Facilitative Folate Transporters SLC19A1 and SLC46A1: Biology and Role in Antifolate Chemotherapy of Cancer[#]

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Running Title: Biology and Therapy of Facilitative Folate Transporters

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List of abbreviations:

AICA, 5-aminoimidazole-4-carboxamide

AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide

AICARFTase, 5-amino-4-imidazole carboxamide ribonucleotide formyltransferase

ALL, acute lymphoblastic leukemia

AMPK, AMP-activated protein kinase

AMT, aminopterin

CDX2, caudal-type homeobox transcription factor 2

C/EBPα, CCAAT/enhancer-binding protein α

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CNS, central nervous system

DHFR, dihydrofolate reductase

FPGS, folylpoly-γ-glutamyl synthetase

FR, folate receptor

GAR, β-glycinamide ribonucleotide

GARFTase, glycinamine ribonucleotide formyl transferase

GI, gastrointestinal

GlpT, glycerol-2-phosphate/ inorganic phosphate antiporter

HA, hemagglutin

HFM, hereditary folate malabsorption

HNF4α, hepatocyte nuclear factor 4α

hPCFT, human proton-coupled folate transporter

HPRT, hypoxanthine phosphoribosyl transferase

hRFC, human reduced folate carrier

KLF4, Krüppel-like factor 4

LacY, lactose/proton symporter (lactose permease)

LMX, lometrexol

MFS, major facilitator superfamily

MRP, multidrug resistance-associated

MTS, methanethiosulfonate

MTSEA, 2-aminoethyl methanethiosulfonate

MTSES, 2-sulfanatoethyl methanethiosulfonate

MTSET, 2-trimethylammonium)ethyl methanethiosulfonate

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MRP, multidrug resistance-associated protein

MTS-1-MTS, 1,1-methanediyl bismethanethiosulfonate

MTX, methotrexate

NHS, N-hydroxysuccinimide

NRF-1, nuclear respiratory factor 1

OAT, organic anion transporter

OATP, organic anion-transporting polypeptide

PCFT, proton-coupled folate transporter

PDX , pralatrexate

pH_e, extracellular pH

pH_i, intracellular pH

PMX, pemetrexed

PRPP, phosphoribosylpyrophosphate

RFC, reduced folate carrier

RTX, raltitrexed

SAM, S-adenosylmethionine

SCAM, scanning cysteine accessibility methods

THF, tetrahydrofolate

TMD, transmembrane domain

TS, thymidylate synthase

UTR, untranslated region

VDR, vitamin D receptor

ABSTRACT

This review summarizes the biology of the major facilitative membrane transporters, the reduced folate carrier (RFC) (SLC19A1) and the proton-coupled folate transporter (PCFT) (SLC46A1). Folates are essential vitamins, and folate deficiency contributes to a variety of heath 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.

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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 synthesize these derivatives *de novo*. Folates are also hydrophilic molecules that are anions at physiologic pH and do not cross biological membranes by diffusion alone.

Genetically distinct systems have evolved in mammalian cells to facilitate membrane transport of folates (Kugel Desmoulin et al., 2012a; Matherly and Goldman, 2003; Zhao et al., 2011a; Zhao and Goldman, 2013). The best characterized folate transporter is the ubiquitously expressed reduced folate carrier (RFC; SLC19A1) (Matherly and Hou, 2008; Matherly et al., 2007). RFC was initially characterized over 35 years ago in relation to its kinetics and thermodynamics (Goldman, 1969; Goldman, 1971; Goldman et al., 1968). Following its cloning in the mid-1990s (Dixon et al., 1994; Moscow et al., 1995; Prasad et al., 1995; Williams and Flintoff, 1995; Williams et al., 1994; 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 for RFC, including its acidic pH optimum and substrate specificity (Qiu et al., 2006). While 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) (Kugel Desmoulin et al., 2012a; Matherly et al., 2007) (*Figure 1*). These drugs are also substrates for PCFT, albeit to different extents (Kugel Desmoulin et al., 2012a; Zhao and Goldman, 2007). 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 (Kugel 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.

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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, the nature of their associated one-carbon unit (methyl, formyl, methylene, methenyl) and its position of substitution (N_{10} or N_5 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 (see below). Further, polyglutamyl folates are preferred substrates for folate-dependent enzymes, although substrate activity varies for different polyglutamyl forms (Moran, 1999; Shane, 1989). 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 (*Figure 2*). Methionine is converted to S-adenosyl methionine (SAM), required for biological 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 or ZMP) formyltransferase (AICARFTase), leading to the purine nucleotides (*Figure 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., 2011a; Zhao et al., 2009a). Although RFC is expressed throughout the intestine (Inoue et al., 2008; Qiu et al., 2006; Qiu et al., 2007), 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., 2011a; Zhao et al., 2009a). 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

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RFC (hRFC) transcripts are detected in placenta and liver, with significant levels in other tissues including leukocytes, kidney, lung, bone marrow, intestine and portions of the CNS and brain (Whetstine et al., 2002a). By immunohistochemistry of mouse tissues, RFC was detected at the apical brush border membrane of 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 (see below). This can result in pathologic conditions such as cardiovascular disease, neural tube defects, neurologic disorders, and cancer (Lucock, 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, thymus, or spleen. (Zhao et al., 2001c).

PCFT is also expressed in many normal tissues, although levels are generally modest (Kugel 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 (Inoue et al., 2008; Qiu et al., 2006; Umapathy et al., 2007; Zhao et al., 2009a; Zhao et al., 2009b). PCFT is also expressed in placenta and spleen. While 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 (below). Human PCFT (hPCFT) is abundantly expressed in human tumor cell lines (e.g., breast, prostate, ovarian, lung) and at very low-to-undetectable levels in leukemias (Gonen et al., 2008; Kugel Desmoulin et al., 2011; Zhao et al., 2004a).

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 (Atabay et al., 2010; Diop-Bove et al., 2013; Geller et al., 2002; Lasry et al., 2008; Mahadeo et al., 2010; Mahadeo et al., 2011; Meyer et al., 2010; Min et al., 2008; Qiu et al., 2006; Shin et al., 2011; Shin et al., 2010; Zhao et al., 2007). Other manifestations of HFM include hypoimmunoglobulinemia, developmental delays, gait disorders, peripheral neuropathies, and seizures. HFM derives from homozygous 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).

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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, the proximal renal tubules, the retinal pigment epithelium, uterus and placenta (Elnakat and Ratnam, 2004). In polarized epithelial cells, FRα is expressed on the apical membrane where 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 non-functional (Reddy et al., 1999). In malignant tissues such as non-mucinous 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 (Pan et al., 2002; Ross et al., 1994). The expression of FRα in the plasma membranes of solid tumors and FRβ in leukemias has prompted 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; Wang et al., 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 bromosulfopthalein, taurocholate, and probenecid, as well as folates, into epithelial tissues such as kidney and intestine (Burckhardt, 2012; Konig et al., 2013). Finally, efflux pumps including MRPs (MRPs 1-5 and 8) and ABCG2 also transport folates

(Gonen and Assaraf, 2012; Kruh et al., 2007; 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., 2011a; Zhao et al., 2009a). Folates are filtered via the glomerulus and are reabsorbed from the urine primarily by a FRα-mediated process, although OATP1 may contribute to folate reabsorption. Whether PCFT might contribute to folate reabsorption is not clear. Folates are transported into the circulation by folate transporters at the basolateral membrane. While 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 homozygous stop codon in the hPCFT coding sequence was recently reported to experience a normal term pregnancy and delivery (Zhao et al., 2011a).

