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

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ABSTRACT

The proton-coupled folate transporter (PCFT) is a folate-proton symporter with an acidic pH optimum, approximating the microenvironments of solid tumors. We tested 6-substituted pyrrolo[2,3-\(d\)]pyrimidine antifolates with one to six carbons in the bridge region for inhibition of proliferation in isogenic Chinese hamster ovary (CHO) and HeLa cells expressing PCFT or reduced folate carrier (RFC). Only analogs with three and four bridge carbons (\(N\)\(-[3-2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-6-yl]propylbenzoyl\)-L-glutamic acid (compound 2) and \(N\)\(-[4-4-[2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-6-yl]butylbenzoyl\)-L-glutamic acid (compound 3), respectively) were inhibitory, with \(2 > 3\). Activity toward RFC-expressing cells was negligible. Compound 2 and pemetrexed (Pmx) competed with \(\text{[3H]}\)methotrexate for PCFT transport in PCFT-expressing CHO (R2/hPCFT4) cells from pH 5.5 to 7.2; inhibition increased with decreasing pH. In Xenopus laevis oocytes microinjected with PCFT cRNA, uptake of 2, like that of Pmx, was electrogenic. Cytotoxicity of 2 toward R2/hPCFT4 cells was abolished in the presence of adenosine or 5-amino-4-imidazolecarboxamide, suggesting that glycynamide ribonucleotide formyltransferase (GARFTase) in de novo purine biosynthesis was the primary target. Compound 2 decreased GTP and ATP pools by \(\sim 50\) and 75%, respectively. By an in situ GARFTase assay, 2 was \(\sim 20\)-fold more inhibitory toward intracellular GARFTase than toward cell growth or colony formation. Compound 2 irreversibly inhibited clonogenicity, although this required at least 4 h of exposure. Our results document the potent antiproliferative activity of compound 2, attributable to its efficient cellular uptake by PCFT, resulting in inhibition of GARFTase and de novo purine biosynthesis. Furthermore, they establish the feasibility of selective chemotherapy drug delivery via PCFT over RFC, a process that takes advantage of a unique biological feature of solid tumors.

Introduction

The biologic role of folate cofactors derives from their participation in one-carbon transfer reactions, leading to nucleotide precursors, serine, and methionine (Stokstad, 1990). Because mammalian cells cannot synthesize folates de novo, membrane transport of extracellular folates is essential. Three major folate uptake systems have been described.
1) The reduced folate carrier (RFC or SLC19A1) is an anion antiporter that is ubiquitously expressed and represents the primary folate transporter in tissues and tumors at physiologic pH. 2) Folate receptors (FRs) are glycosyl phosphatidylinositol-anchored proteins that transport folates by receptor-mediated endocytosis (Elnakat and Ratnam, 2004). 3) The proton-coupled folate transporter (PCFT; SLC46A1) is a proton-folate symporter that functions optimally at acidic pH by coupling the downhill flow of protons to the uphill transport of folates (Qiu et al., 2006; Nakai et al., 2007; Zhao and Goldman, 2007).

Folate-dependent biosynthetic pathways serve as important therapeutic targets for antifolates. Antifolate drugs for cancer include potent inhibitors of dihydrofolate reductase [methotrexate (Mtx) and PT523], thymidylate synthase [ralitrexed (Rtx), GW1843U89, pemetrexed (Pmx)], and the purine biosynthetic enzymes β-glycinamide ribonucleotide formyltransferase (GARFTase) [lometrexol (Lmx), Pmx] and 5-aminouracil ribonucleotide formyltransferase (Pmx) (Hughes et al., 1999; Mendelsohn et al., 1999; Smith et al., 1999; Monahan and Allegra, 2006; Chattopadhyay et al., 2007; Racanelli et al., 2009). Although these agents are all transported by RFC (Matherly et al., 2007), expression of RFC in both normal and tumor cells presents an obstacle to antitumor selectivity. Furthermore, loss of RFC is associated with antifolate resistance (Zhao and Goldman, 2003; Matherly et al., 2007).

Thus, there is compelling rationale for developing cytotoxic antifolates that are substrates for transporters other than RFC with limited expression and/or transport in normal tissues compared with tumors. This reasoning was the impetus to develop drugs that are selectively transported by FRs over RFC (Gibbs et al., 2005; Hilgenbrink and Low, 2005; Salazar and Ratnam, 2007; Deng et al., 2008, 2009; Wang et al., 2010). Such agents can target tumors (e.g., ovarian adenocarcinomas) that express high levels of FRs (Elnakat and Ratnam, 2004). For instance, we described 6-substituted pyrrolo[2,3-d]pyrimidine analogs (1a, 2, 3, respectively) that most inhibitory toward FR-expressing cells.

PCFT is expressed in the proximal small intestine, where it mediates folate absorption at acidic pH (Qiu et al., 2006) and in tissues such as liver and kidney, which do not experience low pH conditions (Zhao et al., 2009). The intestinal pH of solid tumors is often acidic (Heimlinger et al., 1997; Raghunand et al., 1999), which favors PCFT transport. A prominent low-pH transport route was identified in 29 of 32 solid human tumor cell lines (Zhao et al., 2004a), and high levels of human PCFT (hPCFT) transcripts were reported in a broad range of human tumors (Kugel Desmoulin et al., 2010). Whereas the role of hPCFT in antifolate activity and tumor selectivity is still evolving, transport of classic antifolates by PCFT has been described previously (Zhao et al., 2008), although Pmx shows the highest affinity for the carrier at both acidic and neutral pHs (Zhao and Goldman, 2007).

Loss of RFC results in resistance to Mtx and Rtx, yet Pmx cytotoxic activity can be preserved or even increased if PCFT is present (Zhao et al., 2004b). For agents such as Pmx that are transported by both RFC and PCFT, loss of tumor selectivity could be due to RFC transport in normal tissues. Although RFC-selective agents without PCFT transport were described (PT523, GW1843U89) (Zhao and Goldman, 2007; Deng et al., 2009), until our recent report of pyrrolo[2,3-d]pyrimidine thienoyl antifolate substrates for PCFT (Wang et al., 2010), no analogous PCFT-selective cytotoxic agents without RFC transport had been reported.

