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-[4-[3-2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]-pyrimidin-6-yl]propyl]benzylo]-L-glutamic acid (compound 2) and N-[4-[4-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]-pyrimidin-6-yl]butyl]benzylo]-L-glutamic acid (compound 3), respectively) were inhibitory, with 2 \( \geq 3 \). Activity toward RFC-expressing cells was negligible. Compound 2 and pemetrexed (Pmx) competed with \(^{3}H\)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 glycinamide 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.

ABBREVIATIONS: RFC, reduced folate carrier; FR, folate receptor; PCFT, proton-coupled folate transporter; PT523, \( N^\alpha\)-(4-amino-4-deoxypheroyl)-\( N^\beta\)-hemiphthalalloyl-L-ornithine; GW1843U89, \( S\)-2-(5-[[((1,2-dihydro-3-methyl-1-oxo-benzofquinazolin-9-yl) methyl] amino]-1-oxo-2-isonoldylnil)] glutaric acid; GARFTase, glycinamide ribonucleotide formyltransferase; hPCFT, human protein-coupled folate transporter; Mtx, methotrexate; Pmx, pemetrexed; Lmx, lometrexol; Rtx, raltitrexed; HFFC, human reduced folate carrier; LCV, leucovorin; HPLC, high-performance liquid chromatography; CHO, Chinese hamster ovary; MEM, \( \alpha \)-minimal essential media; DPBS, Dulbecco’s phosphate-buffered saline; MES, 2-(N-morpholino)ethanesulfonic acid; DMSO, dimethyl sulfoxide; dFBS, dialyzed fetal bovine serum; AICA, 5-amino-4-imidazolecarboxamide; GAR, glycinamide ribonucleotide; R2 cells, MtxR\( ^{25} \)-CHO cells.
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), GW1843US9, pemetrexed (Pmx)], and the purine biosynthetic enzymes β-glycinamidase, ribonucleotide formyltransferase (GARFTase) [lometrexol (Lmx), Pmx] and 5-amino-4-imidazolecarboxamide 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 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, GW1843US8) (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-(3,4-dihydro-3-methyl-4-oxoquinazolin-6-ylmethyl)-N-methyl-amino]-2-tetany] glutamic acid] was obtained from AstraZeneca Pharmaceuticals (Macclesfield, Cheshire, UK); Lmx (5,10-dideaza-5,6,7,8-tetrahydrofolate), and Pmx (Alimta) were from Eli Lilly and Co. (Indianapolis, IN); GW1843US8 [5S]-2,4S-[1(12-dihydro-3-methyl-1-oxo-benzo)[quinoxalin-9-yl] methyl] amino1-oxo-2-isoinolinyl] glutaric acid] was from GlaxoSmithKline (Research Triangle Park, NC); and N'- (4-amino-4-deoxytroyl)-N''-hemihemathalyl-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.