Folates are concentrated in the cerebral spinal fluid via active transport at the choroid plexus (Geller et al., 2002; Spector and Lorenzo, 1975). 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., 2011a; Zhao et al., 2009a). 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). While RFC is positioned to extract folates from the CSF, 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, 1969; Goldman, 1971; Goldman et al., 1968). 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 (*Figure 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 (Chello et al., 1982; White et al., 1978). 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 K_ts in the low micromolar

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range (Jansen, 1999; Matherly and Hou, 2008; Matherly et al., 2007; Sirotnak et al., 1998; Visentin et al., 2013). The hemiphthaloylornithine antifolate PT523 (Jansen, 1999; Rhee et al., 1994; Rosowsky et al., 1994; Wright et al., 2000) and the benzoquinazoline antifolate GW1843U89 (Duch et al., 1993) (*Figure 1*) are the best substrates for hRFC yet described with binding affinities in the submicromolar range. Interestingly, GW1843U89 is reported to be a comparatively poor substrate for the murine RFC (Duch et al., 1993).

A unifying feature of RFC transport substrates involves their anionic character. Both folate cofactors and certain antifolates include a terminal glutamic acid and at neutral pH, the glutamyl α and γ carboxyl groups are ionized. Interestingly, some amino acid replacements are well tolerated, including valine and 2-aminosuberate analogs of the antifolate ICI198,583 (Westerhof et al., 1995), and both ZD9331 and PT523 (Jansen, 1999; Rhee et al., 1994; Rosowsky et al., 1994; Wright et al., 2000). ICI198,583-D-glutamate is poorly transported by RFC, in contrast to ICI198,583-L-glutamate (Jansen, 1999). In a study of the role of substrate glutamyl α or γ carboxyl groups in RFC transport, diamino furo[2,3-*d*]pyrimidine antifolates with L-glutamate, or with substituted glutamyl α and γ groups, were tested for RFC binding (Deng et al., 2008). The analog with L-glutamate and that with a single α but no γ carboxyl group were bound to RFC, as reflected by competition with [^{3}H]MTX uptake. Conversely, analogs with only a single γ but no α carboxyl, or without either the α or γ group were inert. Thus, only the α carboxyl group of (anti)folate substrates is required for substrate binding and membrane transport by RFC.

The energetics of RFC transport have been studied. Transport of folate substrates by RFC is not directly linked to ATP hydrolysis, nor is it sodium- or proton-dependent (Goldman, 1971; Henderson and Zevely, 1983). Rather, the driving force for concentrative uptake of (anti)folates seems to involve gradients of organic phosphates across the plasma membrane that bind and exit cells via RFC, while at the same time inhibiting folate export by this mechanism (Goldman, 1971). The net result is the transport of (anti)folates into cells by RFC whereby the transmembrane gradient (inside > outside) for one substrate ("organic phosphate") drives uphill transport of another substrate (folate). In support of this model are reports that MTX transport is competitively inhibited by structurally diverse organic anions such as adenine nucleotides and thiamine phosphates (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 (Visentin et al., 2012b; Zhao et al., 2002; Zhao et al., 2001b). Although RFC generates only small transmembrane chemical gradients, when considered in light of the dianionic nature of folates and membrane potentials, RFC generates substantial electrochemical potentials across the plasma membrane (Goldman, 1971; Goldman et al., 1968).

RFC was cloned in the mid-1990s from rodents and humans (Dixon et al., 1994; Moscow et al., 1995; Prasad et al., 1995; Williams and Flintoff, 1995; Williams et al., 1994; Wong et al., 1995). RFC belongs to the major facilitator superfamily (MFS) of transporters including more than 2000 sequenced members (Chang et al., 2004; Matherly and Hou, 2008; Saier et al., 1999). The MFS family proteins include, among others,

transporters of amino acids, sugars, vitamins, nucleosides, and organic phosphates, as well as neurotransmitters. hRFC shows a structure typical of MFS proteins with 591 amino acids arranged in 12 transmembrane domains (TMDs), with cytosolic N- and C-termini and a large non-conserved loop domain between TMDs 6 and 7 facing the cytosol (Matherly and Hou, 2008; Matherly et al., 2007) (*Figure 3*). RFCs from various species are reasonably conserved (64-66% conservation between humans and rodents) with the highest homologies in the transmembrane-spanning regions and the lowest homologies in the N- and C-termini and the connecting loop between TMDs 6 and 7. The C-termini for primate RFCs are 50-86 amino acids longer than those from other species (Matherly and Hou, 2008). hRFC is glycosylated at Asn58 in the extracellular loop domain connecting TMDs 1 and 2 (Matherly et al., 1991; Wong et al., 1998). Mutation of Asn58 to Gln abolishes N-glycosylation, as reflected in a shift from a broadly-migrating ~85 kDa species to 65 kDa, but has minimal effect on either membrane targeting or transport activity (Wong et al., 1998).

Structural determinants of RFC function and cellular trafficking were characterized by deletional mutagenesis. Whereas deletions of N- (positions 1-27) and C-terminal (positions 453-591) amino acids from ectopically expressed hRFC only slightly impacted trafficking to the membrane surface and carrier function, deletion of larger segments (positions 302-591 or 1-301) abolished surface targeting (Marchant et al., 2002). Deletion of major segments (49 or 60 amino acids; positions 215-263 and positions 204-263, respectively) from the loop domain connecting TMDs 6 and 7 of hRFC (*Figure 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- or 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 (SCAM). The 282 cysteine mutants were individually expressed in a hRFC-null (R5) HeLa subline treated with 2-sulfonatoethyl methanethiosulfonate (MTSES) to identify aqueous accessible TMD residues involved in substrate binding and translocation (Hou et al., 2005; Hou et al., 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, Try136, 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; Sadlish et al., 2002; Sharina et al., 2001) or from studies of non-functional hRFC in cells selected for MTX resistance (Ser313)(Zhao et al., 1999).

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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; Drori et al., 2000a; Jansen et al., 1998; Roy et al., 1998; Wong et al., 1999; Zhao et al., 1998; Zhao et al., 2000; Zhao et al., 1999). 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 (NHS) [^{3}H]MTX] (Deng et al., 2008). NHS-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 (LacY) (Abramson et al., 2003) and glycerol-2-phosphate/ inorganic phosphate antiporter (GlpT) (Huang et al., 2003), a 3-dimensional homology model for hRFC was generated with a membrane translocation pathway comprised of TMDs 1, 2, 4, 5, 7, 8, 10, and 11, and mechanistically important roles for Ser281, 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, co-folding of hRFC monomers to form oligomeric hRFC appears to be necessary for intracellular trafficking and surface expression of the

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functional transporter (Hou and Matherly, 2009). Indeed, by co-expression of wild type and inactive mutant Ser138Cys 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 non-coding regions and promoters (Matherly et al., 2007). Five of these (designated A, B, C, D, and E) are separate non-coding exons, whereas the A1/A2 non-coding sequence is fused to the first hRFC coding exon (Flatley et al., 2004; Whetstine et al., 2002a). Promoter activity was localized to the 5'regions proximal to the A1/A2, A, B, C, and D non-coding regions and for 4 of these promoters, ubiquitously expressed (e.g., SP1, USF1) and tissue-specific (e.g., AP2, 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 (see below), 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 non-coding 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 (Flatley et al., 2004; Payton et al., 2007; Whetstine et al., 2002a). 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 physiological 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 sub-physiologic concentrations of reduced folates (Jansen et al., 1990; Matherly et al., 1991; Sirotnak et al., 1984b). In mice fed folate-deficient diets, RFC transcripts and proteins increased in 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 sub-physiologic 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 (ER)-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' non-coding region (T2582G, C2617G), the A1/A2 promoter and 5' non-coding 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 repeat polymorphism identified in hRFC promoter A (Whetstine et al., 2002b). When transport function of Arg27-hRFC was compared to His27-hRFC, there was no significant difference (Whetstine et al., 2001).

