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The proton-coupled folate transporter: physiological and pharmacological roles

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Summary

Recent studies have identified the proton-coupled folate transporter (PCFT) as the mechanism by which folates are absorbed across the apical brush-border membrane of the small intestine and across the basolateral membrane of the choroid plexus into the cerebrospinal fluid. Both processes are defective when there are loss-of-function mutations in this gene as occurs in the autosomal recessive disorder hereditary folate malabsorption. Because this transporter functions optimally at low pH, antifolates are being developed that are highly specific for PCFT in order to achieve selective delivery to malignant cells within the acidic environment of solid tumors. PCFT has a spectrum of affinities for folates and antifolates that narrows and increases at low pH. Residues have been identified that play a role in folate and proton binding, proton coupling, and oscillation of the carrier between its conformational states.

Introduction

Studies on the mechanism of transport of folates date back more than a half-century providing a clear characterization of the properties of the various folate-specific transporters in a variety of different tissues and cell lines. However, information on the molecular basis for these activities emerged more recently and a full understanding of important aspects of transport, in particular, vectorial transport across epithelia, has not as yet been achieved.

There are two folate-specific members of the superfamily of solute carriers, the reduced folate carrier (RFC-SLC19A1) and the proton-coupled folate transporter (PCFT-SLC46A1). The former was cloned in 1994 [1], the latter was identified in 2006 [2]. PCFT, like other proton-coupled processes, is expressed at the acidic microenvironment of the apical brush-border membrane of the proximal small intestine and mediates the intestinal absorption of folates. PCFT is highly specific for folates and folate analogs. This is unlike the proton-coupled amino acid transporter (SLC36A1; hPAT1), the monocarboxylic acid transporter

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(SLC16A1; MCT1), and the peptide transporter (PEPT1; SLC15A1) that mediate the intestinal absorption of a diverse spectrum of substrates and, hence, are of considerable potential utility as drug transporters [3]. PCFT is also expressed in a variety of malignant cells as well as normal tissues [2,4,5]. Hence, the pharmacological potential of PCFT is focused on its role in the intestinal absorption of antifolates and its potential for the delivery of antifolates to tumor cells for the treatment of cancer. This paper will review the physiological role of PCFT, illustrating its functional properties, and the status of studies that address the pharmacological potential of this transporter. Transport of folates has been the subject of recent reviews [6–8].

The physiological role of PCFT as established by loss of function mutations in this gene in humans and mice

The physiological role of PCFT has been established by the phenotype of humans with the rare autosomal recessive disorder, hereditary folate malabsorption (HFM), in which the function of this transporter is lost or severely impaired [2,9]. The pathophysiological consequences are due to two defects: (i) Impaired transport across the apical brush-border membrane of the proximal small intestine where PCFT is highly expressed (Figure 1) resulting in severe systemic folate deficiency with anemia, sometimes pancytopenia, hypo-immunoglobulinemia and gastrointestinal defects. This fully corrects with pharmacological doses of oral folates or low doses of parenteral folate. (ii) Impaired transport of folates across the blood-brain barrier into the cerebrospinal fluid (CSF) apparently due to a defect in transport across the choroid plexus where PCFT is expressed at the basolateral membrane (Figure 1). Infants and children with HFM have very low CSF folate levels even when blood folate levels are corrected. Much higher blood folate levels are required to normalize CSF folate levels [9,10].

A mouse model of HFM in which this gene has been targeted reproduces the pathological changes observed in humans with this disorder [11]. PCFT-null mice appear to have the same phenotype as humans. The neurological deficiency dominates the clinical picture in mice when the anemia and other systemic signs of folate deficiency are corrected. There are many unanswered questions regarding the biological role of PCFT and the pathophysiological changes that occur when PCFT function is lost, such as: (i) What is the mechanism of intestinal absorption of folate in the absence of PCFT? (ii) What is the role of PCFT in folate transport across the choroid plexus? (iii) What is the role of PCFT in transport of folates across the basolateral membrane of hepatocytes? (iv) Does PCFT have a functional role in other tissues in which it is expressed such as the proximal renal tubule (apical) and the retinal pigment epithelium (basolateral) [12]. While it is unclear as to the mechanism of folate transport across the basolateral membrane of the small intestine, there is evidence that MRP3 and other multidrug resistance-associated proteins are involved [6,13].

