Therapeutic targeting of a novel 6-substituted pyrrolo[2,3-d]pyrimidine thienoyl antifolate to human solid tumors based on selective uptake by the proton-coupled folate transporter

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Non-standard abbreviations:
AICARFTase, 5-amino-4-imidazolecarboxamide ribonucleotide
CHO, Chinese hamster ovary cells
dFBS, dialyzed fetal bovine serum
DHFR, dihydrofolate reductase
DPBS, Dulbecco’s phosphate-buffered saline
FITC, fluorescein isothiocyanate
FR, folate receptor
GAPDH, glyceraldehyde-3-phosphate dehydrogenase
GAR, glycinamide ribonucleotide
GARFTase, glycinamide ribonucleotide formyltransferase
HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC, high performance liquid chromatography
hPCFT, human proton-coupled folate transporter
hRFC, human reduced folate carrier
IC50, fifty percent inhibitory concentration
LCV, leucovorin
Lmx, lometrexol
MES, 4-morpholinopropane sulfonic
MTAP, methylthioadenosine phosphorylase
MTRP, 5-deoxy-5-(methylthio)ribose-1-phosphate
Mtx, methotrexate
PCFT, proton-coupled folate transporter
PG, polyglutamate
PI, propidium iodide
PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid)
Pmx, pemetrexed
RFC, reduced folate carrier
SCID, severe combined immunodeficient
SEM, standard error of the mean
TCA, trichloroacetic acid
TS, thymidylate synthase
ABSTRACT

The proton-coupled folate transporter (PCFT) is a proton-folate symporter with an acidic pH optimum. By real-time RT-PCR, PCFT was expressed in the majority of 53 human tumor cell lines, with the highest levels in Caco-2 (colorectal adenocarcinoma), SKOV3 (ovarian) and HepG2 (hepatoma) cells. A novel 6-substituted pyrrolo[2,3-d]pyrimidine thienoyl antifolate (compound 1) was used to establish whether PCFT can deliver cytotoxic drug under pH conditions that mimic the tumor microenvironment. Both 1 and pemetrexed (Pmx) inhibited proliferation of R1-11-PCFT4 HeLa cells engineered to express PCFT without the reduced folate carrier (RFC), and of HepG2 cells expressing both PCFT and RFC. Unlike Pmx, 1 did not inhibit proliferation of R1-11-RFC6 HeLa cells, which express RFC without PCFT. Treatment of R1-11-PCFT4 cells at pH 6.8 with 1 or Pmx inhibited colony formation with dose- and time-dependence. Transport of [3H]compound 1 into R1-11-PCFT4 and HepG2 cells was optimal at pH 5.5 but appreciable at pH 6.8. At pH 6.8, [3H]compound 1 was metabolized to [3H]polyglutamates. Glycinamide ribonucleotide formyltransferase (GARFTase) in R1-11-PCFT4 cells was inhibited by 1 at pH 6.8, as measured by an in situ GARFTase assay and was accompanied by substantially reduced ATP levels. Compound 1 caused S-phase accumulation and a modest level of apoptosis. An in vivo efficacy trial with severe combined immunodeficient mice implanted with subcutaneous HepG2 tumors showed that compound 1 was active. Our findings suggest exciting new therapeutic possibilities to selectively deliver novel antifolate drugs via transport by PCFT over RFC by exploiting the acidic tumor microenvironment.
INTRODUCTION

Antifolates are some of the most versatile and best understood cancer chemotherapy drugs. These agents, notably aminopterin, revolutionized the treatment of acute lymphoblastic leukemia by inducing complete remissions in children with leukemia (Farber and Diamond, 1948). Aminopterin and methotrexate (Mtx) were recognized to disrupt folate metabolism by inhibiting dihydrofolate reductase (DHFR) (Monahan and Allegra, 2006). Subsequent generations of antifolates primarily targeted other key folate-dependent enzymes, including thymidylate synthase (TS) [Pemetrexed (Pmx)] and β-glycinamide ribonucleotide (GAR) formyltransferase (GARFTase) [Lometrexol (Lmx)]. For all these agents, cellular uptake and metabolism to polyglutamates are critical to drug activity (Chattopadhyay et al., 2007; Mendelsohn et al., 1999; Monahan and Allegra, 2006).

The anionic nature of antifolates precludes their diffusion across biological membranes. Three genetically distinct and functionally diverse transport systems have evolved to facilitate their uptake into mammalian cells. (i) The reduced folate carrier (RFC; SLC19A1), a member of the major facilitator superfamily of solute carriers, is an anionic antiporter and the major transport system for reduced folates in mammalian cells and tissues at physiologic pH (Matherly et al., 2007). RFC is ubiquitously expressed in normal and malignant tissues. (ii) Folate receptors (FRs) α and β are glycosylphosphatidylinositol-anchored membrane proteins that transport folates by receptor-mediated endocytosis. FRα is expressed in epithelial cells of the kidney, choroid plexus, retina, uterus, and placenta. Malignant tissues also express FRα, including adenocarcinomas of the cervix, uterus, and ovary (Elnakat and Ratnam, 2004). (iii) The
proton-coupled folate transporter (PCFT; SLC46A1) is a proton-folate symporter that functions optimally at acidic pHs by coupling the downhill flow of protons to the uphill flow of folates into cells (Nakai et al., 2007; Qiu et al., 2006; Zhao and Goldman, 2007). PCFT is expressed in normal mouse and human tissues. High PCFT levels are present at the apical brush-border membrane along the proximal jejunum and duodenum, and in kidney, liver, placenta, and choroid plexus, whereas much lower levels were detected in other tissues (Kugel Desmoulin et al., 2010a; Qiu et al., 2007; Zhao et al., 2009). While a low pH transport activity was described in human tumor cell lines of assorted origins (Zhao et al., 2004), presumably due to PCFT, tumor expression of PCFT has not been systematically studied.

There is now ample precedent for using FRα to selectively target tumors with cytotoxic agents for therapeutic benefit (Deng et al., 2008; Deng et al., 2009; Gibbs et al., 2005; Hilgenbrink and Low, 2005; Kugel Desmoulin et al., 2010b; Salazar and Ratnam, 2007; Wang et al., 2010). PCFT transport function may be enhanced in many solid tumors by the acidic pH of the tumor microenvironment which has been reported to reach as low as pH 6.2-6.8 (Helmlinger et al., 1997; Raghunand et al., 1999; Wike-Hooley et al., 1984). Intracellular pH is normally alkaline, which creates a substantial transmembrane pH gradient directed intracellularly (Fais et al., 2007). Clearly, harnessing this proton-motive gradient to transport cytotoxic antifolates into tumor cells by PCFT offers a uniquely attractive mechanism of therapeutic targeting solid tumors.