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

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.

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BIOLOGY OF PCFT

Transport characteristics and structure/function considerations

hPCFT is comprised of 459 amino acids (*Figure 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 HA-tagged hPCFT and by SCAM with 2-aminoethyl methanethiosulfonate (MTSEA)-biotin (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 MDCK (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 (Deng et al., 2009; Qiu et al., 2006; Zhao and Goldman, 2007). In HEK293 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

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pH 7, transport is very low. Decreased transport reflects both increased K_t and decreased V_{max} values, although this varies for different transport substrates (Kugel Desmoulin et al., 2011; Kugel Desmoulin et al., 2010; Wang et al., 2010; Wang et al., 2011; Zhao and Goldman, 2007; Zhao et al., 2008). 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 K_ts (Deng et al., 2009; Kugel Desmoulin et al., 2012a; Menter et al., 2012; Qiu et al., 2007; Zhao and Goldman, 2007; Zhao et al., 2008). PCFT shows similar K_ts 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 K_ts. From growth inhibition patterns in Chinese hamster ovary or HeLa cell lines engineered to express hPCFT without RFC, both RTX and lometrexol (LMX) are transported by hPCFT (Deng et al., 2009; Kugel Desmoulin et al., 2010; Zhao et al., 2008); however, PT523 and GW1843U89 are not PCFT substrates (Deng et al., 2009; Zhao and Goldman, 2007). 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 was described as excellent PCFT substrates with K_ts comparable to that for PMX (Cherian et al., 2013; Kugel Desmoulin et al., 2012a; Kugel Desmoulin et al., 2011; Kugel Desmoulin et al., 2010; Wang et al., 2010; Wang et al., 2012; Wang et al., 2011). 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.

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PCFT transport activity was not affected by removal of Na^+, K^+, Ca^{+2}, Mg^{+2}, or Cl^- (Qiu et al., 2006). Treatment with carbonylcyanide p-trifluoromethoxyphenylhydrazone (a proton ionophore) (Qiu et al., 2006) or nigericin (a K^+/H^+-exchanging ionophore) (Inoue et al., 2008) 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; Umapathy 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 (Mahadeo et al., 2010; Unal et al., 2009a).

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) (*Figure 4*). A conserved stretch of amino acids linking TMDs 2 and 3 (DXXGRR; positions 109-114) including a β-turn was

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implicated as functionally important for transport (Lasry et al., 2008; Shin et al., 2010; Subramanian et al., 2008; Zhao et al., 2007). 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 Arg113Cys mutant, homology modeling based on the GlpT template was used and predicted that Arg113 protrudes into a hydrophobic cavity comprised 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 V_{max}) and increased affinities (decreased K_t) for folate substrates compared to wild-type hPCFT (Unal et al., 2009a). In a 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 Ser172Ala hPCFT mutant showed a similar transport phenotype to that for His247Ala 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) (*Figure 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, Gly189 and Gly192, located in TMD5, were implicated as functionally important (Zhao et al., 2012).

Cysteine-scanning mutagenesis, combined with reactivity with membrane-impermeable sulfhydryl-reactive methanethiosulfonate (MTS) reagents [MTSES (2-sulfanatoethyl methanethiosulfonate), MTSET [(2-trimethylammonium)ethyl methanethiosulfonate), MTSEA-biotin] were 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 (Shin et al., 2013; Zhao et al., 2012).

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, co-binding of co-expressed epitope-tagged (HA and His_{10}) hPCFT monomers to nickel affinity columns, and fluorescence resonance energy transfer between co-expressed YPet- and ECFP*-tagged hPCFT monomers. Oligomerization was functionally significant as co-expression of wild-type and mutant Pro425Arg 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 hPCFT, was proposed which incorporates a functionally important role for hPCFT oligomerization (Hou et al., 2012).

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The hPCFT primary sequence includes GXXXG motifs in TMD 2 (amino acids 93-97) and TMD 4 (amino acids 155-159), analogous to dimerization motifs reported for other amphipathic proteins (Duan et al., 2011; Polgar et al., 2010). While 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. individually mutated each of the seven cysteine residues in wild type hPCFT in order to assess the impact on PCFT oligomerization (Zhao et al., 2012). Whereas serine replacement of Cys21, -66, -151, -298, -328, and -397 had no impact on cross-linking with MTS-1-MTS, Cys229Ser in TMD6 abolished cross-linking (Zhao et al., 2012). This implies that TMD6 provides a structural interface between individual hPCFT monomers.

In contrast to aforementioned evidence that oligomeric hPCFT is structurally and functionally important, a study by Duddempudi et al. 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 (Duddempudi et al., 2013). As this latter report used entirely different methods and metrics than the earlier study of Hou et al. (Hou et al., 2012), it is not possible to reconcile their disparate conclusions.

Regulation of PCFT expression

The hPCFT gene consists of 5 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, CCRF-CEM) cells (Diop-Bove et al., 2009; Gonen et al., 2008). 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 to 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 (Eloranta et al., 2009; Furumiya et al., 2013; Gonen and Assaraf, 2010; Gonen et al., 2008; Stark et al., 2009). Three nuclear respiratory factor-1 (NRF-1) sites (positions -108 to -97, -93 to -82, and -10 to +1) were identified in the hPCFT minimal promoter and NRF-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). While these results suggested that vitamin D3 could affect bioavailability of dietary folates via PCFT transactivation, in VDR homozygous knock-out 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 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 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 (KLF4) and this was further enhanced by hepatocyte nuclear factor 4α (HNF4α). Conversely, caudal-type homeobox transcription factor 2 (CDX2) and CCAAT/enhancer-binding protein α (C/EBPα) suppressed hPCFT promoter activity. Western blots of rat small intestine proteins demonstrated uniform expression of KLF4 along the entire length of the intestinal tract, proximally expressed HNF4α, and distal expression of CDX2 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 (Gonen and Assaraf, 2012; Kugel Desmoulin et al., 2012a; Monahan and Allegra, 2011; Visentin et al., 2012a). MTX was introduced more than 60 years ago (Farber, 1949; Farber and Diamond, 1948) yet remains a vital drug for both cancer (Gonen and Assaraf, 2012; Monahan and Allegra, 2011; Visentin et al., 2012a) and non-malignant 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 (Marchi et al., 2013; Sirotnak et al., 1998; Thompson, 2009), RTX (Wilson and Malfair Taylor, 2009), and PMX (Cohen et al., 2009; Hazarika et al., 2005) (*Figure 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-*d*]pyrimidine antifolates designed to selectively target solid tumors by virtue of their substantial PCFT expression and their acid microenvironments which favor membrane transport by PCFT (Kugel Desmoulin et al., 2012a) (*Figure 5*).

Classical 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; Gonen and Assaraf, 2012; Zhao and Goldman, 2003). 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 (Gonen and Assaraf, 2012; Kugel Desmoulin et al., 2012a; Matherly et al., 2007). The relative contributions of these routes 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 these agents.

The role of membrane transport in MTX antitumor activity has been extensively documented (Goldman and Matherly, 1985; Gonen and Assaraf, 2012; Kugel Desmoulin

et al., 2012a; Matherly et al., 2007; Monahan and Allegra, 2011; Zhao and Goldman, 2003). 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 in spite of 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; Gonen and Assaraf, 2012; Monahan and Allegra, 2011; Zhao and Goldman, 2003). 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 (see below). 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 non-polyglutamyl drug forms (Chattopadhyay et al., 2007; Hughes et al., 1999; Mendelsohn et al., 1999; Shih and Thornton, 1999).