In this report, we further explore the potential of PCFT to selectively deliver cytotoxic antifolates for the chemotherapy of tumors. We expand upon the transport activity profile for the earlier series of 6-substituted pyrrolo[2,3-d]pyrimidine antifolates (Deng et al., 2008) to include hPCFT, and we document potent antiproliferative activities attributable to nearly complete selectivity for hPCFT over human RFC (hRFC), resulting in inhibition of GARFTase and de novo purine biosynthesis. Our results establish that hPCFT is an efficient means of delivering cytotoxic antifolate drugs and suggest that hPCFT provides a unique opportunity to selectively target solid tumors with cytotoxic antifolates that are not substrates for the ubiquitously expressed hRFC.

**Methods and Materials**

**Materials.** [3,5,7,2H]Mtx (20 Ci/mmol), [3H]Pmx (3.7 Ci/mmol), and [14C(U)]glycine (87 mCi/mmol) were purchased from Moravek Biochemicals (Brea, CA). Unlabeled Mtx and leucovorin ([6R,S]-5-formyl tetrahydrofolate) (LCV) were provided by the Drug Development Branch, National Cancer Institute (Bethesda, MD). Both labeled and unlabeled Mtx were purified by HPLC before use (Fry et al., 1982). The sources of the antifolate drugs were as follows. Rtx [N-(5-[N-(4,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-N-methyl-amino]-2-thenoyl)-L-glutamic acid] was obtained from AstraZeneca Pharmaceuticals (Macclesfield, Cheshire, UK); Lmx (5,10-dioxa-5,6,7,8-tetrahydrofolate), and Pmx (Alimta) were from Eli Lilly and Co. (Indianapolis, IN); GW1843U89 ([S]-2-4-5-[(1,2-dihydro-3-methyl-1-oxo-benzox/[quinoxalin-9-yl]-methyl)aminol]-2-oxo-2-indololyl) glutaric acid] was from GlaxoSmithKline (Research Triangle Park, NC); and N4-(4-amino-4-deoxyxypertol)-N6-hemiphtalaxy1-1-ornithine (PT523) was a gift of Dr. Andre Rosowsky (Dana-Farber Cancer Institute, Boston, MA). Restriction and modifying enzymes were purchased from Promega (Madison, WI). Other chemicals were obtained from commercial sources in the highest available purities. Synthesis and properties of the substituted pyrrolo[2,3-d]pyrimidine antifolate compounds 1 to 5 were described previously (Deng et al., 2008) and structures are provided in Fig. 1. Preparation of compound 1a was done by an analogous synthesis and is described in detail in the supplemental material.

**Cell Lines.** PCFT-, RFC-, and FR-null MTXRIIOuaR2–4 (R2) Chinese hamster ovary (CHO) cells (Flintoff and Nagai, 1983) were a gift of Dr. Wayne Flintoff (University of Western Ontario, London, ON, Canada) and were cultured in α-minimal essential

![Fig. 1. Structures of previously reported 6-substituted pyrrolo[2,3-d]pyrimidines 1 to 5 (Deng et al., 2008) and newly synthesized 2 methylene bridge pyrrolo[2,3-d]pyrimidine analog 1a.](at://molpharm.aspetjournals.org)
media (MEM) supplemented with 10% bovine calf serum (Invitrogen, Carlsbad, CA), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM 1-glutamine at 37°C with 5% CO₂. PC43-10 cells are R2 cells transfected with hRFC (Wong et al., 1995) and were cultured in complete α-MEM plus 1 mg/ml G418. With the use of LookOut, a polymerase chain reaction-based Mycoplasma spp. detection kit from Sigma Chemical Co. (St. Louis, MO), cell lines were periodically determined to be free of Mycoplasma spp. Generation and culture of hPCFT-expressing R2/hPCFT4 and vector control R2/VC cells are described below. HeLa R1-11-RFC6 and R1-11-PCFT4 cells were derived from hRFC- and hPCFT-null R1-11 cells by stable transfection with hemagglutinin-tagged pZeosV2+/-RFC and pZeosSV2+/-PCFT constructs, respectively (Zhao et al., 2008), and were gifts of Dr. J. David Goldman (Albert Einstein School of Medicine, Bronx, NY).

Preparation of a Myc-His₆-Tagged Human PCFT Construct and Generation of Stable Transfectants. Total RNA from wild-type HeLa cells was reverse-transcribed and polymerase chain reaction-amplified with EasyA proof-reading polymerase (Agilent Technologies, La Jolla, CA) using the following primers: 5′-AACTT GGA TCC gca cat gga ggg gag cgc gag cc-3′; and 5′-AAGCT TCT ACC ggg ggt tgt cgg aaa ctg ctg gaa cga g-3′ (bold capital letters designate the BamHI and KpnI restriction sites, respectively). The 1403-base pair amplicon was subcloned into pCDNA3.1 (Invitrogen) in-frame with a Myc-His₆ sequence inserted at the C-terminal amino acid 466 (hereafter designated hPCFTMyc-His₆/pCDNA3.1). The construct was confirmed by automated DNA sequencing at the Wayne State University Sequencing Core.

R2 cells were transfected with hPCFTMyc-His₆/pCDNA3.1 by electroporation (200 V, 1000 μF capacitance). After 24 h, the cells were cultured in the presence of G418 (1.5 mg/ml). Stable clones were selected by plating for individual colonies in the presence of 1.5 mg/ml G418. Colonies were isolated, expanded, and screened for expression of hPCFTMyc-His₆ protein by Western blotting and transfection assays at pH 5.5 (see Gel Electrophoresis and Western Blotting). One clone (R2/hPCFT4) was selected for further study. Vector control R2 cells (R2/VC) transfected with empty pCDNA3.1 were also prepared and used as a negative control. R2/hPCFT4 and R2/VC cells were cultured in complete α-MEM with G418.

Gel Electrophoresis and Western Blotting. For characterizing hPCFT protein expression in R2/hPCFT4 cells, crude plasma membranes were prepared by differential centrifugation. In brief, cells were suspended in 10 mM Tris-HCl, pH 7, containing X1 protease inhibitor cocktail tablets (Roche, Indianapolis, IN), and disrupted with a probe sonicator. The cell homogenate was centrifuged (600g, 10 min) to remove cell debris and nuclei; the supernatant was then centrifuged at 200,000g (45,000 rpm) in an ultracentrifuge (TL100; Beckman Coulter, Fullerton, CA) for 30 min. The particulate fraction was solubilized in 10 mM Tris-HCl, pH 7, with 2% SDS in the presence of proteolytic inhibitors, and proteins were quantified with Folin-Pirolen reagent (Lowry et al., 1951). Membrane proteins were electrophoresed on 7.5% polyacrylamide gels in the presence of SDS (Laemmli, 1970) and electroblotted onto polyvinyldene difluoride membranes (Pierce, Rockford, IL) (Matsudaia, 1987). PCFTMyc-His₆ protein was detected with Mysc-specific mouse antibody (Covance, Berkeley, CA) and secondary IRDye 800-conjugated antibody (Rockland Immunochemicals, Gilbertsville, PA). Detection of immunoreactive proteins used the Odyssey Infrared Imaging System (LI-COR, Lincoln, NE).

