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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Received May 27, 2011; accepted September 22, 2011

ABSTRACT

The proton-coupled folate transporter (PCFT) is a proton-folate symporter with an acidic pH optimum. By real-time reverse transcription-polymerase chain reaction, 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]-labeled 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.

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This study was supported by the National Institutes of Health National Cancer Institute [Grants CA65335, CA152316, CA125153]; the Barbara Ann Karmanos Cancer Institute; the Mesothelioma Applied Research Foundation; and a Doctoral Research Award from the Canadian Institutes of Health Research (to S.K.D.).

L.H.M. and A.G. contributed equally to this work.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.111.073833.

[1] The online version of this article (available at http://molpharm.aspetjournals.org) contains supplemental material.

ABBREVIATIONS: Mtx, methotrexate; Pmx, pemetrexed; GAR, β-glycinamide ribonucleotide; GARFTase, β-glycinamide ribonucleotide formyltransferase; RFC, reduced folate carrier; FR, folate receptor; PCFT, proton-coupled folate transporter; h, human; LCV, leucovorin; RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; dFBS, dialyzed fetal bovine serum; DMSO, dimethyl sulfoxide; PIPES, 25 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (HPLC, high-performance liquid chromatography; FITC, fluorescein isothiocyanate; PI, propidium iodide; SCID, severe combined immunodeficiency; ALL, acute lymphoblastic leukemia; CHO, Chinese hamster ovary; MTAP, methylthioadenosine phosphorylase.

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 (Monahan and Allegra, 2006). Subsequent generations of antifolates...
primarily targeted other key folate-dependent enzymes, including thymidylate synthase [5-ethynyl-2′-deoxyuridine (5-Edu)] and β-glycinamidase ribonucleotide formyltransferase (GARFTase) (lometrexol). For all these agents, cellular uptake and metabolism to polyglutamates are critical to drug activity (Mendelsohn et al., 1999; Monahan and Allegra, 2006; Chattopadhay et al., 2007).

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. 1) 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. 2) 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 (El-Nakat and Ratnam, 2004). 3) The proton-coupled folate transporter (PCFT; SLC46A1) is a proton-folylate symporter that functions optimally at acidic pH by coupling the downhill flow of protons to the uphill flow of folates into cells (Qiu et al., 2006; Nakai et al., 2007; 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 jejenum and duodenum, and in kidney, liver, placenta, and choroid plexus, whereas much lower levels were detected in other tissues (Qiu et al., 2007; Zhao et al., 2009; Kugel Desmoulin et al., 2010a). Although a low pH transport activity was described in human tumor cell lines of assorted origins (Zhao et al., 2004), presumably as a result of 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 (Gibbs et al., 2005; Hilgenbrink and Low, 2005; Salazar and Ratnam, 2007; Deng et al., 2008, 2009; 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 to 6.8 (Wike-Hooley et al., 1984; Helmlinger et al., 1997; Raghu-nand et al., 1999). 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 have been developed that are specifically transported by FRs and/or PCFT and not by RFC (Gibbs et al., 2005; Hilgenbrink and Low, 2005; Salazar and Ratnam, 2007; Deng et al., 2008, 2009; Kugel Desmoulin et al., 2010b; Wang et al., 2010). This strategy is necessary because antifolate membrane transport by RFC precludes tumor selectivity, in that RFC is expressed in both normal and tumor cells, and RFC transport is optimal at neutral pH characterizing most normal tissues (Zhao and Goldman, 2003; Matherly et al., 2007). Indeed, a major obstacle in implementing this approach has been a lack of FR- or PCFT-selective antifolates, because all of the clinically useful antifolates with significant FR- and PCFT substrate activity (e.g., Mtx and Pnx) 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 four carbons (compound 1) (Fig. 1) (Wang et al., 2010). Cellular uptake of compound 1 by FRα was substantial in the absence of its membrane transport by RFC, resulting in potent antitumor activity both in vitro and in vivo because of inhibition of GARFTase in de novo purine nucleotide biosynthesis. Although 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]Pnx (2.5 Ci/mmol), and custom-radiolabeled [3H](compound 1 (1.3 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA). Leucovorin (LCV) ([6R,5S]-5-formyl tetrahydrofolate) was provided by the Drug Development Branch, National Cancer Institute (Bethesda, MD). Pnx [N-(4-[2-(2-amino-3,4-dihydro-4-oxo-7H-pyrrolo[2,3-d]pyrimidin-5-yl)ethyl]benzoyl)-L-glutamic acid (Alima) was provided by Eli Lilly and Co. (Indianapolis, IN). Synthesis and properties of the substituted pyrrolo[2,3-d]pyrimidine antifolate compound 1 were described previously (Wang et al., 2010). Other chemicals were obtained from commercial sources with previous availability.