For tumor targeting of cytotoxic drugs via FR or PCFT, ideally, therapeutic agents could be developed that are specifically transported by FRs and/or PCFT and not by RFC.
(Deng et al., 2008; Deng et al., 2009; Gibbs et al., 2005; Hilgenbrink and Low, 2005; Kugel Desmoulin et al., 2010b; Salazar and Ratnam, 2007; Wang et al., 2010). This strategy is necessary since antifolate membrane transport by RFC precludes tumor selectivity, as RFC is expressed in both normal and tumor cells, and RFC transport is optimal at neutral pH characterizing most normal tissues (Matherly et al., 2007; Zhao and Goldman, 2003). Indeed, a major obstacle in implementing this approach has been a lack of FR- or PCFT-selective antifolates, as all of the clinically useful antifolates with significant FR- and PCFT substrate activity (e.g., Mtx and Pmx) are also transported by RFC (Matherly et al., 2007).

We recently described a novel 2-amino-4-oxo-6-substituted pyrrolo[2,3-d]pyrimidine antifolate with a thienoyl for benzoyl replacement and a bridge length of 4 carbons (compound 1) (Figure 1) (Wang et al., 2010). Cellular uptake of compound 1 by FRα was substantial in the absence of evidence of its membrane transport by RFC, resulting in potent antitumor activity both in vitro and in vivo due to inhibition of GARFTase in de novo purine nucleotide biosynthesis. While transport of compound 1 by PCFT was also inferred, neither this nor the capacity of PCFT to deliver a cytotoxic dose of compound 1 under conditions relevant to the solid tumor microenvironment was directly tested.

This report describes such experiments with compound 1 to establish the feasibility of selectively targeting chemotherapy to human solid tumors based upon drug membrane transport by PCFT. Experiments are also described documenting the PCFT transport and polyglutamylation characteristics of compound 1, which account for its potent inhibition of GARFTase leading to tumor cell death in vitro and in vivo.
MATERIALS AND METHODS

Materials. [3',5',7-3H]Mtx (20 Ci/mmol), [3H]Pmx (2.5 Ci/mmol) and custom-radiolabeled [3H]compound 1 (1.3 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA). Leucovorin (LCV) [(6R,S) 5-formyl tetrahydrofolate] was provided by the Drug Development Branch, National Cancer Institute, Bethesda, MD. Pmx [N-{4-[2-(2-amino-3,4-dihydro-4-oxo-7H-pyrrolo[2,3-d]pyrimidin-5-yl)ethyl]benzoyl}-L-glutamic acid] (Alimta) was provided by Eli Lilly and Co. (Indianapolis, IN). Synthesis and properties of the substituted pyrrolo[2,3-d]pyrimidine antifolate compound 1 were previously described (Wang et al., 2010). Other chemicals were obtained from commercial sources in the highest available purities.

Cell lines. The sources and cell culture conditions for the panel of human solid tumor and leukemia cell lines used for qRT-PCR assays of transcript levels for FRα, hPCFT, and hRFC are summarized in Table 1S (Supplement). HeLa R1-11-RFC6 and R1-11-PCFT4 cells were derived from human RFC (hRFC)- and human PCFT (hPCFT)-null R1-11 cells by stable transfection with HA-tagged pZeoSv2(+)RFC and pZeoSv2(+)PCFT constructs, respectively (Zhao et al., 2008). These HeLa sublines along with R1-11-mock transfected cells were gifts from Dr. I. David Goldman (Albert Einstein School of Medicine, Bronx, NY). Characteristics and maintenance of the HeLa sublines were previously described (Zhao et al., 2008).

Real-time RT-PCR analysis of RFC, FRα, and PCFT transcripts. RNAs were isolated from a variety of human cell lines, including solid tumor (n=53) and leukemia (n=27) sublines (Supplement, Table 1S), and engineered R1-11 HeLa sublines (R1-11
mock, R1-11-RFC6, and R1-11-PCFT4), using TRIZOL reagent (Invitrogen). cDNAs were synthesized using Superscript reverse transcriptase III kit (Invitrogen). cDNAs were purified with the QIAquick PCR Purification Kit (Qiagen). Quantitative real-time RT-PCR was performed on a Roche LightCycler 480 using Universal Probes (Roche, Indianapolis, IN) and gene-specific primers. Primers are included in Table 2S (Supplement). Transcript levels for FRα, hPCFT, and hRFC genes were normalized to those for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using commercial probes and primers (Roche 05190541001). External standard curves were constructed for each gene of interest using serial dilutions of linearized templates, prepared by amplification from suitable cDNA templates, subcloning into a TA-cloning vector (PCR-Topo; Invitrogen), and restriction digestions.

**Proliferation and colony-forming assays.** For growth inhibition assays, R1-11-PCFT4 and R1-11-RFC6 HeLa cells, and HepG2 cells were cultured in folate-free RPMI 1640 (pH 7.2) containing 25 nM LCV, supplemented with 10% dialyzed fetal bovine serum (dFBS), 2 mM L-glutamine and 100 units/ml penicillin/100 µg/ml streptomycin for at least 2 weeks. Cells were plated in 96 well culture dishes (5000 cells/well; 200 µl/well) in the above medium with a broad concentration range of drugs (depending on the compound, drug dilutions were in DMSO or water with appropriate vehicle controls); cells were incubated for up to 96 h at 37°C in a CO₂ incubator. Metabolically active cells (a measure of cell viability) were assayed with CellTiter-blue cell viability assay (Promega) and a fluorescent plate reader (590 nm emission, 560 nm excitation) for determining IC₅₀s, corresponding to drug concentrations that result in 50% loss of cell growth.
For colony-forming assays, folate-depleted R1-11-PCFT4 cells (500 cells) in log-phase were plated into 60 mm dishes in folate-free RPMI 1640 medium, supplemented with 25 nM LCV, 10% dFBS, penicillin-streptomycin, and 2 mM L-glutamine (pH 7.2), and allowed to adhere for 48 h. Cells were then treated with compound 1 or Pmx in the above media, supplemented with 25 mM piperazine-N,N′-bis(2-ethanesulfonic acid (PIPES) and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) to maintain the pH at 6.8. After 16, 24, or 48 h, cells were rinsed with Dulbecco’s phosphate-buffered saline (DPBS), then incubated in drug-free, complete folate-free RPMI 1640 medium, supplemented with 25 nM LCV (pH 7.2). Cells were allowed to outgrow for 12 days, at which time the dishes were rinsed with DPBS, 5% trichloroacetic acid (TCA), and borate buffer (10 mM, pH 8.8), followed by 1% methylene blue (in borate buffer). The dishes were again rinsed with borate buffer, and colonies were counted for calculating percent colony formation relative to the DMSO controls.

**Transport assays.** To determine the pH-dependent uptake of [³H]compound 1 and [³H]Pmx (both at 0.25 µM) in R1-11-PCFT4, R1-11-mock and HepG2 cells, uptake was assayed at 37°C in cell monolayers over 2-30 min at 37°C in complete folate-free RPMI 1640 (pH 5.5, 6.8 and 7.2), supplemented with 10% dFBS and 25 mM HEPES/25 mM PIPES. At the end of the incubations, transport was quenched with ice-cold DPBS, cells were washed 3 times with ice-cold DPBS, and cellular proteins were solubilized with 0.5 N NaOH. Levels of drug uptake were expressed as pmol/mg protein, calculated from direct measurements of radioactivity and protein contents of cell homogenates. Proteins were quantified using Folin-phenol reagent (Lowry et al., 1951).
For PCFT transport kinetic analyses, R1-11-PCFT4 cells were grown in suspension using spinner flasks at densities of 2-5 x 10^5 cells/mL. Cells were collected by centrifugation, washed with DPBS, and suspended (at 1.5 x 10^7 cells) in 2 ml transport buffer (below) for cellular uptake assays. To determine [3H]compound 1 and [3H]Pmx kinetic constants for PCFT (K_t and V_max), initial uptake rates were measured at 37°C over 2 min in HEPES-buffered saline (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 2 mM MgCl_2, and 5 mM glucose) at pH 6.8, or in 4-morpholinopropane sulfonic (MES)-buffered saline (20 mM MES, 140 mM NaCl, 5 mM KCl, 2 mM MgCl_2, and 5 mM glucose) at pH 5.5 (Zhao et al., 2004), using substrate concentrations from 0.04 to 5 μM. K_t and V_max values were determined from Lineweaver-Burke plots.