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 (Gonen and Assaraf, 2012; Matherly et al., 2007; Zhao and Goldman, 2003). Impaired RFC transport has also been described for other antifolate inhibitors (Gonen and Assaraf, 2012). In non-small cell lung cancer and malignant

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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 (Gonen and Assaraf, 2012; Matherly et al., 2007; Zhao and Goldman, 2003). Loss of transport frequently accompanies other cellular alterations including decreased polyglutamate synthesis and/or increased levels of intracellular target enzymes (Gonen and Assaraf, 2012; Zhao and Goldman, 2003). For LMX which is an especially good substrate for FPGS and is extensively converted to polyglutamates (far exceeding levels for MTX; below), sensitivity can be preserved toward MTX resistant cells in spite of substantial losses of hRFC, as long as FPGS activity is preserved (Matherly et al., 1993).

In the following sections, we describe the biological 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 hypothesized that folate antagonists could inhibit the proliferation of cancer cells (Farber et al., 1947). A series of folate analogs was synthesized, one of which (AMT) (*Figure 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 (*Figure 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 (Gonen and Assaraf, 2012; Monahan and Allegra, 2011; Visentin et al., 2012a). MTX is also used for treating other conditions ranging from rheumatoid arthritis and psoriasis, to Crohn's disease (Chladek et al., 1998; Feagan et al., 1995; Wessels et al., 2008).

Both AMT and MTX are potent inhibitors of DHFR (Gonen and Assaraf, 2012; Monahan and Allegra, 2011; Visentin et al., 2012a; Zhao and Goldman, 2003). Inhibition of DHFR results in accumulation of dihydrofolate from 5,10-methylene THF, generated during synthesis of thymidylate by TS (*Figure 2*). Dihydrofolate is reduced to THF by DHFR such that in the absence of DHFR the build-up 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) (Matherly and Muench, 1990; Tibbetts and Appling, 2010; Trent et al., 1991a).

AMT is better substrate than MTX for RFC transport and polyglutamylation by FPGS (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 (*Figure 1*) or 10-propargyl-10-deaza-AMT was a result of the collaboration between F.M. Sirotnak (Memoral 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 3rd generation analog of this series, was a less potent DHFR inhibitor than AMT, MTX, or edatrexate but exhibited better RFC-mediated transport and polyglutamylation 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 (Marchi et al., 2013; O'Connor et al., 2009), PDX showed efficacy and safety. The FDA approved the use of PDX in 2009 for the treatment of relapsed, refractory peripheral T-cell lymphoma (Thompson, 2009).

PT523 (Talotrexin) is a hemiphaloylornithine antifolate (*Figure 1*) synthesized by A. Rosowsky and colleagues (Dana Farber) (Rosowsky et al., 1988). PT523 is a potent DHFR inhibitor (Rhee et al., 1994; Rosowsky et al., 1988) and is among the best substrates for RFC with a sub-micromolar K_t for the human carrier (Rhee et al., 1994; Rosowsky et al., 1994; Wright et al., 2000). PT523 is a very poor substrate for PCFT (Kugel Desmoulin et al., 2010; Wang et al., 2010; Zhao and Goldman, 2007). Reflecting

the absence of a terminal glutamate, PT523 is not a substrate for polyglutamylation 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) (*Figure 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 (Hughes et al., 1999; Jackman and Calvert, 1995). Early efforts to develop a TS-targeted antifolate resulted in N_{10}-propargyl-5,8-didazafolic acid (CB3717). 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 N_{10}-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 polyglutamylation by FPGS. These properties resulted in more potent anti-tumor efficacy *in vitro* and *in vivo* (Hughes et al., 1999; Jackman et al., 1991). 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) (*Figure 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). While 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 non-polyglutamylated antifolates was that such compounds would be active against tumors expressing low FPGS or high γ-glutamyl hydrolase 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; Louvet et al., 2004; Rader et al., 2003; Schulz et al., 2004; Smith and Gallagher, 2003). ZD9331 showed a manageable toxicity profile and some evidence of activity in patients with relapsed or refractory disease.

GW1843U89 (*Figure 1*) was an outgrowth of a program at the Burroughs Welcome Company to discover folate inhibitors as anti-microbial agents (Smith et al., 1999). This resulted in a series of benzo[f]quinazolin-1(2H)-ones including GW1843U89. GW1843U89 is an extremely potent non-competitive inhibitor of human TS with a K_i of 0.09 nM (Duch et al., 1993). TS binds GW1843U89 in a binary complex

which is further stabilized upon binding of its dUMP substrate. GW1843U89 was reported to be an excellent substrate for hRFC with a K_t of 0.33 μM, whereas unlike other RFC substrates, RFC transport of GW1843U89 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 polyglutamylation. GW1843U89 showed potent inhibitory activity against a number of human tumor cell lines including human tumor xenografts engrafted into mice (Smith et al., 1995; Smith et al., 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-7904L. OSI-7904L showed better bioavailability and superior antitumor 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-7904L 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 (Ciuleanu 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 (*Figure 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 (Mendelsohn et al., 1999; Moran et al., 1989; Taylor et al., 1985) (*Figure 1*). LMX is a substrate for RFC (Jansen, 1999; Matherly et al., 1993), although it can also be transported by both FRs and PCFT (Jansen, 1999; Kugel Desmoulin et al., 2012a). Following internalization, LMX is extensively polyglutamylated (Matherly et al., 1993; Moran et al., 1989). Polyglutamyl forms of LMX are potent inhibitors of GARFTase, and result in ATP and GTP depletion (Beardsley et al., 1989; Mendelsohn et al., 1999; Moran et al., 1989). 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 (Beardsley et al., 1989; Mendelsohn et al., 1999; Moran et al., 1989; Taylor et al., 1985). 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, AG2034, and AG2037 (*Figure 1* shows structures of LY309882 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) (*Figure 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; Kugel Desmoulin et al., 2012a; Matherly et al., 2007; Zhao and Goldman, 2007). For PCFT, PMX is among the best substrates and its transport is much less sensitive to pH than other (anti)folate substrates. Within cells, PMX is extensively polyglutamylated and its polyglutamylation is negatively impacted by cellular folate status (Kugel Desmoulin et al., 2011; Shih et al., 1997; Zhao et al., 2001a; Zhao et al., 2004b). In contrast to antifolates such as MTX or RTX, anti-tumor 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

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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 (Shih et al., 1997; Taylor et al., 1992). 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 since 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 K_i for PMX pentaglutamate of 1.3 nM, compared to a K_i of 109 nM for unmetabolized PMX (Shih et al., 1997). Inhibitions of GARFTase, AICARFTase, and DHFR were all confirmed, albeit less than for TS. PMX was originally termed a "multi-targeted 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 pleural mesothelioma (Hazarika et al., 2005). In 2008, PMX was approved as a first-line treatment for non-squamous non-small cell lung cancer in combination with cisplatin (Cohen et al., 2009), and in 2009, PMX was approved for maintenance therapy of patients with locally advanced or metastatic non-squamous non-small cell lung cancer (Cohen et al., 2010).

R.G. Moran (Virginia Commonweath 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.

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ZMP acts as an AMP mimetic that activates AMPK which, in turn, phosphorylates target proteins involved in initiation of cap-dependent translation, lipid synthesis, and energy metabolism. Tuberous sclerosis complex 2 and raptor (component of mTORC1 complex) proteins are AMPK targets, such that AMPK activation results in inhibition of mTOR signaling (Gwinn et al., 2008; Inoki et al., 2003). While this could contribute to the anti-tumor efficacy of PMX, particularly in the absence of a primary inhibition on TS, in KB tumor cells, AMPK activation in response to PMX or direct AMPK activators (e.g., metformin) did not result in anti-proliferative effects (Mitchell-Ryan et al., 2013).

