Published in final edited form as:

Biochem J. 2016 October 15; 473(20): 3545–3562. doi:10.1042/BCJ20160399.

Functional and mechanistic roles of the human proton-coupled folate transporter transmembrane domain 6–7 linker

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Abstract

The proton-coupled folate transporter (PCFT; SLC46A1) is a folate–proton symporter expressed in solid tumors and is used for tumor-targeted delivery of cytotoxic antifolates. Topology modeling suggests that the PCFT secondary structure includes 12 transmembrane domains (TMDs) with TMDs 6 and 7 linked by an intracellular loop (positions 236–265) including His247, implicated as functionally important. Single-cysteine (Cys) mutants were inserted from positions 241 to 251 in Cys-less PCFT and mutant proteins were expressed in PCFT-null (R1–11) HeLa cells; none were reactive with 2-aminoethyl methanethiosulfonate biotin, suggesting that the TMD6–7 loop is intracellular. Twenty-nine single alanine mutants spanning the entire TMD6–7 loop were expressed in R1–11 cells; activity was generally preserved, with the exception of the 247, 250, and 251 mutants, partly due to decreased surface expression. Coexpression of PCFT TMD1–6 and TMD7–12 half-molecules in R1–11 cells partially restored transport activity, although removal of residues 252–265 from TMD7–12 abolished transport. Chimeric proteins, including a nonhomologous sequence from a thiamine transporter (ThTr1) inserted into the PCFT TMD6–7 loop (positions 236–250 or 251–265), were active, although replacement of the entire loop with the ThTr1 sequence resulted in substantial loss of activity. Amino acid replacements (Ala, Arg, His, Gln, and Glu) or deletions at position 247 in wild-type and PCFT–ThTr1 chimeras resulted in differential effects on transport. Collectively, our findings suggest that the PCFT TMD6–7 connecting loop confers protein stability and may serve a unique functional role that depends on secondary structure rather than particular sequence elements.

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Author Contribution

Experiments were primarily executed by M.R.W., with assistance from L.J.W. and Z.H.; M.R.W., Z.H., and L.H.M. participated in research design, data analysis, and manuscript writing. J.Y. consulted on structure homology modeling and docking.

Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

Introduction

The proton-coupled folate transporter (PCFT; SLC46A1) is a folate–proton symporter, which is characterized by an acidic pH optimum [1–4]. Although PCFT is the major folate transporter in the proximal jejunum and duodenum, recent interest in PCFT reflects its high level of expression in a broad range of human solid tumors and its potential for targeted chemotherapy drug delivery [5,6]. Antifolates typified by methotrexate (MTX) have long been used in the clinic to treat various cancer types, including childhood leukemia and osteogenic sarcoma [7]. Newer antifolates have been approved for clinical use, including pralatrexate for treating cutaneous T-cell lymphoma and pemetrexed for treating malignant pleural mesothelioma and non-squamous non-small-cell lung cancer [8–10]. All of these agents are substrates for the ubiquitously expressed reduced folate carrier (RFC), the major tissue folate transporter, and variously for PCFT [11,12]. RFC transport into normal tissues probably contributes to the adverse side effects of classic antifolates. Most recently, novel 6-substituted pyrrolo[2,3-*d*]pyrimidine antifolates related to pemetrexed were empirically developed with specificities for membrane transport by PCFT over RFC [6,13–17]. Several of these compounds exhibited potent antitumor efficacies in preclinical tumor models, directly attributable to their high levels of membrane transport by PCFT, and are candidates for clinical development.

PCFT belongs to the major facilitator superfamily (MFS) of transporters [18,19]. Human PCFT (hPCFT) is an oligomeric protein [20,21], with each monomer comprising 459 amino acids including 12 transmembrane domains (TMDs), including cytosolic N- and C-termini and a large loop domain connecting TMDs 6 and 7 (Figure 1A). Several studies have used MFS homology considerations and loss-of-function mutations in hPCFT in patients with hereditary folate malabsorption syndrome as a guide for interrogating particular residues in hPCFT that may be structurally or functionally important [22–29]. Amino acids implicated as functionally important in hPCFT were further studied by site-directed mutagenesis and include Glu185, His281, and Arg376 [24,30,31]. In other studies, residues mapping to TMD2 (Gly93 and Phe94), TMD4 (Phe157, Gly158, and Leu161), and TMD5 (Ile188) were identified by cysteine (Cys)-scanning accessibility methods as comprising key substrate-binding domains [32–34]. A novel TMD2–3 re-entrant loop was identified as functionally important and includes Asp109 and Arg113, neither of which can be replaced by other amino acids [23,27,34,35].

Of particular interest in the present study is the TMD6–7 loop domain, which includes a stretch of 30 mostly non-conserved amino acids (positions 236–265) with the exception of a conserved stretch of amino acids (RLFXXRH) from positions 241 to 247, Leu254 and Arg264 (Figure 1B). His247 was previously implicated as functionally important, as amino acid substitutions (Ala, Arg, Gln, or Glu) at this position were associated with decreased transport rates (decreased $V_{\max}$) and increased substrate-binding affinities (decreased K_t) compared with wild-type hPCFT [30]. By molecular modeling, His247 was predicted to reside at the cytoplasmic end of a solute pathway with hydrogen bonding to Ser172 where it restricts substrate access to the folate-binding pocket [30].

In the present study, we systematically explore the functional and mechanistic importance of the hPCFT TMD6–7 connecting loop by site-directed, deletion, and insertion mutagenesis. Our findings suggest that the hPCFT TMD6–7 connecting loop provides for protein stability and may serve a unique functional role by potentially restricting substrate access to the folate translocation pathway in a manner that depends principally on secondary structure rather than individual sequence elements.

Experimental

Materials

[3',5',7-³H]MTX (20 Ci/mmol) was purchased from Moravsek Biochemicals (Brea, CA). Unlabeled MTX was obtained from the Drug Development Branch, NCI, National Institutes of Health (Bethesda, MD). Folic acid, leucovorin [(6*R*,5*S*)-formyltetrahydrofolate], and fetal bovine serum (FBS) were purchased from Sigma Chemical Co. (St. Louis, MO). Other tissue culture supplies were purchased from various vendors.

hPCFT mutagenesis

Wild-type hPCFT constructs with various epitope tags, including *wthPCFT*^{HA} and *w^{FLAG}hPCFT*^{Myc-His10}, and a Cys-less hPCFT (*chPCFT*^{HA}) were previously described and used as templates for site-directed, deletion, and insertion mutagenesis [20,34]. Mutations were generated with the QuikChange™ Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA). (i) Twenty-nine individual alanine insertions from positions 236 to 266 were introduced using *wthPCFT*^{HA} in the pcDNA3 vector as a template. (ii) Eleven Cys residues were introduced from positions 241 to 251 using *chPCFT*^{HA} in pcDNA3 as a template. (iii) Additional mutants of His247 and His248, including amino acid replacements (Ala, Arg, Gln, and Glu) or deletions [H247 (deletion of His247), H247/ H248 (deletions of both His247 and His248), and H247/H248A (His247 deletion, Ala248 replacement)], were generated using *wthPCFT*^{HA} as a template. (iv) *wthPCFT*^{HA} was used as a template for PCR to remove the TMD6–7 loop including amino acids 236–265, generating *dthPCFT*^{HA}. With *dthPCFT*^{HA} as a template, we reintroduced 30 amino acids (positions 236–265) from hPCFT (designated *pthPCFT*^{HA}; as a control) or from SLC19A2 (ThTr1; positions 250–279) (designated *thPCFT*^{HA}), or we replaced hPCFT sequence in 15 amino acid segments (positions 236–250 or positions 251–265) with ThTr1 sequence (positions 250–264 or positions 265–279, respectively), generating *tphPCFT*^{HA} and *pthPCFT*^{HA}, respectively. (v) Additional mutagenesis generated several new replacements at position 247 (Glu, Arg, Ala, and His) or position 247 deletions, using *tphPCFT*^{HA}, *pthPCFT*^{HA}, and *thPCFT*^{HA} as templates.

To generate the hPCFT-TMD1–6^{HA} half-molecule construct, we used PCR to introduce a hemagglutinin (HA) tag, followed by a stop codon after amino acid 251 of *wthPCFT*^{HA} in pcDNA3. To create *FLAGhPCFT*-TMD7–12^{Myc-His10}, we introduced EcoRI cleavage sites following amino acids 24 and 249 in full-length *w^{FLAG}hPCFT*^{Myc-His10} by PCR. The mutant construct was digested with EcoRI (New England Biolabs, Ipswich, MA) and the larger linearized fragment was gel-purified from a 1% agarose gel with a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). The fragment was religated (Promega, Fitchburg,

WI) to reassemble $\text{FLAG}^{\text{hPCFT-TMD7-12Myc-His10}}$ in pCDNA3, which contained an FLAG epitope following Met1, followed by amino acids 2–24 and 252–459 from wild-type hPCFT sequence. We used additional mutagenesis to remove the TMD6–7 loop residues from the PCFT half-molecules, generating hPCFT-TMD1–6^{HA}236–251 (residues 1–235) and $\text{FLAG}^{\text{hPCFT-TMD7-12Myc-His10}}$ 252–265 (residues 1–24 and 266–459).

