The human proton-coupled folate transporter (hPCFT): modulation of intestinal expression and function by drugs

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Urquhart BL, Gregor JC, Chande N, Knauer MJ, Tirona RG, Kim RB. The human proton-coupled folate transporter (hPCFT): modulation of intestinal expression and function by drugs. Am J Physiol Gastrointest Liver Physiol 298: G248–G254, 2010. First published September 17, 2009; doi:10.1152/ajpgi.00224.2009.—Folic acid is a vitamin essential for thymidylate and purine synthesis. The human proton-coupled folate transporter (hPCFT) has recently been identified as a pH-dependent folate acid transporter, and mutations in this transporter have been linked to hereditary folic acid malabsorption. In this study, we assessed hPCFT-mediated transport activity in vitro, intersubject variability of intestinal expression in relation to blood folates, and the relationship of proton-pump inhibitor (PPI) therapy on hPCFT expression in vivo. We created a Madin-Darby canine kidney strain II (MDCKII) cell line stably expressing hPCFT to evaluate its drug substrates and inhibitors. Intestinal pinch biopsies (duodenum, ileum, colon) were collected from patients undergoing routine endoscopy procedures, and expressed levels of hPCFT were determined by RT-PCR. When assessed using MDCKII-hPCFT cells, folate acid and methotrexate were found to be high-affinity hPCFT substrates. Sulfasalazine and pyrimethamine were noted to inhibit hPCFT activity with Ki values of 42.3 and 161.7 μmol/L, respectively. hPCFT was localized to the brush-border membrane of enterocytes with highest expression in the duodenum and reduced levels in the ileum and colon. When we assessed hPCFT expression in a subset of patients who were receiving PPI therapy, a near 50% reduction in duodenal hPCFT mRNA expression was noted. These results suggest that hPCFT transporter activity can be modulated by many drugs in clinical use, and expression of this transporter in the gastrointestinal tract is higher in the duodenum than more distal sites (duodenum > ileum > colon). Importantly, we note that PPI drug use appears to be associated with reduced hPCFT expression in vivo.

Folic acid absorption; vitamin

FOLIC ACID IS AN ESSENTIAL vitamin that functions as a carbon donor for various methylation reactions and is therefore obligatory for thymidylate and purine biosynthesis. In North America, mandatory fortification of grain with folic acid has resulted in a decline in the incidence of folic acid deficiency states, but there is still evidence to suggest inadequate intake in certain populations (2). Given its role in DNA synthesis, it is not surprising to note that rapidly proliferating cells have the greatest demand for folic acid, thus are most greatly affected by folic acid deficiency. The primary signs of folic acid deficiency are megaloblastic anemia and intestinal mucosa deterioration. Folic acid intake is especially important to woman of child-bearing age as adequate folic acid has been shown to significantly reduce the risk of neural tube defects (12). Unlike bacteria and yeast, mammals are unable to synthesize folic acid and thus must obtain it from dietary sources such as leafy vegetables, legumes, or fortified cereal grains.

Intestinal folic acid absorption is known to occur in the proximal intestine (16). Furthermore, studies in humans clearly demonstrate that folic acid absorption increases as intestinal pH decreases, implicating a proton gradient as an important determinant of folic acid absorption (18, 19). Dietary folic acid exists as the polyglutamated form with the pteroic acid moiety bound to as many as nine glutamic acid residues. Upon ingestion, dietary folates are enzymatically hydrolyzed to the monoglutamate form before intestinal absorption in the duodenum and jejunum. It has been long noted that intestinal folic acid absorption appeared to be mediated by a carrier-mediated process most notable in the upper intestine with optimal transport function at low pH (22, 28). However, it was not until recently that the human proton-coupled folate transporter (hPCFT/SLC46A1) was identified through genetic association studies where loss of function mutations were linked to hereditary folate malabsorption (15). Although it was originally identified as a low-affinity heme transporter and initially named heme carrier protein 1 (21), subsequent studies clearly demonstrate the primary role of this transporter as a proton-dependent, high-affinity folic acid transporter.