**Cell Lines.** PCFT-, RFC-, and FR-null MXTRIIOu4a-g (R2) Chinese hamster ovary (CHO) cells (Flintoff and Nagainis, 1983) were a gift from Dr. Wayne Flintoff (University of Western Ontario, London, ON, Canada) and were cultured in α-minimal essential...
media (MEM) supplemented with 10% bovine calf serum (Invitrogen, Carlsbad, CA), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-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 pZeocSV2(+)-RFC and pZeocSV2(+)–PCFT constructs, respectively (Zhao et al., 2008), and were gifts of Dr. I. 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'-AACTG GGA TCC gca cat gga ggg gag cgc cc-3' and 5'-AACTC GGT ACC ggg gct cgg aaa etg cta gaa cta gc-3' (bold capital letters designate the BamHl 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 transport 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 1X protease inhibitor cocktail tablets (Roche, Indianapolis, IN), and disrupted with a probe sonicator. The cell homogenate was centrifuged at 200,000 g with a probe sonicator. The cell homogenate was centrifuged (600 g) 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 a protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA). Cell lysates were electrophoresed on 7.5% polyacrylamide gels in the presence of SDS (Laemmli, 1970) and electroblotted onto polyvinylidene difluoride membranes (Pierce, Rockford, IL) (Matsudaira, 1987). PCFTMyc-His₆ protein was detected with Myc-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 transfected to Cytostrin spinners and maintained in suspension at densities of 2 to 5 × 10⁶ cells/ml. Cells were collected by centrifugation and washed with Dulbecco’s phosphate-buffered saline (DPBS), and the cells 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 a Folin-phenol reagent (Lowry et al., 1951). To determine Kᵢ and Vₘₐₓ kinetic constants for hPCFT in R2/hPCFT4 cells (Kᵢ and Vₘₐₓ), transport rates were measured at pH 6.8 and 5.5, as described above, using substrate concentrations from 0.04 to 5.0 μM. Kᵢ and Vₘₐₓ values were determined from Lineweaver-Burke plots. Inhibition of hRFC transport by unlabeled antifolates (reflecting binding to the carrier) was measured in PC43-10 cells over 2 min at 37°C in Hanks’ balanced salts solution, pH 7.2, 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 × 10⁻⁵ 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 Vₘₐₓ values for Mtx, and the concentration of [³H]Mtx, using equation Kᵢ = Kᵢ(Vₘₐₓ(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, which is the same protocol 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 0.1 M sodium cacodylate, pH 7.4, containing 30 mM NaCl and 2.5 mM KCl) for 20 min at room temperature, rinsed twice in DPBS, and permeabilized with 0.1% Triton X-100 (in phosphate-buffered saline, pH 7.4) for 10 min. Cells were rinsed with DPBS supplemented with 0.5% BSA and then incubated in 100 μl of blocking solution (10% Fetal Bovine Serum, 0.1% Tween 20, 1% BSA) at room temperature for 90 min. Cells were incubated with the primary antibody (1:500 dilution) for 3 h at room temperature. Then, cells were washed 3 times with DPBS and incubated with the secondary antibody (1:1000 dilution) for 1 h at room temperature. After washing 3 times with DPBS, cells were mounted with 60% glycerol in PBS and visualized in 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 l-glutamine, 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 CellTitertiter-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...
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 anticofacts. Growth inhibition assays for the PC43-10 CHO and R1-11-RFC6 HeLa sublines were routinely performed in complete RPMI 1640 with 10% dPBS, 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 nM LCV, 10% dPBS, penicillin-streptomycin, and 2 mM L-glutamine, in the presence of assorted anticofate 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 then 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 anticofate 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 GARF Tase. Incorporation of [14C(U)]glycine into [14C]formyl β-glucaminidase ribonucleotide (GAR) as an in situ measure of endogenous GARFTase activity in R2/hPCFT4 cells was performed using a modification of published methods (Beardsey 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 nM LCV, 10% dPBS, 2 mM L-glutamine, and penicillin-streptomycin in T25 flasks, or DMSO (control) was added to the culture medium, then 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.

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, we previously generated novel sublines derived from the RFC-, FR-, and PCPT-null MtxRIOuaR2-4 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 subline (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.
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 37- and 11-fold higher transport at pH 5.5 and 6.8, 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 hPCRFT- 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 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 mM, 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 μM) and AICA (320 μM) were completely protective toward R2/hPCFT4 cells with the most potent agent of the 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 μM) 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 μM) and thymidine (10 μM). 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 timerequirement 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.

Transport Characteristics for 6-Substituted Pyrrolo[2,3-d]Pyrimidine Antifolate 2. Although growth inhibition results (Table 2) strongly suggested selective membrane transport by hPCFT and not hRFC for the 6-substituted pyrrolo[2,3-d]pyrimidine 2, for further confirmation, we tested compound 2 (10 μM) as a direct competitor for inhibition of hPCFT-mediated uptake of [3H]Mtx (0.5 μM) in R2/hPCFT4 cells from pH 5.5 to 7.2, and compared the results with those for Pmx and PT523. A