Development of tumor-targeted antifolates with selective membrane transport by PCFT

The extracellular pH (pH_e) of the microenvironment of solid tumors has been reported to be as low as pH ~6.7 to ~7.1, whereas the intracellular pH (pH_i) is $\geq$ 7.4 (Gallagher et al., 2008; Gillies et al., 2002; Webb et al., 2011). By comparison, the pH_e is ~7.3 and the pH_i is ~7.2 for normal differentiated cells. hPCFT is detected at substantial levels in many human tumors (Kugel Desmoulin et al., 2011) and can show appreciable transport activity at pH 6.5 to 6.8, depending on the substrate, although maximal transport occurs at pH 5 to pH 5.5 (Deng et al., 2009; Zhao and Goldman, 2007). It was this reasoning, following upon evidence of clinical efficacy with PMX (likely due in part to its tumor uptake by PCFT), that prompted intensive efforts to develop novel cytotoxic folate analogs with transport specificity for PCFT over RFC (Kugel Desmoulin et al., 2012a). It was reasoned that should PCFT-targeted agents be developed without substrate activity for RFC, these would exhibit greater anti-tumor selectivity and less toxicity toward normal tissues than drugs such as PMX or MTX, since PCFT is expressed at modest levels in normal tissues other than liver, kidney and the upper GI, and most

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normal tissues are unlikely to experience the acidic pH conditions conducive to PCFT transport (Kugel Desmoulin et al., 2012a).

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 (*Figure 1*). The 6-pyrrole regioisomer of PMX is inert, although when the bridge region was lengthened to 3- (compound **3**) or 4- (compound **4**) carbons so as to provide greater conformational flexibility (*Figure 5*), compounds with anti-tumor activities at nanomolar concentrations and PCFT-selectivity over RFC resulted (Kugel Desmoulin et al., 2010). Longer bridge lengths (Figure 5) resulted in reduced antitumor effects. Synthesis of 6-substituted pyrrolo[2,3-*d*]pyrimidines analogous to compounds **3** and **4** with a thienoyl-for-benzoyl replacement (based in part on earlier GARFTase inhibitors LY309887 and AG2034) afforded the most potent PCFT-selective agents yet described (compounds **9** and **10**, respectively) (*Figure 5*) (Cherian et al., 2013; Kugel Desmoulin et al., 2011; Kugel Desmoulin et al., 2012b; Wang et al., 2010; Wang et al., 2011). hPCFT selectivity over hRFC was confirmed in HeLa sublines expressing hPCFT or hRFC, and direct transport assays with radiolabeled compounds **9** and **10** established detailed kinetics and pH dependencies consistent with those expected for hPCFT (Cherian et al., 2013; Kugel Desmoulin et al., 2011; Kugel Desmoulin et al., 2012b). Substrate activities with **9** and **10** were at least equivalent to those for PMX. Further, compounds **9** and **10** were metabolized to polyglutamyl conjugates in HeLa cells incubated with the radiolabeled compounds, with 7- to 8-fold higher levels of polyglutamates for compound **9** over compound **10** (Cherian et al., 2013; Kugel Desmoulin et al., 2011; Kugel Desmoulin et al., 2012b). In hRFC-null HeLa cells expressing hPCFT, the antiproliferative effects of **9**

and **10** were greater compared to wild-type HeLa cells expressing hPCFT with intact hRFC, due to the depletion of intracellular folate cofactors (Kugel 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 (Cherian et al., 2013; Kugel Desmoulin et al., 2011; Kugel Desmoulin et al., 2010; Wang et al., 2010; Wang et al., 2011). 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) (Cherian et al., 2013; Kugel Desmoulin et al., 2011; Kugel Desmoulin et al., 2012b). 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. While 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 posttranscriptional regulation of hPCFT is certainly warranted in order 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 5'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 posttranslational 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. In spite of extensive research, the functional or clinical significance of high frequency hRFC polymorphisms remains largely unresolved and at best controversial.

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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 multi-parameter 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 MRP1 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 potential 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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Wrote or contributed to the writing of the manuscript: Matherly, Wilson, Hou

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Established Antifolate Drugs. Structures are shown for clinically relevant antifolates including methotrexate (MTX), pemetrexed (PMX), raltitrexed (RTX), and pralatrexate (PDX), the original antifolate, aminopterin (AMT), and antifolates that were advanced to clinical trials [lometrexol (LMX), ZD9331, GW1843U89, PT523, LY309887, AG2034], as described in the text.

Figure 2. Folate metabolism and targets of antifolate drugs. The schematic shows folate interconverting and biosynthetic steps. Intracellular folates include tetrahydrofolate (THF), dihydrofolate (DHF), 10-formyl tetrahydrofolate (10-CHO-THF), 5, 10-methylene tetrahydrofolate (5,10-CH_2-THF), 5,10-methenyl tetrahydrofolate (5,10-CH^+-THF), and 5-methyl tetrahydrofolate (5-CH_3-THF). Biosynthetic steps are catalyzed by dihydrofolate reductase (DHFR), thymidylate synthase (TS), serine hydroxymethyltransferase (SHMT), glycinamide ribonucleotide formyltransferase (GARFTase), and 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase (AICARFTase), and methionine synthetase (MS). Folate-dependent enzyme targets for cytotoxic antifolates, as described in the text, are indicated.

Figure 3. Membrane Topology of the Human Reduced Folate Carrier. The predicted membrane topology for the human reduced folate carrier or hRFC is shown. Much of this has been experimentally validated. Functionally important residues, as described in the text, are highlighted in blue, and the N-glycosylation consensus site is highlighted in green. Undefined abbreviations include: EL, extracellular loop; IL, intracellular loop.

Figure 4. Membrane Topology of the Human Proton Coupled Folate Transporter. The predicted membrane topology of the human proton-coupled folate transporters or

hPCFT is shown. Functionally important residues as described in the text are highlighted in blue. The β-turn formed by residues 109-114 is highlighted in orange. Cys229 which is important for crosslinking PCFT monomers is highlighted in yellow. The two N-glycosylation consensus sites, Asn58 and Asn68, are highlighted in green.

Figure 5. Development of Solid Tumor-Targeted Antifolate Drugs. The structures of novel 6-substituted pyrrolo[2,3-*d*]pyrimidine antifolates, including compounds with hPCFT selectivity over hRFC (compounds **3**, **4**, **9** and **10**), as described in the text, are shown.

Figure 6. The *de novo* Purine Nucleotide Biosynthesis Pathway. The ten steps from phosphoribosyl pyrophosphate (PRPP) to inosine monophosphate (IMP) are shown. Antifolate drugs that inhibit the folate-dependent enzymes, GARFTase and AICARFTase, are noted in red, as described in the text. Undefined abbreviations: AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; AIR, aminoimidazole ribonucleotide; CAIR, carboxyaminoimidazole ribonucleotide; FAICAR, formyl 5-aminoimidazole-4-carboxamide ribonucleotide; FGAM, N-formylglycinamidine ribonucleotide; FGAR, formyl glycinamide ribonucleotide; GAR, β-glycinamide ribonucleotide; SAICAR, 5-aminoimidazole-4-(*N*-succinylocarboxamide.