**Cell lines.** The sources and cell culture conditions for the panel of human solid tumor and leukemia cell lines used for quantitative RT-PCR assays of transcript levels for FRα, hPCFT, and hRFC are summarized in Supplemental Table 1S. 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(R1-11-RFC6) and pZeoSV2(R1-11-PCFT4) 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 described previously (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 (Supplemental Table 1S) and engineered R1-11 HeLa sublines (R1-11 mock, R1-11-RFC6, and R1-11-PCFT4), using TRIzol reagent

![Fig. 1. Structure of 6-substituted pyrrolo[2,3-d]pyrimidine thienyl antifolate compound (Wang et al., 2010).](image-url)
(Invitrogen, Carlsbad, CA). cDNAs were synthesized using SuperScript reverse transcriptase III kit (Invitrogen). cDNAs were purified with the QIAquick PCR Purification Kit (Qiagen, Valencia, CA). 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 Supplemental Table 2S. Transcript levels for FRα, hPCFT, and hRFC genes were normalized to those for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using commercial probes and primers (Universal ProbeLibrary Human GAPD Gene Assay; Roche Applied Science, Indianapolis, IN). 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-PCT4, R1-11-RFC6 HeLa, and HepG2 cells were cultured in folate-free RPMI 1640 medium, pH 7.2, containing 25 nM LCV, supplemented with 10% diazoylated folate bovine serum (dFBS; Invitrogen), 2 mM l-glutamine, 100 units/ml penicillin, and 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 assessed with CellTiter-Blue cell viability assay (Promega, Madison, WI) and a fluorospectrometer (emission at 590 nm, excitation at 560 nm) for determining IC₅₀ values, corresponding to drug concentrations that result in 50% loss of cell growth.

For colony-forming assays, folate-depleted R1-11-PCT4 cells (500 cells in 2 ml of transport buffer) and HepG2 cells were cultured 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 PIPES and 25 mM 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 plus dFBS, 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, and borate buffer (10 mM, pH 9.8), followed by 1% methylene blue (in borate buffer). The dishes were again rinsed with borate buffer, and colonies were counted for calculating percentage colony formation relative to the DMSO controls.

**Transport Assays.** To determine the pH-dependent transport of [³H]compound 1 and [³H]Pmx (both at 0.25 μM) in R1-11-PCT4, R1-11-mock, and HepG2 cells, uptake was assayed at 37°C in cell monolayers over 2 to 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 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 based on lineages in the peaks from the HPLC chromatogram and total picomoles per milligram 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 0.1 ml of 0.1 M sodium borate, pH 7.8, 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]Glucose into [¹⁴C]Formyl GAR as an in situ measure of endogenous GARFTase activity in folate-depleted R1-11-PCT4 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-PCT4 cells were seeded in 5 ml of complete folate-free RPMI 1640 medium/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 of complete folate-free RPMI 1640 medium/10% dFBS with 25 mM PIPES/25 mM HEPES, pH 6.8, and 25 mM 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 of complete folate-free, L-glutamine-free RPMI 1640 medium/10% dFBS plus 25 mM PIPES/25 mM HEPES, pH 6.8, and 25 mM 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 of complete folate-free, L-glutamine-free RPMI 1640 medium/10% dFBS plus 25 mM PIPES/25 mM HEPES, pH 6.8, and 25 mM LCV with or without 0.5 to 100 nM antifolate and azaserine (final concentration, 4 μM), and incubated for 30 min. L-Glutamine (final concentration, 2 mM) and [¹⁴C]Glucose (final specific activity, 0.1 μCi/μl) 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% trichloroacetic acid 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 ex-
tracted 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, Hercules, CA), washed with 10 ml of 0.5 N formic acid, followed by 10 ml of 4 N formic acid, and eluted with 8 ml of 1 N HCl solution. The eluents were collected as 1-ml fractions and determined for radioactivity.