Hereditary folate malabsorption (OMIM 229050) is a rare autosomal recessive familial condition that is symptomatic within months after birth. The disorder is characterized by severe folic acid deficiency in serum, erythrocytes, and cerebrospinal fluid (CSF), and signs may include megaloblastic anemia, diarrhea, seizures, and mental retardation. Elegant studies by Goldman and colleagues (13, 15, 26) have clearly identified the cause of hereditary folate malabsorption to be mutations in the hPCFT gene, underscoring the crucial role of this transporter in mediating intestinal folic acid absorption. In Goldman’s original report identifying hPCFT, a G to A nucleotide change at position 5882 was shown to be a null function mutation and the cause of hereditary folate malabsorption. This mutation causes a deletion of exon 3 and defective trafficking of a shorter variant protein to the cell surface. Subsequent studies in patients with hereditary folate malabsorption have resulted in the identification of seven additional mutations in hPCFT, demonstrating the essential role that hPCFT plays in the intestinal absorption of dietary folic acid.

Aside from inadequate dietary intake and hereditary folate malabsorption, several prescription medications and disease states are known to be associated with folic acid deficiency.
The most prevalent examples of drug-induced folic acid deficiency states arise from the use of dihydrofolate reductase inhibitors, such as methotrexate, in the treatment of certain cancers, arthritis, and inflammatory bowel disease. Although methotrexate may not decrease total folates, it blocks the recycling of tetrahydrofolate from dihydrofolate. In addition, drugs such as phenytoin, valproic acid, pyrimethamine, and sulfasalazine have all been implicated in drug-induced folic acid deficiency and often require folic acid supplementation to prevent the emergence of folate deficiency states (11). It should also be noted that intestinal disorders such as celiac disease (8), Crohn’s disease (23), and ulcerative colitis (3) are also commonly associated with low serum and erythrocyte folate.

Accordingly, we conducted mechanistic studies of hPCFT in vivo and in vitro to better define intersubject variation in expression in the intestine of human subjects and to also systematically assess the longitudinal expression of hPCFT in human intestine. We then compared intestinal expression of hPCFT to folic acid homeostasis. Furthermore, in vitro studies of hPCFT function, extent of substrate specificity, and the potential to be inhibited by drugs were determined. Finally, we assessed hPCFT expression in patients being treated with proton-pump inhibitors (PPIs) and note that such therapy is associated with significant reduction in intestinal hPCFT expression, suggesting that chronic PPI therapy may also be an unrecognized risk factor for folic acid deficiency.

Materials and Methods

Materials. [1H]Folic acid (33 Ci/mmol, 97.9% purity) and [1H]methotrexate (31.8 Ci/mmol, 99.5% purity) were obtained from Moravek Biochemicals (Brea, CA). The source and specific activity of all other radiolabeled chemicals are summarized in Supplemental Table S1. Supplemental material for this article is available online at the American Journal of Physiology Gastrointestinal and Liver Physiology website. All other reagents were from Sigma-Aldrich (Oakville, ON) unless otherwise specified.

hPCFT cDNA cloning and stable cell line generation. The full open-reading frame of hPCFT was obtained by PCR using AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA) from cDNA synthesized from human liver using the following primers: 5’-GGAGCCACATAAATGTATAGCAATGG-3’ (forward) and 5’-GGTCCTGAGGTCCATGCAG-3’ (reverse). The PCR product was ligated into pcDNA3.1 (Invitrogen, Carlsbad, CA) and transfected into MDCK vector control cells. After selection with neomycin (1 mg/ml) and resistant clones were tested for PCFT expression and transfection reagent (F. Hoffman-LaRoche, Basel, Switzerland). The pcDNA3.1 vector lacking any cDNA insert was transfected into MDCKII cells using the following equation: $P_{app} = (dX/dT)(SA \times C_0)$, where $dX/dT$ is the slope of the line fit through the [1H]folic acid or [1H]methotrexate vs. time plot, SA is the surface area of the cell culture insert, and $C_0$ is the initial concentration.

