Functional Loss of the Reduced Folate Carrier Enhances the Antitumor Activities of Novel Antifolates with Selective Uptake by the Proton-Coupled Folate Transporter[S]

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ABSTRACT

Uptake of 6-substituted pyrrolo[2,3-d]pyrimidine thienoyl antifolates with four or three bridge carbons [compound 1 (C1) and compound 2 (C2), respectively] into solid tumors by the proton-coupled folate transporter (PCFT) represents a novel therapeutic strategy that harnesses the acidic tumor microenvironment. Although these compounds are not substrates for the reduced folate carrier (RFC), the major facilitative folate transporter, RFC expression may alter drug efficacies by affecting cellular tetrahydrofolate (THF) cofactor pools that can compete for polyglutamylation and/or binding to intracellular enzyme targets. Human tumor cells including wild-type (WT) and R5 (RFC-null) HeLa cells express high levels of PCFT protein. C1 and C2 inhibited proliferation of R5 cells 3 to 4 times more potently than WT cells or R5 cells transfected with RFC. Transport of C1 and C2 was virtually identical between WT and R5 cells, establishing that differences in drug sensitivities between sublines were independent of PCFT transport. Steady-state intracellular [H]THF cofactors derived from [3H]5-formyl-THF were depleted in R5 cells compared with those in WT cells, an effect exacerbated by C1 and C2. Whereas C1 and C2 polyglutamates accumulated to similar levels in WT and R5 cells, there were differences in polyglutamyl distributions in favor of the longest chain length forms. In severe combined immunodeficient mice, the antitumor efficacies of C1 and C2 were greater toward subcutaneous R5 tumors than toward WT tumors, confirming the collateral drug sensitivities observed in vitro. Thus, solid tumor-targeted antifolates with PCFT-selective cellular uptake should have enhanced activities toward tumors lacking RFC function, reflecting contraction of THF cofactor pools.

Introduction

Classic antifolates such as methotrexate (MTX) and pemetrexed (PMX), like folate cofactors, have minimal lipid solubility and therefore require specific transport mechanisms to enter mammalian cells. There are three primary folate transporters, including the reduced folate carrier (RFC), the proton-coupled folate transporter (PCFT), and folate receptor (FR) α (Assaraf, 2007; Goldman et al., 2010). RFC is the predominant transport route for the major circulating folate, 5-methyl-tetrahydrofolate (THF), and (6S)-5-formyl THF (5-CHO-THF) in mammalian cells and tissues (Matherly et al., 2007). RFC also mediates cellular uptake of MTX and is essential to MTX antitumor activity (Matherly et al., 2007). Impaired RFC function is a major mechanism of MTX resistance in cultured tumor cells selected in vitro (Zhao and Goldman, 2003; Matherly et al., 2007) and in murine leuko-

ABBREVIATIONS: MTX, methotrexate; PMX, pemetrexed; RFC, reduced folate carrier; PCFT, proton-coupled folate transporter; FR, folate receptor; THF, tetrahydrofolate; 5-CHO-THF, 5-formyl-tetrahydrofolate; C1, compound 1; C2, compound 2; hRFC, human reduced folate carrier; hPCFT, human proton-coupled folate transporter; HA, hemagglutinin; WT, wild-type; RT, reverse transcription; PCR, polymerase chain reaction; MES, 4-morpholinopropane sulfonic acid; DPBS, Dulbecco’s phosphate-buffered saline; PIPES, piperazine-N,N’-bis(2-ethanesulfonic acid); HPLC, high-performance liquid chromatography; SCID, severe combined immunodeficient; LMX, lonetrexol; RTX, raltitrexed; PT523, N’-(4-amino-4-deoxypteroyl)-N’-hemipthaloyl-L-ornithine; PG, polyglutamate; FPGS, folylpolyglutamate synthetase; ABC, ATP-binding cassette.

Received March 29, 2012; accepted June 26, 2012

[88x732]82:591–600, 2012

http://dx.doi.org/10.1124/mol.112.079004.

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

This study was supported by the National Institutes of Health National Cancer Institute [Grants CA53535, CA152316, CA125153]; the Barbara Ann Karmanos Cancer Institute; and the Mesothelioma Applied Research Foundation. S.K.D. was supported by a Doctoral Research Award from the Canadian Institute of Health Research. A.G. and L.H.M. contributed equally to this work.

Article, publication date, and citation information can be found at http://dx.doi.org/10.1124/mol.112.079004.
mia cells in vivo (Sirotnak et al., 1981). Loss of RFC function in clinical specimens has also been reported (Gorlick et al., 1997; Guo et al., 1999; Yang et al., 2003). RFC transport of cytotaxic antifolates can also be undesirable because RFC is ubiquitously expressed and exhibits a high level of activity at the neutral pH values characterizing most normal tissues (Matherly et al., 2007). Thus, transport of antifolates by RFC could easily preclude tumor selectivity and cause toxicity to normal tissues.

The novel 6-substituted pyrrolo[2,3-d]pyrimidine thienoyl antifolates with four [compound 1 (C1)] or three [compound 2 (C2)] carbon bridge lengths (Fig. 2A) represent a new class of antitumor agents that exhibit a lack of significant membrane transport by RFC (Wang et al., 2010, 2011; Kugel Desmoulin et al., 2011). Cellular uptake of C1 and C2 by PCFT and FRs is efficient and offers a promising new strategy for solid tumor targeting (Anderson and Thwaites, 2010; Kugel Desmoulin et al., 2011). Because PCFT functions optimally at acidic pH values (Qiu et al., 2006; Umapathy et al., 2007; Zhao et al., 2008), transport of C1 and C2 by PCFT may lead to further enhancement of tumor selectivity owing to the acidic microenvironments of many solid tumors (Helmingler et al., 1997; Gillies et al., 2002; Anderson and Thwaites, 2010; Webb et al., 2011). Our previous results established that C1 and C2 are potent inhibitors of tumor cell proliferation both in vitro and in vivo (Wang et al., 2010, 2011; Kugel Desmoulin et al., 2011).