In all cases, mutagenesis primers were designed with the QuikChange™ Primer Design program and are available upon request. PCR conditions were 95°C for 30 sec, 55°C for 1 min, and 68°C for 8 min for 16 cycles. All hPCFT mutant constructs were confirmed by DNA sequencing (Genewiz, Inc., South Plainfield, NJ).

Cell culture

The human RFC- and hPCFT-null R1–11 HeLa cell line was a gift from Dr I. David Goldman (Albert Einstein Cancer Center, Bronx, NY) [36]. R1–11 cells were grown in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin/100 µg/ml streptomycin, and 600 µg/ml G418, and 500 nM MTX. For transfections with the hPCFT constructs, R1–11 cells were seeded in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS in 60 mm culture dishes at a density of 0.8 million cells per dish. After 24 h, R1–11 cells were transiently transfected with hPCFT constructs (in pcDNA3) (above) at 1 µg of DNA/plate using Lipofectamine 2000 (10 µl/plate) in Opti-MEM, as per the manufacturer's instructions (Life Technologies, Carlsbad, CA). The transfection medium was replaced with DMEM, containing 10% FBS, 4 h after transfection. To observe the effects of inhibiting proteasomal or lysosomal degradation, transfected R1–11 cells were treated 24 h post-transfection with either 3 µM MG132 (Calbiochem, San Diego, CA) or 10 nM bafilomycin (Sigma), respectively [37].

Membrane transport

Forty-eight hours after transfection of R1–11 cells, cellular uptake of [³H]MTX (0.5 µM) was measured at 37°C over 2 min. Uptake assays were performed in 60 mm dishes using MES-buffered saline (20 mM MES, 140 mM NaCl, 5 mM KCl, 2 mM MgCl₂, and 5 mM glucose; pH 5.5), as previously described [20]. Dishes were washed three times with Dulbecco's phosphate-buffered saline (PBS) and cells were solubilized in 0.5 N NaOH. Intracellular radioactivity was calculated in units of pmol/mg of protein, based on measurements of radioactivity and protein concentrations of the alkaline cell homogenates [38]. To measure MTX transport kinetics for R1–11 cells transfected with *w*hPCFT^{HA} and hPCFT mutant constructs, [³H]MTX uptake was measured over 2 min at 37°C from 0.33 to 5 µM [³H]MTX, and results were analyzed using Lineweaver–Burk plots to calculate K_t and V_{max} values. To determine K_i values for non-radioactive substrates (folic acid and leucovorin), transfected cells were incubated with 0.5 µM [³H]MTX and unlabeled inhibitors from 0.3 to 1.5 µM, with results compared with those without inhibitors. K_i values were calculated from Dixon plots. To compare transport pH dependences for *w*hPCFT^{HA} and hPCFT mutants, transport assays were conducted at pHs from 7.2 to 5.5. HEPES-buffered saline (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 2 mM MgCl₂, and 5 mM glucose) was used for pHs 7.2 and 6.8, and MES-buffered saline (20 mM MES, 140 mM NaCl, 5 mM KCl, 2 mM MgCl₂, and 5 mM glucose) was used for pHs 6.5, 6.0, 5.8, and 5.5.

Surface biotinylation with 2-aminoethyl methanethiosulfonate-biotin

Forty-eight hours after transfection of R1–11 cells with hPCFT Cys mutants in *c^ΔhPCFT^{HA}* (above), cells were treated with 2-aminoethyl methanethiosulfonate (MTSEA)-biotin (Biotium, Hayward, CA) to biotinylate aqueous accessible Cys residues [34]. MTSEA-biotin was freshly prepared in DMSO at 2 mg/100 μ l and then diluted 1:100 in PBS. R1–11 cells were washed twice with PBS and then treated with 2 ml of the diluted MTSEA-biotin solution at room temperature for 30 min. The MTSEA-biotin solution was aspirated, and cells were treated with 14 mM β -mercaptoethanol in PBS for 5 min to quench the reactions, followed by two washes with PBS. The dishes were then placed at 4°C and the cells were treated with 1.4 ml of hypotonic buffer (0.5 mM Na₂HPO₄, 0.1 mM EDTA, pH 7.0), containing a protease inhibitor mixture (Roche Applied Science). The cells were removed from the dishes with a rubber policeman, followed by centrifugation at 16 000 rpm for 10 min at 4°C. The supernatants were removed, and the pellets were resuspended in 400 μ l of cell lysis buffer (50 mM Tris base, 150 mM NaCl, 1% Nonidet P-40, and 0.5% sodium deoxycholate, pH 7.4) by vortexing, then mixed on a rotisserie shaker at 4°C for 1 h. The samples were centrifuged at 16 000 rpm for 15 min at 4°C, leaving the detergent-solubilized membrane proteins in the supernatant. An aliquot (25 μ l) of this membrane fraction was used as a loading control, whereas the remainder (375 μ l) was added to 50 μ l of streptavidin–agarose resin (Thermo Scientific, Waltham, MA; prewashed three times with lysis buffer). Samples were mixed overnight at 4°C with a rotisserie shaker. The next day, this mixture was centrifuged at 16 000 rpm for 1 min, and the supernatant was aspirated. The resin-containing bound proteins was washed three times with regular lysis buffer, then with lysis buffer supplemented with 2% SDS. Bound proteins were released from the streptavidin resin by treatment at 95°C for 5 min in 2 $\times$ Laemmli sample buffer [38] containing 50 mM dithiothreitol.

Surface protein biotinylation with sulfo-NHS (*N*-hydroxysuccinimide)-SS-biotin

We used the Cell Surface Labeling Accessory Pack (Thermo Scientific) to biotinylate and isolate surface membrane proteins. Forty-eight hours after transfection of R1–11 cells with the hPCFT constructs, the cells were treated with 0.25 mg/ml sulfo-NHS-SS-biotin in PBS at 4°C for 30 min. The cells were treated with lysis buffer, followed by sonication and centrifugation to remove the insoluble fraction. The supernatants were incubated with immobilized NeutrAvidin™ gel slurry for 1 h at room temperature, and the beads were washed five times with wash buffer. Proteins were eluted with 1 $\times$ Laemmli sample buffer [38] containing 50 mM dithiothreitol and analyzed by SDS/polyacrylamide gel electrophoresis (PAGE)/western blotting (see below). All buffers (i.e. lysis, washing, and elution) were supplemented with a protease inhibitor mixture (Roche Applied Science).

Western blotting

Protocols for plasma membrane preparation, SDS/PAGE, and electrotransfer on to polyvinylidene difluoride membranes (Pierce, Rockford, IL) were identical with those previously described [20,39]. For fractionation of crude membrane fractions, 10% SDS/PAGE gels were used. For fractionation of biotinylated surface membrane proteins, Novex™ 4–20% Tris/glycine mini-protein gels were used. Anti-HA antibody (Covance; Princeton,

NJ), anti-Myc antibody (Covance), and IRDye800-conjugated secondary antibody were used with an Odyssey Infrared Imaging System (LI-COR, Lincoln, NE) to identify HA- and Myc-tagged hPCFT proteins. Anti- β -actin (Sigma) or anti- Na^+/K^+ -ATPase (Novus Biologicals, Littleton, CO) antibodies were used to establish equal protein loading. Densitometry values were calculated using the Odyssey Infrared Imaging System and software package.

Confocal microscopy

R1–11 cells were seeded in Lab-Tek II Chamber Slides (Nalge Nunc International, Naperville, IL) at a density of 6.9×10^4 cells/well. After 24 h, cells were transfected with hPCFT constructs in pcDNA3, as described above, using 88.7 ng of DNA and 0.86 μl of Lipofectamine 2000 per sample. Forty-eight hours post-transfection, cells were fixed with 3.3% paraformaldehyde (in PBS) and permeabilized with 0.1% Triton X-100 (in PBS). Chamber slides were stained with primary antibodies, followed by incubation with secondary antibodies. The primary antibodies used were goat anti-HA polyclonal antibodies (Abcam, Cambridge, MA) and mouse anti-FLAG antibody (Sigma). Fluorescent secondary antibodies included were Alexa Fluor[®] 568-conjugated donkey anti-goat IgG (H + L) and Alexa Fluor[®] 488-conjugated donkey anti-mouse IgG (H + L) (Life Technologies). Slides were viewed with a Zeiss LSM-510 META NLO microscope, using a $\times 63$ water-immersion lens and the same parameters for all samples. Confocal microscopy was performed in the Microscopy, Imaging, and Cytometry Resources Core at the Wayne State University School of Medicine.

Molecular modeling

We used the crystal structures of members of MFS transporters GlpT (glycerol 3-phosphate transporter; 1PW4; for *pphPCFT*) [40] and YajR (3WDO; for *pt*, *tp*, and *thPCFT*) [41] as templates for homology modeling of hPCFT monomers containing varied 6–7 loop sequence replacements with Robetta Server (<http://robetta.bakerlab.org>). Modeling was based on the notion that all crystal structures from members of MFS members have preserved tertiary structural elements during evolution, thus enabling published structures to extrapolate to other MFS members [42–44]. The top hPCFT homology model retrieved through Robetta for each hPCFT form was superimposed by the SALIGN server (<http://modbase.compbio.ucsf.edu/salign/>). Structures were visualized with PyMol. The secondary structure of the 6–7 loops from each hPCFT form was predicted with GOR4 [45].