### Table 1

**Kinetic constants for hPCFT**

<table>
<thead>
<tr>
<th>Substrates and Parameters</th>
<th>pH 5.5</th>
<th>pH 6.8</th>
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<tbody>
<tr>
<td>Mtx</td>
<td>$K_i$ (μM)</td>
<td>0.280 ± 0.022</td>
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<td></td>
<td>$V_{max}$ (pmol/mg/min)</td>
<td>32.3 ± 4.31</td>
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<tr>
<td></td>
<td>$V_{max}/K_i$</td>
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<tr>
<td>Pmx</td>
<td>$K_i$ (μM)</td>
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<tr>
<td></td>
<td>$V_{max}$ (pmol/mg/min)</td>
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<td></td>
<td>$V_{max}/K_i$</td>
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<tr>
<td>Compound 2</td>
<td>$K_i$ (μM)</td>
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</tr>
<tr>
<td>Pmx</td>
<td>$K_i$ (μM)</td>
<td>0.0960 ± 0.012</td>
</tr>
<tr>
<td>Lmx</td>
<td>$K_i$ (μM)</td>
<td>0.249 ± 0.013</td>
</tr>
</tbody>
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### 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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<tr>
<td>Mtx</td>
<td>120.5 ± 16.8</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>12 ± 1</td>
<td>216 ± 9</td>
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<td>GW1843U89</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>11 ± 3.3</td>
<td>&gt;1000</td>
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<tr>
<td>Lmx</td>
<td>38.6 ± 5.0</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>12 ± 2.3</td>
<td>&gt;1000</td>
<td>36.1 ± 9.9</td>
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<tr>
<td>Pmx</td>
<td>13.2 ± 2.4</td>
<td>&lt;1000</td>
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<td>138 ± 13</td>
<td>894 ± 93</td>
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<td>PT523</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>1.53 ± 0.16</td>
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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.

**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.

**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.
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. GTP and ATP 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;

Fig. 6. Electrophysiology studies of antifolate transport by hPCFT in X. laevis oocytes. Substrate-induced currents (nanoamperes) were recorded in individual oocytes injected with wild-type hPCFT and voltage-clamped to a holding potential (Vₒ) of ~90 mV. Oocytes were perfused with ND90 solution at pH 5.5 with LCV followed by compound 2 (A) and with Pmx followed by compound 2 (B). For all substrates, concentrations were maintained at a level of 5 μM.

Fig. 7. Analysis of ATP and GTP pools after treatment of R2/hPCFT4 cells with compound 2 and lometrexol. For analysis of ATP (A) and GTP (B) levels, cells were treated with various concentrations of compound 2 or Lmx for 24 h. Nucleotides were extracted, and ATP/GTP pools were determined by a modification of the HPLC method of Huang et al. (2003). Details are provided under Materials and Methods.
Monahan and Allegra, 2006; Chattopadhyay et al., 2007; Zain and Marchi, 2010) more than 60 years since the original report that aminopterin could induce remissions in childhood acute lymphoblastic leukemia (Farber and Diamond, 1948). 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 (Qu 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 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 PC4310 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 Ki 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]glycine 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 prove 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 folytpolyglutamate 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 (Illei 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 FC 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 Pmx that are normally transported by hRFC that compete for polyglutamylated metabolites, 5,10-dideaza-5,6,7,8-tetrahydrofolate is a potent inhibitor of de novo purine synthesis. J Biol Chem 264:322–332.

References

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).
SUPPLEMENT
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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Scheme 1S. Synthesis of two carbon chain classical 6-substituted pyrrolo[2,3-d]pyrimidine 1a

Compounds 1 (N-{4-[1-(2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]-pyrimidin-6-yl)methyl]benzoyl}-L-glutamic acid), 2 (N-{4-[3-(2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]-pyrimidin-6-yl)propyl]benzoyl}-L-glutamic acid), 3 (N-{4-[4-(2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]-pyrimidin-6-yl)butyl]benzoyl}-L-glutamic acid), 4 (N-{4-[5-(2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]-pyrimidin-6-yl)pentyl] benzoyl}-L-glutamic acid), and 5 (N-{4-[6-(2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]-pyrimidin-6-yl)hexyl]benzoyl}-L-glutamic acid) were made as we reported before (Deng et al., 2008). Compound 1a was obtained via a Sonogashira coupling reaction between the intermediates 10 and 15 as the key step as described by Taylor et al. (Taylor et al., 1993). The synthesis of intermediate 10 began with the coupling of commercially available 4-iodobenzoic acid 6 and diethyl-L-glutamate hydrochloride 7 to afford diethyl 4-iodobenzoyl-L-glutamate 8. The yield of 8 was 95%, which was better than that reported by Taylor et al. (75%) (Taylor et al., 1993). Palladium-catalyzed coupling of 8 with trimethylsilyl acetylene, in the presence of tetrakis-(triphenylphosphine) palladium (0) (Pd(PPh₃)₄), copper(I) iodide (CuI) and triethylamine, gave 9 as a reddish oil, which was immediately desilylated using n-Bu₄NF to afford the acetylene 10 (74% over two steps). The other intermediate 15 was obtained by a four-step synthesis from the commercially available 2,4-diamino-6-hydroxy primidine 11. Compound 11 was condensed with α-chloro acetaldehyde 12 to afford the pyrrolo[2,3-d]pyrimidine 13 (Secrist et al., 1978). Protection of 13 with a pivaloy group provided compound 14,
which was converted to the 6-substituted mercury salt and immediately reacted with iodine to give the desired 6-iodo pyrrolo[2,3-d]pyrimidine 15. With the 6-iodo pyrrolo[2,3-d]pyrimidine 15 in hand, a palladium catalyzed carbon-carbon coupling reaction with the aryl iodide 15 and the acetylene 10 led to the 6-substituted compound 16. Instead of using the reaction condition of 5% Pd/C, 48 hours described by Taylor et al (Taylor et al., 1993) to get 72% yield of the hydrogenation of 16, a 10% Pd/C, 5 hour-condition was employed to get complete transformation (100% yield of 17) without any partial reduction. Compound 17 was then converted to the target compound 1a by a convenient deprotection with 1N sodium hydroxide solution.