**Determination of Intracellular ATP levels.** For analysis of ATP levels after antifolate treatments, R1-11-PCFT4 cells were seeded in 10 ml of complete folate-free RPMI 1640 medium/10% dFBS, with 25 mM PIPES/25 mM HEPES, pH 6.8, and 25 mM LCV. After 24 h, 10 μM compound 1 or DMSO (final concentration, 0.5%) (control) was added to the culture medium. Cells were incubated for an additional 24 to 72 h, after which they were trypsinized and washed twice with ice-cold DPBS. Nucleotides were extracted and ATP levels quantitated by HPLC exactly as described previously (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 medium/10% dFBS with 25 mM PIPES/25 mM HEPES, pH 6.8, and 25 mM 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 (−10⁶) with fluorescein isothiocyanate (FITC)-conjugated annexin V and propidium iodide (PI) with the apoptotic cell cycle distribution (widespread fluorescent isothiocyanate (FITC)-conjugated annexin V and propidium iodide (PI) with the apoptotic cell cycle distribution (Wang et al., 2010). The following quantitative end points were used to assess antitumor activities: 1) TIC and T − C (tumor growth delay) where T is the median time in days required for the treated 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 2) calculation of tumor cell kill [log10 cell kill total (gross) = (T − C)/3.32(TD)2], where (T − C) is the tumor growth delay, as described above, and T(D) 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- to 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.** On the basis of a report of a low pH transport activity in solid tumor cell lines (Zhao et al., 2004), presumably reflecting PCFT, we turned our attention to establishing an expression profile for hPCFT compared with 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 real-time RT-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.) (Fig. 2A), and uniformly low hPCFT transcript levels in human leukemias, including both ALL and acute myeloid leukemia (Supplemental Fig. 1S, C). 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 (Fig. 2B and Supplemental Fig. 1S, D). High levels of FRα were detected in a small subset of ovarian, cervical, and breast cancer cell lines, and low but detectable FRβ levels were measured in ALL (mostly T-cell) sublines (Supplemental Fig. 1S, A and 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 acute myeloid leukemia and T-cell ALL sublines (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 (Fig. 1) was a potent (nanomolar) 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 from 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) (Fig. 3A). 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 (Fig. 2) without FRα (expression levels for hPCFT and hRFC in HepG2 cells are compared with those for the R1-11 sublines in Fig. 3A).

We measured inhibition of cell proliferation by compound 1 and results were compared with those for Pmx. Pmx inhibited cell growth in both the R1-11-PCFT4 and R1-11-RFC6 lines with IC₅₀ values (mean ± S.E.M.) of 59.3 ± 7.37 and 81.7 ± 5.49 nM, respectively (Fig. 3B), demonstrating its lack of specificity for hPCFT over hRFC despite its high PCFT substrate activity (Zhao et al., 2008; Kugel Desmoulin et al., 2010b). Conversely, compound 1 inhibited cell growth in R1-11-PCFT4 cells (IC₅₀ 99.2 ± 20.2 nM) but not R1-11-RFC6 (Fig. 3C), 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 with R1-11-PCFT4 cells probably 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 (Fig. 3D) or Pmx (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. 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. Despite the latter activity, 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 antifo-
lates 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 (Fig. 4, A–C). Uptake in R1-11-PCFT4 cells exceeded that of its hPCFT-null isogenic counterpart (R1-11-mock transfected), 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 min and probably reflects the presence of low levels of FR in these cells (Fig. 3A).