Statistical analysis

GraphPad v.6.0 software was used for plotting and data analysis including descriptive statistics.

Results and discussion

Homology analysis and Cys accessibilities of positions 241–251 in the hPCFT TMD6–7 loop region

Based on computer-predicted hydropathy models [12,46], hPCFT contains 12 TMDs with cytosol-oriented C- and N-termini and segments comprising TMDs 1–6 and TMDs 7–12, connected by a long *cytosol*-facing loop domain (30 amino acids) between TMDs 6 and 7

(positions 236–265; Figure 1A). Portions of this topology structure have been confirmed experimentally [34,46]. We compared the amino acid sequences across the TMD6–7 loop in PCFTs from five species from *Xenopus* to humans. Of the 30 amino acids, seven residues were completely conserved among all five species, including Arg241, Leu242, Phe243, Arg246, His247, Leu254, and Arg264 (numbering corresponds to hPCFT; Figure 1B). His247 was previously implicated from site-directed mutagenesis and molecular homology modeling to be important in hPCFT transport by virtue of its location at the putative cytoplasmic opening to the membrane translocation pathway [30].

Previous studies used Cys-accessibility methods and MTSEA-biotin reactivity with Cys insertions at Thr240 and Glu261 in the TMD6–7 loop of *c*hPCFT^{HA} to establish the likely cytosolic orientations at these positions [46]. To further establish the aqueous accessibilities (i.e., membrane topology) of amino acids comprising the TMD6–7 loop, particularly the *conserved stretch from positions 241 to 247*, we generated 11 Cys replacements from positions 241 to 251, using *c*hPCFT^{HA} as a template for site-directed mutagenesis. As we previously reported, *c*hPCFT^{HA} exhibits functional characteristics virtually identical with those for *w*hPCFT^{HA} [34]. The Cys mutant constructs were transfected into hPCFT-null R1–11 cells and were assayed for [³H]MTX transport at pH 5.5 and 37°C (Figure 2A) and for hPCFT protein expression on western blots (Figure 2B,C). Results were compared with those for *c*hPCFT^{HA}-transfected cells and for untransfected R1–11 cells. We found that all hPCFT Cys mutants were expressed in crude membrane fractions (as broadly banding glycosylated proteins on 10% gels; ~21 to ~54% of *c*hPCFT^{HA} levels by densitometry; Figure 2B) and that most mutants were active for [³H]MTX transport [~55–100% of *c*hPCFT^{HA} transport (Figure 2A); all uptakes were significantly greater than for R1–11 cells], although activities for two Cys mutants, L242C and H247C, were notably decreased (18.3 and 12.4%, respectively, of *c*hPCFT^{HA} uptake; $P < 0.0002$). These decreases were accompanied by reduced surface protein expression relative to *c*hPCFT (28% for L242C and 29% for H247C; Figure 2C).

To establish aqueous accessibilities, we treated the 11 Cys mutants with membrane-impermeant MTSEA-biotin, using G207C in the TMD5–6 external loop and S110C in the TMD2–3 re-entrant loop as positive controls [34]. Following MTSEA-biotin treatments, biotinylated proteins were ‘pulled down’ with streptavidin beads and analyzed on western blots with HA-specific antibody. Whereas G207C and S110C were biotinylated, *none* of the 11 Cys mutants from positions 241 to 251 were biotinylated (Supplementary Figure S1). These results suggest that residues in the TMD6–7 loop are not accessible to the extracellular aqueous milieu, and that the TMD6–7 loop does indeed have a cytosolic orientation, as predicted by the hPCFT hydropathy model [12], analogous to other MFS proteins such as RFC [47].

Scanning alanine mutagenesis across the hPCFT TMD6–7 loop region

To explore the functional importance of the TMD6–7 loop region in hPCFT membrane transport, we used scanning alanine mutagenesis with *w*hPCFT^{HA} in pcDNA3 to generate alanine replacement mutants from positions 236 to 266 (positions 257 and 259 are already alanines). Each of the 29 alanine mutant constructs was transfected into R1–11 HeLa cells,

and after 48 h, the transfectants were assayed for [3 H]MTX transport (Figure 3A), and for hPCFT membrane protein expression (Figure 3B), compared with *wt*hPCFT^{HA}. All of the hPCFT mutants were expressed, although there were some variations (measured by densitometry), ranging from ~28% (for S250A) to ~128% (for P260A) of the *wt*hPCFT^{HA} level. Twenty-six of the alanine mutants showed high levels of transport (~60–110% of *wt*hPCFT^{HA}). Although three mutants (H247A, S250A, and I251A) were notably less active (13, 15, and 13%, respectively, of *wt*hPCFT^{HA}; $P < 0.0001$), they were nonetheless significantly more active than the non-transfected negative control (R1–11; $P < 0.01$; Figure 3A). In each case, loss of transport activity and hPCFT expression in crude membranes were related to loss of surface membrane expression (Figure 3C; relative surface expression by densitometry of 51% for H247A, 11% for S250A, and 63% for I251A, compared with the *wt*hPCFT level).

Taken with the results of the Cys-scanning mutagenesis (Figure 2), these results imply that most of the residues comprising the hPCFT TMD6–7 loop region are *not essential* for transport. When comparing results for Cys and alanine substitutions across the TMD6–7 loop, His247 in the conserved RLFXXRH motif (positions 241–247; Figure 1B) was the *only* amino acid for which its replacement consistently caused a significant loss of transport activity.

Coexpression of hPCFT TMD1–6 and TMD7–12 half-molecules

To further investigate the functional and structural roles of the hPCFT TMD6–7 connecting loop, we generated hPCFT ‘half-molecule’ constructs. These include hPCFT-TMD1–6^{HA}, comprising amino acids 1–251 (TMDs 1–6) with a C-terminal HA epitope, and ^{FLAG}hPCFT-TMD7–12^{Myc-His10}, comprising amino acids 252–459 (TMDs 7–12) and an N-terminal FLAG epitope just after Met1, followed by 23 N-terminal amino acids (residues 2–24) and a C-terminal Myc-His₁₀ (see schematic diagram in Figure 4A).

The hPCFT half-molecule constructs were expressed in R1–11 HeLa cells both individually and together, which were then assayed for restoration of [3 H]MTX transport and protein expression. Results were compared with those for *wt*hPCFT^{HA} and *wt*^{FLAG}hPCFT^{Myc-His10} transfectants, and for untransfected R1–11 cells. When transfected separately, individual half-molecules were detected on western blots (Figure 4D), but were unable to restore transport activity above the low residual level in R1–11 cells (Figure 4B). The HA signal on the western blots for hPCFT-TMD1–6^{HA} was notably decreased (48%) compared with that for *wt*hPCFT^{HA} (Figure 4D, left panel); the Myc signal for ^{FLAG}hPCFT-TMD7–12^{Myc-His10} was ~12% compared with *wt*^{FLAG}hPCFT^{Myc-His10} (Figure 4D, right panel). Co-transfection of hPCFT-TMD1–6^{HA} with ^{FLAG}hPCFT-TMD7–12^{Myc-His10} resulted in an increased level of ^{FLAG}hPCFT-TMD7–12^{Myc-His10} over the individual transfectant, accompanying a modest restoration of transport activity (5.3% of *wt*hPCFT^{HA}) that was significantly higher than for the non-transfected R1–11 cells ($P < 0.05$; Figure 4B). By confocal microscopy, hPCFT-TMD1–6^{HA} and ^{FLAG}hPCFT-TMD7–12^{Myc-His10} were coexpressed and at least a portion colocalized to the cell surface, although substantial intracellular fluorescence for both hPCFT-TMD1–6^{HA} and ^{FLAG}hPCFT-TMD7–12^{Myc-His10} was detected (Supplementary Figure S2). Surface expression of coexpressed hPCFT-TMD1–6^{HA} and ^{FLAG}hPCFT-TMD7–

$12^{\text{Myc-His10}}$ was confirmed by surface biotinylation (Figure 4E). Transport activity for the hPCFT-TMD1–6^{HA}- and FLAGhPCFT-TMD7–12^{Myc-His10}-co-transfected cells showed the characteristic pH dependence for PCFT transport that was essentially identical with that for *wh*PCFT^{HA} (Figure 4C).

Since hPCFT-TMD1–6^{HA} and FLAGhPCFT-TMD7–12^{Myc-His10} included the TMD6–7 loop sequence (residues 236–251 and 252–265, respectively), we created and expressed two variant half-molecule constructs in which segments of the TMD6–7 loop were deleted. These constructs are hPCFT-TMD1–6 236–251^{HA} (TMD1–6), comprising PCFT amino acids 1–235, and FLAGhPCFT-TMD7–12 252–265^{Myc-His10} (TMD7–12), comprising of PCFT amino acids 1–24 and 266–459 (Figure 4A). Low levels of hPCFT-TMD1–6 236–251^{HA} and FLAGhPCFT-TMD7–12 252–265^{Myc-His10} were detected on western blots when transfected both singly and in combination (Figure 4D). Surface biotinylation confirmed surface expression of the coexpressed half-molecules (Figure 4E), paralleling patterns seen in crude membrane fractions (Figure 4D). Deletion of the residual loop sequence from *both* half-molecule constructs (hPCFT-TMD1–6 236–251^{HA} and FLAGhPCFT-TMD7–12 252–265^{Myc-His10}) abolished the modest transport activity recorded for combined hPCFT-TMD1–6^{HA} and FLAGhPCFT-TMD7–12^{Myc-His10} (Figure 4B). Whereas hPCFT-TMD1–6^{HA} (including amino acids 236–251) combined with FLAGhPCFT-TMD7–12 252–265^{Myc-His10} failed to restore transport above that for R1–11 cells, co-transfection of hPCFT-TMD1–6 236–251^{HA} with FLAGhPCFT-TMD7–12^{Myc-His10} (including amino acids 252–265) restored low-level transport activity (4.2% of *wh*PCFT^{HA}), greater than for R1–11 cells ($P < 0.05$), and equivalent to that for combined hPCFT-TMD1–6^{HA} and FLAGhPCFT-TMD7–12^{Myc-His10}.