Figure 1

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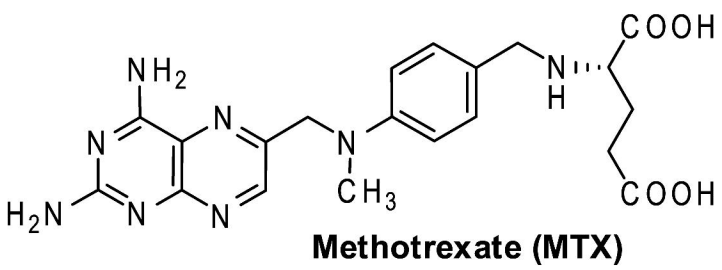

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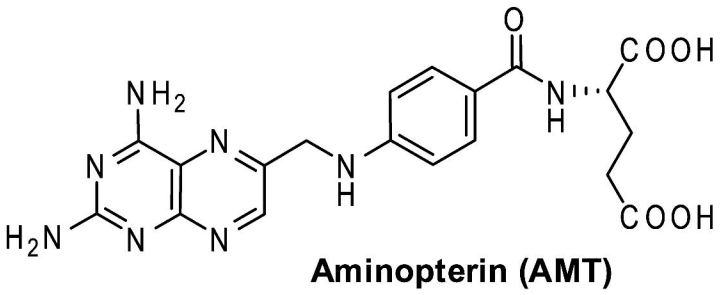

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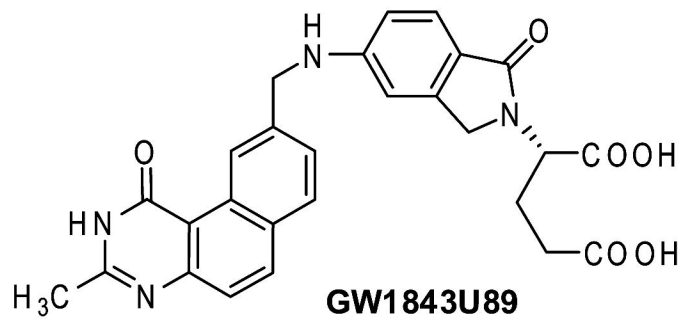

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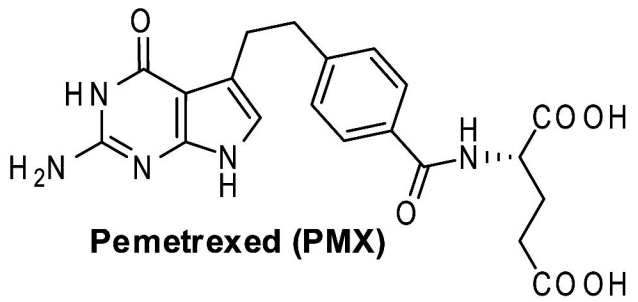

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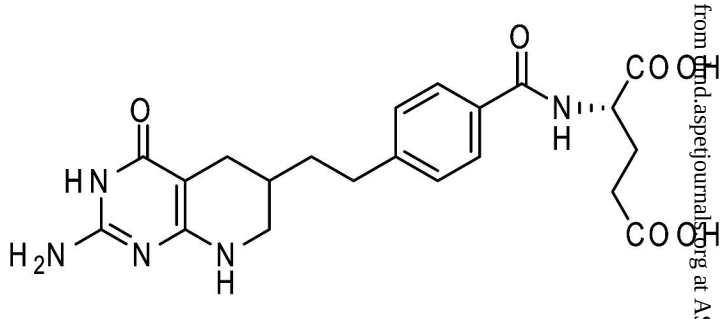
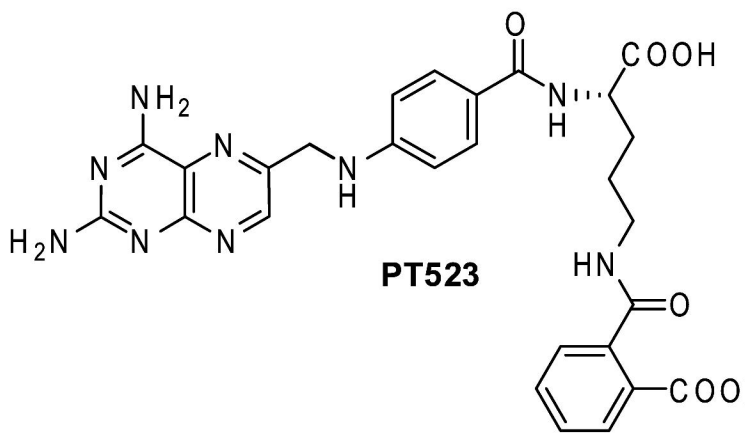
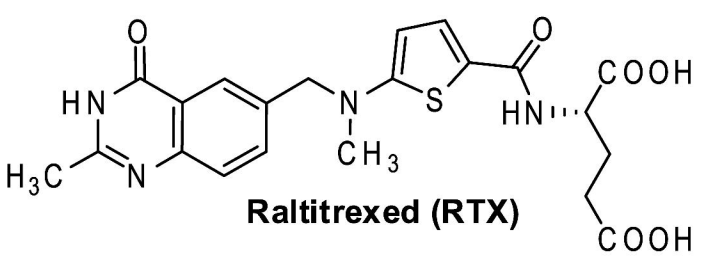


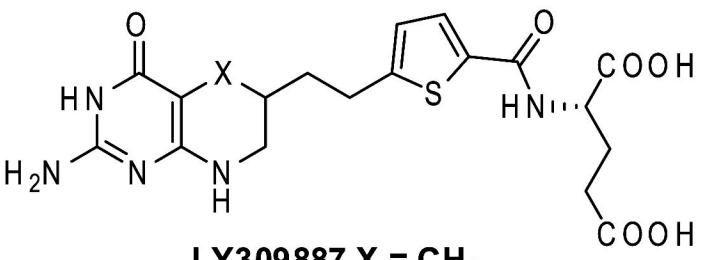
Lometrexol (LMX)



PT523



Raltitrexed (RTX)



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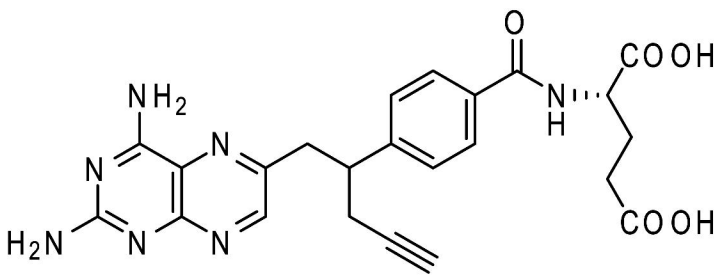
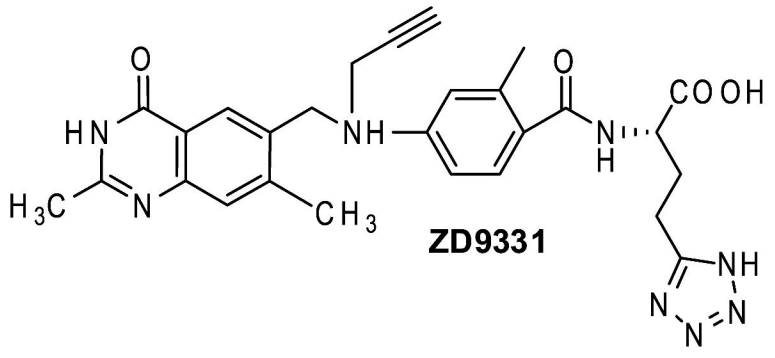
AG2034 X = S



Pralatrexate (PDX)