Human intestinal PCFT expression. Fasted subjects undergoing diagnostic esophagastroduodenoscopy or colonoscopy were invited to participate in the study. Patients were excluded from the study if they were taking folic acid or multivitamin supplements, taking drugs known to effect the metabolism and/or absorption of folic acid (i.e., dihydrofolate reductase inhibitors, antiepileptic medications, sulfasalazine), or found to have gastrointestinal diseases such as Crohn’s disease, ulcerative colitis, or celiac disease on the basis of the reports of the gastroenterologist and the pathologist. On the basis of these criteria, 22 subjects having esophagastroduodenoscopy were recruited to the study for determination of duodenal hPCFT expression and erythrocyte folate. An additional 10 subjects undergoing diagnostic colonoscopy were recruited to the study for determination of terminal ileum and colon hPCFT expression. For each subject, three pinch biopsies were taken in addition to biopsies collected for diagnostic purposes. One biopsy was immediately placed in RNAlater (Qiagen, Valencia, CA), and two were placed in 10% neutral-buffered formalin. The study protocol was approved by the Health Sciences Research Ethics Board at the University of Western Ontario, and all patients provided written informed consent before the procedure.

Samples for intestinal PCFT mRNA expression were homogenized in Trizol (Invitrogen), and RNA extraction was performed by standard methods. The cDNA synthesis was performed with a total of 750 ng of RNA. Quantitative RT-PCR of hPCFT was performed using a SYBR green method (Applied Biosystems) using the following primers: 5’-ATGAGAAGTTGGACTGTGGT-3’ (forward) and 5’-GGACCGCCACATA GACTGAGAC-3’ (reverse). All samples were compared with a standard curve of the hPCFT ampiclon cloned into pCR2.1 TOPO vector (Invitrogen) for accurate determination of copy number. Expression was normalized to the copy number of the enterocyte marker protein villin, which controls for the depth, integrity, and presence of submucosa in the biopsy specimen. Villin was amplified with the following primers: 5’-GGAGCCACATAAATGTATAGCAATGG-3’ (forward) and 5’-GGTCCTGAGGTCCATGCAG-3’ (reverse) and similarly cloned into pCR2.1 TOPO. In a subset of patients, fasting serum (n = 8) and erythrocyte (n = 22)
folic acid and methotrexate were transport substrates of hPCFT (Fig. 1).

Because PCFT is thought to be apically expressed in enterocytes (24), expression of hPCFT in an MDCK cell line allowed for the evaluation of vectorial transport of its substrates, folic acid, and methotrexate. We note a marked enhancement in the apical to basal transport of \[^{3}H\]folic acid (Fig. 2A) and \[^{3}H\]methotrexate (Fig. 2B) in the MDCK-hPCFT, whereas the extent of apical-basal transport in the MDCK vector only transfected control cell line was modest. These observations are consistent with the significantly increased PCFT expression in the MDCK-PCFT cell line compared with the lower endogenous level of PCFT expression in vector control cells (data not shown). Basal to apical transport of \[^{3}H\]folic acid and \[^{3}H\]methotrexate did not differ significantly between hPCFT-overexpressing cells compared with the MDCK vector only transfected control cell line. Calculated apparent permeability (P_{app}) for \[^{3}H\]folic acid and \[^{3}H\]methotrexate were significantly greater in MDCK-hPCFT compared with MDCK vector control-transfected cells. Similarly the net uptake ratio (P_{app} apical to basal/ P_{app} basal to apical) was significantly greater for MDCK-hPCFT compared with vector control for both \[^{3}H\]folic acid and \[^{3}H\]methotrexate. Apparent permeability data and net vectorial transport for \[^{3}H\]folic acid, \[^{3}H\]methotrexate, along with \[^{14}C\]inulin control are summarized in Table 1.

**RESULTS**

**Characterization of hPCFT-mediated folate and methotrexate uptake.** We characterized hPCFT-mediated folic acid and methotrexate uptake through the creation of a MDCK-hPCFT stable cell line. We screened an array of radiolabeled drugs and steroid conjugates as potential substrates and note that only
CHARACTERIZATION AND EXPRESSION OF hPCFT

Table 1. Apparent permeability of [3H]folic acid and [3H]methotrexate across MDCK-hPCFT and MDCK-vector control monolayers