Collectively, these results demonstrate that transport function can be partly restored by expressing hPCFT as separate TMD1–6 and TMD7–12 half-molecules and suggest that the 252–265 loop segment (but not amino acids 236–251) is essential for this restored transport activity.

Expression of hPCFT–ThTr1 TMD6–7 loop chimeric transporters

The thiamine transporter ThTr1 (SLC19A2) is <20% homologous to hPCFT at the amino acid level and there is an almost complete loss of amino acid identity across the TMD6–7 loop domains for hPCFT and ThTr1 (Figure 5A). To further examine the primary sequence requirement for the hPCFT TMD6–7 loop (amino acids 236–265), we replaced this stretch in hPCFT with *structurally analogous but non-homologous* sequence from the TMD6–7 loop of ThTr1 (amino acids 250–279; Figure 5A).

To achieve this, we first removed the nucleotide sequence encoding amino acids 236–265 from *wh*PCFT^{HA} by PCR, generating *d*hPCFT^{HA} (Figure 5A). Using *d*hPCFT^{HA} as a template, we then reintroduced hPCFT 236–265 sequence (designated *p*hPCFT^{HA}; as a control) or ThTr1 250–279 sequence (*t*hPCFT^{HA}). Two additional mutant constructs were generated with *partial* (15 amino acids) TMD6–7 loop replacements. These include *p*hPCFT^{HA}, with amino acids 236–250 from hPCFT, followed by amino acids 265–279 from ThTr1, and *t*hPCFT^{HA}, with amino acids 250–264 from ThTr1, followed by amino acids 251–265 from hPCFT (Figure 5A). All hPCFT mutant constructs were transiently

expressed in R1–11 HeLa cells and were assayed for hPCFT levels on western blots and for [3 H]MTX uptake compared with *wthPCFT*^{HA} (Figures 5B,C).

The *re-engineered* full-length hPCFT (*pphPCFT*^{HA}) showed a modest decrease in transport activity (~85% of *wthPCFT*^{HA}; Figure 5B), accompanying slightly decreased hPCFT protein expression (Figure 5C), including surface hPCFT (Figure 5D). The complete removal of the hPCFT TMD6–7 loop sequence (residues 236–265) in *dthPCFT*^{HA} abolished [3 H]MTX transport (<2% of *wthPCFT*^{HA}), reflecting a significant loss of membrane (Figure 5C) and surface (Figure 5D) hPCFT protein. Complete replacement of the 236–265 hPCFT sequence with amino acids 250–279 from ThTr1 (*thPCFT*^{HA}) likewise resulted in low expression and activity (~2% of *wthPCFT*^{HA} activity, although this was significantly higher than for R1–11 transport; $P < 0.0001$; Figure 5B–D). Replacement of either the C-terminal TMD6–7 loop fragment (amino acids 251–265) in *pthPCFT*^{HA} or the N-terminal fragment (amino acids 236–250) in *tphPCFT*^{HA} with ThTr1 sequence preserved significant transport (23 and 11%, respectively; Figure 5B). Compared with *wthPCFT*^{HA}, *pthPCFT*^{HA} and *tphPCFT*^{HA} showed insignificant differences in binding affinities for assorted transport substrates (as reflected in K_t and K_i values; Table 1) and only minor differences in pH dependence for transport (Figure 5E). The reduced transport activity relative to *wthPCFT*^{HA} appeared to be entirely due to decreased $V_{\max}$ values (measured with [3 H]MTX; Table 1) and was reflected in levels of membrane (Figure 5C) and surface (Figure 5D) hPCFT^{HA} proteins on western blots.

To assess contributions of protein stability *vis-à-vis* intracellular trafficking for this series of mutants, cells were treated with MG132, an inhibitor of proteasomal degradation [37]. Membrane expression of *dthPCFT*^{HA}, *tphPCFT*^{HA}, *pthPCFT*^{HA}, and *thPCFT*^{HA}, but not of *wthPCFT*^{HA}, was increased by 24 h treatment with 3 μ M MG132 (Supplementary Figure S3A). Treatment with the lysosomal degradation inhibitor bafilomycin [37] (24 h, 10 nM) had little impact on protein expression (Supplementary Figure S3A). For *pthPCFT*^{HA}, *tphPCFT*^{HA}, and *wthPCFT*^{HA}, cell-surface expression was measured following treatment with MG132 with identical results (Supplementary Figure S3B). These results suggest that the reduced surface expression for the chimeric hPCFT–ThTr1 proteins was principally due to decreased protein stability compared with *wthPCFT*^{HA} and was attributable to proteasomal degradation. Notably, *increased* surface expression of misfolded *pthPCFT*^{HA} and *tphPCFT*^{HA} proteins by MG132 was accompanied by *decreased* transport activity (Supplementary Figure S3C).

To better understand functional differences between wild-type hPCFT and the hPCFT–ThTr1 chimeric transporters with various TMD6–7 loop sequence replacements in relation to structure, we used molecular homology modeling. Superimposition of structural models for the *wt*-, *pt*-, *tp*-, and *thPCFT* proteins gave a composite RMSD value of 1.2 Å for 234 C α atoms from ten transmembrane helices (TMDs 1–10), implying that the overall structures of these various hPCFT forms were almost identical (Figure 6A). This suggests that the sequence composition of the TMD6–7 loop has a minimal impact on *overall* folding of the hPCFT protein. We also considered isolated conformational changes involving the TMD6–7 loop domain that may affect transport activity for the individual hPCFT insertion mutants compared with wild-type PCFT. We explored the secondary structure changes in the hPCFT TMD6–7 loop domain for the hPCFT–ThTr1 chimeric proteins, using the secondary

structure prediction tool GOR4 [45]. Whereas *whhPCFT^{HA}* showed significant α -helix (16.7%) for this region, this was lost for the insertion mutants (*tphPCFT^{HA}*, *pthPCFT^{HA}*, and *thPCFT^{HA}*). This loss of α -helical structure for the TMD6–7 mutant proteins was accompanied by progressively increased random coil structures, as reflected in major conformational changes (Figure 6B), from 60% for *whhPCFT* to 70% for *pthPCFT*, 80% for *tphPCFT*, and 87% for *thPCFT*. Thus, there is an inverse relationship between the extent of random coil for the TMD6–7 loop region and hPCFT transport activity.

These results establish that the TMD6–7 loop is important for hPCFT transport activity. However, this functional role probably depends on preservation of secondary structure rather than particular sequence motifs.

Characterization of His247 substitution and deletion mutants in wild-type hPCFT and hPCFT–ThTr1 chimeric transporters

While the above studies imply a relative independence of the TMD6–7 loop primary sequence for transport activity, scanning alanine, and Cys mutagenesis (Figures 2 and 3), nonetheless His247 was identified as a residue for which its replacement consistently caused a significant loss of transport activity. Unal et al. [30] previously showed that amino acid substitutions (Ala, Arg, Gln, and Glu) at position 247 had various effects on hPCFT transport activity. All of these mutants were expressed (Figure 7B) and, relative to *whhPCFT^{HA}*, activity ranged from ~5% for *wt-H247R* to ~13% for *wt-H247A*, and ~26 and ~28% for *wt-H247E* and *wt-H247Q*, respectively (Figure 7A).

Interestingly, the impact of position 247 replacements was much different for the hPCFT–ThTr1 chimeric transporters. For the partially active *tphPCFT^{HA}* mutant with a glutamate at position 247, replacement with alanine (*tp-E247A*) resulted in decreased transport (3% of *whhPCFT*), whereas reintroduction of histidine (*tp-E247H*) or replacement with glutamine (*tp-E247Q*) subtly increased transport over *tphPCFT^{HA}* (~19 and ~22.4% of *whhPCFT*, respectively; Figure 7A). Arginine insertion at position 247 of *tphPCFT* restored transport activity at ~50% of the *whhPCFT* level. These effects on activity for *tp-E247H* and *tp-E247R* were accompanied by disproportionately elevated $V_{\max}$ values compared with *tphPCFT* (Table 1), even when normalized to surface expression levels on western blots (Figure 5D). While the K_i for leucovorin was increased for *tp-E247H* compared with *tphPCFT*, for both *tp-E247H* and *tp-E247R*, there were minor differences in pH dependences for transport compared with *tphPCFT* (Figure 7F).