Transport Assays. CHO (R2, R2/VC, R2/hPCFT4, and PC43-10) sublines were routinely grown as monolayers (see Cell Lines). Three days before transport experiments, cells were transferred to Cytostir spinners and maintained in suspension at densities of 2 to 5 × 10⁵ cells/ml. Cells were collected by centrifugation and washed with Dulbecco’s phosphate-buffered saline (DPBS), and the cell pellets (~2 × 10⁷ cells) were suspended in transport buffer (2 ml) for cellular uptake assays.

pH-dependent uptake of 0.5 μM [3H]Mtx was assayed in cell suspensions over 2 min at 37°C in HEPES-buffered saline (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 2 mM MgCl₂, and 5 mM glucose) at pH 6.8 or 7.2, or in MES-buffered saline (20 mM MES, 140 mM NaCl, 5 mM KCl, 2 mM MgCl₂, and 5 mM glucose) at pH 5.5, 6.0, or 6.5 (Zhao et al., 2004a). At the end of the incubations, transport was quenched with ice-cold DPBS, cells were washed three times with ice-cold DPBS, and cellular proteins were solubilized with 0.5 N NaOH. Levels of drug uptake were expressed as picomoles per milligram of protein, calculated from direct measurements of radioactivity and protein contents of cell homogenates. Radioactivity was measured with a scintillation counter (model LS6500; Beckman Coulter), and proteins were quantified using Folin-Pirolen reagent (Lowry et al., 1951). To determine Kᵢ values for hPCFT and inhibition of transport was determined from pH 5.5 to 7.2 in the above MES and HEPES buffers over 2 min at 37°C with 0.5 μM [3H]Mtx and 10 μM inhibitor. For hPCFT, inhibition of transport was determined from pH 5.5 to 7.2 in the above MES and HEPES buffers over 2 min at 37°C with 0.5 μM [3H]Mtx and 10 μM inhibitors. Kᵢ values for hPCFT were calculated from Dixon analysis by plotting reciprocal transport velocities measured over a range (1–5 μM) of inhibitor concentrations and 0.5 μM [3H]Mtx at pH 5.5 and pH 6.8. Kᵢ values were calculated from the slopes, Kᵢ and Vₘ₃ values for Mtx, and the concentration of [3H]Mtx, using equation Kᵢ = Kᵢ/Vₘ₃(slope)/S, where S is the substrate concentration.

Electrophysiology Experiments. X. laevis oocytes were used to assess currents associated with transport of the antifolate substrates. PCFT cRNA (50 nl of 0.5 μg/μl; i.e., 25 ng) or water (50 nl) was injected into stage VNI oocytes, and electrophysiological measurements were made 3 to 5 days later (Unal et al., 2009). Oocytes were voltage-clamped to −90 mV to maximize folate-induced currents, a technique that was used in studies on the divalent metal transporter DMT1 (Gunshin et al., 1997; Mackenzie et al., 2006) and PCFT (Unal et al., 2009). Oocyte solutions were adjusted to pH 5.5 using MES, pH 5.5. During these experiments, oocytes were continuously superfused with solution (with and without antifolates as indicated) at 5 ml/min.

Indirect Immunofluorescence and Confocal Microscopy. For confocal microscopy, R2/hPCFT4 and R2/VC CHO cells were plated in Lab-Tek II chamber slides (Nalge Nunc International, Naperville, IL). Cells were fixed with 3.3% paraformaldehyde (in DPBS), permeabilized with 0.1% Triton X-100 (in phosphate-buffered saline), and stained with mouse anti-c-myc monoclonal antibody (Covance) and Alexa Fluor 488 donkey anti-mouse IgG (H + L) secondary antibody (Invitrogen). Slides were visualized with a Zeiss laser-scanning microscope 510 with a 63× water immersion lens with exactly the same parameter setting for all samples. Confocal analysis was performed at the Imaging Core of the Karmanos Cancer Institute.

Proliferation and Colony-Forming Assays. For growth inhibition assays, R2/hPCFT4 CHO and R1-11-PCFT4 HeLa cells were plated in 96-well culture dishes (2500 and 5000 cells/well, respectively; total volume of 200 μl of medium) with a broad concentration range of drugs. The drugs were dissolved in DMSO such that after dilution, the DMSO concentration did not exceed 1%. The medium was folate-free RPMI 1640, pH 7.2, containing 25 mM LCV, supplemented with 10% dialyzed fetal bovine serum (dFBS; Invitrogen), 2 mM l-glutamine, and 100 units/ml penicillin/100 μg/ml streptomycin. Cells were routinely incubated for up to 96 h, and metabolically active cells (a measure of cell viability) were assayed with CellTiter blue cell viability assay (Promega). Fluorescence was measured (emission at 590 nm, excitation at 560 nm) with a fluorescence plate reader (Molecular Devices, Sunnyvale, CA). Data were exported from SoftMax Pro software (Molecular Devices) to a spreadsheet (Excel; Microsoft Corp., Redmond, WA) for analysis and determinations of

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IC_{50} values corresponding to drug concentrations that result in 50% loss of cell growth. In some experiments, the protective effects of adenosine (60 μM), thymidine (10 μM), and 5-amino-4-imidazolecarboxamide (AICA) (320 μM) were tested to validate the intracellular inhibitory locus for the cytotoxic antifolates. Growth inhibition assays for the PC43-10 CHO and R1-11-RFC6 HeLa sublines were routinely performed in complete RPMI 1640 with 10% dFBS, although for a few experiments, PC43-10 and R1-11-RFC6 cells were cultured exactly as for the R2/hPCFT cells. To follow changes in pH accompanying cell outgrowth, cells were seeded into T75 flasks, using the same media, cell number-to-volume ratio, and incubation times as for the cytotoxicity assays. Media pH values were measured daily with an Orion 2 Star benchtop pH meter.