**COMPOUND 1a EXPERIMENTAL SECTION.**

All evaporations were carried out in vacuo with a rotary evaporator. Analytical samples were dried in vacuo (0.2 mmHg) in a CHEM-DRY drying apparatus over P₂O₅ at 80°C. Melting points were determined on a MEL-TEMP II melting point apparatus with FLUKE 51 K/J electronic thermometer and are uncorrected. Nuclear magnetic resonance spectra for proton (¹H NMR) were recorded on a Bruker 400MHz/52 MM (400 MHz) spectrometer. The chemical shift values are expressed in ppm (parts per million) relative to tetramethylsilane as internal standard: s ) singlet, d ) doublet, t ) triplet, q ) quartet, m ) mutiplet, br ) broad singlet. The relative integrals of peak areas agreed with those expected for the assigned structures. Thin-layer chromatography (TLC) was performed on Whatman Sil G/UV254 silica gel plates with fluorescent indicator, and the spots were visualized under 254 and 366 nm illumination. Proportions of solvents used for TLC are by volume. Column chromatography was performed on 230-400 mesh silica gel purchased from Aldrich (Milwaukee, WI). Elemental analyses were performed by Atlantic Microlab, Inc. (Norcross, GA). Element compositions are within ±0.4% of the calculated values. Fractional moles of water or organic solvents frequently found in some analytical samples of antifolates could not be prevented despite 24-48 h of drying in vacuo and were confirmed where possible by their presence in the ¹H NMR spectra. All solvents and chemicals were purchased from Aldrich Chemical Co. or from Fisher Scientific and were used as received.