For *pthPCFT*, the effects of alanine, arginine, glutamine, and glutamic acid substitutions for His247 on transport activity (Figure 7A) and expression (Figure 7B) were nominal. Histidine insertion at position 247 in *thPCFT* resulted in a subtle but significant decrease in activity (from 2% of *whhPCFT^{HA}* for *thPCFT* to 1% for *tt-E247H*; $P < 0.05$; Figure 7A).

To further characterize the functional importance of His247 to hPCFT transport, we deleted this residue entirely, using *whhPCFT^{HA}* as a template, generating *wt- H247*. Since His247 in hPCFT is followed by His248 (thus effectively replacing His247 in the *wt- H247* mutant), we also created three additional mutants in the *whhPCFT^{HA}* background. These include *wt-H247A/H248A*, in which both His247 and His248 are mutated to alanine, *wt-*

H247/ H248, in which both histidines are deleted, and *wt*- H247/H248A, in which His247 is deleted and His248 is replaced by alanine. H247 and E247 deletion mutants were also prepared for *p*hPCFT^{HA} and *t*pPCFT^{HA}, respectively.

All of the wild-type hPCFT His deletion mutants showed similar, albeit slightly decreased levels of PCFT^{HA} protein from that for *wt*hPCFT^{HA} (Figure 7D). Interestingly, for *wt*- H247, *wt*- H247/ H248, and *wt*- H247/H248A, transport activity was significantly increased over that for *wt*-H247A ($P < 0.05$; Figure 7C); for *wt*- H247 and *wt*- H247/ H248, transport approximated ~46 and ~64%, respectively, of *wt*hPCFT^{HA} levels. Increased activity for *wt*- H247 and *wt*- H247/ H248, compared with *wt*-H247A hPCFT, was accompanied by slightly increased surface protein (Figure 7E). When transport was normalized to *surface* hPCFT levels, this gave ~75 and ~93% of that of *wt*hPCFT^{HA} for *wt*- H247 and *wt*- H247/ H248, respectively. While position 247 deletion in *t*pPCFT (*t*p-E247) also increased transport activity compared with *t*pE247A (23% of *wt*hPCFT), position 247 deletion in *p*hPCFT (*p*t- H247) resulted in a subtle decrease in transport activity (14% of *wt*hPCFT^{HA} for *p*t- H247 compared with 23% for *p*hPCFT; $P < 0.05$; Figure 7A).

We examined transport characteristics for the restored transport activity of the *wt*- H247, and *wt*- H247/ H248 mutants compared with *wt*-H247A and *wt*hPCFT^{HA}, including pH dependences and transport kinetics. There were no differences in the pH profiles between mutant and wild-type PCFTs, as maximal transport activity was measured at pH 5.5 and decreased with increasing pH (Figure 7F). By kinetic analysis, *wt*-H247A showed decreases in both K_t (4.2-fold) and V_{max} (26.7-fold) for MTX compared with *wt*hPCFT^{HA} (Table 1), similar to published results by Unal et al. [30]. Interestingly, for the deletion mutants (*wt*- H247 and *wt*- H247/ H248), the MTX K_t s were restored to approximate the wild-type values, accompanying increased V_{max} values, compared with H247A (Table 1). The latter reflect increased surface hPCFT, as noted above (Figure 7E). Analogous patterns were seen in the K_t s for both folic acid and leucovorin for the *wt*-H247A, *wt*- H247, and *wt*- H247/ H248 mutants, determined by Dixon analysis from the inhibition of [³H]MTX uptake.

Overall, our results suggest that neither His247 nor His248 is absolutely essential for hPCFT transport. Whereas *wt*-H247A resulted in increased affinity for transport substrates, His247 deletion had no impact on substrate binding (as reflected in the K_t and K_i values for various substrates). The decreased V_{max} values for *wt*-H247A, *wt*- H247, and *wt*- H247/ H248 mutants were all accompanied by reduced surface hPCFT levels compared with wild-type hPCFT.

Conclusions

hPCFT is predicted to be formed from distinct TMD1–6 and TMD7–12 segments, with the longest of its 11 connecting loop domains linking TMDs 6 and 7 comprising amino acids 236–265. In this study, we explored the structure and transport function of the TMD6–7 connecting loop using a range of complementary approaches.

- i. Ala and Cys substitutions mutagenesis across the TMD6–7 loop identified His247 as the only amino acid residue in this stretch for which its

replacement consistently and substantially suppressed transport activity. However, deletion of His247 significantly restored transport activity compared with *wt*-H247A. This implies that His247 is not in itself essential to high levels of transport.

- ii. Further studies established that large (15 amino acid) segments of the hPCFT TMD6–7 loop including His247 could be replaced with non-homologous sequence from ThTr1 (in *p*hPCFT^{HA} and *t*p^hPCFT^{HA}), resulting in significant preservation of transport, although complete deletion of the loop (in *d*hPCFT^{HA}) or its replacement with non-homologous sequence from ThTr1 (in *t*hPCFT^{HA}) abolished transport activity. These differences in transport activity between the *p*hPCFT^{HA} and *t*p^hPCFT^{HA}, compared with *w*hPCFT^{HA}, were independent of substrate binding, but were associated with a pronounced impact on transport $V_{\max}$, probably due to a loss of protein stability, resulting in decreased hPCFT protein expression. Interestingly, the increased surface expression for *p*hPCFT and *t*p^hPCFT when MG132 was added to inhibit proteasomal degradation resulted in decreased rather than increased transport activity, probably due to an excess of misfolded protein at the membrane surface. These results are consistent with the notion of a ‘dominant-negative’ interaction between correctly folded and misfolded hPCFT proteins in the context of hPCFT oligomerization [20,21].
- iii. While His247 replacements with Ala, Arg, Gln, and Glu in *w*hPCFT replicated previous findings of Unal et al. [30], substitutions for Glu247 in *t*p^hPCFT followed a distinctly different pattern. The same His247 replacements in *p*hPCFT showed no obvious impact on transport activity. Analogous results were obtained for the His247 deletion mutants (*wt*-H247 and *t*p^h-E247 versus *p*t^h-H247). Thus, the impact of position 247 alterations is *context-dependent* and varies with flanking sequence and secondary structure. Indeed, the role of the TMD6–7 loop domain in hPCFT function appears to be largely unrelated to specific primary sequence elements, as long as sufficient secondary structure is preserved and proper spacing between the TMD1–6 and TMD7–12 segments is ensured to maintain protein stability and to facilitate optimal membrane translocation. (iv) Consistent with the latter notion, hPCFT could be coexpressed as TMD1–6 and TMD7–12 half-molecules to restore transport activity, albeit in low levels.

Collectively, our results demonstrate that the TMD6–7 loop structure is critical for intracellular trafficking and protein stability essential for transport function. Our biochemical and molecular modeling results further suggest that while absolute primary sequence elements of the TMD6–7 loop are not essential to transport function, as long as sufficient secondary structure is preserved, the loop may nonetheless serve a unique functional role, even possibly restricting substrate access to the folate-binding site and membrane translocation pathway, as previously suggested [30]. While our results imply that His247 can play a role in hPCFT transport, this is subtle, as loss of His247 can be compensated for by

other primary sequence elements, as indicated by our results with *p^hPCFT* His-replacement mutants. Future studies will continue to explore key structural features of hPCFT as a means of understanding the transport mechanism, including identifying critical determinants of substrate binding and membrane translocation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding

The present study was supported by a grant from the National Cancer Institute, National Institutes of Health [R01 CA53535] (to L.H.M. and Z.H.), and the Eunice and Milt Ring Endowed Chair for Cancer Research (to L.H.M.). The Microscopy, Imaging and Cytometry Resources Core is supported, in part, by NIH Center Grant P30CA22453 (to the Barbara Ann Karmanos Cancer Institute) and the Perinatology Research Branch of NICHD, NIH, Wayne State University. M.R.W. was supported by a pre-doctoral training grant [T32 CA009531].

We thank Dr I. David Goldman for his gift of hRFC- and hPCFT-null R1–11 HeLa cells.