ZD9331

Figure 1

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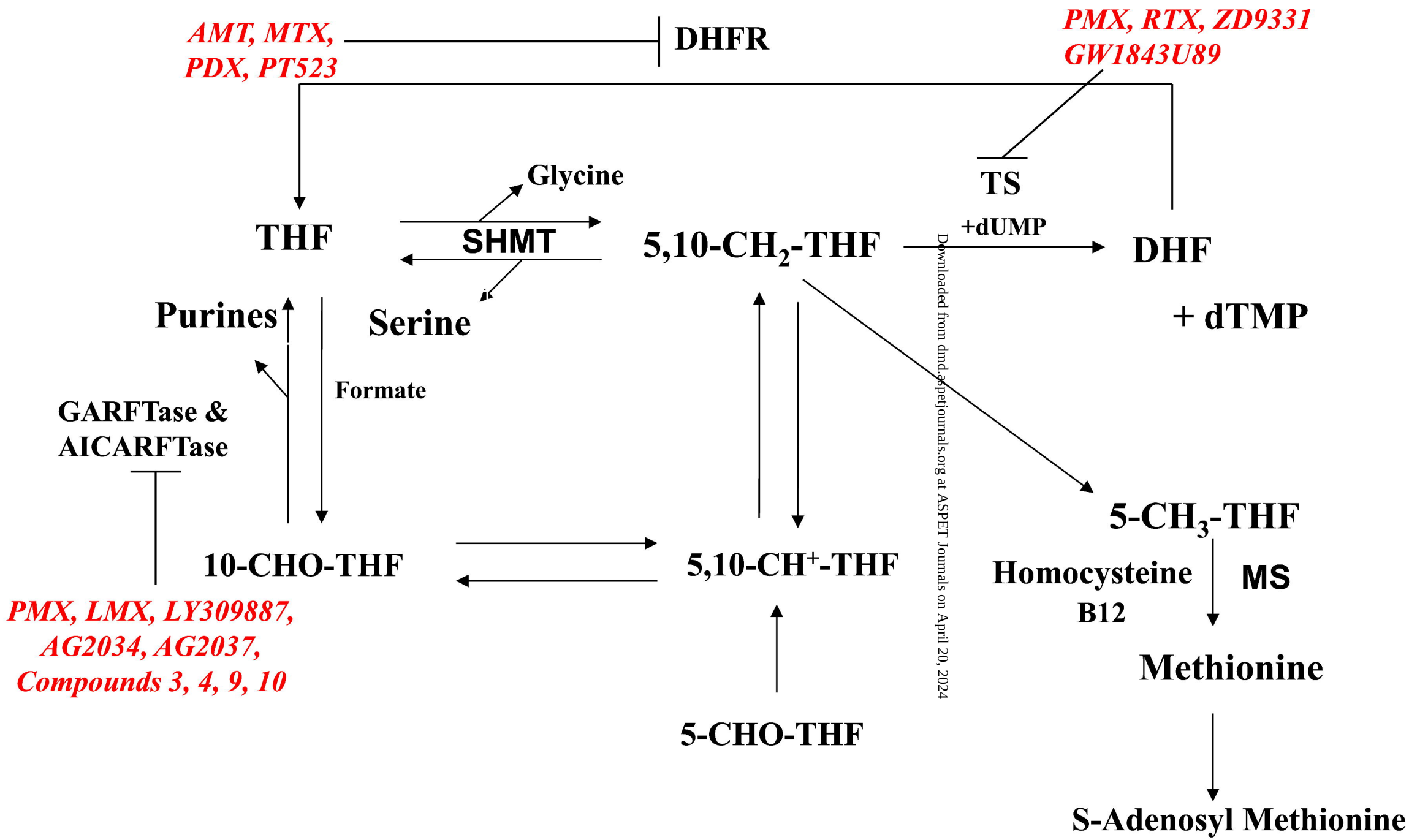