**HPLC analysis of polyglutamyl derivatives of compound 1 and Pmx.** Folate-depleted R1-11-PCFT4 and HepG2 cells were grown in complete folate-free RPMI 1640 medium, supplemented with 25 nM LCV and 10% dFBS. Cells were washed with DPBS and incubated in complete RPMI 1640 with dFBS and 25 mM PIPES/25 mM HEPES (pH 6.8) with 1 μM [3H]compound 1 or [3H]Pmx at 37°C in the presence of 60 μM adenosine, or 60 μM ade plus 10 μM thymidine, respectively. After 16 h, cells were washed three times with ice-cold DPBS, then scraped mechanically into 5 ml of ice-cold DPBS, pelleted and flash frozen. The cell pellets were resuspended into 0.5 ml of 50 mM sodium phosphate buffer (pH 6)/100 mM 2-mercaptoethanol including unlabeled compound 1 (or Pmx) and Mtx-diglutamate, -triglutamate, and –tetraglutamate standards (Schircks Laboratories, Jona, Switzerland) (50 μM each). A portion (50 μL) was used to determine total [3H]compound 1 or Pmx (in pmol/mg protein). Proteins were measured by the Bio-
Rad protein assay (Bio-Rad Laboratories, Richmond, CA). The remaining extract was boiled (10 min), the supernatant containing radiolabeled compound 1 (or Pmx) and its metabolites was centrifuged, then (250 μl) injected into a Waters 4μm Nova-Pak C-18 column (3.9 mm x 150 mm) with a Nova-Pak 4 μm C-18 guard column. A Varian 9012 ternary gradient programmable pump was used for gradient development and a 9050 Varian UV/Vis detector set to 313 nm was used for detection of compound 1, Pmx, or Mtx polyglutamate standards. HPLC analysis involved a binary gradient. Mobile phase A consisted of 100 mM sodium acetate at pH 5.5; mobile phase B consisted of 100% acetonitrile. The flow rate was set at 1.6 ml/min. The gradient consisted of 100% A from 0-5 minutes, then changed to 85%A/15% B from 5 to 27.5 minutes. Fractions were mechanically collected every min for the first 10 min and then every 10 sec for the duration of the run. Radioactivity of the fractions was measured with a scintillation counter. Intracellular levels of radiolabeled compounds are expressed as pmol/mg protein, based on calculated percentages in the peaks from the HPLC chromatogram and total pmol/mg of cellular [3H]antifolate. To confirm the identities of the early-eluting peaks as polyglutamate metabolites of compound 1, samples were hydrolyzed to their parent drug forms by an overnight treatment at 32˚C with a preparation of partially purified chicken pancreas conjugase in 0.5 mL 0.1 M sodium borate containing 10 mM 2-mercaptoethanol (Matherly et al., 1985). Samples were deproteinized by boiling (5 min), then analyzed by HPLC.

**In situ GARFT enzyme inhibition assay.** Incorporation of [14C(U)]glycine into [14C]formyl GAR as an *in situ* measure of endogenous GARFTase activity in folate-depleted R1-11-PCFT4 cells at pH 6.8 was performed using a modification of published
methods (Beardsley et al., 1989; Deng et al., 2008). For these experiments, R1-11-PCFT4 cells were seeded in 5 ml of complete folate-free RPMI 1640/10% dFBS, plus 25 nM LCV in 60 mm dishes and allowed to adhere overnight. Cells were washed twice with DPBS and resuspended in 5 mL complete folate-free RPMI 1640/10% dFBS with 25 mM PIPES/25 mM HEPES (pH 6.8) and 25 nM LCV. Antifolate inhibitor or an equivalent amount of vehicle (e.g., DMSO) (“control”) was added to the culture medium and the cells were incubated for another 16 h. Cells were washed twice with DPBS and resuspended in 5 mL complete folate-free, L-glutamine-free RPMI 1640/10% dFBS plus 25 mM PIPES/25 mM HEPES (pH 6.8) and 25 nM LCV with or without 0.5-100 nM antifolate and azaserine (4 μM final), and incubated for 30 min. L-glutamine (2 mM final concentration) and [14C]glycine (final specific activity, 0.1 mCi/L) were added, followed by incubation at 37°C for 8 h, after which time cells were washed three times with ice-cold DPBS and trypsinized. Cell pellets were suspended in 2 mL of 5% TCA at 0°C. Cell debris was removed by centrifugation; 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. The aqueous layer was passed through a 1 cm column of AG1x8 (chloride form, 100-200 mesh) (BioRad Laboratories), 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 elutants were collected as 1 mL fractions and determined for radioactivity.

**Determination of intracellular ATP levels.** For analysis of ATP levels following antifolate treatments, R1-11-PCFT4 cells were seeded in 10 ml of complete folate-free RPMI 1640/10% dFBS, with 25 mM PIPES/25 mM HEPES (pH 6.8) and 25 nM LCV. After 24 h, 10 μM compound 1 or DMSO (0.5% final) (control) was added to the culture
medium. Cells were incubated for an additional 24-72 h, after which they were trypsinized and washed (2x) with ice-cold DPBS. Nucleotides were extracted and ATP levels quantitated by HPLC exactly as previously described (Kugel Desmoulin et al., 2010b).

**Assessment of apoptosis and cell cycle distribution.** R1-11-PCFT4 cells were treated with 10 μM compound 1 for 48 h at pH 6.8 in complete folate-free RPMI 1640/10% dFBS with 25 mM PIPES/25 mM HEPES (pH 6.8) and 25 nM LCV. Cells were trypsinized, pelleted, and washed once with ice-cold DPBS. Samples were divided so that the cell cycle profile and apoptosis analysis could be performed on the same sample. The amount of apoptosis was measured by staining cells (~1 x 10^6) with fluorescein isothiocyanate (FITC)-conjugated annexin V and propidium iodide (PI) with the apoptotic cells determined using the CELL LAB ApoScreen™ Annexin V-FITC Apoptosis Kit (Beckman Coulter), as recommended by the manufacturer. Cells were analyzed for the presence of viable (annexin V and PI), early apoptotic (annexin V^+ and PI), and late apoptotic/necrotic (annexin V^+ and PI^+) cells by flow cytometry.