For colony-forming assays, R2/hPCFT4 cells (500 cells) were harvested in log-phase and plated into 60-mm dishes in folate-free RPMI 1640 medium, supplemented with 25 mM LCV, 10% dFBS, penicillin-streptomycin, and 2 mM L-glutamine, in the presence of assorted antifolate drugs. The dishes were incubated at 37°C with 5% CO₂ for 10 days. At the end of the incubations, the dishes were rinsed with DPBS, 5% trichloroacetic acid, and borate buffer (10 mM, pH 8.8), followed by 1% methylene blue (in borate buffer; 30 min). The dishes were again rinsed with borate buffer, and colonies were counted for calculating percentage of colony formation relative to the DMSO control. To test the reversibility of drug effects, as reflected in inhibition of colony formation over time, R2/hPCFT4 cells were harvested in log phase and 500 cells were plated, allowed to adhere for 48 h, then cultured in the presence or absence of 1 μM antifolate compounds and thymidine (10 μM) plus adenosine (60 μM) for 2, 4, 8, 24, or 48 h, before rinsing with DPBS and adding medium with or without thymidine (10 μM) plus adenosine (60 μM). The dishes were incubated for 10 days, and colonies were stained with methylene blue and counted for calculating percentage of colony formation compared with control.

In Situ Assays for GARFTase. Incorporation of [14C(U)]glycine into [14C]formyl β-glycaminde ribonucleotide (GAR) as an in situ measure of endogenous GARFTase activity in R2/hPCFT4 cells was performed using a modification of published methods (Beardsley et al., 1989; Deng et al., 2008). For these experiments, R2/hPCFT4 cells were seeded in 5 ml of folate-free RPMI 1640 plus 25 mM LCV, 10% dFBS, 2 mM L-glutamine, and penicillin-streptomycin in T25 flasks at a density of 2 × 10⁵ cells per flask. After 48 h, antifolate inhibitor or DMSO (control) was added to the culture medium, and the cells were incubated for another 15 h, after which the pH of the media was determined. Cells were washed twice with DPBS and resuspended in 5 ml of folate-free, 10% L-glutamine-free RPMI 1640 (Sigma) plus penicillin-streptomycin, 10% dFBS, 0.46 g/liter NaHCO₃ and 1.21 g/liter NaCl medium, with or without 0.5 to 100 nM antifolate and azaserine (4 μM final concentration), and incubated for 30 min. l-Glutamine (final concentration, 2 mM) and [14C]glycine (final specific activity, 0.1 μCi/liter) were added, followed by incubation at 37°C for 8 h, after which cells were trypsinized and washed twice with ice-cold DPBS. Cell pellets were treated with 2 ml of 5% trichloroacetic acid at 0°C. Cell debris was removed by centrifugation, and samples were solubilized in 0.5 N NaOH and assayed for protein contents (Lowry et al., 1951). The supernatants were extracted twice with 2 ml of ice-cold ether to remove the trichloroacetic acid. The aqueous layer was passed through a 1-cm column of AG1 × 8 (chloride form), 100 to 200 mesh (Bio-Rad Laboratories, Hercules, CA), washed with 10 ml of 0.5 N formic acid followed by 10 ml of 4 N formic acid, and eluted with 8 ml of 1 N HCl solution. The eluants were collected as 1-ml fractions and determined for radioactivity.

Determination of Intracellular ATP/GTP Levels. For analysis of ATP and GTP levels after antifolate treatments, R2/hPCFT4 cells were seeded in 10 ml of folate-free RPMI 1640 plus 25 mM LCV, 10% dFBS, 2 mM L-glutamine, and penicillin-streptomycin in T75 flasks at a density of 7 × 10⁵ cells per flask. After 48 h, antifolates or DMSO (control) were added to the culture medium. After another 24 h, the cells were trypsinized and washed (2×) with ice-cold DPBS, with a final additional wash with ice-cold DPBS containing 1 mM EDTA. The final cell pellet (2.5 × 10⁶ cells) was resuspended in 100 μl of 155 mM NaCl containing 1 mM EDTA, and 100 μl of ice-cold 0.6 M trichloroacetic acid was added drop-wise while vortexing. Samples were incubated 10 min on ice with occasional mixing, then centrifuged (14,000 rpm, 5 min). The supernatant was removed, whereas the protein pellet was solubilized in 0.2 ml of 0.5 N NaOH for protein determinations. Tri-n-octylamine (0.5 M) in trichlorotrifluoroethane (Freon) (1 ml) was added to the supernatant, and the mixtures were vortexed and incubated for 20 min on ice. Samples were centrifuged, and the trichlorotrifluoroethane amine (lower) layer was discarded. One ml of methylene chloride was added to the upper layer, followed by mixing, incubation (ice, 10 min), centrifugation, and removal of the organic (lower) layer. Samples were stored at −80°C until analysis. Intracellular ATP and GTP were measured by a modification of the HPLC method of Huang et al. (2003). The chromatography system consisted of a Varian 9010 ternary gradient pump, a 9050 variable wavelength detector, and a Varian Star 5.3 data handling system. A 50-μl injection loop was used. The analytical column was a Waters Symmetry C18 (5 μm, 150 × 4.6 mm) equipped with a Waters Novapak phenyl prep column (Waters, Milford, MA). The detection wavelength was set at 254 nm. The flow rate was 1 ml/min. The gradient elution was as follows: 0 to 30 min at 60% A/40% B; 30 to 50 min linear at 1%/min to 40% A/60% B; and 50 to 60 min at 40% A/60% B. Buffer A was composed of 10 mM tetrabutylammonium hydroxide, 10 mM KH₂PO₄, and 0.25% MeOH; the pH was adjusted to 6.9 with 1 N H₃PO₄. Buffer B consisted of 5.6 mM tetrabutylammonium hydroxide, 50 mM KH₂PO₄, and 30% MeOH; the pH was adjusted to 7 with 1 N KOH. Both solutions were freshly prepared before each experiment and degassed. External standards were used for each assay to construct a standard curve from which cellular levels were calculated. Standards ranged from 0 to 75 μM for ATP and 0 to 30 μM for GTP in the initial mobile phase. Variations between standards were 5% or less. Extraction efficiencies were established by adding known amounts of ATP and GTP standards (200 and 50 μM, respectively) to a control sample before extraction.

Results

Generation of hPCFT Stable Transfectants in Transport-Impaired CHO Cells. As part of our larger drug discovery endeavor to establish pharmacophores for all the major folate transporters (Deng et al., 2008, 2009) and to develop transporter-specific drugs, we previously generated novel sublines derived from the RFC-, FR-, and PCFT-null MtxRIIOuA2-4 CHO cells (hereafter, simply R2) that ectopically express hRFC (designated PC43-10) (Wong et al., 1995) and human FRs (Deng et al., 2008).