Abbreviations

Cys	cysteine
DMEM	Dulbecco's modified Eagle's medium
FBS	fetal bovine serum
GlpT	glycerol 3-phosphate transporter
HA	hemagglutinin
hPCFT	human proton-coupled folate transporter
MFS	major facilitator superfamily
MTSEA	2-aminoethyl methanethiosulfonate
MTX	methotrexate
NHS	<i>N</i> -hydroxysuccinimide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCFT	proton-coupled folate transporter
RFC	reduced folate carrier
ThTr1	thiamine transporter
TMD	transmembrane domain

References

1. Inoue K, Nakai Y, Ueda S, Kamigaso S, Ohta K-y, Hatakeyama M, et al. Functional characterization of PCFT/HCP1 as the molecular entity of the carrier-mediated intestinal folate transport system in the rat model. *Am. J. Physiol. Gastrointest. Liver Physiol.* 2008; 294:G660–G668. [PubMed: 18174275]
2. Nakai Y, Inoue K, Abe N, Hatakeyama M, Ohta K-y, Otagiri M, et al. Functional characterization of human proton-coupled folate transporter/heme carrier protein 1 heterologously expressed in mammalian cells as a folate transporter. *J. Pharmacol. Exp. Ther.* 2007; 322:469–476. [PubMed: 17475902]
3. Umapathy NS, Gnana-Prakasam JP, Martin PM, Mysona B, Dun Y, Smith SB, et al. Cloning and functional characterization of the proton-coupled electrogenic folate transporter and analysis of its expression in retinal cell types. *Invest. Ophthalmol. Vis. Sci.* 2007; 48:5299–5305. [PubMed: 17962486]
4. Qiu A, Jansen M, Sakaris A, Min SH, Chattopadhyay S, Tsai E, et al. Identification of an intestinal folate transporter and the molecular basis for hereditary folate malabsorption. *Cell.* 2006; 127:917–928. [PubMed: 17129779]
5. Desmoulin SK, Hou Z, Gangjee A, Matherly LH. The human proton-coupled folate transporter: biology and therapeutic applications to cancer. *Cancer Biol. Ther.* 2012; 13:1355–1373. [PubMed: 22954694]
6. Kugel Desmoulin S, Wang L, Hales E, Polin L, White K, Kushner J, et al. Therapeutic targeting of a novel 6-substituted pyrrolo [2,3-d]pyrimidine thienoyl antifolate to human solid tumors based on selective uptake by the proton-coupled folate transporter. *Mol. Pharmacol.* 2011; 80:1096–1107. [PubMed: 21940787]
7. Monahan, BA.; Allegra, CJ. Antifolates. In: Chabner, BA.; Longo, DL., editors. *Cancer Chemotherapy and Biotherapy*. Philadelphia: Lippincott-Raven; 2001. p. 109-148.
8. Cohen MH, Justice R, Pazdur R. Approval summary: pemetrexed in the initial treatment of advanced/metastatic non-small cell lung cancer. *Oncologist.* 2009; 14:930–935. [PubMed: 19737998]
9. Hazarika M, White RM Jr, Booth BP, Wang YC, Ham DY, Liang CY, et al. Pemetrexed in malignant pleural mesothelioma. *Clin. Cancer Res.* 2005; 11:982–992. PMID:15709163. [PubMed: 15709163]
10. Thompson CA. FDA approves pralatrexate for treatment of rare lymphoma. *Am. J. Health Syst. Pharm.* 2009; 66:1890. [PubMed: 19850775]
11. Matherly LH, Hou Z, Deng Y. Human reduced folate carrier: translation of basic biology to cancer etiology and therapy. *Cancer Metastasis Rev.* 2007; 26:111–128. [PubMed: 17334909]
12. Matherly LH, Wilson MR, Hou Z. The major facilitative folate transporters solute carrier 19A1 and solute carrier 46A1: biology and role in antifolate chemotherapy of cancer. *Drug Metab. Dispos.* 2014; 42:632–649. [PubMed: 24396145]
13. Desmoulin SK, Wang Y, Wu J, Stout M, Hou Z, Fulterer A, et al. Targeting the proton-coupled folate transporter for selective delivery of 6-substituted pyrrolo[2,3-d]pyrimidine antifolate inhibitors of de novo purine biosynthesis in the chemotherapy of solid tumors. *Mol. Pharmacol.* 2010; 78:577–587. [PubMed: 20601456]
14. Wang L, Cherian C, Kugel Desmoulin S, Polin L, Deng Y, Wu J, et al. Synthesis and antitumor activity of a novel series of 6-substituted pyrrolo[2,3-d]pyrimidine thienoyl antifolate inhibitors of purine biosynthesis with selectivity for high affinity folate receptors and the proton-coupled folate transporter over the reduced folate carrier for cellular entry. *J. Med. Chem.* 2010; 53:1306–1318. [PubMed: 20085328]
15. Wang L, Kugel Desmoulin S, Cherian C, Polin L, White K, Kushner J, et al. Synthesis, biological, and antitumor activity of a highly potent 6-substituted pyrrolo[2,3-d]pyrimidine thienoyl antifolate inhibitor with proton-coupled folate transporter and folate receptor selectivity over the reduced folate carrier that inhibits β -glycinamide ribonucleotide formyltransferase. *J. Med. Chem.* 2011; 54:7150–7164. [PubMed: 21879757]
16. Wang L, Wallace A, Raghavan S, Deis SM, Wilson MR, Yang S, et al. 6-Substituted pyrrolo[2,3-d]pyrimidine thienoyl regioisomers as targeted antifolates for folate receptor alpha and the proton-

- coupled folate transporter in human tumors. *J. Med. Chem.* 2015; 58:6938–6959. [PubMed: 26317331]
17. Wilson MR, Hou Z, Yang S, Polin L, Kushner J, White K, et al. Targeting non-squamous nonsmall cell lung cancer via the proton-coupled folate transporter with 6-substituted pyrrolo[2,3-d]pyrimidine thienoyl antifolates. *Mol. Pharmacol.* 2016; 89:425–434. [PubMed: 26837243]
 18. Reddy VS, Saier MH Jr. BioV Suite — a collection of programs for the study of transport protein evolution. *FEBS J.* 2012; 279:2036–2046. [PubMed: 22568782]
 19. Saier MH Jr. Eukaryotic transmembrane solute transport systems. *Int. Rev. Cytol.* 1999; 190:61–136. [PubMed: 10331239]
 20. Hou Z, Kugel Desmoulin S, Etnyre E, Olive M, Hsiung B, Cherian C, et al. Identification and functional impact of homo-oligomers of the human proton-coupled folate transporter. *J. Biol. Chem.* 2012; 287:4982–4995. [PubMed: 22179615]
 21. Wilson MR, Kugel S, Huang J, Wilson LJ, Wloszczynski PA, Ye J, et al. Structural determinants of human proton-coupled folate transporter oligomerization: role of GXXXG motifs and identification of oligomeric interfaces at transmembrane domains 3 and 6. *Biochem. J.* 2015; 469:33–44. [PubMed: 25877470]
 22. Lasry I, Berman B, Glaser F, Jansen G, Assaraf YG. Hereditary folate malabsorption: a positively charged amino acid at position 113 of the proton-coupled folate transporter (PCFT/SLC46A1) is required for folic acid binding. *Biochem. Biophys. Res. Commun.* 2009; 386:426–431. [PubMed: 19508863]
 23. Lasry I, Berman B, Straussberg R, Sofer Y, Bessler H, Sharkia M, et al. A novel loss-of-function mutation in the proton-coupled folate transporter from a patient with hereditary folate malabsorption reveals that Arg 113 is crucial for function. *Blood.* 2008; 112:2055–2061. [PubMed: 18559978]
 24. Mahadeo K, Diop-Bove N, Shin D, Unal ES, Teo J, Zhao R, et al. Properties of the Arg376 residue of the proton-coupled folate transporter (PCFT-SLC46A1) and a glutamine mutant causing hereditary folate malabsorption. *Am. J. Physiol. Cell Physiol.* 2010; 299:C1153–C1161. [PubMed: 20686069]
 25. Mahadeo KM, Diop-Bove N, Ramirez SI, Cadilla CL, Rivera E, Martin M, et al. Prevalence of a loss-of-function mutation in the proton-coupled folate transporter gene (PCFT-SLC46A1) causing hereditary folate malabsorption in Puerto Rico. *J. Pediatr.* 2011; 159:623.e1–627.e1. e621. [PubMed: 21489556]
 26. Shin DS, Mahadeo K, Min SH, Diop-Bove N, Clayton P, Zhao R, et al. Identification of novel mutations in the proton-coupled folate transporter (PCFT-SLC46A1) associated with hereditary folate malabsorption. *Mol. Genet. Metab.* 2011; 103:33–37. [PubMed: 21333572]
 27. Shin DS, Min SH, Russell L, Zhao R, Fiser A, Goldman ID. Functional roles of aspartate residues of the proton-coupled folate transporter (PCFT-SLC46A1); a D156Y mutation causing hereditary folate malabsorption. *Blood.* 2010; 116:5162–5169. [PubMed: 20805364]
 28. Shin DS, Zhao R, Yap EH, Fiser A, Goldman ID. A P425R mutation of the proton-coupled folate transporter causing hereditary folate malabsorption produces a highly selective alteration in folate binding. *Am. J. Physiol. Cell Physiol.* 2012; 302:C1405–C1412. [PubMed: 22345511]
 29. Zhao R, Min SH, Qiu A, Sakaris A, Goldberg GL, Sandoval C, et al. The spectrum of mutations in the PCFT gene, coding for an intestinal folate transporter, that are the basis for hereditary folate malabsorption. *Blood.* 2007; 110:1147–1152. [PubMed: 17446347]
 30. Unal ES, Zhao R, Chang M-H, Fiser A, Romero MF, Goldman ID. The functional roles of the His247 and His281 residues in folate and proton translocation mediated by the human proton-coupled folate transporter SLC46A1. *J. Biol. Chem.* 2009; 284:17846–17857. [PubMed: 19389703]
 31. Unal ES, Zhao R, Goldman ID. Role of the glutamate 185 residue in proton translocation mediated by the proton-coupled folate transporter SLC46A1. *Am. J. Physiol. Cell Physiol.* 2009; 297:C66–C74. [PubMed: 19403800]
 32. Zhao R, Shin DS, Fiser A, Goldman ID. Identification of a functionally critical GXXG motif and its relationship to the folate binding site of the proton-coupled folate transporter (PCFT-SLC46A1). *Am. J. Physiol. Cell Physiol.* 2012; 303:C673–C681. [PubMed: 22785121]