To determine compound 1 concentration-dependent effects on cell-cycle progression, R1-11-PCFT4 cells (1 x 10^6) treated with 0, 0.5, 1, 5, and 10 μM compound 1 in complete folate-free RPMI 1640/10% dFBS with 25 mM PIPES/25 mM HEPES (pH 6.8) and 25 nM LCV for 48 h at pH 6.8. Cells (~1 x 10^6) were fixed in ethanol (at least 1 h), then stained by resuspension in 0.5 ml DPBS containing 50 μg/ml PI and 100 μg/ml RNAse type I-A (Sigma Aldrich). The cells were analyzed by flow cytometry for determining the percent of cells in each phase of the cell cycle.
Flow cytometry was performed at the Karmanos Cancer Institute Imaging and Cytometry Core using the BD FACSCanto™ II operated with BD FACSDiva™ software (v6.0) (Becton Dickinson; San Jose, California). In each experiment, 20,000 cells were assessed for apoptosis and cell cycle distribution. Data were analyzed with the FlowJo (v7.6.1) software (Tree Star, Inc; Oregon).

**In vivo efficacy study of compound 1 in HepG2 xenografts.** Cultured HepG2 human hepatoma tumor cells were implanted subcutaneously (~ $1 \times 10^7$ cells/flank) to establish a solid tumor xenograft model in female ICR SCID mice (NIH DCT/DTP Animal Production Program, Frederick, MD). For the efficacy study, mice were 8 weeks old on day 0 (tumor implant) with an average body weight of 17.6 g. Mice were provided food and water *ad libitum*. Study mice were maintained on either a folate-deficient diet (Harlan-Teklad; Product ID: TD.00434) or a folate-replete diet (Lab Diet; 5021; autoclavable mouse breeder diet) starting 16 days before subcutaneous tumor implant to ensure serum folate levels would approximate those of humans. Folate serum levels were determined prior to tumor implantation and post-study with a *Lactobacillus casei* bioassay (Varela-Moreiras and Selhub, 1992). The animals were pooled and implanted bilaterally subcutaneously with 30-60 mg tumor fragments by a 12-gauge trocar and again pooled before unselective distribution to the various treatment and control groups. Chemotherapy was begun 4 days after tumor implantation, when the number of cells was relatively small ($10^7$-$10^8$ cells; before the established limit of palpation). Tumors were measured with a caliper two or three times weekly. Mice were sacrificed when the cumulative tumor burden reached 1500 mg. Tumor weights were estimated from two
dimensional measurements [i.e., tumor mass (in mg) = (ab²)/2, where a and b are the tumor length and width in mm, respectively]. For calculation of end points, both tumors on each mouse were added together, and the total mass per mouse was used. The following quantitative end points were used to assess antitumor activities: (i) T/C and T-C (tumor growth delay) [where T is the median time in days required for the treatment group tumors to reach a predetermined size (e.g., 500 mg) and C is the median time in days for the control group tumors to reach the same size; tumor-free survivors are excluded from these calculations]; and (ii) calculation of tumor cell kill \[ \log_{10} \text{cell kill total (gross)} = \frac{(T - C)}{(3.32)(T_d)}, \] where (T – C) is the tumor growth delay, as described above, and Td is the tumor volume doubling time in days, estimated from the best fit straight line from a log-linear growth plot of control group tumors in exponential growth (100-800 mg range). With the exception of the xenograft model, these methods are essentially identical to those described previously (Wang et al., 2010).

RESULTS

Expression and function of RFC and PCFT in human solid tumor and leukemia cell lines. Based on a report of a low pH transport activity in solid tumor cells lines (Zhao et al., 2004), presumably due to PCFT, we turned our attention to establishing an expression profile for hPCFT compared to hRFC and FRs in a number of cell lines derived from human solid tumors and leukemias. Transcript levels for hPCFT, hRFC, and FRs α and β were measured by qRT-PCR and normalized to GAPDH. Our results showed significant levels of hPCFT transcripts in the majority of human solid tumor cell lines of different origins (e.g., breast, prostate, ovarian, etc.) (Figure 2A), and uniformly low hPCFT transcript levels in human leukemias, including both ALL and AML (Supplement, Figure
hPCFT levels were highest in Caco-2 (colorectal adenocarcinoma), SKOV3 (ovarian carcinoma), HepG2 (hepatoma), and H69 (small cell lung cancer) cells, with appreciable hPCFT levels in numerous other tumor sublines. hRFC transcripts were detected in all solid tumor and leukemia cell lines with the exception of MDA-MB-231 breast cancer cells (Figure 2B and Supplement, Figure 1S, panel D). High levels of FRα were detected in a small subset of ovarian, cervical, and breast cancer cell lines and low but detectable FRα levels were measured in ALL (mostly T-cell) sublines (Supplement, Figure 1S, panel B). FR β transcripts were consistently low to undetectable in both solid tumors and leukemias, with the highest levels restricted to a small number of AML and T-cell ALLs (not shown).

**Effects of compound 1 on cell growth inhibition and colony formation in HeLa and HepG2 human tumor sublines.** Our previous studies (Wang et al., 2010) established that the novel pyrrolo[2,3-d]pyrimidine thienoyl antifolate compound 1 (Figure 1) was a potent (nM) inhibitor of proliferation of a Chinese hamster ovary (CHO) subline engineered to express hPCFT in the absence of other folate transporters (RFC and FRs), and of [3H]Mtx transport by hPCFT, suggesting competitive binding to the carrier (and transport by this mechanism). Conversely, the data strongly suggested that compound 1 was not transported by hRFC in a CHO subline similarly engineered to exclusively express hRFC.

To begin to establish the therapeutic potential of hPCFT as a selective approach for chemotherapy drug delivery to human solid tumors, we used isogenic HeLa sublines derived by stable transfections of hRFC- and hPCFT-null R1-11 HeLa cells, designated
R1-11-PCFT4 (express physiologic levels of hPCFT in the absence of hRFC, as measured by real-time RT-PCR) and R1-11-RFC6 (engineered to express hRFC without hPCFT), (Zhao et al., 2008) (Figure 3, panel A). Low levels of FRα were detected in all the R1-11 sublines. As a tumor prototype, we used HepG2 cells, established from our tumor cell line screen to express significant levels of hPCFT and hRFC (Figure 2) without FRα (expression levels for hPCFT and hRFC in HepG2 cells are compared to those for the R1-11 sublines in Figure 3, panel A).

We measured inhibition of cell proliferation by compound 1 and results were compared to those for Pmx. Pmx inhibited cell growth in both the R1-11-PCFT4 and R1-11-RFC6 lines with IC₅₀ values of 59.3 nM (± 7.37; standard error mean (SEM)) and 81.7 nM (± 5.49), respectively (Figure 3, panel B), demonstrating its lack of specificity for hPCFT over hRFC in spite of its high PCFT substrate activity (Kugel Desmoulin et al., 2010b; Zhao et al., 2008). Conversely, compound 1 inhibited cell growth in R1-11-PCFT4 cells (IC₅₀ = 99.2 ± 20.2 nM) but not R1-11-RFC6 (Figure 3, panel C), indicating selective hPCFT transport. In HepG2 cells, both Pmx (IC₅₀ = 40.63 ± 4.52 nM) and compound 1 (IC₅₀ = 227.50 ± 8.98 nM) were growth inhibitory. The decreased sensitivity to compound 1 for HepG2 cells compared to R1-11-PCFT4 cells likely reflects the presence of hRFC in HepG2 cells. Although not active for transport with compound 1, hRFC still transports folates and elevates intracellular folate pools, resulting in decreased cytotoxic drug effects on this basis.