We compared the uptake of [3H]Pmx with that of [3H]com-
compound 1 in HepG2 cells (Fig. 4, D and E). For compound 1, pH-dependent uptake in HepG2 cells showed a profile (despite the ~2-fold increased net uptake) similar to that of R1-11-PCFT4 HeLa cells. Net uptake of [3H]Pmx exceeded that of [3H]compound 1 in HepG2 cells by ~50 to 100% and showed a greater uptake fraction at neutral pH, most likely due to the presence of hRFC in HepG2 cells (Fig. 3A).

We measured transport kinetics over 2 min for [3H]comp-
TABLE 1
Kinetic constants for hPCFT

<table>
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<tr>
<th>Substance and Parameter</th>
<th>pH 5.5</th>
<th>pH 6.8</th>
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<tbody>
<tr>
<td>Pmx</td>
<td></td>
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<tr>
<td>$K_v$, μM</td>
<td>0.03 ± 0.003</td>
<td>4.43 ± 0.253</td>
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<tr>
<td>$V_{max}$, pmol · mg⁻¹ · min⁻¹</td>
<td>1.27 ± 0.023</td>
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<tr>
<td>$V_{max}/K_v$</td>
<td>42.3</td>
<td>0.49</td>
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<tr>
<td>Compound 1</td>
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<tr>
<td>$K_v$, μM</td>
<td>0.02 ± 0.013</td>
<td>5.91 ± 1.36</td>
</tr>
<tr>
<td>$V_{max}$, pmol · mg⁻¹ · min⁻¹</td>
<td>1.76 ± 0.154</td>
<td>3.08 ± 0.451</td>
</tr>
<tr>
<td>$V_{max}/K_v$</td>
<td>88</td>
<td>0.52</td>
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</table>

Compound 1 and $[3H]$Pmx in R1-11-PCFT4 cells using a range of drug concentrations at pH 5.5 and 6.8 (Table 1). The data show nearly identical $K_v$ values for compound 1 and Pmx at pH 5.5 and only modest (within ~40%) differences in $V_{max}$. Increases in both $K_v$ (increased ~300- to 400-fold, respectively, compared with values at pH 5.5) and $V_{max}$ values (~70% increased) were measured at pH 6.8. $V_{max}/K_v$ 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-d]pyrimidine Thienoyl Antifolate Compound 1 in R1-11-PCFT4 and HepG2 Cells. Polyglutamylation of classic antifolates is a critical factor in drug activity, because these conjugated drug forms are retained within cells and they typically inhibit folate-dependent enzyme targets to a greater extent than their nonpolyglutamyl 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 $[3H]$compound 1 for 16 h at pH 6.8 in the presence of adenosine (60 μM). For HepG2 cells, parallel incubations were performed with $[3H]$Pmx (in presence of thymidine (10 μM) and adenosine). $[3H]$-labeled metabolites were extracted and analyzed by reversed-phase HPLC (Fig. 5) shows an HPLC chromatogram for compound 1 in HepG2 cells; additional results for R1-11-PCFT4 and HepG2 cells are included in the Supplemental Fig. 2S, A and B). Up to five polyglutamyl metabolites of $[3H]$compound 1 and $[3H]$Pmx (PG₂₋₆) were resolved by HPLC. The identities of the peaks were confirmed by comparing retention 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 (Supplemental Fig. 2S, 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 Fig. 4), in both cases, compound 1 was predominantly polyglutamylated (64 and 84% of the total intracellular drug, respectively). For HepG2 cells, the increased accumulation of $[3H]$compound 1 over that of R1-11-PCFT4 cells was reflected in the polyglutamate levels. Analogous results were obtained with $[3H]$Pmx in HepG2 cells, although the net extent of drug uptake and metabolism of $[3H]$Pmx was elevated over that of $[3H]$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 that quantifies incorporation of $[14C]$glycine into $[14C]$formyl GAR as a measure of GARFTase inhibition. Results were compared with 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 (Racanelli et al., 2009) (Fig. 6). IC₅₀ values for GARFTase inhibition in R1-11-PCFT4 cells by compound 1 and Pmx were 43.6 and 69.7 nM, respectively. Although the IC₅₀ for GARFTase inhibition by compound 1 closely approximated the IC₅₀ for growth inhibition of R1-11-PCFT4 cells (Fig. 3C), GARFTase inhibition by Pmx was incomplete up to 5 μM. Analogous results were described for Pmx with hPCFT-expressing CHO cells (R2/...
with 10 pools, we measured intracellular ATP levels in cells treated with 10% dFBS, and 25 mM HEPES and 25 mM PIPES before incubating 16 h at pH 6.8 in complete folate-free RPMI 1640 medium supplemented with subcutaneous HepG2 tumors that express hPCFT and hRFC, was widely and highly expressed in an extensive 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.