33. Shin DS, Zhao R, Fiser A, Goldman ID. Role of the fourth transmembrane domain in proton-coupled folate transporter function as assessed by the substituted cysteine accessibility method. *Am. J. Physiol. Cell Physiol.* 2013; 304:C1159–C1167. [PubMed: 23552283]
34. Wilson MR, Hou Z, Matherly LH. Substituted cysteine accessibility reveals a novel transmembrane 2–3 reentrant loop and functional role for transmembrane domain 2 in the human proton-coupled folate transporter. *J. Biol. Chem.* 2014; 289:25287–25295. [PubMed: 25053408]
35. Subramanian VS, Marchant JS, Said HM. Apical membrane targeting and trafficking of the human proton-coupled transporter in polarized epithelia. *Am. J. Physiol. Cell Physiol.* 2008; 294:C233–C240. [PubMed: 18003745]
36. Zhao R, Qiu A, Tsai E, Jansen M, Akabas MH, Goldman ID. The proton-coupled folate transporter: impact on pemetrexed transport and on antifolates activities compared with the reduced folate carrier. *Mol. Pharmacol.* 2008; 74:854–862. [PubMed: 18524888]
37. Polgar O, Ierano C, Tamaki A, Stanley B, Ward Y, Xia D, et al. Mutational analysis of threonine 402 adjacent to the GXXXG dimerization motif in transmembrane segment 1 of ABCG2. *Biochemistry.* 2010; 49:2235–2245. [PubMed: 20088606]
38. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 1951; 193:265–275. PMID:14907713. [PubMed: 14907713]
39. Matherly LH, Czajkowski CA, Angeles SM. Identification of a highly glycosylated methotrexate membrane carrier in K562 human erythroleukemia cells up-regulated for tetrahydrofolate cofactor and methotrexate transport. *Cancer Res.* 1991; 51:3420–3426. PMID:2054782. [PubMed: 2054782]
40. Huang Y, Lemieux MJ, Song J, Auer M, Wang DN. Structure and mechanism of the glycerol-3-phosphate transporter from *Escherichia coli*. *Science.* 2003; 301:616–620. [PubMed: 12893936]
41. Jiang D, Zhao Y, Wang X, Fan J, Heng J, Liu X, et al. Structure of the YajR transporter suggests a transport mechanism based on the conserved motif A. *Proc. Natl Acad. Sci. USA.* 2013; 110:14664–14669. [PubMed: 23950222]
42. Abramson J, Kaback HR, Iwata S. Structural comparison of lactose permease and the glycerol-3-phosphate antiporter: members of the major facilitator superfamily. *Curr. Opin. Struct. Biol.* 2004; 14:413–419. [PubMed: 15313234]
43. Lemieux MJ. Eukaryotic major facilitator superfamily transporter modeling based on the prokaryotic GltT crystal structure (Review). *Mol. Membr. Biol.* 2007; 24:333–341. [PubMed: 17710637]
44. Vardy E, Arkin IT, Gottschalk KE, Kaback HR, Schuldiner S. Structural conservation in the major facilitator superfamily as revealed by comparative modeling. *Protein Sci.* 2004; 13:1832–1840. [PubMed: 15215526]
45. Garnier J, Gibrat J-F, Robson B. GOR method for predicting protein secondary structure from amino acid sequence. *Methods Enzymol.* 1996; 266:540–553. [PubMed: 8743705]
46. Zhao R, Unal ES, Shin DS, Goldman ID. Membrane topological analysis of the proton-coupled folate transporter (PCFT-SLC46A1) by the substituted cysteine accessibility method. *Biochemistry.* 2010; 49:2925–2931. [PubMed: 20225891]
47. Liu XY, Matherly LH. Analysis of membrane topology of the human reduced folate carrier protein by hemagglutinin epitope insertion and scanning glycosylation insertion mutagenesis. *Biochim. Biophys. Acta.* 2002; 1564:333–342. [PubMed: 12175915]

Figure 1. hPCFT topology structure
(A) Membrane topology of hPCFT. Amino acid residues (236–265) in the TMD6–7 connecting loop are colored according to their conservation in hPCFT among species as in (B). IL, intracellular loop; EL, extracellular loop. (B) Sequence alignment of the PCFT TMD6–7 connecting loop across five species including zebrafish, *xenopus*, cattle, mouse, and human. The color codes are as follows: red, residues are conserved across all species; green, residues are conserved across three or four species; blue, residues are similar across species; and black, residues are not similar.

Figure 2. Scanning Cys insertion mutagenesis for positions 241–251 in the TMD6–7 connecting loop

(A) hPCFT single-Cys mutants from positions 241 to 251 in a c/hPCFT^{HA} background were transiently transfected into hPCFT-null R1–11 cells. Transport activity was measured after 48 h with [³H]MTX (0.5 μM) over 2 min at pH 5.5 and at 37°C. Results are expressed as a percentage of c/hPCFT^{HA} activity and reported as mean values ± standard errors (error bars) from triplicate experiments. All single-Cys mutants were significantly more active than the non-transfected R1–11 control ($P < 0.05$). (B) Western blots are shown for membrane proteins (10 μg) from HA-tagged hPCFT Cys mutants, compared with c/hPCFT^{HA}-transfected and untransfected R1–11 cells. Densitometry results (as relative percentages, noted below each lane, including ranges in parentheses) are shown for duplicate experiments and were normalized to β-actin expression. Migrations of molecular mass standard proteins (in kDa) are shown. (C) HA-tagged hPCFT surface membrane proteins (40 μg) were labeled with sulfo-NHS-SS-biotin (0.25 mg/ml) and isolated on immobilized NeutrAvidin™ gel. Biotinylated proteins were analyzed by SDS/PAGE and western blotting. Densitometry

results (as relative percentages, noted below each lane, including ranges in parentheses) are shown for duplicate experiments and were normalized to Na⁺/K⁺-ATPase expression. Migrations of molecular mass standard proteins (in kDa) are shown.

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Figure 3. Alanine-scanning mutagenesis of residues 236–266 in the TMD6–7 connecting loop (A) Alanine mutants were generated in *whPCFT*^{HA} from positions 236 to 266 spanning the TMD6–7 connecting loop and transfected into hPCFT-null R1–11 cells. Transport activity was measured after 48 h with [³H]MTX over 2 min at pH 5.5 and at 37°C. Results are expressed as a percentage of *whPCFT*^{HA} activity and are reported as mean values ± standard errors (error bars) from triplicate experiments. All of the alanine mutants were significantly more active than the non-transfected control, R1–11 ($P < 0.05$). (B) Western blots are shown for membrane proteins (10 µg) from HA-tagged hPCFT alanine mutants. Densitometry results (as relative percentages, noted below each lane, including ranges in parentheses) are shown for duplicate experiments and were normalized to β-actin expression. Migrations of molecular mass standard proteins (in kDa) are shown. (C) HA-tagged hPCFT surface membrane proteins (40 µg) were labeled with sulfo-NHS-SS-biotin (0.25 mg/ml) and isolated on immobilized NeutrAvidin™ gel. Biotinylated proteins were analyzed by SDS/PAGE and western blotting. Densitometry results (as relative percentages, noted below each lane, including range in parentheses) are shown for duplicate experiments and were normalized to Na⁺/K⁺-ATPase expression. Migrations of molecular mass standard proteins (in kDa) are shown.

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Figure 4. Expression of TMD1–6 and TMD7–12 hPCFT half-molecules

(A) A schematic diagram of all half-molecule constructs/proteins. (B) Half-molecule constructs were transiently transfected into R1–11 cells both individually and together. Transport activity was measured after 48 h by [³H]MTX uptake assays over 2 min at pH 5.5 and at 37°C. Results are expressed as a percentage of wthPCFT^{HA} activity and are reported as mean values ± standard errors (error bars) from triplicate experiments. Transport by the individual hPCFT-TMD1–6^{HA} and FLAGhPCFT-TMD7–12^{Myc-His10} transfectants was not significantly greater than for negative control R1–11 cells ($P > 0.05$). However, when transfected together, hPCFT-TMD1–6^{HA} and FLAGhPCFT-TMD7–12^{Myc-His10} showed significantly increased transport activity, exceeding that for R1–11 cells. hPCFT-TMD1–6^{HA} 236–251/FLAGhPCFT-TMD7–12^{Myc-His10} co-transfection also restored transport activity over R1–11 cells ($P < 0.05$), which was not statistically different from that for the hPCFT-TMD1–6^{HA}/FLAGhPCFT-TMD7–12^{Myc-His10}-co-transfected cells ($P > 0.05$) (asterisks indicate an increased level of transport over R1–11; $P < 0.05$). (C) [³H]MTX transport activity of wthPCFT^{HA} and hPCFT-TMD1–6^{HA}/FLAGhPCFT-TMD7–12^{Myc-His10} transfectants at pHs from 5.5 to 7.2 is shown. Uptake for each transfectant (wthPCFT^{HA} and hPCFT-TMD1–6^{HA}/FLAGhPCFT-TMD7–12^{Myc-His10}) was normalized to its own uptake at pH 5.5. (D) Western blots are shown for membrane proteins (10 µg) from HA-tagged or Myc-tagged hPCFT half-molecules. Densitometry results (as relative percentages, noted below each lane, including ranges in parentheses) are shown for duplicate experiments and were normalized to β-actin. Migrations of molecular mass standard proteins (in kDa) are shown. (E) HA- and Myc-tagged hPCFT surface membrane proteins (40 µg) were labeled with sulfo-NHS-SS-biotin (0.25 mg/ml) and isolated on an immobilized NeutrAvidinTM gel. Proteins were analyzed by SDS/PAGE and western blotting. Densitometry results (as relative percentages, noted below each lane, including ranges in parentheses) are shown for duplicate experiments and were normalized to Na⁺/K⁺-ATPase expression. Migrations of molecular mass standard proteins (in kDa) are shown.