Figure 3

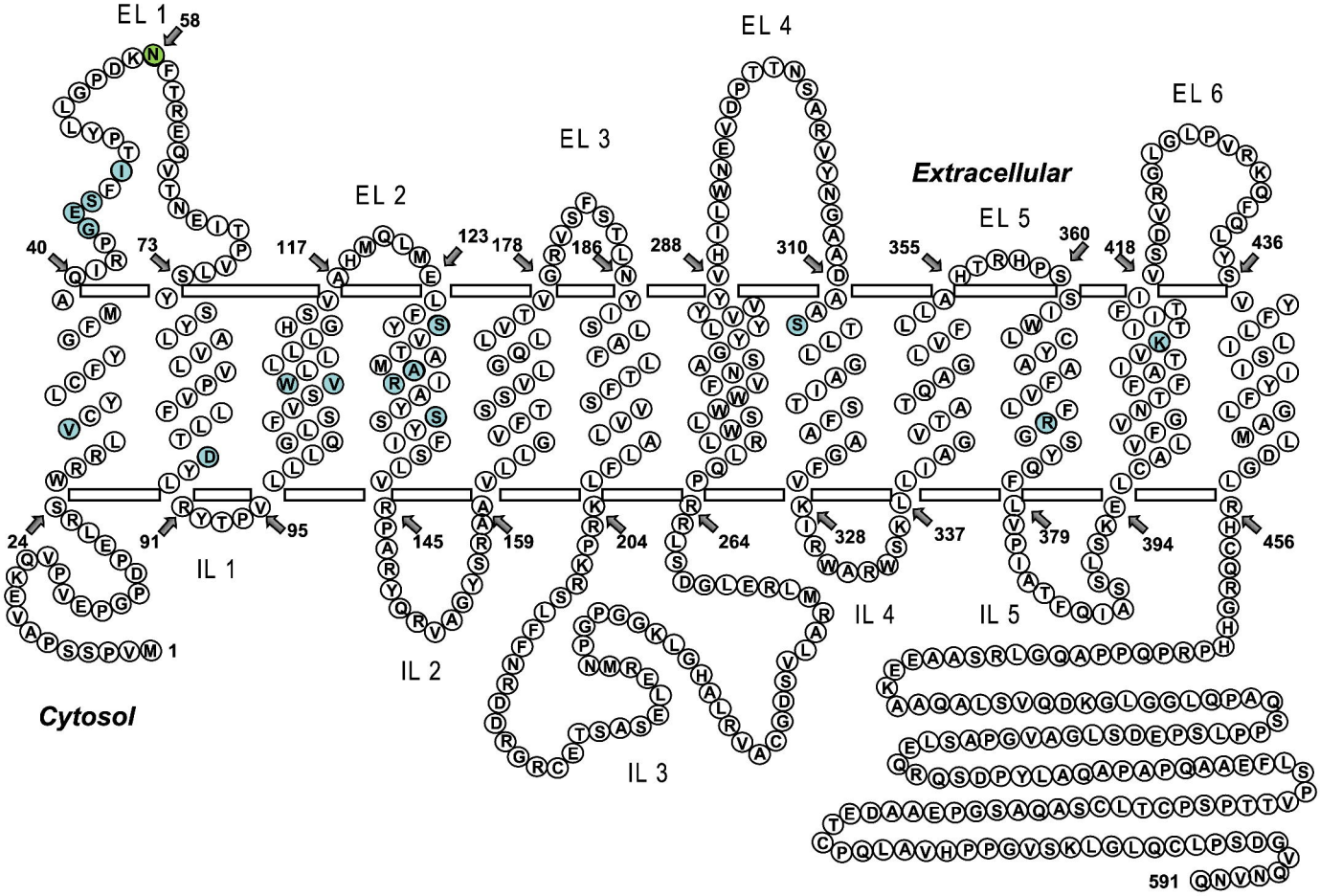


Figure 3

Figure 4

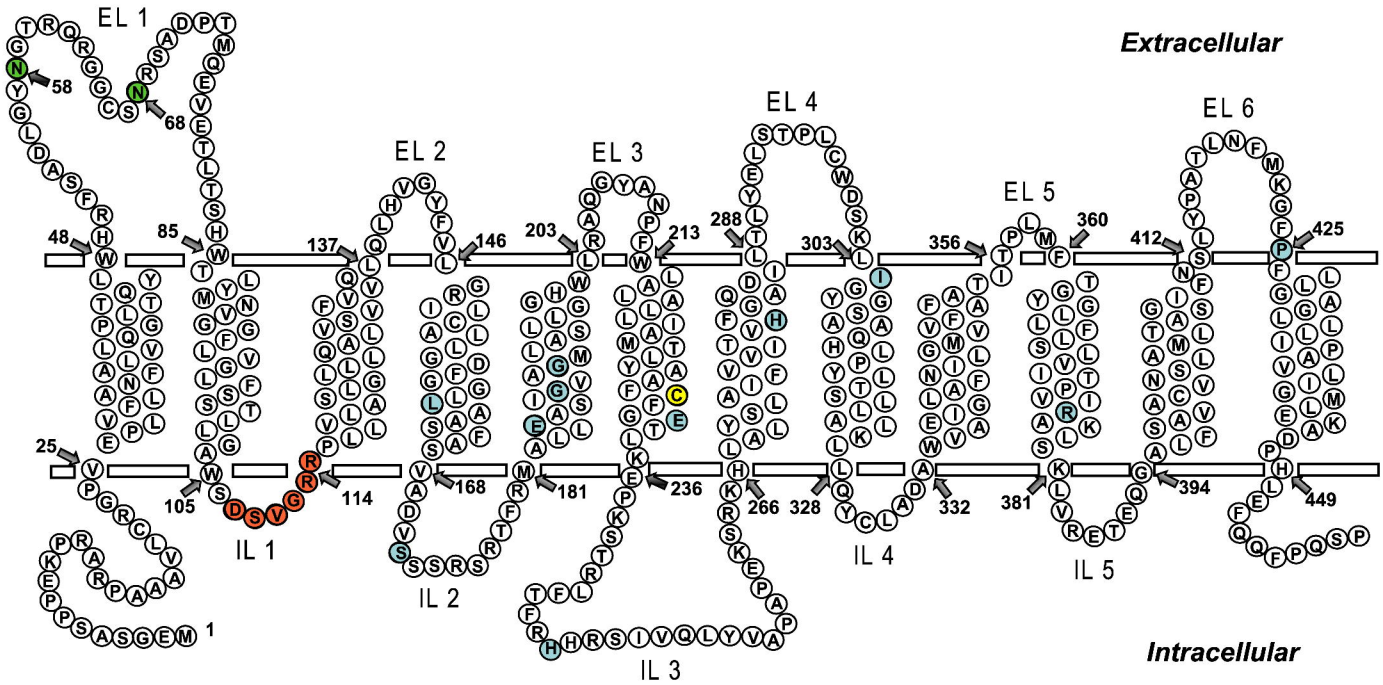
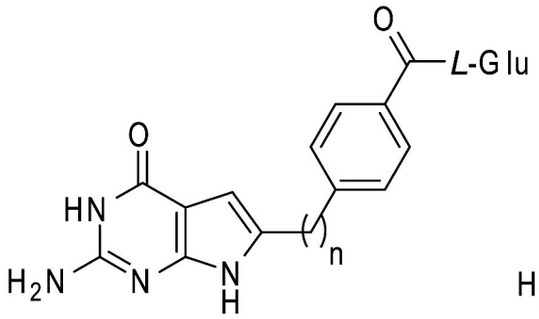
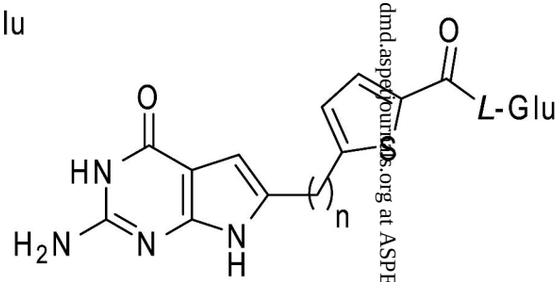


Figure 4

Figure 5



1 n=1
2 n=2
3 n=3
4 n=4
5 n=5
6 n=6



7 n=1
8 n=2
9 n=3
10 n=4
11 n=5
12 n=6

Figure 5

Figure 6

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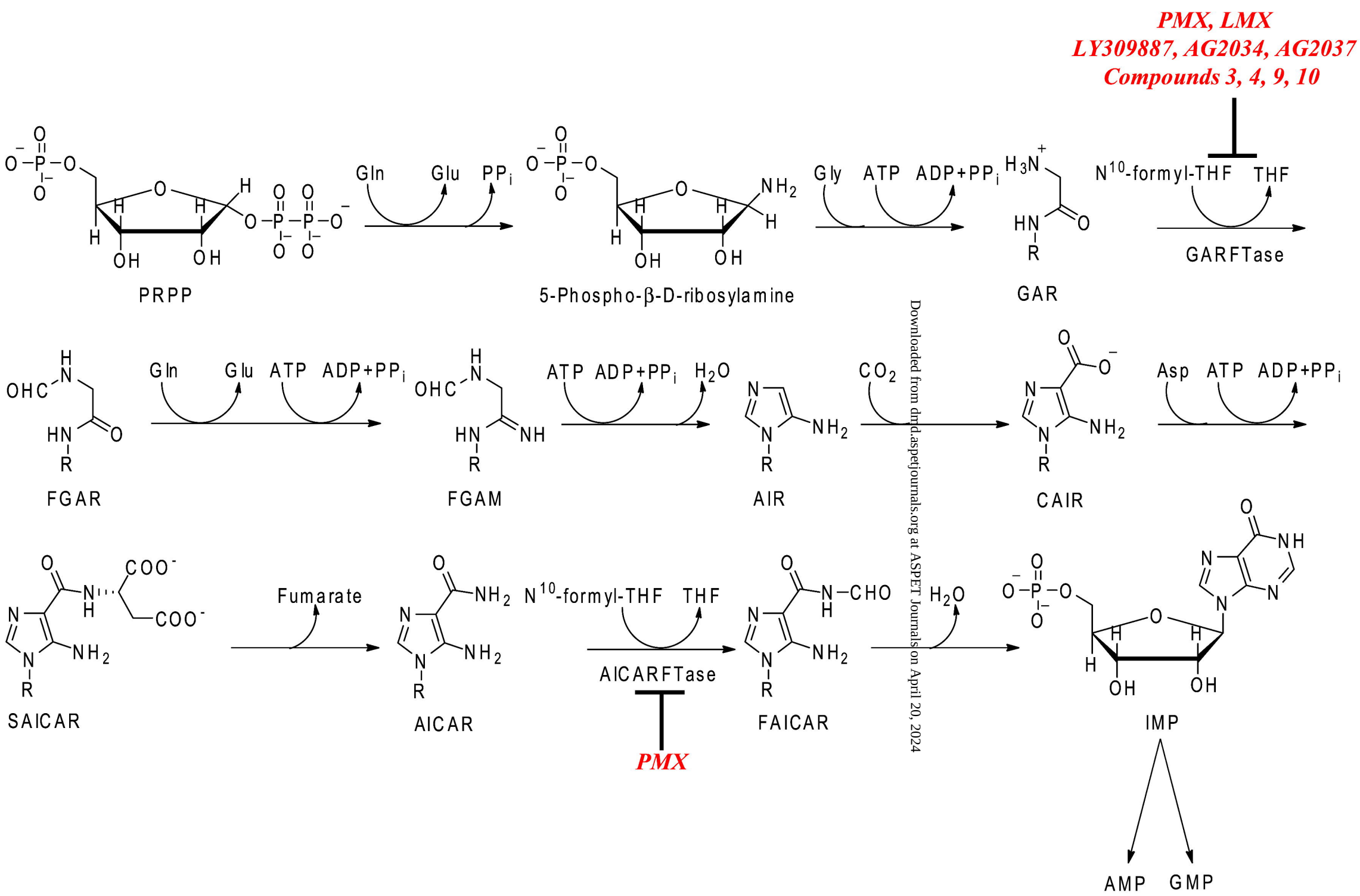

Figure 6