**(S)-diethyl 2-(4-iodobenzamido)pentanedioate (8).**

To a solution of 4-iodobenzoic acid 6 (1.24 g, 5 mmol) in anhydrous DMF (40 mL) was added 6-chloro-2,4-dimethoxy-1,3,5-triazine (1.05 g, 6 mmol) and N-methylmorpholine (0.65 mL, 6 mmol). After the mixture was stirred at r.t. for 2 h, N-methylmorpholine (0.65 mL, 6 mmol) and dimethyl L-glutamate hydrochloride 7 (1.44 g, 6 mmol) were added all at once. The mixture was stirred at r.t. for 4 h. TLC showed the formation of one major spot at R_f = 0.42 (Hexane/EtOAc, 2:1 ). The reaction mixture was evaporated to dryness under reduced pressure. The residue was chromatographed on a silica gel column (2 cm x 15 cm) with Hexane/EtOAc, 3:1 as the eluent. Fractions were pooled and evaporated to dryness to afford 8 2.05g, yield 95% as white crystals, mp 105-106 °C (lit.² mp 105-106 °C), R_f = 0.42 (Hexane/EtOAc, 2:1 ). ¹H NMR (CDCl₃) δ 1.18-1.26(t, 3 H, J = 7.2 Hz, γ-COOCH₂CH₃), 1.27-1.33 (t, 3 H, J = 7.2 Hz, α-COOCH₂CH₃), 2.10-2.35 (m, 2 H, β-C₃H₂), 2.38-2.56 (m, 2 H, γ-CH₂), 4.06-4.16 (m, 2 H, γ-COOCH₂CH₃), 4.20-4.28 (m, 2 H, α-COOCH₂CH₃), 4.72-4.77 (m, 1 H, α-CH), 7.10 (d, 1 H, J = 6.7 Hz, CONH, exch), 7.55 (d, 2 H, J = 8.6 Hz, C₆H₄), 7.80 (d, 2 H, J = 8.6 Hz, C₆H₄).
(S)-diethyl 2-(4-((trimethylsilyl)ethynyl)benzamido)pentanedioate (9).
A mixture of 8 (1.30 g, 3 mmol), trimethylsilyl acetylene (0.87 g, 9 mmol), tetrakis(triphenylphosphine)palladium (0.35 g, 0.3 mmol), copper iodide (0.114 g, 0.6 mmol), and triethylamine (0.6 mL) in 1,2-dichloroethane (15 mL) was stirred at room temperature under nitrogen in the dark overnight. Methylene chloride (20 mL) was added to the reaction mixture, and the mixture was washed with brine (20 mL x 2). The organic layer was separated and the solvent evaporated. The residue obtained was loaded onto a silica gel column and eluted with 4:1 hexanes/ethyl acetate. Fractions containing the product (TLC, $R_f = 0.44$, Hexane/EtOAc, 2:1) were pooled and the solvent evaporated to afford 1.22 g (100%) of 9 as a red oil. 1H NMR (CDCl$_3$) δ 0.26 (s, 9H, -Si(CH$_3$)$_3$), 1.20-1.24 (t, 3 H, $J =$ 7.2 Hz, $\gamma$-COOCH$_2$C$_2$H$_3$), 1.28-1.32 (t, 3 H, $J =$ 7.2 Hz, $\alpha$-COOCH$_2$C$_2$H$_3$), 2.10-2.36 (m, 2 H, $\beta$-C$_2$H$_2$), 2.38-2.56 (m, 2 H, $\gamma$-C$_2$H$_2$), 4.06-4.15 (m, 2 H, $\gamma$-COOC$_2$H$_2$C$_3$), 4.20-4.28 (m, 2 H, $\alpha$-COOC$_2$H$_2$C$_3$), 4.74-4.81 (m, 1 H, $\alpha$-CH), 7.06 (d, 1 H, $J =$ 7.4 Hz, CONH, exch), 7.52 (d, 2 H, $J =$ 8.5 Hz, C$_6$H$_4$), 7.76 (d, 2 H, $J =$ 8.5 Hz, C$_6$H$_4$).

(S)-diethyl 2-(4-ethynylbenzamido)pentanedioate (10).
Compound 9 (1.21 g, 3mmol) was dissolved in THF (15 mL), to which tetrabutylammonium fluoride (3 mL of a 1 M solution in THF) was added, and the solution stirred at room temperature for 2 h. Methylene chloride (20 mL) was added to the reaction mixture and washed with brine (20 mL x 2), then the organic layer was separated and dried over Na$_2$SO$_4$ and the solvent was evaporated. The crude residue was flash chromatographed on silica gel and eluted with 2:1 hexanes/ethyl acetate. Fractions containing the desired product (TLC) were pooled and evaporated to afford 0.72 g (72%) of 10 as a red oil: TLC $R_f$ 0.25 (hexanes/ethyl acetate, 2:1); 1H NMR (CDCl$_3$) δ 1.20-1.24 (t, 3 H, $J =$ 7.2 Hz, $\gamma$-COOCH$_2$C$_2$H$_3$), 1.28-1.32 (t, 3 H, $J =$ 7.2 Hz, $\alpha$-COOCH$_2$C$_2$H$_3$), 2.09-2.37 (m, 2 H, $\beta$-C$_2$H$_2$), 2.38-2.56 (m, 2 H, $\gamma$-C$_2$H$_2$), 3.20 (s, 1H, -CH), 4.06-4.15 (m, 2 H, $\gamma$-COOC$_2$H$_2$C$_3$), 4.20-4.28 (m, 2 H, $\alpha$-COOC$_2$H$_2$C$_3$), 4.74-4.81 (m, 1 H, $\alpha$-CH), 7.13 (d, 1 H, $J =$ 7.4 Hz, CONH, exch), 7.55 (d, 2 H, $J =$ 8.5 Hz, C$_6$H$_4$), 7.78 (d, 2 H, $J =$ 8.5 Hz, C$_6$H$_4$).