More recently, we described another R2 (R2/hPCFT4) that expressed hPCFT, although few details were provided (Deng et al., 2009). R2/hPCFT4 cells were generated by electroporating R2 CHO cells with a Myc-His6-tagged hPCFT (hPCFT'Myc-His₆) cDNA construct. Stable transfectants were selected with G418. Clones were isolated, expanded, and screened by Western blotting. A clonal R2/hPCFT4 subline was established that expressed a high level of hPCFT'Myc-His₆ protein (Fig. 2A). By indirect immunofluorescence staining with Myc-specific antibody and Alexa Fluor 488-tagged secondary antibody, hPCFT'Myc-His₆ protein was targeted to the cell surface of R2/hPCFT4 cells (Fig. 2B). Expression of hPCFT'Myc-His₆ protein was accompanied by substantial [3H]Mtx transport at pH 5.5 during 2 min over the low level measured in vector control R2/Vc cells (Fig. 2C). Transport at pH 7.2 was ~14% of that at pH 5.5; at pH 6.8.

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transport increased to ~35% of that at pH 5.5. For hRFC-expressing PC43-10 cells, \([3H]\)Mtx transport was active at pH 7.2, as reported previously (Wong et al., 1995), then fell with decreasing pH and was essentially indistinguishable from the residual low level in R2 cells at pH 5.5 (Fig. 2D).

We measured the kinetics for \([3H]\)Mtx and \([3H]\)Pmx transport in R2/hPCFT4 cells over a range of concentrations at pH 5.5 and 6.8. Data were analyzed by Lineweaver-Burke plots and are summarized in Table 1. Results for Mtx showed a 16-fold decrease in \(K_t\) and a 2.3-fold increase in \(V_{max}\) when the pH was decreased from 6.8 to 5.5, whereas for Pmx, only the \(K_t\) was changed (10.7-fold) with pH. \(V_{max}/K_t\) values, a reflection of overall transport efficiency, were calculated for both \([3H]\)Mtx and \([3H]\)Pmx and were 37- and 11-fold higher, respectively, at pH 5.5 than at pH 6.8.

Thus, as previously reported (Qiu et al., 2006; Zhao and Goldman, 2007), transport by hPCFT shows an extraordinary pH dependence, with the greatest activity at acidic pH. Furthermore, the impact of pH on kinetic parameters for hPCFT membrane transport varies with different transport substrates (Zhao et al., 2009).

**Chemosensitivities to Classic Antifolate Inhibitors and Identification of a 6-Substituted Pyrrolo[2,3-d]pyrimidine Antifolate with hPCFT Selectivity over hRFC.** We screened hPCRF- and hRFC-expressing cell lines for growth inhibition in the continuous presence of established antifolates including Mtx, GW1843U89, Lmx, Pmx, PT523, and Rtx (Table 2). Assays were performed at pH 7.2 in standard RPMI 1640/10% dFBS (for hRFC-expressing R2/hPCFT4 cells over respective controls (Table 2). For most of the antifolates, drug sensitivities, as reflected in decreased IC_{50} values for inhibition of growth over 96 h, were increased in both R2/hPCFT4 and PC43-10 cells over respective controls (Table 2). Neither GW1843U89 nor PT523 showed any activity toward hPCFT-expressing R2/hPCFT4 cells. Because hPCFT is optimally active at acidic pH (Fig. 2C), we measured the changes in media pH during the interval of drug exposure. Over 96 h, the pH of the media decreased linearly and reached pH 6.7 to 6.9 by day 4 (not shown).

Thus, at extracellular pH approximating that associated with solid tumor microenvironments (Helmlinger et al., 1997; Raghunand et al., 1999), several of these classic agents seemed to be substrates for hPCFT in R2/hPCFT4 cells, as reflected in patterns of growth inhibition. PT523 and GW1843U89 were completely selective toward hRFC over hPCFT. Only Pmx showed any indication of selective activity toward hPCFT over hRFC, (i.e., 74-fold increased activity for R2/hPCFT4 cells versus 6.5-fold for the PC43-10 cells, compared with respective controls). However, this was incomplete; i.e., Pmx was appreciably active toward both hPCFT- and hRFC-expressing cells.

We previously tested a series of 6-substituted pyrrolo[2,3-d]pyrimidine antifolates with increasing numbers (one and three to six) of methylene groups in the bridge region connecting the pyrrolo[2,3-d]pyrimidine moiety to p-aminobenzoate (compounds 1-5, respectively; Fig. 1) as growth inhibi...
tors of FR-expressing CHO and human tumor cells (Deng et al., 2008). Compounds 2 and 3 were the most potent of this series toward FR-expressing CHO and KB cells and inhibited de novo purine nucleotide biosynthesis at the level of GARFTase. For the present study, we synthesized the additional pyrrolo[2,3-\(d\)]pyrimidine analog with a 2-carbon bridge (compound 1a in Fig. 1) to complete the series.

Compounds 1 to 5 and 1a were all effectively inert as inhibitors of cell growth for RFC-expressing PC43-10 cells, either in standard RPMI 1640 (Table 2) or folate-free RPMI supplemented with 25 nM LCV (not shown). Although compounds 1, 1a, 4, and 5 were likewise inactive toward hPCFT-expressing R2/hPCFT4 cells, compounds 2 and 3 were both active, with IC\(_{50}\) values of ~23 and ~213 nM, respectively. With human HeLa R1-11 cells transfected with hRFC (R1-11-RFC6) and hPCFT (R1-11-PCFT4) and treated with assorted antifolates (Mtx, Pmx, Lmx, 2), only compound 2 was selective for hPCFT (Table 2). The decreased (~7-fold) sensitivity in the R1-11-PCFT4 cells versus R2/hPCFT4 CHO cells probably reflects differences (~10-fold) in hPCFT transport activity at pH 5.5 between these engineered sublines (Supplemental Fig. 1S).

As in our previous FR-targeting studies with compounds 2 and 3 (Deng et al., 2008), both adenosine (60 \(\mu\)M) and AICA (320 \(\mu\)M) were completely protective toward R2/hPCFT4 cells with the most potent agent of this series (compound 2), further establishing GARFTase as the likely cytotoxic target (Supplemental Fig. 2S).

Cytotoxicity assays were extended to include colony-forming assays, in which R2/hPCFT4 cells were continuously exposed to a range of concentrations of compound 2, Pmx, or Lmx for 10 days. As an inhibitor of colony formation, compound 2 gave an IC\(_{50}\) of 17.14 ± 0.74 nM (S.E.M.), whereas IC\(_{50}\) values for Pmx and Lmx were 4.94 ± 0.48 and 29.70 ± 0.59 nM, respectively (Fig. 3 and Supplemental Fig. 3S).