The secondary structure of PCFT

Based largely on the substituted cysteine accessibility method, along with hemagglutin tagging, PCFT has been shown to consist of twelve transmembrane domains with both N-

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and C-termini directed to the cytoplasm [14–16]. The human protein is glycosylated at two sites (N58 and N68 in the 1st extracellular loop); neither is required for trafficking nor function in individual cells [15]. PCFT contains seven Cys residues, three are within transmembrane domains. Two of the Cys residues are in extracellular loops, (C66 1st and C298 4th extracellular loops), and form a disulfide bond [14]. None of these residues, nor the bond that links two extracellular loops, are required for function. However, the loss of the Cys residues makes the protein less tolerant of additional amino acid substitutions [17]. PCFT appears to form an oligomer based upon detection of a high molecular weight species on protein electrophoresis [18,19]. However, the monomeric structure may represent the functional unit in the plasma membrane [20].

Residues that play a role in folate and proton binding, proton coupling, and oscillation of the carrier

Notable findings that relate to the impact of protons on PCFT function are emerging: His 281 in the 7th TMD is an important determinant of proton binding. When mutated to Ala there is a marked increase in the folate influx K_t (concentration at which influx is one-half the maximum rate). This is reversed when the pH is decreased [21]. This is in contrast to Glu185 in the 5th TMD which appears to be required for proton coupling. In this case, when mutated to Ala, there is impaired function at low pH but no change in function at pH 7.4 in the absence of a pH gradient. This mutation results in a marked fall in the influx V_{max} without any change in the influx K_t. The defect here was attributed to impaired proton dissociation from the carrier at the inner cell membrane interface, a rate-limiting step in carrier cycling [22]. A variety of residues and domains have been identified recently that play a role in folate substrate binding and oscillation of the carrier between its conformational states [19,23–28].

Regulation of PCFT expression

The basal *PCFT* promoter has been defined [29,30]. Transcription of the *PCFT* gene is regulated by the nuclear respiratory factor (NRF1) and NRF1 binding sites are present in the *PCFT* promoter [31]. *PCFT* expression is increased by vitamin D3 in Caco-2 cells and in rat duodenal biopsies and at least one vitamin D receptor response element has been identified in the *PCFT* gene [32]. Hypermethylation of the GC-rich PCFT promoter appears to be responsible for the lack of *PCFT* expression in T leukemia cell lines and in methotrexate-resistant HeLa cells [29,33]. *PCFT* mRNA in the small intestine of folate deficient mice is markedly increased, as are the other folate-specific transporters; however, the underlying molecular basis is not known [16,34].

The pharmacological role of PCFT

Four folate analogs are currently employed for the treatment of cancer. All these antifolates form active polyglutamate derivatives, mediated by folylpolyglutamate synthetase (FPGS). This results in: (i) their retention and build up within cells and (ii) broadens and intensifies their inhibitory effects on tetrahydrofolate-cofactor dependent enzymes. Methotrexate and pralatrexate are dihydrofolate reductase inhibitors that deplete cellular tetrahydrofolate

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cofactors. This results in the cessation of downstream tetrahydrofolate cofactor-dependent purine, thymidylate, and methionine synthesis. Pemetrexed, raltitrexed and pralatrexate have much higher affinities for FPGS than methotrexate; the longer polyglutamates of the former two are potent inhibitors of thymidylate synthase and, in the case of pemetrexed, inhibition of one of the enzymes required for formation of the purine ring [7]. These antifolates, similar to naturally-occurring tetrahydrofolate cofactors, are negatively charged molecules that diffuse poorly across cell membranes. Hence, they require folate transporters to enter cells, reach their intracellular targets and achieve their pharmacological effects. These antifolates are excellent substrates for RFC with influx K_ts in the 0.5–5 μM range. While transport efficiency mediated by PCFT varies considerably among folates and antifolates at neutral pH, this divergence decreases as the pH is decreased to 5.8–6.0, levels found at the intestinal absorptive surface [35,36]. The PCFT-mediated influx K_t for these drugs decreases, and influx V_{max} increases, further as the pH is decreased to 5.5. Methotrexate and raltitrexed have low affinity for PCFT at neutral pH (influx $K_t \geq 100$ μM); however, pemetrexed retains an influx K_t of ~ 15 μM at pH 7.4 and only a 50% decrease in the influx V_{max} from its maximal level at pH 5.5 [2,35,37].

Antifolates are administered intravenously within the context of their use in cancer treatment. Methotrexate is also utilized for the treatment of autoimmune/inflammatory disorders and in this context is usually administered orally. Methotrexate and other antifolates are good substrates for PCFT at low pH as assessed in cell systems *in vitro*; hence, PCFT is assumed to be the mechanism by which methotrexate is, and other antifolates could be, absorbed from the intestine. Since pemetrexed is the favored substrate for this transporter, it should be highly bioavailable by the oral route if it is stable following passage through the stomach.