<table>
<thead>
<tr>
<th></th>
<th>Papp (A→B) X 10⁻⁶, cm/s (SD)</th>
<th>Papp (B→A)</th>
<th>Uptake Ratio, Papp (A→B)/Papp B→A</th>
</tr>
</thead>
<tbody>
<tr>
<td>[3H]Folic acid MDCK-hPCFT</td>
<td>3.0 (0.4)*</td>
<td>0.5 (0.05)</td>
<td>6.0*</td>
</tr>
<tr>
<td>[3H]Folic acid MDCK-vector control</td>
<td>1.1 (0.1)</td>
<td>0.5 (0.04)</td>
<td>2.2</td>
</tr>
<tr>
<td>[3H]Methotrexate MDCK-hPCFT</td>
<td>7.1 (1.5)*</td>
<td>0.2 (0.06)</td>
<td>35.5†</td>
</tr>
<tr>
<td>[3H]Methotrexate MDCK-vector control</td>
<td>0.8 (0.1)</td>
<td>0.2 (0.02)</td>
<td>4.0</td>
</tr>
<tr>
<td>[14C]Inulin</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*P < 0.05 compared with vector control (folic acid), †P < 0.05 compared with vector control (methotrexate). A→B represents flux from the apical (A) to basal (B) chamber, and B→A represents flux from the basal to apical chamber of the cell culture insert. MDCK, Madin-Darby canine kidney cells; hPCFT, human proton-coupled folate transporter; Papp, apparent permeability.

Inhibition of hPCFT-mediated folic acid uptake. We assessed the specific role of hPCFT inhibition to drug-induced folic acid deficiency through a systematic screen of drugs for their ability to inhibit hPCFT-mediated folic acid transport in our model cell line. Interestingly, we found that a number of drugs were capable of significantly decreasing hPCFT-mediated folic acid uptake. These drugs included methotrexate, sulfasalazine, pyrimethamine, aminopterin, probenecid, dipyridamole, and MK571 (Fig. 3). Detailed inhibition analysis revealed that the most potent inhibitor among the tested compounds was methotrexate, with a Ki of 2.7 μM. Other drugs that showed appreciable inhibition of folic acid uptake included sulfasalazine, pyrimethamine, aminopterin, and the leukotriene antagonist and well-known multidrug resistance-associated protein inhibitor MK571. The Ki value for these compounds is summarized in Table 2.

Intestinal hPCFT expression and folic acid status among folate-replete subjects. Although apical membrane localization of PCFT has been shown in mouse and rat duodenum (16, 21), we wanted to determine the expression of hPCFT in human duodenum. We collected pinch biopsies from subjects undergoing diagnostic biopsies of upper and/or lower gastrointestinal tract. We ascertained that hPCFT is indeed localized to the apical/brush-border membrane in human duodenum (Fig. 4A). Because the majority of folic acid absorption occurs in the proximal intestine (4, 6), we hypothesized that PCFT expression would be the highest in the duodenum compared with more distal intestinal sites. To confirm our hypothesis, we characterized the longitudinal hPCFT expression in the intestine by quantitative RT-PCR of patients who underwent diagnostic biopsies of upper and/or lower gastrointestinal tract. Expression of hPCFT was markedly higher in the duodenum than in the terminal ileum or colon (Fig. 4B). This pattern of expression is fully consistent with data that show the majority of folic acid absorption occurs in the duodenum and jejunum with low to absent absorption in the more distal regions of the intestine (4, 6).

Given the central role of hPCFT to intestinal folic acid absorption and the severe folic acid malabsorption observed in patients with mutations in hPCFT, we assessed for a correlation between duodenal hPCFT expression with fasting erythrocyte and serum folate levels. Fasting erythrocyte folate ranged between 634 to over 2,500 nM in the twenty-two subjects investigated. hPCFT mRNA expression varied 25-fold between subjects; however, we did not observe a statistically significant correlation between erythrocyte folate (r = 0.22, P = 0.33) or serum folate (r = −0.06, P = 0.90) and hPCFT expression in our study population. It should be noted that serum folate levels were available in a limited number (n = 8) of subjects who underwent the biopsy procedure; however, serum folate is more reflective of recent folic acid intake, whereas erythrocyte folate is a better predictor of folate status in humans. Importantly, none of our subjects had clinical folate deficiency or anemia suggestive of folic acid deficiency.