Proliferation assays were extended to include colony-forming assays, in which R1-11-PCFT4 cells were exposed to a range of concentrations (1-10 μM) of compound 1 (Figure
3, panel D) or Pmx (panel E) for 16, 24 or 48 h. Drug exposures were performed at pH 6.8, after which drugs were removed and colonies allowed to outgrow for 12 days in standard culture media. As an inhibitor of colony formation, Pmx and compound 1 showed both concentration- and time-dependence, although this effect was more pronounced for compound 1, and Pmx was more active at 16 h for the lower drug concentrations. In spite of the latter, the maximum extent of inhibition after 48 h at 10 μM drug was greater for compound 1 (95%) than for Pmx (87%).

Collectively, these results demonstrate that compound 1, like Pmx, is cytotoxic toward cells that express hPCFT and under acidic conditions (pH 6.8) achievable in solid tumors. Unlike Pmx, compound 1 is selectively active toward cells expressing hPCFT and is inactive toward cells expressing exclusively hRFC.

Transport characteristics for [3H]6-substituted pyrrolo[2,3-d]pyrimidine thienoyl antifolate compound 1 in HeLa R1-11-PCFT4 and HepG2 cells. To directly measure hPCFT membrane transport of the cytotoxic antifolates into HeLa R1-11-PCFT4 and HepG2 cells, we used radiolabeled compound 1 and Pmx. For R1-11-PCFT4 cells, uptake of [3H]compound 1 (0.25 μM) was time- and pH-dependent with maximum drug accumulation at pH 5.5 (Figure 4, panels A-C). Uptake in R1-11-PCFT4 cells exceeded that of its hPCFT-null isogenic counterpart (R1-11-mock transfectant), unequivocally establishing transport of compound 1 by hPCFT. The modest time-dependent uptake in the hPCFT-null R1-11-mock transfected subline was particularly obvious at 30 minutes and likely reflects the presence of low levels of FR in these cells (Figure 3, panel A).
We compared the uptake of [³H]Pmx to that of [³H]compound 1 in HepG2 cells (Figure 4, panels D and E). For compound 1, pH-dependent uptake in HepG2 cells showed a similar profile (despite the ~2-fold increased net uptake) to R1-11-PCFT4 HeLa cells. Net uptake of [³H]Pmx exceeded that of [³H]compound 1 in HepG2 cells by ~50-100% and showed a greater uptake fraction at neutral pH, most likely due to the presence of hRFC in HepG2 cells (Figure 3, panel A).

We measured transport kinetics over 2 minutes for [³H]compound 1 and [³H]Pmx in R1-11-PCFT4 cells using a range of drug concentrations at pH 5.5 and pH 6.8 (Table 1). The data show nearly identical Kᵣ values for compound 1 and Pmx at pH 5.5 and only modest (within ~40%) differences in V_max. Increases in both Kᵣ (increased ~300- to 400-fold, respectively, compared to values at pH 5.5) and V_max values (~70% increased) were measured at pH 6.8. V_max/Kᵣ ratios for compound 1 and Pmx were similar (within ~2-fold) at both pH 5.5 and pH 6.8.

These results establish that for both R1-11-PCFT4 and HepG2 cells, compound 1 is an excellent substrate for hPCFT, essentially equivalent to Pmx.

**Polyglutamylation of the 6-substituted pyrrolo[2,3-\textit{d}]pyrimidine thienoyl antifolate compound 1 in R1-11-PCFT4 and HepG2 cells.** Polyglutamylation of classical antifolates is a critical factor in drug activity, since these conjugated drug forms are retained within cells and they typically inhibit folate-dependent enzyme targets to a greater extent than their non-polyglutamyl forms (Goldman and Matherly, 1985; Shane, 1989). To assess the extent of this metabolism for compound 1 in R1-11-PCFT4 and
HepG2 cells, cells were treated with 1 μM \([^3\text{H}]\)compound 1 for 16 h at pH 6.8 in the presence adenosine (60 μM). For HepG2 cells, parallel incubations were performed with \([^3\text{H}]\)Pmx [in presence of thymidine (10 μM) and/or adenosine] . \([^3\text{H}]\) metabolites were extracted and analyzed by reverse phase HPLC (Figure 5 shows an HPLC chromatograph for compound 1 in HepG2 cells; additional results for R1-11-PCFT4 and HepG2 cells are included in the Supplement, Figure 2S, panels A and B). Up to five polyglutamyl metabolites of \([^3\text{H}]\)compound 1 and \([^3\text{H}]\)Pmx (PG2-6) were resolved by HPLC. The identities of the peaks were confirmed by comparing elution times with those for Mtx polyglutamyl standards and by treatment with chicken pancreas conjugase which reverted the majority of the polyglutamyl metabolites to the parental drug (Supplement, Figure 2S, panel C).

The distributions of the individual compound 1 and Pmx drug forms in R1-11-PCFT4 and HepG2 cells are summarized in Table 2. Although there were differences in the relative amounts of total intracellular compound 1 between the R1-11-PCFT4 and HepG2 sublines (as expected from the transport results in Figure 4), in both cases compound 1 was predominately polyglutamylated (64% and 84% of the total intracellular drug, respectively). For HepG2 cells, the increased accumulation of \([^3\text{H}]\)compound 1 over that of R1-11-PCFT4 cells was reflected in the polyglutamate levels. Analogous results were obtained with \([^3\text{H}]\)Pmx in HepG2 cells, although the net extent of drug uptake and metabolism of \([^3\text{H}]\)Pmx was elevated over that of \([^3\text{H}]\)compound 1.
Collectively, these results establish that, like Pmx, compound 1 is an excellent substrate for polyglutamylation under conditions (pH 6.8) that favor its membrane transport by hPCFT.

Validation of GARFTase and de novo purine nucleotide biosynthesis as primary cellular targets for compound 1 in R1-11-PCFT4 cells. We previously reported that the principal intracellular target of compound 1 in hPCFT-expressing CHO cells is GARFTase (Wang et al., 2010), the first folate-dependent enzyme in de novo purine nucleotide biosynthesis. To confirm this result in R1-11-PCFT4 HeLa cells under acidic conditions (pH 6.8) that favor PCFT transport, we used an in situ metabolic assay which measures incorporation of \([^{14}\text{C}]\text{glycine into }^{[14}\text{C}]\text{formyl GAR as a read-out for GARFTase inhibition. Results were compared to those of Pmx, an established GARFTase inhibitor, along with its documented effects on thymidylate synthase (Chattopadhyay et al., 2007) and 5-amino-4-imidazolecarboxamide ribonucleotide formyltransferase (AICARFTase) (Racanelli et al., 2009) (Figure 6). IC}_{50}s for GARFTase inhibition in R1-11-PCFT4 cells by compound 1 and Pmx were 43.6 nM and 69.7 nM, respectively. While the IC}_{50} for GARFTase inhibition by compound 1 closely approximated the IC}_{50} for growth inhibition of R1-11-PCFT4 cells (Figure 3, panel C), GARFTase inhibition by Pmx was incomplete up to 5 \(\mu\)M. Analogous results were described for Pmx with hPCFT-expressing CHO cells (R2/hPCFT4) (Kugel Desmoulin et al., 2010b) and in CCRF-CEM cells by Racanelli et al. (Racanelli et al., 2009).