Because treatment with compound 1 (10 μM, 48 h at pH 6.8) causes loss of clonogenicity in R1-11-PCFT4 cells (Fig. 3D) and a modest increase in the sub-G1 fraction (Fig. 7B), we were interested in measuring apoptosis under these same conditions using annexin V/PI staining. Results were compared with 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 with the negative controls (2.9 and 10.3%, respectively), compound 1 was less apoptotic (8.4 and 15.7%, respectively) (Fig. 7C). These results are consistent with previous reports that GARFTase inhibitors are distinctly cytotoxic, yet modestly apoptotic (Smith et al., 1993; Deng et al., 2008).

### 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 that express hPCFT and hRFC but not FRα (Fig. 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) reported previously 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 nonselectively randomized (five mice per group); compound 1 was administered intravenously on a schedule of every 4 days for three treatments (180 mg/kg per injection) on days 4, 8, and 12 after implantation (total dose 540 mg/kg). Results were compared with those for paclitaxel (Taxol; every 2 days for six treatments, 7.5 mg/kg per injection). Mice were weighed daily and tumors were measured 2 to 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; 14 gross log kill) (Fig. 8), exceeding that for paclitaxel (*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 Fig. 8 are summarized in Supplemental Table 3S.

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 (Zhao and Goldman, 2007; Kugel Desmoulin et al., 2010b) 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 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 antitumor drug selectivity. Compound 1 is not transported by the ubiquitously expressed RFC (Wang et al., 2010). This is particularly important because 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; 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.

Fig. 7. Compound 1 treatment depletes ATP levels and induces an S-phase cell accumulation, 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 described previously (Kugel Desmoulin et al., 2010b). Details are provided under Materials and Methods. B, the percentages of cells in each phase of the cell cycle (G1, S, and G2), including those in the sub-G1 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 (Fig. 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 h at pH 6.8 to induce apoptosis.
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 pH values characterizing the tumor microenvironment. For R1-11-PCFT4 and HepG2 cells, after 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, high hPCFT levels being 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., 2010). 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 probably exhibit a neutral pH (Martin and Jain, 1994), so 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 with 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. Likewise, for PCFT-targeted agents in solid tumors, if sufficient RFC were present, enough transport of folates might occur even at slightly acidic pH values 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 is also affected 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 from methylthioadenosine formed during polyamine biosynthesis (Illei et al., 2003). Adenine is used in purine salvage, and 5-deoxy-5-(methylthio)ribose-1-phosphate 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 codelleted 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 $IC_{50}$ for GARFTase inhibition in R1-11-PCFT4 cells by the in situ GARFTase assay is virtually identical to the $IC_{50}$ for inhibition of cell proliferation. This result differs somewhat from our previous finding with an analogous 6-substituted pyrrolo[2,3-d]pyrimidine benzoyl antifolate in CHO cells for which the $IC_{50}$ 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 with etoposide. This could (at least in part) reflect the requirement of ATP for apoptosis, because 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.

Acknowledgments
We thank Dr. I. David Goldman for the generous gift of the R1-11 HeLa cell line series (R1-11-mock, R1-11-RFC6, and R1-11-PCFT4). We thank Kelly Haagenson for editorial assistance in preparing the manuscript.

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