PCFT as an alternative route for antifolate transport

All the antifolates noted above are transported into tumor cells via RFC. When there is a loss of RFC expression, or mutations in the carrier that result in loss-of-function, there is resistance to methotrexate and raltitrexed [35,38]. This is not the case for pemetrexed. While PCFT is not sufficient to sustain a normal rate of pemetrexed transport in the absence of RFC, the residual transport at neutral pH is sufficient to sustain full activity of the drug *in vitro* because there is concurrent depletion of cellular folates when RFC function is lost which is not compensated by PCFT [35,38]. This enhances the rate of polyglutamation of the drug by decreasing the level of folates that compete with pemetrexed at the level of FPGS [35,39]. This has enormous pharmacological ramifications because it means that resistance to pemetrexed due to impaired transport would require the loss of two genetically distinct transporters. Indeed, transport-associated resistance to pemetrexed has not, as yet, been observed.

A role for PCFT in receptor-mediated endocytosis

Folates are also transported by an endocytic process mediated by folate receptors; FRα (in epithelial tissues and tumors) and FRβ (hematopoietic malignancies, some normal hematopoietic cells, and hepatoma) [40]. There is evidence that PCFT plays a role in the

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export of folates from acidified endosomes, where it co-localizes with FRα, into the cytoplasm [41,42]. However, the endocytic process for at least some folate substrates is functional in the absence of PCFT [43,44]. Another proton-coupled transporter, DMT1, plays a similar role in the endocytosis of dimetal ions [45].

The potential impact of PCFT on the pharmacokinetics of antifolates through its role in the enterohepatic circulation

Methotrexate and other folates/antifolates participate in the entero-hepatic circulation and this impacts on their pharmacokinetics. This process involves a role for other transporters that are not specific for folates/antifolates as illustrated in Figure 1. Excretion in the bile requires transport across two epithelial surfaces: (i) the sinusoidal (basolateral) membrane from blood to hepatocyte and (ii) across the bile canalicular (apical) membrane from hepatocyte to the biliary duct. OATP1B1 and OATP1B3 are expressed at the basolateral membrane with K_t's for MTX of 25–40 μM. The role for OATPB1 in biliary excretion of methotrexate is supported by the observation that when this transporter is over-expressed in mice, clearance of methotrexate from the blood is increased [46]. PCFT is also expressed in the basolateral membrane; however, its role in transport across this interface is not clear. MRP2 and the breast cancer resistance protein (BCRP) are expressed at the bile canalicular membrane and loss of function of these proteins also results in decreased methotrexate clearance from the blood [47]. The intestinal reabsorptive component of this cycle is mediated by PCFT. Substrates with a low affinity for this transporter would likely have increased fecal excretion, and a more rapid rate of clearance from the blood than methotrexate or pemetrexed, when administered intravenously.

The development of drugs designed specifically as substrates selective for PCFT

Of particular interest is a new class of folate analogs being developed as highly specific substrates for PCFT. The rationale here is that RFC is known to be the vehicle that delivers antifolates to normal tissues and is therefore responsible for the major antifolate toxicities to replicating bone marrow and intestinal cells. While PCFT is widely expressed, it has limited function within the neutral pH that surrounds normal tissues, but would be active within the acidic environment of solid tumors due to the comprised blood supply that results in hypoxia which, along with the glycolytic shift in tumors (Warburg Effect), results in increased production of lactate and a local acidosis [48]. Accordingly, antifolates are being developed as anticancer agents that have a very low affinity for RFC but a high affinity for PCFT. The agents currently in development are inhibitors of glycinamide ribonucleotide formyltransferase, one of the two enzyme required for the synthesis of the purine ring [5,8,44,49]. These drugs result in a marked fall in cellular ATP levels that, if selective for tumor cells alone, could have considerable therapeutic potential.

PCFT-associated drug interactions

While PCFT is highly specific for folates and their analogs, a number of molecules have been found to be inhibitory when present at sufficiently high concentrations. Of particular

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interest is the inhibitory effect of bicarbonate which, at physiological concentrations at neutral pH is markedly inhibitory. Other univalent anions such as bisulfite and nitrite are also inhibitory but this is due largely to their PCFT-independent diffusion into cells causing the collapse of the transmembrane pH gradient. On the other hand, sulfate and nitrate are not inhibitory [50]. A number of drugs inhibit PCFT-mediated transport when present at high concentrations, such as sulfasalazine [51]. Since this agent is co-administered with methotrexate in the treatment of rheumatoid arthritis and other inflammatory disorders, it could impact on the oral bioavailabiity of methotrexate. There is also evidence that proton pump inhibitors decrease the expression of PCFT [52].