To establish the time-dependent requirements for loss of clonogenicity upon exposure to compound 2, Lmx, or Pmx, R2/hPCFT4 cells were exposed to the drugs (each at 1 \(\mu\)M) for different times (2, 4, 8, 24, 48, and 72 h), after which drug was removed and cells were incubated in the presence or absence of adenosine (60 \(\mu\)M) and thymidine (10 \(\mu\)M). A parallel incubation was performed in which cells were treated with drugs and nucleosides, after which drugs were washed out and cells were incubated in the presence of adenosine and thymidine. Colonies were counted after 10 days, with results compared with those for the untreated vehicle (DMSO) control. Nucleoside protection, both during and after drugs were washed out, completely protected R2/hPCFT4 cells from loss of colony formation. When cells were treated during the initial incubation with antifolates without nucleoside protection, colony formation was significantly inhibited whether or not nucleosides were included after the drug was removed (Fig. 4 and Supplemental Fig. 4S). The difference between these two conditions established a time-requirement for irreversible drug effects resulting in loss of clonogenicity and ranged from greater than 4 h for Pmx (A) and compound 2 (B) to greater than 8 h for Lmx (C). For all drugs thereafter, there was a progressive diminution of the protective effects such that by 48 to 72 h, loss of colony formation was essentially complete.

### TABLE 1

Kinetic constants for hPCFT

<table>
<thead>
<tr>
<th>Substrates and Parameters</th>
<th>pH 5.5</th>
<th>pH 6.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mtx</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(K_c) ((\mu)M)</td>
<td>0.280 ± 0.022</td>
<td>4.52 ± 0.19</td>
</tr>
<tr>
<td>(V_{max}) (pmol/mg/min)</td>
<td>31.23 ± 4.31</td>
<td>13.72 ± 2.26</td>
</tr>
<tr>
<td>(V_{max}/K_c)</td>
<td>111.5</td>
<td>3.0</td>
</tr>
<tr>
<td>Pmx</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(K_c) ((\mu)M)</td>
<td>0.124 ± 0.014</td>
<td>1.33 ± 0.26</td>
</tr>
<tr>
<td>(V_{max}) (pmol/mg/min)</td>
<td>27.11 ± 4.27</td>
<td>27.59 ± 6.38</td>
</tr>
<tr>
<td>(V_{max}/K_c)</td>
<td>218.6</td>
<td>20.7</td>
</tr>
<tr>
<td>Compound 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(K_c) ((\mu)M)</td>
<td>0.223 ± 0.017</td>
<td>4.07 ± 0.34</td>
</tr>
<tr>
<td>Lmx</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(K_c) ((\mu)M)</td>
<td>0.0960 ± 0.012</td>
<td>1.54 ± 0.17</td>
</tr>
<tr>
<td>N.D.</td>
<td></td>
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</table>

### TABLE 2

Growth inhibition by antifolate drugs toward hPCFT- and hRFC-stable R2 CHO transfectants

<table>
<thead>
<tr>
<th>Antifolate</th>
<th>R2/hPCFT4</th>
<th>R2/VC</th>
<th>PC43-10</th>
<th>R2</th>
<th>R1-11-RFC6</th>
<th>R1-11-PCFT4</th>
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</thead>
<tbody>
<tr>
<td>GW1843U89</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>12 ± 1.1</td>
<td>216 ± 9</td>
<td>35.7 ± 7.1</td>
</tr>
<tr>
<td>Lmx</td>
<td>38.6 ± 5.0</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>278.3 ± 26.2</td>
</tr>
<tr>
<td>Pmx</td>
<td>13.2 ± 2.4</td>
<td>974.0 ± 18.1</td>
<td>138 ± 13</td>
<td>894 ± 93</td>
<td>817 ± 5.5</td>
<td>59.3 ± 7.4</td>
</tr>
<tr>
<td>PT523</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>1a</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>2</td>
<td>23.0 ± 3.3</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>155.0 ± 12.9</td>
</tr>
<tr>
<td>3</td>
<td>212.9 ± 28.0</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
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<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

N.D., not determined.
parallel experiment was performed with PC43-10 cells to assess the inhibitory effects of compound 2 (10 μM) on hRFC-mediated 
\[^{3}H\]Mtx uptake (at pH 7.2) compared with other established hRFC transport substrates. As shown in Fig. 5A, with R2/hPCFT4 cells, compound 2 was a potent inhibitor of hPCFT transport, only slightly less so than Pmx and with substantially increased potency at pH values less than 7.2. As expected (Zhao and Goldman, 2007), PT523 did not inhibit \[^{3}H\]Mtx uptake at any pH for R2/hPCFT4 cells. For hRFC-expressing PC43-10 cells at pH 7.2, PT523, Pmx, Rtx, Lmx, and LCV all potently inhibited \[^{3}H\]Mtx transport (Fig. 5B). However, compound 2 was completely inert as an inhibitor of hRFC.

We used Dixon analysis at pH 5.5 and 6.8 with R2/hPCFT4 cells and \[^{3}H\]Mtx to calculate \(K_i\) values for hPCFT competitors, including compound 2 and Pmx (Table 1). We also determined the \(K_i\) for Lmx. Transport of 0.5 μM \[^{3}H\]Mtx was measured over a range of inhibitor concentrations. Compound 2 showed an 18-fold lower \(K_i\) at pH 5.5 than at pH 6.8, approximating the 16-fold difference in \(K_i\) values for Pmx. Lmx was potently inhibitory at pH 5.5; however, transport inhibition by Lmx was substantially reduced at pH 6.8. For Pmx, the \(K_i\)s closely approximated the \(K_i\)s recorded with \[^{3}H\]Pmx.