2-amino-3H-pyrrolo[2,3-d]pyrimidin-4(7H)-one (13).
To a solution of 2,4-diamino-6-hydroxypyrimidine 11 (5.0 g, 40 mmol) and sodium acetate (4.88 g, 60 mmol) in water (200 mL) at 100 °C was added a 50% solution of chloroacetaldehyde in water (5.0 mL, 40 mmol), dropwise, over a period of 15 min. The reaction mixture was refrigerated overnight, and the precipitate obtained was filtered, washed with cold water (25 mL x 2), then with cold acetone (20 mL x 2), and dried to afford 4.15 g (69%) of 13 as a grey solid: TLC $R_f$ 0.45 (CHCl$_3$/MeOH, 3:1); mp 322 °C (lit$^\text{2}$ mp 323-324 °C); 1H NMR (DMSO-d$_6$) δ 6.04 (bs, 2 H, 2-NH$_2$, exch), 6.18 (q, 1 H, 5-H), 6.60 (q, 1 H, 6-H), 10.22 (bs, 1 H, 3-NH, exch), 10.96 (bs, 1 H, 7-NH, exch).

$N$-(4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-2-yl)pivalamide (14).
A mixture of 13 (3.67 g, 24.5 mmol), pyridine (40 mL) and pivaloyl chloride (10.5 mL) was heated at 80-90 °C for 2 h. Volatiles were removed under vacuum, and the residue was dissolved in THF (15 mL), to which tetrabutylammonium fluoride (3 mL of a 1 M solution in THF) was added, and the solution stirred at room temperature for 2 h. Methylene chloride (20 mL) was added to the reaction mixture and washed with brine (20 mL x 2), then the organic layer was separated and dried over Na$_2$SO$_4$ and the solvent was evaporated. The crude residue was flash chromatographed on silica gel and eluted with 2:1 hexanes/ethyl acetate. Fractions containing the desired product (TLC) were pooled and evaporated to afford 0.72 g (72%) of 10 as a red oil: TLC $R_f$ 0.25 (hexanes/ethyl acetate, 2:1); 1H NMR (CDCl$_3$) δ 1.20-1.24 (t, 3 H, $J =$ 7.2 Hz, $\gamma$-COOCH$_2$C$_2$H$_3$), 1.28-1.32 (t, 3 H, $J =$ 7.2 Hz, $\alpha$-COOCH$_2$C$_2$H$_3$), 2.09-2.37 (m, 2 H, $\beta$-C$_2$H$_2$), 2.38-2.56 (m, 2 H, $\gamma$-C$_2$H$_2$), 3.20 (s, 1H, -CH), 4.06-4.15 (m, 2 H, $\gamma$-COOC$_2$H$_2$C$_3$), 4.20-4.28 (m, 2 H, $\alpha$-COOC$_2$H$_2$C$_3$), 4.74-4.81 (m, 1 H, $\alpha$-CH), 7.13 (d, 1 H, $J =$ 7.4 Hz, CONH, exch), 7.55 (d, 2 H, $J =$ 8.5 Hz, C$_6$H$_4$), 7.78 (d, 2 H, $J =$ 8.5 Hz, C$_6$H$_4$).
was dissolved in methanol (20 mL), silica gel (5 g) was added, and the solvent was evaporated to form a plug which was dried, loaded on top of a silica gel column and eluted with 2:1 ethyl acetate/hexanes. Fractions containing the product (TLC) were pooled and the solvent evaporated to afford 2.07 g (36%) of 14 as an yellow solid: TLC Rf 0.50 (acetate/hexanes, 5:1); mp 293 °C (lit2 295 °C); 1H NMR (DMSO-d6) δ 1.24 (s, 9 H, -C(CH3)3), 6.40 (q, 1 H, 5-H), 6.95 (q, 1 H, 6-H), 10.80 (bs, 1 H, 2-NH2Piv or 3-NH, exch), 11.58 (bs, 1 H, 2-NH2Piv or 3-NH, exch), 11.84 (bs, 1 H, 7-NH, exch).

N-(6-iodo-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-2-yl)pivalamide (15).
To a solution of 14 (150 mg, 0.64 mmol) in glacial acetic acid (5 mL) was added mercuric acetate (239 mg, 0.75 mmol) that was completely dissolved in glacial acetic acid (15 mL). The mixture was stirred at room temperature for 10 min, poured into saturated NaCl (10 mL), and stirred for 30 min. The precipitate was filtered, washed with water (3 mL), followed by MeOH (3 mL), and dried. The precipitate was combined with MeOH (5 mL) and stirred at room temperature for 1 h to remove starting material and filtrated to afford the product. A mixture of this product, iodine (0.19 g, 0.75 mmol) and CH2Cl2 (5 mL) was stirred at room temperature overnight. The solvent was removed under reduced pressure. The residue was washed with 3 M Na2S2O3 (5 mL x 2), followed by water (5 mL x 2), and dried in vacuo. The crude product was purified by column chromatography on silica gel and eluted with 1:1 ethyl acetate/hexanes. The fractions containing the desired product (TLC) were pooled and evaporated to afford 175 mg (78%) of 15 as a white solid: TLC Rf 0.60 (ethyl acetate/hexanes, 2:1); mp 210 °C dec (lit.2 mp 211 °C dec); 1H NMR (DMSO-d6) δ 1.23 (s, 9 H, -C(CH3)3), 6.61 (s, 1 H, 5-H), 10.88 (s, 1 H, 2-NH2Piv or 3-NH, exch), 11.88 (s, 1 H, 2-NH2Piv or 3-NH, exch), 12.10 (s, 1 H, 7-NH, exch).