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Highlights

- PCFT is the mechanism of intestinal absorption of folates and antifolates
- Inactivating mutations of the pcft gene result in hereditary folate malabsorption
- PCFT activity is enhanced within the acidic microclimate of solid tumors
- PCFT is a potential route for the selective delivery of antifolates to tumor cells
- PCFT plays a role in folate transport across the choroid plexus

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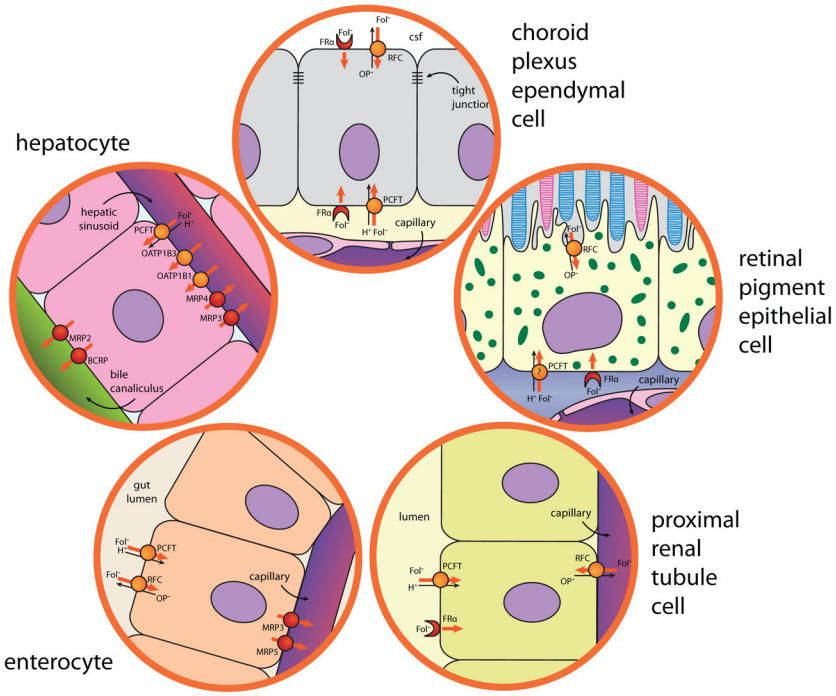

Figure 1.

The expression pattern of folate-specific and other transporters in epithelia. (i) Enterocyte: Both PCFT and RFC are expressed at the apical brush-border membrane. However, RFC does not contribute significantly to folate absorption under physiological conditions due, at least in part, to its neutral pH optimum and the low pH at the microenvironment of the surface of the proximal small intestine. Hence, when PCFT is absent, as occurs in HFM and PCFT-null mice, but RFC is expressed, there is a failure of intestinal folate absorption. Transport across the serosal membrane is likely mediated by several multidrug resistance-associated proteins (MRPs). (ii) Hepatocyte: PCFT is expressed at the sinusoidal membrane. RFC is also expressed but its location not defined. Their role in blood to bile transport is not clear. A variety of other transporters, that are not folate-specific, play a role in this process based upon studies in knock-out mice and the consequences of polymorphisms and mutations in these transporter genes. (iii) Choroid Plexus: FRα is highly expressed at the apical membrane in apposition to the cerebrospinal fluid (CSF), to a much lesser extent at the basolateral membrane in apposition to the capillaries. PCFT is expressed at the basolateral membrane. RFC is also expressed at the apical membrane. RFC is a bidirectional transporter and since it is an organic anion antiporter driven by the organic phosphate gradient, it favors transport from CSF into the ependymal cells. Vectorial transport of folate from blood to CSF requires both PCFT and FRα since deletion of either one leads to a marked decrease in CSF folate (6). (iv) Retinal Pigment Epithelium: All three folate-specific transporters are expressed in this epithelium. The location of PCFT has not been confirmed. Neither PCFT nor FRα is required for visual function since vision is intact in humans who have lost either transporter. (v) Proximal Renal Tubule: PCFT and FRα are expressed in the apical membrane. Folate clearance is increased in FRα-null mice. The roles of PCFT and RFC are not clear. Again, RFC's location favors transport into the cells (6). Cancer cells

express both RFC and PCFT. FRs are also expressed in most malignant cells but to varying degrees.

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