To confirm that compound 2 is transported by hPCFT, electrophysiological studies were performed in \(X. laevis\) oocytes injected with hPCFT cRNA. Uptake was assessed in oocytes clamped to −90 mV at a bath pH of 5.5. A substrate concentration of 5 μM was used, which is saturating for LCV and Pmx. These experiments show that the current induced

![Fig. 3. Inhibition of colony formation by compound 2. R2/hPCFT4 cells were plated into 60-mm dishes at a density of 500 cells per dish in the presence or absence of different concentrations of compound 2, Pmx, and Lmx from 1 to 100 nM. Colonies were scored by counting visible colonies after 10 days and are presented as a percentage of the vehicle control. Results are presented as mean values ± S.E. from three experiments.](image)

![Fig. 4. Time dependence for loss of clonogenicity in R2/hPCFT4 cells treated with compound 2, Pmx, and Lmx. R2/hPCFT4 cells were plated into 60-mm dishes at 500 cells per dish and allowed to adhere for 48 h, after which cells were treated with or without 1 μM drug in the presence or absence of adenosine (60 μM) and thymidine (10 μM) for 2, 4, 8, 24, and 48 h. After drug treatment, cells were washed with DPBS and drug-free media with or without adenosine (60 μM) and thymidine (10 μM) were added. Colonies were enumerated after 10 days, and results are presented as a percentage of vehicle control. A, Pmx. B, compound 2. C, Lmx. Results are presented as mean values ± S.E. from three experiments. Ade, adenosine; Thd, thymidine.](image)

![Fig. 5. Competitive inhibition of hPCFT and hRFC transport of \[^{3}H\]Mtx by compound 2, classical antifolates, and folates. A, R2/hPCFT4 cells ectopically expressing hPCFT but no FR or RFC were assayed for \[^{3}H\]Mtx transport in the presence of 10 μM compound 2, Pmx, and PT523 at pH 5.5 to 7.2. B, PC43-10 cells expressing hRFC but no PCFT or FR were assayed for \[^{3}H\]Mtx transport in the presence of 10 μM compound 2, PT523, Pmx, Rtx, Lmx, or LCV at pH 7.2. Results are presented as mean values ± S.E. from more than three experiments.](image)
by compound 2 was comparable with that produced by LCV (Fig. 6A) or Pmx (Fig. 6B).

**Identification of De Novo Purine Nucleotide Biosynthesis and GARFTase As Primary Cellular Targets for Compound 2.** Our protection studies further identified de novo purine nucleotide biosynthesis as the primary targeted pathway after hPCFT transport of the 6-substituted pyrrolo[2,3-d]pyrimidine antifolate 2. By HPLC analysis, absolute levels of ATP and GTP in untreated R2/hPCFT4 cells were 43.85 and 9.43 μmol/mg, respectively. ATP and GTP pools were severely depleted (approximately 50 and 75%, respectively) during a 24-h exposure of R2/hPCFT4 cells to either compound 2 or Lmx. For ATP pools, IC50 values of 58 and 166 nM were measured for compound 2 and Lmx, respectively (Fig. 7). For GTP pools, IC50 values were 441 and 579 nM, respectively.

To confirm GARFTase inhibition and to provide a metabolic “read-out” for hPCFT transport of compound 2 in R2/hPCFT4 cells, we used an in situ assay for GARFTase. GARFTase catalyzes formylation of the glycine-derived nitrogen of GAR, producing formyl GAR with 10-formyl tetrahydrofolate as the one-carbon donor. The in situ GARFTase assay measures incorporation of [14C]glycine into [14C]formyl GAR in the presence of azaserine (4 μM) (Beardsley et al., 1989; Deng et al., 2008). R2/hPCFT4 cells were cultured for 48 h in complete folate-free media supplemented with 25 nM LCV. The 48 h incubation allowed the cells to adhere and the pH of the culture media to decrease to ~6.9 accompanying cell growth. Cells were then treated for 15 h with or without a range of concentrations of compound 2, or with Pmx or Lmx, after which cells were washed, resuspended in L-glutamine- and folate-free medium, then treated with azaserine, L-glutamine, and [14C]glycine. After an additional 8 h, cells were washed, proteins were precipitated with trichloroacetic acid, and the supernatants were ether-extracted and fractionated on anion exchange columns so that [14C]formyl GAR could be measured and IC50 values calculated.

Our results demonstrate that Pmx, Lmx, and compound 2 all inhibited [14C]formyl GAR accumulation in R2/hPCFT4 cells at pH 6.9 when hPCFT was the sole mode of antifolate drug delivery (Fig. 8). Compound 2 was by far the most potent of these drugs, with an impressive IC50 of 0.97 nM for GARFTase inhibition, compared with IC50 values of 7.3 nM for Pmx and 31.5 nM for Lmx. For Lmx, which inhibits GARFTase as its primary intracellular target, the IC50 for in situ inhibition was similar to that recorded for loss of clonogenicity (Fig. 3). However, for compound 2, there was a 17.5-fold differential.

**Discussion**

Antifolates continue to comprise an important component of the chemotherapy arsenal for cancer (Hughes et al., 1999;...
Chemotherapy activity of classic antifolates has traditionally been interpreted in terms of their active membrane transport into tumors by RFC (Matherly et al., 2007). Furthermore, impaired membrane transport due to loss or mutations of RFC was reported to result in antifolate resistance (Zhao and Goldman, 2003; Assaraf, 2007; Matherly et al., 2007). However, after reports of a novel low-pH transporter termed PCFT (Qiu et al., 2006; Nakai et al., 2007; Zhao and Goldman, 2007) and the recognition that cells can efficiently transport antifolates at pH values approximating those of solid tumors (Zhao et al., 2004a), it becomes necessary to examine the possibility that PCFT could represent an important mode of chemotherapy drug transport.

Because most cultured cells endogenously express more than one folate transport system (Kugel Desmoulin et al., 2010), to explore the role of hPCFT in delivery of cytotoxic antifolates we engineered the R2/hPCFT4 CHO subline from R2/PCFT-, RFC-, and FR-null R2 cells to express hPCFT without either RFC or FRs (Deng et al., 2009). R2/hPCFT4 cells exhibited optimal transport activity at acidic over neutral pHs, reflecting high-affinity transport of substrates with decreasing pH. The impact of pH on transport by PCFT was substantially different between (antifolate substrates. By growth inhibition assays, R2/hPCFT4 cells were sensitive to classic antifolates, including Mtx, Rtx, Pmx, and Lmx, suggesting their membrane transport by hPCFT; Pmx was most active and neither GW1843U89 nor PT523 was growth inhibitory. Although hPCFT exhibits limited transport at neutral pH typical of culture media, transport of these cytotoxic antifolates by hPCFT was enhanced by the progressively decreasing pH that accompanies cell outgrowth.