Figure 5. Expression of hPCFT–ThTr1 chimeric transporters with replacement of the hPCFT TMD6–7 loop with ThTr1 sequence
(A) A schematic diagram of the TMD6–7 loop sequence replacements for *pphPCFT*^{HA}, *pthPCFT*^{HA}, *tphPCFT*^{HA}, and *tthPCFT*^{HA}. Chimeras were generated first by removing amino acid residues 236–265 through PCR mutagenesis (*dthPCFT*^{HA}) and then inserting new sequence. *pphPCFT*^{HA} serves as a positive control and contains an exact replacement of the hPCFT residues 236–265. *tthPCFT*^{HA} contains ThTr1 residues 250–279 in place of hPCFT residues 236–265. The TMD6–7 loop of *pthPCFT*^{HA} contains hPCFT residues 236–

250, followed by ThTr1 residues 265–279. The TMD6–7 loop of *tp*hPCFT^{HA} contains ThTr1 residues 250–264, followed by hPCFT residues 251–265. **(B)** hPCFT–ThTr1 chimera mutants were transiently transfected into R1–11 cells. Transport activity was measured after 48 h by [³H]MTX uptake assays over 2 min at pH 5.5 and at 37°C. Results are expressed as a percentage of *wh*hPCFT^{HA} activity and are reported as mean values ± standard errors (error bars) from triplicate experiments. All hPCFT–ThTr1 chimera proteins were significantly more active than that for the non-transfected control, R1–11. Transport for *d*hPCFT^{HA} was insignificantly different from that for R1–11 cells (asterisks indicate that transport activity is *not* significantly different from R1–11; *P* > 0.05). **(C)** Western blots are shown for membrane proteins (10 µg) from HA-tagged hPCFT chimera mutants. Densitometry results (as relative percentages, noted below each lane, including ranges in parentheses) are shown for duplicate experiments and were normalized to β-actin levels. Migrations of molecular mass standard proteins (in kDa) are shown. **(D)** HA-tagged hPCFT surface membrane proteins (40 µg) were labeled with sulfo-NHS-SS-biotin (0.25 mg/ml) and isolated on immobilized NeutrAvidin™ gel. Biotinylated proteins were analyzed by SDS/PAGE and western blotting. Densitometry results (as relative percentages, noted below each lane, including ranges in parentheses) are shown for duplicate experiments and were normalized to Na⁺/K⁺-ATPase expression. Migrations of molecular mass standard proteins (in kDa) are shown. **(E)** R1–11 cells expressing hPCFT–ThTr1 chimera mutant proteins were tested for [³H]MTX transport activity as in **(B)**, using MES- and HEPES-buffered saline, as appropriate, at different pHs. Uptake for each mutant at the individual pHs was normalized to its uptake at pH 5.5 (asterisk indicates that mutant PCFT transport showed a greater decrease from pH 5.5 to 6.0 than *wh*hPCFT; *P* < 0.05).

Figure 6. Protein modeling of PCFT-ThTr1 chimera mutants

hPCFT protein modeling using Robetta and SALIGN software is described in the Experimental Procedures section. (A) Cytosolic view of hPCFT TMDs 1-12 (including TMDs 1, 2, 4, 5, 7, 8, 10, and 11, predicted to form the transmembrane translocation pathway), as a composite structure from *pphPCFT*^{HA} (green), *phPCFT*^{HA} (pink), *tphPCFT*^{HA} (cyan), and *thPCFT*^{HA} (yellow). In (B), the TMD6-7 loop is highlighted and can be distinguished from TMDs 1-12 (grey) as a side view including *pphPCFT*^{HA} (green), *phPCFT*^{HA} (pink), *tphPCFT*^{HA} (cyan), and *thPCFT*^{HA} (yellow).

Figure 7. Analysis of 247/248 substitution and deletion mutants

(A and C) Amino acid substitution and deletion mutants were generated from *wthPCFT*^{HA}, *pHPCFT*^{HA}, *tpHPCFT*^{HA}, and *ttHPCFT*^{HA}, as described in the text, and were transfected into R1-11 cells. Transport activity was measured after 48 h with [³H]MTX (0.5 μM) over 2 min at pH 5.5 and at 37°C. Results are expressed as a percentage of *wthPCFT*^{HA} activity and are reported as mean values ± standard errors (error bars) from triplicate experiments. With the exception of *tt-E247H*, all mutants were significantly more active than the non-transfected control, R1-11 ($P < 0.05$) [asterisks indicate where transport activity is significantly different from parent *wt*-, *pt*-, *tp*-, or *ttHPCFT*^{HA}, as appropriate; $P < 0.05$]. (B and D) Western blots are shown for membrane proteins (10 μg) from HA-tagged hPCFT 247/248 substitution and deletion mutants. Densitometry results (as relative percentages, noted below

each lane, including ranges in parentheses) are shown for duplicate experiments and were normalized to β -actin expression. Migrations of molecular mass standard proteins (in kDa) are shown. (E) HA-tagged hPCFT surface membrane proteins (40 μ g) were labeled with sulfo-NHS-SS-biotin (0.25 mg/ml) and isolated on immobilized NeutrAvidin™ gel. Biotinylated proteins were analyzed by SDS/PAGE and western blotting. Densitometry results (as relative percentages, noted below each lane, including ranges in parentheses) are shown for duplicate experiments and results for hPCFT were normalized to Na^+/K^+ -ATPase expression. Migrations of molecular mass standard proteins (in kDa) are shown. (F) R1–11 cells expressing hPCFT His247 mutant proteins were tested for [^3H]MTX transport activity as in (A), using MES- and HEPES-buffered saline, as appropriate, at various pHs. Uptake for each mutant at the individual pHs was normalized to its own uptake at pH 5.5 (asterisk indicates that mutant PCFT transport showed a greater decrease from pH 5.5 to 6.0 than *wt*PCFT; $P < 0.05$).

Kinetic analysis of *w*hPCFT^{HA}, His247 hPCFT substitution and deletion mutants, and hPCFT–ThTr1 chimera proteins

Table 1

HA-tagged *w*hPCFT and hPCFT mutants were transiently transfected into R1–11 cells, and 48 h later, [³H]MTX uptake was assayed at pH 5.5 over 2 min at 37°C. To determine *K_i* and *V_{max}* values, cells were treated with [³H]MTX with concentrations between 0.33 and 5 μM, and results were analyzed by Lineweaver–Burk plots. To determine *K_i* values, cells were incubated with 0.5 μM [³H]MTX with folic acid (FA) and leucovorin (LCV) as competitors from 0.3 to 1.5 μM, with results analyzed by Dixon plots. Data are presented as mean values ± standard errors from four independent experiments.

hPCFT variant	<i>K_i</i> (μM)	<i>V_{max}</i> (pmol/mg/min)	MTX	<i>V_{max}</i> / <i>K_i</i>	FA		LCV	
					<i>K_i</i> (μM)		<i>K_i</i> (μM)	
<i>w</i> hPCFT ^{HA}	0.75 ± 0.10	261.5 ± 28.3		414.3 ± 80.1	0.59 ± 0.08		0.38 ± 0.08	
<i>w</i> ^Δ H247A	0.18 ± 0.01 *	10.8 ± 1.06 *		60.8 ± 10.7 *	0.12 ± 0.03 *		0.13 ± 0.02	
<i>w</i> ^Δ H247	0.54 ± 0.05	67.6 ± 12.2 *		122.8 ± 24.8	0.27 ± 0.07		0.25 ± 0.06	
<i>w</i> ^Δ H247/ H248	0.82 ± 0.07	101.6 ± 7.7 *		125.8 ± 15.3	0.46 ± 0.12		0.46 ± 0.13	
<i>tp</i> hPCFT	0.99 ± 0.21	63.0 ± 10.1 *		73.6 ± 20.9 *	0.71 ± 0.16		0.29 ± 0.06	
<i>tp</i> -E247H	1.00 ± 0.10	141.6 ± 20.0 *,#		150.2 ± 31.1	0.47 ± 0.06		0.72 ± 0.11 #	
<i>tp</i> -E247R	1.50 ± 0.17 *	276.8 ± 30.5 #		194.5 ± 30.1 #	0.60 ± 0.12		0.53 ± 0.18	
<i>p</i> hPCFT	0.77 ± 0.07	68.0 ± 10.9 *		92.0 ± 20.0 *	0.55 ± 0.13		0.17 ± 0.06	

Values significantly different from those for *w*hPCFT^{HA} (*P* < 0.05) are marked with *. Values significantly different from *tp*hPCFT^{HA} (*P* < 0.05) are marked with # (only *tp*-E247H and *tp*-E247R were compared with *tp*hPCFT).