PPI use is associated with reduced duodenal hPCFT expression. Because PCFT activity is dependent on a proton gradient, we hypothesized that patients who are treated with PPIs will have lower duodenal hPCFT expression. We recruited nine control subjects and nine subjects taking a PPI undergoing upper endoscopy procedures for determination of duodenal hPCFT expression and fasting erythrocyte folate levels. Baseline patient characteristics are summarized in Table 3 (control subjects) and Table 4 (subjects taking a PPI). Interestingly, patients taking a PPI had significantly decreased duodenal hPCFT expression compared

Table 2. Ki of compounds found to significantly inhibit PCFT-mediated [3H]folic acid uptake

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Ki, μM</th>
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<tbody>
<tr>
<td>Methotrexate</td>
<td>2.7</td>
</tr>
<tr>
<td>Aminopterin</td>
<td>7.3</td>
</tr>
<tr>
<td>MK571</td>
<td>9.7</td>
</tr>
<tr>
<td>Sulfasalazine</td>
<td>42.3</td>
</tr>
<tr>
<td>Pyrimethamine</td>
<td>161.7</td>
</tr>
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</table>
with PPI-free controls (Table 4; Fig. 5). This appeared to be a class effect, as expression was decreased in patients taking omeprazole, pantoprazole, and lansoprazole (Fig. 5A) compared with PPI-free controls. Although hPCFT expression was decreased in patients taking PPIs, there was no significant difference in erythrocyte folate between the two groups (mean erythrocyte folate 1,108 nmol/l in control vs. 1,221 nmol/l in PPI, \(P = 0.60\)).

DISCUSSION

Folic acid is a single carbon donor for a number of biologically critical methylation reactions and is essential for DNA biosynthesis (20). The importance of folate status has been highlighted by mandated fortification of cereal grains in the United States and Canada, which resulted in a marked lowering of the incidence of neural tube defects (5, 17), a devastating consequence of maternal folic acid deficiency. The recent identification of the long sought after intestinal PCFT (hPCFT/SLC46A1) (15) has provided indisputable mechanistic insight governing how dietary folic acid is absorbed from the intestine. The importance of this transporter as a rate-limiting step to the maintenance of normal folic acid status is further exemplified in subjects with hereditary folate malabsorption, a rare autosomal recessive condition characterized by low CSF and blood folic acid levels, mental retardation, diarrhea, and anemia. A number of recent reports have clearly linked hPCFT as the molecular basis of hereditary folate malabsorption.

We created a hPCFT-MDCK cell line to demonstrate that hPCFT is a high-affinity, proton-dependent transporter for folic acid and methotrexate. Although PCFT is endogenously expressed in MDCKII cells (15), our results clearly demonstrate that overexpression significantly increases substrate uptake. Importantly, we show that this transporter can maintain the vectorial transport of folic acid across the apical cell membrane domain and requires a proton gradient as the driving force. It should be noted that methotrexate is widely used in the treatment of Crohn’s disease, rheumatoid arthritis, and several types of cancer. Methotrexate pharmacokinetics are variable with the oral bioavailability ranging between 67% and 92% (7, 10, 25), suggesting that variation in hPCFT expression or activity may play a role in this regard. Because methotrexate is a high-affinity substrate for hPCFT, it was expected to show a strong inhibition of PCFT-mediated folic acid uptake. Our results, along with those of others, confirm that methotrexate is a potent PCFT inhibitor (6, 14, 15, 27). Similarly, when we assessed drugs such as sulfasalazine, widely reported as capable of causing drug-induced folic acid deficiency (9), our findings suggest that sulfasalazine-mediated inhibition of hPCFT can occur at a concentration that is readily attained in the gastrointestinal tract. On the basis of an estimated intestinal folic acid concentration of 3.0 \(\mu\)M following a standard supplement and a Ki of 42.3, the calculated IC\(_{50}\) for sulfasalazine-mediated PCFT inhibition is 140 \(\mu\)M (14). Therefore, taken together, these results suggest that inhibition of hPCFT is the mechanism that results in folic acid

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Table 3. Baseline patient characteristics and endoscopy outcome of control patients