To confirm that potent inhibition of GARFTase in R1-11-PCFT4 cells by compound 1 also results in decreased ATP pools, we measured intracellular ATP levels in cells treated
with 10 μM compound 1 for 16, 24 and 48h under acidic conditions (pH 6.8), analogous to those used for our clonogenicity studies (Figure 3D). Compound 1 caused a time-dependent decrease in cellular ATP levels, such that treatment for 48 h led to an 88% decrease in ATP pools (Figure 7, panel A).

These results demonstrate that hPCFT-delivery of compound 1 is an efficient mode of drug uptake which effects a potent inhibition of GARFTase and ATP depletion in R1-11-PCFT4 cells.

**Effect of compound 1 on cell cycle progression and apoptosis induction in R1-11-PCFT4 cells.** To determine the impact of GARFTase inhibition and ATP depletion on cell cycle progression, we treated R1-11-PCFT4 cells with compound 1 (10 μM) for 48 h at pH 6.8, along with a vehicle control. Cells were fixed, stained with PI, and analyzed for cell cycle distribution by flow cytometry. Treatment with 10 μM compound 1 caused an accumulation of cells in S-phase such that 38.9% of cells were in S-phase, compared to 16.8% of the control (Figure 7, panel B and Supplement 3S). When a range of concentrations (0.5, 1, 5, and 10 μM) of compound 1 were tested for their abilities to induce S-phase accumulation, we found that maximal arrest was achieved at 1 μM.

Since treatment with compound 1 (10 μM, 48 h at pH 6.8) causes loss of clonogenicity in R1-11-PCFT4 cells (Figure 3D) and a modest increase in the sub-G1 fraction (Figure 7B), we were interested in measuring apoptosis under these same conditions using annexin V/PI staining. Results were compared to those for R1-11-PCFT4 cells treated with etoposide (5 μM) and with a no-drug control. Whereas etoposide strongly induced
apoptosis (12.2% early apoptotic and 22.3% late apoptotic/necrotic) compared to the negative control (2.9% and 10.3%, respectively), compound 1 was less apoptotic (8.4% and 15.7%, respectively) (Figure 7, panel C). These results are consistent with previous reports that GARFTase inhibitors are distinctly cytotoxic, yet modestly apoptotic (Deng et al., 2008; Smith et al., 1993).

**In vivo efficacy study of compound 1 against HepG2 xenografts.** As proof-of-concept that *in vivo* antitumor efficacy can result from tumor targeting of compound 1 via its transport by hPCFT, an *in vivo* efficacy trial was performed with 8 week old female ICR SCID mice implanted with subcutaneous HepG2 tumors, which express hPCFT and hRFC but not FRα (Figure 3A). Mice were maintained *ad libitum* on folate-deficient or standard folate-replete diets. Serum folate concentrations were measured in mice after 14 days on the folate-deficient diet by an *L. casei* bioassay; the value was 90.2 nM (median) [range = 79.2-120.7 nM (n=3)]. This value slightly exceeds serum folate levels (31 and 35 nM, respectively) previously reported in humans (Ganji and Kafai, 2009). With the standard diet, by comparison, serum folate was 715.2 nM (median) [range = 652.8-742.8 nM (n=3)]. For the trial, control and drug treatment groups were non-selectively randomized (five mice/group); compound 1 was administered intravenously on a Q4dx3 day schedule (180 mg/kg per injection) on days 4, 8, and 12 post-implantation (total dose 540 mg/kg). Results were compared to those for taxol (Q2dx6; 7.5 mg/kg per injection). Mice were weighed daily and tumors were measured 2-3 times per week. For the mice maintained on the folate-deficient diet, appreciable antitumor activity was recorded with compound 1 (T/C of 0% on day 21; T-C = 13 days; 1.4 gross log kill) (Figure 8), exceeding that for taxol (T/C=16%; 0.8 gross log kill). Antitumor drug efficacy for 1 was
completely abolished (99% T/C) for the standard folate-replete diet. The treatment regimen was well tolerated with dose-limiting symptoms manifesting as reversible body weight loss for mice maintained on the folate-deficient diet. Results for the *in vivo* efficacy experiment shown in Figure 8 are summarized in Table 3S in the supplement.

The results of the *in vivo* efficacy trial demonstrate potent antitumor activity for compound 1 toward subcutaneously engrafted HepG2 tumors associated with significant transport by hPCFT and a lack of membrane transport by hRFC.

**DISCUSSION**

In this study, we significantly expand upon previous reports (Kugel Desmoulin et al., 2010b; Zhao and Goldman, 2007) suggesting that PCFT may be therapeutically exploitable for treating solid tumors. We found that hPCFT, like hRFC, was widely and highly expressed in an extensive panel of human solid tumor cell lines but not in human leukemias. Another group showed that low pH transport activity of Mtx was prominent in human tumor cell lines (Zhao et al., 2004), in direct support of the findings reported herein. Twelve of the human sublines were included in both studies and for these there was reasonable correlation between hPCFT and hRFC transcript levels and transport activity at pH 5.5 and 7.4, respectively. We further show herein that the novel 6-substituted pyrrolo[2,3-\(d\)]pyrimidine thienoyl antifolate compound 1 can be selectively transported by hPCFT in a pH- and time-dependent manner. The tumor models employed, R1-11-PCFT4 HeLa and HepG2 cells, express similar levels of hPCFT, although they differ in the presence of hRFC and FRα.
The premise behind our drug discovery efforts, exemplified by compound 1, is that membrane transport of cytotoxic antifolates is a critical determinant of anti-tumor drug selectivity. Compound 1 is not transported by the ubiquitously expressed RFC (Wang et al., 2010). This is particularly important since drugs, such as compound 1, that target FRα and/or PCFT, yet are not substrates for RFC, have the potential to selectively target tumor cells and decrease toxicity to normal tissues. This is a substantial advantage over chemotherapy drugs currently in use and indeed pursuing the development of these novel antifolates could yield a new class of clinically relevant antitumor agents. Our previous work used engineered CHO models, as well as KB (nasopharyngeal) and IGROV1 (ovarian) human tumor cells, that express FRα and/or hPCFT to deliver cytotoxic antifolates, including compound 1, that are not substrates for hRFC (Deng et al., 2008, 2009; Wang et al., 2010). The present report significantly expands upon this concept by demonstrating exclusive transport of compound 1 by hPCFT into human tumor cell lines at pHs reflecting those characterizing the tumor microenvironment. For R1-11-PCFT4 and HepG2 cells, following its internalization at pH 6.8, compound 1 was extensively polyglutamylated, such that the predominant metabolite was the pentaglutamate form (compound 1 conjugated to 4 glutamate residues). Moreover, compound 1 potently inhibited GARFTase, leading to R1-11-PCFT4 HeLa cell death in vitro and HepG2 tumor growth delay in vivo.