(S)-diethyl 2-(4-((4-oxo-2-pivalamido-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-6-yl)ethynyl)benzamido)pentanedioate (16).
To a 50-mL round-bottom flask covered with aluminum foil were added 15 (180 mg, 0.5 mmol) and acetylene 10 (248 mg, 0.75 mmol), copper(I) iodide (25 mg, 0.1 mmol) and tetrakis(triphenyl phosphine)palladium (0) (30 mg, 0.025 mmol)dissolved in anhydrous DMF (10 mL), followed by the addition of triethylamine (0.25 mL). The dark brown solution was stirred at 60 °C under nitrogen for 4h. The volatiles were removed in vacuo and the crude residue was flash chromatographed on silica gel and eluted with 3% MeOH in CH2Cl2 to afford the compound 16 (135 mg) (48%) as a yellow solid: TLC Rf 0.65 (MeOH/CH2Cl2, 1:9); mp 260 °C dec; 1H NMR (DMSO-d6) δ 1.15-1.22 (m, 6H, -2CH3), 1.25 (s, 9 H, -C(CH3)3), 1.99-2.15 (m, 2 H, β–CH2), 2.43-2.45 (m, 2 H, γ–CH2), 4.02-4.15 (m, 4 H, α–COOC2H5CH3), 4.42-4.48 (m, 1 H, α–CH), 6.85 (s, 1 H, 5-H), 7.65 (d, 2 H, C6H4, J = 8.5 Hz), 7.94 (d, 2 H, C6H4, J = 8.5 Hz), 8.85 (d, 1 H, CONH, J = 7.5 Hz), 10.98 (s, 1 H, 2-NH2Piv or 3-NH, exch), 11.97 (s, 1 H, 2-NH2Piv or 3-NH, exch), 12.27 (s, 1 H, 7-NH, exch).

(S)-diethyl 2-(4-(2-(4-oxo-2-pivalamido-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-6-yl)ethyl)benzamido)pentanedioate (17).
To a solution of 16 (50 mg) in MeOH/CH2Cl2 (1:1, 30 mL) was added 10% Pd/C (50 mg). The resulting suspension was hydrogenated in a Parr apparatus for 5h at 50 psi
hydrogen pressure. The reaction mixture was filtered through Celite and washed with methanol (30 mL). After evaporation of the solvent, 50 mg (100%) of 17 was obtained as a grey solid: mp 142 °C, \( R_f \) 0.65 (MeOH/CH\(_2\)Cl\(_2\), 1:9). \(^1\)H NMR (DMSO-\(d_6\)) \( \delta \) 1.13-1.21 (m, 6H, -2CH\(_3\)), 1.24 (s, 9H, -C(CH\(_3\))\(_3\)), 1.96-2.13 (m, 2H, \( \beta\)-CH\(_2\)), 2.41-2.45 (t, 2H, \( \gamma\)-CH\(_2\), \( J = 7.6 \) Hz), 2.91-3.04 (m, 4H, -CH\(_2\)CH\(_2\)\(_2\)), 4.00-4.14 (m, 4H, \( \alpha\), \( \gamma\)-COOCH\(_2\)CH\(_3\)), 4.38-4.45 (m, 1H, \( \alpha\)-CH), 6.10 (s, 1H, 5-H), 7.33 (d, 2H, C\(_6\)H\(_4\), \( J = 8.2 \) Hz), 7.79 (d, 2H, C\(_6\)H\(_4\), \( J = 8.2 \) Hz), 8.65 (d, 1H, CONH), \( J = 7.5 \) Hz), 10.76 (s, 1H, 2-NH\(_{Piv}\) or 3-NH, exch), 11.40 (s, 1H, 2-NH\(_{Piv}\) or 3-NH, exch), 11.82 (s, 1H, 7-NH, exch).