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age, yr</th>
<th>Sex</th>
<th>Reason for Scope</th>
<th>Scope Result</th>
<th>Medications</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>54</td>
<td>F</td>
<td>Reflux Disease</td>
<td>Normal</td>
<td>Domperidone, metformin</td>
</tr>
<tr>
<td>2</td>
<td>53</td>
<td>M</td>
<td>Family history of polyposis</td>
<td>Polyps removed</td>
<td>None</td>
</tr>
<tr>
<td>3</td>
<td>80</td>
<td>F</td>
<td>Epigastric Pain</td>
<td>Normal</td>
<td>None</td>
</tr>
<tr>
<td>4</td>
<td>53</td>
<td>F</td>
<td>Epigastric Pain</td>
<td>Normal</td>
<td>None</td>
</tr>
<tr>
<td>5</td>
<td>44</td>
<td>M</td>
<td>Abdominal Pain</td>
<td>Stomach Ulcer</td>
<td>Acetylsalicylic acid (discontinued)</td>
</tr>
<tr>
<td>6</td>
<td>52</td>
<td>F</td>
<td>Abdominal Pain</td>
<td>Normal</td>
<td>None</td>
</tr>
<tr>
<td>7</td>
<td>52</td>
<td>M</td>
<td>Abdominal Pain</td>
<td>Normal</td>
<td>None</td>
</tr>
<tr>
<td>8</td>
<td>42</td>
<td>F</td>
<td>Potential Celiac</td>
<td>Normal</td>
<td>None</td>
</tr>
<tr>
<td>9</td>
<td>23</td>
<td>M</td>
<td>Bowel Infection (follow-up)</td>
<td>Moderate villous atrophy (jejunum only)</td>
<td>Calcium, VitD, azathioprine</td>
</tr>
</tbody>
</table>
deficiency because sulfasalazine is typically administered at a dose of 3–4 g per day, which results in intestinal concentrations several fold higher than the calculated Ki. Pyrimethamine and trimethoprim are dihydrofolate reductase inhibitors used in the treatment of malaria/toxoplasmosis and urinary tract infections, respectively. High doses of these drugs have been shown to cause symptoms of folic acid deficiency although it is unclear whether this is attributable to dihydrofolate reductase inhibition or blocked folic acid absorption (11, 29). Interestingly, we now show that such drugs can also inhibit hPCFT. Pyrimethamine is typically given orally at doses between 50–75 mg per day for 3 wk for the treatment of toxoplasmosis. On the basis of this dose range, the estimated intestinal concentration would range from 670–1,000 μM, well above the calculated Ki for pyrimethamine-mediated inhibition of hPCFT. Therefore, inhibition of hPCFT by drugs in clinical use may be a mechanism of variable folic acid and methotrexate absorption and efficacy.

Previous studies have demonstrated PCFT expression in the gastrointestinal tract (15) as well as in MDCK and Caco-2 cell lines (24). In this report, we show that hPCFT is expressed on the apical membrane of duodenal villi in human intestine, similar to observations previously noted for the rat (16). We also show the variability of intestinal hPCFT expression in patients as opposed to commercially available RNA or cDNA. Indeed we observed a 25-fold variation in duodenal hPCFT expression with significantly higher expression in the duodenum compared with ileum and colon. We also hypothesized that PPI therapy would be associated with reduced hPCFT expression in the duodenum. Indeed this was the case (Fig. 5B) among patients on PPI therapy. Our findings clearly suggest that, not only does the proton gradient drive the activity of this transporter, but also hPCFT transcriptional regulation may be either directly or indirectly regulated by PPI therapy. Surprisingly, we did not find a readily observable link between intestinal hPCFT expression with either serum or erythrocyte folate levels. One possible explanation for this observation is the naturally high folic acid intake by all subjects in North America attributable to mandated folic acid fortification of cereal grains that started in the late 1990s. Thus it is likely that folic acid intake in our study subjects was sufficiently high enough to mask the large variability in the expression of intestinal hPCFT. Nevertheless our findings of drug inhibitors of hPCFT or drugs that lead to reduced hPCFT expression suggest that, in patients whose intake of folic acid is marginal, concomitant administration of such drugs may result in frank deficiency. Overall, our findings provide important new insights to hPCFT expression, function, and mechanisms of drug-induced folate deficiency.

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DISCLOSURES

No conflicts of interest are declared by the author(s).
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