Pmx is a 5-substituted pyrrolo[2,3-d]pyrimidine analog and is the best substrate yet described for PCFT (Zhao and Goldman, 2007). Because Pmx was a potent inhibitor of R2/hPCFT4 cell growth, we compared the growth inhibitory effects of a number of structurally related 6-substituted pyrrolo[2,3-d]pyrimidine antifolates with bridge lengths from one to six methylenes as inhibitors of R2/hPCFT4 cell proliferation. The analogs with three and four methylenes (2 and 3, respectively) were potent inhibitors of R2/hPCFT4 cell growth or clonogenicity, compound 2 showing an IC50 ≥ 2-fold higher than that for Pmx. However, compound 2 was essentially inert toward hRFC-expressing PC43-10 cells. These findings were extended to hRFC- and hPCFT-expressing HeLa cell line models. It is noteworthy that compound 2 selectively inhibited transport of [3H]Mtx by hPCFT with potency only slightly less than that for Pmx, as reflected in K values, and with nearly identical pH dependence. After microinjection of hPCFT cRNA into X. laevis oocytes, perfusion with a saturating concentration of compound 2 elicited a current, confirming that compound 2 is electrogenically transported by hPCFT. Collectively, these results establish that the cytotoxic 6-substituted pyrrolo[2,3-d]pyrimidine compound 2 is a bona fide transport substrate for hPCFT, essentially on par with Pmx. However, unlike Pmx, compound 2 has nominal transport activity with hRFC.

Compound 2 was previously reported to be cytotoxic toward cells that express high levels of FRα, reflecting inhibition of GARFTase, the trifunctional enzyme that catalyzes the second, third, and fifth reactions of de novo purine nucleotide biosynthesis, including the first folate-dependent step (Deng et al., 2008). Consistent with primary inhibition of GARFTase after transport by hPCFT, both adenosine and AICA protected R2/hPCFT4 cells from growth inhibition by compound 2. By an in situ GARFTase assay, which measures [14C]glycin incorporation into formyl GAR, compound 2 was disproportionately inhibitory, with an IC50 less than 1 nM, far lower than the IC50 for Lmx. Although Pmx is primarily an inhibitor of thymidylate synthase and was recently reported to inhibit AICA ribonucleotide formyltransferase in CCRF-CEM cells (Racanelli et al., 2009), in R2/hPCFT4 CHO cells, appreciable GARFTase inhibition was detected, albeit less than that for compound 2.

Our results establish that hPCFT is a surprisingly efficient means of cytotoxic drug delivery. The much higher concentrations of compound 2 needed to inhibit colony formation/cell proliferation or to significantly suppress ATP/GTP pools versus those required to inhibit GARFTase in cells must reflect the nature of the enzyme target and requirement for sustained inhibition of GARFTase and de novo purine biosynthesis for cell killing. Indeed, sustained exposures to GARFTase inhibitors were required to irreversibly inhibit colony formation of R2/hPCFT4 cells, although an analogous time-dependence was obtained with Pmx. Similar results were previously reported when comparing effects on clonogenicity of GARFTase inhibition by Lmx versus TS inhibition by Rtx in WiDr colonic carcinoma cells (Smith et al., 1993).

The higher concentrations of Lmx over compound 2 needed to inhibit GARFTase in cells relative to those required to manifest cytotoxicity provide further evidence that GARFTase inhibition is not limiting to cell killing. The decreased GARFTase inhibition for Lmx in R2/hPCFT4 cells probably reflects its reduced transport by hPCFT compared with compound 2, although factors such as differences in the extent of polyglutamate synthesis may also contribute. We previously reported high-level substrate activity for compound 2 for human folylpolyglutamate synthetase (Gangjee et al., 2004).
The delay in irreversible drug effects upon inhibition of GARFTase may reflect salvage of purines generated from breakdown of nucleic acids (Smith et al., 1993; Bronder and Moran, 2002). Accordingly, the delay for irreversible cell death by GARFTase inhibitors may be substantially shortened in cells that have defects in purine salvage, increasing dependence on de novo purine synthesis. For instance, deletions of S-methyl-5′-thioadenosine phosphorylase have been described in human malignancies, including ~70% of pleural mesotheliomas (Illies et al., 2003) and 38% of non–small-cell lung cancers (Schmid et al., 1998). On this basis, GARFTase inhibitors such as compound 2 may show far greater potency in S-methyl-5′-thioadenosine phosphorylase-deficient tumors, especially if there are high levels of hPCFT.

Finally, the present results, combined with earlier studies of 6-substituted pyrrolo- and thieno[2,3-d]pyrimidine antifolates (Deng et al., 2009; Wang et al., 2010), shed light on the impact of both aromatic ring systems and the length of the bridge domain on transport by PCFT versus other folate transporters. Thus, antifolates with thieno[2,3-d]pyrimidine and benzoyl rings (designated A and B rings, respectively) (labeled ‘I’ in Fig. 9) are effective transport substrates for FRs but not for RFC or PCFT, with optimal activity for the 3- and 4-carbon bridge analogs (Deng et al., 2009). Replacement of the thieno[2,3-d]pyrimidine A ring with a pyrrolo[2,3-d]pyrimidine system favors binding and transport by both PCFT and FRs regardless of whether the B ring is a benzoyl (e.g., compound 2) or thiophene (‘II’ in Fig. 9). For the pyrrolo[2,3-d]pyrimidine benzoyl series (Fig. 1), the 3-carbon bridge analog (compound 2) was the most potent toward cells expressing PCFT. For other analogs differing in lengths of the carbon bridge, PCFT activity was reduced (compound 3) or abolished (compounds 1, 1a, and 4 to 6). It is noteworthy that the bridge length requirement for FR uptake for the pyrrolo[2,3-d]pyrimidine benzoyl series was less restrictive (Deng et al., 2008).

In conclusion, we document hPCFT-selective transport over hRFC for the potent 6-substituted pyrrolo[2,3-d]pyrimidine antifolate compound 2. Our results strongly suggest the therapeutic potential of hPCFT for targeting drugs to tumors. The notion of hPCFT drug targeting is appealing given the acidic pH optimum for this system and the low pH microenvironment of many solid tumors (Helmlinger et al., 1997; Raghunand et al., 1999). In tumors that express both hRFC and hPCFT, loss of one or the other transporter should not affect net sensitivity to antifolates such as Pnx that are normally transported by hRFC that compete for polyglutamylated metabolite, 5,10-dideaza-5,6,7,8-tetrahydrofolate is a potent inhibitor of de novo purine synthesis. J Biol Chem 264:322–332.


Fig. 9. Structures of 6-substituted thieno- and pyrrolo[2,3-d]pyrimidine antifolates. The novel antifolate analogs include those described by Deng et al. (2009) and Wang et al. (2010).


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