(\(S\))-2-(4-(2-(2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-6-yl)ethyl)benzamido)pentanedioic acid (1a).

To a solution of the diester (17) (50 mg) was added 1 N NaOH (4 mL), and the mixture was stirred under N\(_2\) at room temperature for 3 days. TLC (CH\(_2\)Cl\(_2\)/MeOH, 9:1) showed the disappearance of the starting material (\( R_f \) = 0.65) and formation of one major spot at the origin. The reaction mixture was evaporated to dryness under reduced pressure. The residue was dissolved in water (3 mL), the resulting solution was cooled in an ice bath, and the pH was adjusted to 3-4 with dropwise addition of 1 N HCl. The resulting suspension was frozen in a dry ice/acetone bath, thawed in a refrigerator to 4-5 °C, and filtered. The residue was washed with a small amount of cold water and ethyl acetate and dried in vacuo using P\(_2\)O\(_5\) to afford 30 mg (80%) of 1a as a pale white powder: mp 209 °C (lit.\(^2\) mp 210-213 °C), \( R_f \) = 0.05 (CHCl\(_3\)/MeOH, 5:1). \(^1\)H NMR (DMSO-\(d_6\)) \( \delta \) 1.90-2.11 (m, 2H, CH\(_2\)), 2.31-2.37 (t, 2H, CH\(_2\)), 2.77-2.84 (t, 2H, CH\(_2\)), 2.92-2.98 (t, 2H, CH\(_2\)), 4.34-4.41 (m, 1H, CH), 5.86 (s, 1H, CH), 5.97 (s, 2H, 2-NH\(_2\)), 7.31 (d, 2H, C\(_6\)H\(_4\), \( J = 8.0 \) Hz), 7.79 (d, 2H, C\(_6\)H\(_4\), \( J = 8.0 \) Hz), 8.51 (d, 1H, CONH), \( J = 8.0 \) Hz), 10.12 (s, 1H, 3-NH), 10.89 (s, 1H, 7-NH), 12.60 (br, 2H, 2 COOH). Anal. (C\(_{20}\)H\(_{21}\)N\(_5\)O\(_6\) \( \cdot \) 0.75 H\(_2\)O) Cal. C: 54.48, H: 5.14, N: 15.88. Found C: 54.49, H: 5.04, N: 15.53.

REFERENCES


Figure 1S. Comparison of PCFT transport activity in R2/hPCFT4 and R1-11-PCFT4 cells. hPCFT transport activity in R2/VC, R2/hPCFT4, R1-11-mock and R1-11-PCFT4 cells was assessed by measuring uptake of 0.5 μM [³H]Mtx at 37°C for 5 min at pH 5.5 or 7.2 in MES-buffered saline, and at 7.2 in HEPES-buffered saline. Internalized [³H]Mtx was normalized to total protein.

Figure 2S. Inhibition of cell growth and protection by nucleosides. Cell proliferation inhibition was measured on 96-well plates with 2500 R2/hPCFT4 cells per well and a range of antifolate concentrations (1-1000 nM) in the presence or absence of adenosine (60 μM), thymidine (10 μM) or 5-amino-4-imidazole carboxamide (AICA) (320 μM). Cell densities were measured with CellTiter Blue fluorescence dye (Promega) and a fluorescence plate reader. Results were normalized to cell density in the absence of drug. Results shown are representative data of experiments performed in triplicate.
Figure 3S. Inhibition of colony formation by compound 2 and Pmx. 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 and Pmx from 1 to 100 nM. Colonies were stained with 1% methylene blue in 10 mM borate buffer pH 8.8 for counting. Three separate experiments were performed and representative images are displayed.
Figure 4S. Time-dependent loss of clonogenicity by compound 2, Pmx and Lmx. 
R2/hPCFT4 CHO 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 (60μM; “A”) and thymidine (10μM; “T”) protection for 
2, 4, 8, 24 and 48 h. Following drug treatment, cells were washed with PBS and 
resuspended with drug free media with or without adenosine (60μM) and thymidine 
(10μM) protection. A parallel control treatment was included in which adenosine and 
thymidine were present during the period of drug exposure and cells were subsequently 
plated in the absence of drug. Colonies were stained with 1% methylene blue in 10 mM 
borate buffer pH 8.8 and counted. Three separate experiments were performed and 
representative pictures are presented.