Expression of hPCFT transcripts and protein in normal human tissues is more restrictive than for hRFC, with high hPCFT levels observed in the liver, kidney, and small intestine and very low levels in the bone marrow and colon (Kugel Desmoulin et al., 2010a). This pattern of PCFT transcripts was generally observed in mouse tissues (Qiu et al., 2007).
Our finding that hPCFT transcripts are low in human bone marrow (Kugel Desmoulin et al., 2010a) is particularly significant and suggests that hPCFT-targeted therapeutics may be less marrow toxic than antifolates presently in clinical use.

The microenvironments for most normal tissues likely exhibit a neutral pH (Martin and Jain, 1994), such that even if PCFT is present, the electrochemical proton gradient is reduced, leading to less accumulation of PCFT substrates such as compound 1. Conversely, RFC would exhibit a far greater activity under these conditions. This, when combined with the greater capacity of RFC to transport reduced folates across the cell membrane compared to PCFT (Zhao et al., 2008), would result in elevated levels of cellular folates in normal tissues. The increased availability of reduced folates would result in competition with internalized antifolates for polyglutamylation and/or for binding to intracellular drug targets (e.g., GARFTase), thus protecting normal cells from drug cytotoxicity. Similarly, for PCFT-targeted agents in solid tumors, if sufficient RFC was present, enough transport of folates may occur even at slightly acidic pHs to decrease drug efficacy on this basis. This implies that the ratio of PCFT to RFC in tumors is critical to antitumor activities of PCFT-selective cytotoxic antifolates and that Mtx resistant tumors that have substantially lost RFC function may be exquisitely sensitive to the effects of PCFT-selective drugs such as compound 1. Thus, for compound 1 and related agents, tumor selectivity is not only reliant upon differential PCFT levels between normal tissues and solid tumors, but it is also impacted by interstitial pH, and activity of RFC.
Another consideration involves the purine salvage pathway. Methylthioadenosine phosphorylase (MTAP) is an enzyme that releases adenine and 5-deoxy-5-(methylthio)ribose-1-phosphate (MTRP) from methylthioadenosine formed during polyamine biosynthesis (Illei et al., 2003). Adenine is used in purine salvage and MTRP is subsequently recycled to methionine. Whereas MTAP has been reported to be abundantly expressed in normal tissues, in many solid tumors the MTAP gene is co-deleted with CDKN2A (encodes p16INK4A) (Illei et al., 2003). Thus, many solid tumors are deficient in purine salvage and functional purine salvage in normal tissues would theoretically protect cells from cell death caused by GARFTase inhibition, increasing tumor cell selectivity for agents such as compound 1 (Hori et al., 1996).

It is interesting that under nearly the same conditions, the IC\textsubscript{50} for GARFTase inhibition in R1-11-PCFT4 cells by the \textit{in situ} GARFTase assay is virtually identical to the IC\textsubscript{50} for inhibition of cell proliferation. This result differs somewhat from our previous finding with an analogous 6-substituted pyrrolo[2,3-\textit{d}]pyrimidine benozyl antifolate in CHO cells, for which the IC\textsubscript{50} for GARFTase inhibition was substantially lower, suggesting that sustained GARFTase inhibition was necessary to manifest as cytotoxicity (Kugel Desmoulin et al., 2010b). This quantitative difference may reflect differences in the size of purine pools between the human and hamster sublines such that R1-11-PCFT4 HeLa cells would be more sensitive to the inhibition of GARFTase. Of course, other factors could also contribute. For instance, differences in drug polyglutamylation and polyglutamate turnover could result in disparate potencies for sustained GARFTase inhibition in different cell lines.
Finally, our studies with compound 1 assess the impact of GARFTase inhibition on ATP levels and the mechanism(s) of tumor cell death. Treatment of R1-11-PCFT4 cells with compound 1 substantially reduced ATP levels and caused S-phase accumulation. Apoptosis resulting from compound 1 was reduced compared to etoposide. This could (at least in part) reflect the requirement of ATP for apoptosis, as ATP levels must be maintained above a minimal level for apoptosis induction (Tsujimoto, 1997).

In conclusion, our in vitro studies suggest the feasibility of using hPCFT and the acidic tumor microenvironment to selectively deliver a novel PCFT-targeted antifolate to human solid tumors. Our in vivo results with HepG2 tumor cells that express only hPCFT and hRFC provide compelling proof-of-principle validation and rationale for developing drugs whose transport by PCFT, but not RFC, allows for GARFTase inhibition.

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AUTHORSHIP CONTRIBUTIONS


Wrote or contributed to the writing of the manuscript: S. Kugel Desmoulin, L. Wang, E. Hales, L. Polin, A. Gangjee, L.H. Matherly
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Racanelli AC, Rothbart SB, Heyer CL and Moran RG (2009) Therapeutics by cytotoxic metabolite accumulation: pemetrexed causes ZMP accumulation, AMPK


FOOTNOTES

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L.H. Matherly and A. Gangjee contributed equally to this work.

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FIGURE LEGENDS

Figure 1. Structure of 6-substituted pyrrolo[2,3-d]pyrimidine thienoyl antifolate compound (Wang et al., 2010).

Figure 2. PCFT and RFC expression in human solid tumor cell lines. hPCFT (upper panel) and hRFC (lower panel) transcripts were measured in 53 human solid tumor cell lines by real-time RT-PCR from total RNAs using a Roche480 Light-cycler. Transcript levels were normalized to GAPDH transcripts. Experimental details are provided in the Materials and Methods. Results for FRα and for transporter levels in 27 human leukemia cell lines are included in Figure 1S, panels B-E (Supplement). A table summarizing the characteristics of the 53 tumor and 27 leukemia cell lines is also included in Table 1S (Supplement).

Figure 3. Characterization of folate transporter expression and compound 1 and Pmx growth inhibition and inhibition of colony formation in R1-11 sublines and HepG2 cells. (A) FRα, hPCFT and hRFC transcript levels in R1-11 sublines and HepG2 cells was measured by real-time reverse transcriptase PCR. (B and C) Growth inhibition curves for folate-depleted R1-11-PCFT4 and -RFC6 cells treated with Pmx or compound 1 for 96 h are shown. (D and E) R1-11-PCFT4 cells were plated in 60 mm dishes at a density of 500 cells per dish and allowed to adhere overnight. Cells were treated at pH 6.8 in the presence or absence of different concentrations of compound 1 or Pmx from 0 to 10 µM for 16, 24 and 48 h, followed by drug wash-out. Plates were scored by counting
visible colonies after 12 days (by staining with methylene blue) and presented as a percent of vehicle control.

**Figure 4. pH- and time-dependent transport of compound 1 and Pmx into R1-11-PCFT4 and HepG2 cells.** Direct hPCFT transport activity of compound 1 (A-D) and Pmx (E) in R1-11-PCFT4 (A-C) and HepG2 (D and E) cells was assessed by measuring uptake of 0.25 μM [3H]compound 1 or [3H]Pmx over 2-30 min at 37°C in complete folate-free RPMI 1640 (pH 5.5, 6.8 and 7.2), supplemented with 10% dFBS, and 25 mM HEPES/25 mM PIPES. Internalized [3H]compound 1 and [3H]Pmx were normalized to total protein and expressed as pmol/mg protein.

**Figure 5. HPLC analysis of polyglutamyl derivatives of compound 1 in HepG2 cells at pH 6.8.** HepG2 cells were treated with 1 μM [3H]compound 1 at pH 6.8 in the presence of adenosine (60 μM) for 16 h. Polyglutamates were extracted by boiling in 50 mM phosphate buffer (pH 6.0) containing 100 mM 2-mercaptoethanol and separated on a 5 μm Spherisorb C-18 ODS-2 column (4.6 mm x 250mm) with a Nova-Pak 4 μm C-18 guard column. Fractions were collected and radioactivity was measured. Percent monoglutamate and polyglutamate drug forms were determined by chromatographic analysis and the total intracellular radiolabeled drug calculated in units of pmol/mg protein (Table 2).

**Figure 6. In situ GARFTase inhibition by compound 1 and Pmx in R1-11-PCFT4 cells.** GARFTase activity and inhibition were evaluated in situ with R1-11-PCFT4 cells. R1-11-PCFT4 cells were treated with drug for 16 h at pH 6.8 in complete folate-free
RPMI 1640 supplemented with 10% dFBS, and 25 mM HEPES and 25 mM PIPES before incubating in the presence of 4 μM azaserine for 30 min, followed by [14C]glycine and L-glutamine treatment. After 8 h, radioactive metabolites were extracted and fractionated on 1 cm columns of AG1x8(Cl–) and the fractions were collected and radioactivity measured. Accumulation of [14C]formyl GAR was calculated as a percent of vehicle control over a range of antifolate concentrations.

Figure 7. Compound 1 treatment depletes ATP levels and induces an S-phase cell arrest, accompanied by a modest level of apoptosis in R1-11-PCFT4 cells. (A) For analysis of ATP levels, cells were treated with 10 μM compound 1 or left untreated (DMSO) for 48 h at pH 6.8. Nucleotides were extracted and ATP pools were determined by a modification of the HPLC method of Huang et al. (Huang et al., 2003), as previously described (Kugel Desmoulin et al., 2010b). Details are provided in the Materials and Methods. (B) The percentages of cells in each phase of the cell cycle (G1, S, and G2), including those in the subG1 fraction, were determined in R1-11-PCFT4 cells treated with a range of concentrations of compound 1 for 48 h by measuring the cellular DNA content with PI staining and flow cytometry. Representative cell cycle profiles are shown in the Supplement (Figure 3S). (C) Pseudo-color dot plots show the flow cytometric analysis of cells stained with annexin V-FITC and PI. The percentages of viable cells (annexin V-/PI−), early apoptotic cells (annexin V+/PI−), and late apoptotic/necrotic cells (annexin V+/PI+) are noted. As a positive control, cells were treated with 5 μM etoposide for 48 hrs at pH 6.8 to induce apoptosis.
Figure 8. *in vivo* efficacy trial of compound 1 in HepG2 xenografts. Female ICR SCID mice were maintained on a folate-deficient diet *ad libitum*. Human HepG2 tumors were implanted bilaterally and mice were non-selectively randomized into 5 mice/group. Compound 1 [dissolved in 5% ethanol (v/v), 1% Tween-80 (v/v), 0.5% NaHCO₃] was administered on a Q4dx3 schedule intravenously on days 4, 8, and 12 (indicated with arrows) at 180 mg/kg/injection. Taxol (dissolved in water) was administered on a Q2dx6 schedule (7.5 mg/kg/injection) beginning on day 4. Mice were observed and weighed daily; tumors were measured twice per week. For the mice maintained on the folate-deficient diet and treated with compound 1, appreciable antitumor activity was recorded (T/C=0%; T-C=13 days; 1.4 gross log kill). Data are shown for the median tumor burdens and are summarized in Table 3S in the Supplement.
Table 1. Kinetic constants for hPCFT. Kinetic constants for compound 1 and Pmx (K_t and V_max) were determined with [3H]compound 1 and [3H]Pmx respectively, and calculated from Lineweaver Burke plots with R1-11-PCFT4 HeLa cells. Results are presented as mean values ± standard errors from 3 experiments.

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<td>K_i (μM)</td>
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### Table 2. Distribution of compound 1 and Pmx polyglutamates in R1-11-PCFT4 and HepG2 cells.

Cells were incubated for 16 h with 1 μM [³H]compound 1 or [³H]Pmx. Drug accumulations and HPLC analysis of [³H] polyglutamate metabolites were performed as described in Materials and Methods.

<table>
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<th>Metabolites</th>
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<th>Compound 1 HepG2 (pmol/mg)</th>
<th>Pmx HepG2 (pmol/mg)</th>
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<td>Total Drug</td>
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*Parent (unmetabolized) drug.
Figure 1.
Figure 2A and B.
Figure 3A, B, and C.

A

B

Pemetrexed

C

Compound 1

Relative transcripts

Concentration (nM)

% Cell growth

Concentration (nM)

% Cell growth
Figure 3D and E.
Figure 4 A, B, and C.

A. pH 5.5 - [3H]Compound 1

B. pH 6.8 - [3H]Compound 1

C. pH 7.2 - [3H]Compound 1
Figure 4D and E.

D  
HepG2 - $[^3]$HCompound 1

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pmol/mg protein

E  
HepG2 - $[^3]$HPmx

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</tr>
</tbody>
</table>

pmol/mg protein
Figure 5.

HepG2
Compound 1

CPM

Fraction number

PG₅
PG₄
PG₃
PG₂
PG₁

38%
17%
11%
9%
16%
10%

PG₆

Cpd 1

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Figure 6.

% of Control

0.00 0.05 0.10 0.50 1.00 5.00

Drug concentration (μM)

0.00
0.05
0.10
0.50
1.00
5.00

R1-11-PCFT4 - pH 6.8

Cpd 1

Pmx
Figure 7A and B.

A

% of Control

DMSO 48 h

B

Percent

Cpd1 (μM)

SubG1 G1 S G2

21.49 17.43 16.79 21.68 19.04
16.81 25.99 34.01 35.26 38.91
52.43 42.51 31.44 28.77 26.99
9.27 14.07 17.75 14.29 15.06
21.49 17.43 16.79 21.68 19.04
16.81 25.99 34.01 35.26 38.91
52.43 42.51 31.44 28.77 26.99
9.27 14.07 17.75 14.29 15.06
Figure 7C

- **DMSO**
  - 84.2% in lower left quadrant
  - 2.93% in upper right quadrant

- **Compound 1 (10 µM)**
  - 75.6% in lower left quadrant
  - 8.40% in upper right quadrant

- **Etoposide (5 µM)**
  - 64.5% in lower left quadrant
  - 12.2% in upper right quadrant
Figure 8.

180 mg/kg/inj IV (540 mg/kg total dose)

Days of Injection: 4, 8, 12

- No Rx
- Compound 1
- Taxol
- limit of palpation

Median Tumor Burden (in mg)

Time Post Tumor Implant (Days)