For agents such as PMX that are excellent substrates for both RFC and PCFT, loss of RFC has a limited effect on overall activity, because PMX uptake is maintained by PCFT (Zhao et al., 2004c, 2008). Paradoxically, RFC loss has been shown to enhance antitumor activity (i.e., collateral sensitivity) of PMX via decreased intracellular THF cofactor pools (Zhao et al., 2004c; Chattopadhyay et al., 2006, 2007). This response to RFC loss can be further affected by the type and amount of extracellular folate (Zhao et al., 2004b,c; Chattopadhyay et al., 2006).

An analogous effect may exist for PCFT-selective substrates such as C1 and C2, although this has never been systematically tested. In this report, we examine the complex interplay between RFC and extracellular reduced folates. Specifically, we investigate the mechanistic ramifications of loss of RFC function on in vitro and in vivo tumor efficacies of these PCFT-targeted antifolates. Our results strongly imply that levels of folate transport by RFC in tumors are critical determinants of drug efficacy for this novel class of PCFT-selective antitumor agents.

Materials and Methods

Materials. [3',5',7',9'-H1]MTX (20 Ci/mmol), [3'H]PMX (2.5 Ci/ mmol), [3',5',7,9-3H(A)]6(S)-5-formyl tetrahydrofolate (16.6 Ci/ mmol), and custom-radiolabeled [3'H]C1 (1.3 Ci/mmol) and [3'H]C2 (16 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA). Sources of the nonradioactive folate and antifolate drugs are summarized in Supplemental Table 1S.

Cell Lines. The sources and cell culture conditions for assorted human solid tumor cell lines are summarized in Supplemental Table 2S. HeLa cells were obtained commercially. RFC-null R5 HeLa cells were described previously (Zhao et al., 2004b). R11-mock and R11-PCFT4 HeLa cells were derived from humanRFC(−)- and -null R1-11 cells by stable transfection with pZeosV2(+) vector (Invitrogen, Carlsbad, CA) only or with hemagglutinin (HA)-tagged hPCFT in pZeosV2(+), respectively. Characteristics and culture conditions of the engineered HeLa sublines were described previously (Zhao et al., 2008) and are also in Supplemental Table 2S.

Preparation of hRFC[CH3]/pZeosV2 and Generation of Stable hRFC R5 Transfectants. Full-length hRFC was subcloned using BamHI and XhoI into pZeosV2(+) in-frame with a C-terminal HA sequence to generate hRFC[CH3]/pZeosV2. The plasmid was transformed into XL10-Gold ultracompotent cells (Agilent Technologies, Santa Clara, CA) and selected using low-salt LB agar plates containing 25 μg/ml phleomycin (Zeocin). Plasmids were isolated and the wild-type (WT) hRFC insert was confirmed by DNA sequencing by Genewiz Corp. (South Plainfield, NJ).

R5 cells were transfected with pZeosV2 vector control or hRFC[CH3] pZeosV2 with Lipofectamine 2000 and Opti-MEM (Invitrogen). After 24 h, the cells were cultured with phleomycin (0.1 mg/ml). Stable clones were selected by plating for individual colonies in the presence of 0.1 mg/ml phleomycin. Colonies were isolated, expanded, and screened for expression of hRFC[CH3] by real-time reverse transcription (RT)-polymerase chain reaction (PCR), Western blotting, and transport assays at pH 7.2 (below). A representative clone (R5-RFC2) was selected for further study. R5 cells transfected with empty pZeosV2 (R5-mock) were also prepared.

Gel Electrophoresis and Western Blotting. To characterize hPCFT and hRFC protein levels in assorted human solid tumor cell lines and the R5-RFC2 transfecant, plasma membranes were prepared by differential centrifugation and sucrose density centrifugation (Matherly et al., 1991). Proteins were quantified with the Folin phenol reagent (Lowry et al., 1951).

Membrane proteins were electrophoresed on 7.5% polyacrylamide gels with SDS (Laemmli, 1970) and electroblotted onto polyvinylidene difluoride membranes (Thermo Fisher Scientific, Waltham, MA) (Matsudaaira, 1987). For detecting total immunoreactive hPCFT and hRFC proteins on polyvinylidene difluoride membranes, hPCFT- or hRFC-specific polyclonal antibodies raised in rabbits to carboxyl termini hPCFT or hRFC (Wong et al., 1998; Hou et al., 2012) peptides and an IRDye800WC-conjugated goat anti-rabbit IgG secondary antibody (Rockland Immunoccheamicals, Gilbertsville, PA) were used. For detecting HA-tagged proteins (i.e., R5-RFC[CH3]), HA-specific mouse monoclonal antibody (Covance Research Products, Princeton, NJ) and an IRDye800WC-conjugated goat anti-mouse IgG secondary antibody (Rockland Immunoccheamicals) were used. Membranes were scanned with the Odyssey infrared imaging system. hPCFT and hRFC levels were normalized to levels of Na+/K+-ATPase (Novus Biologicals, Inc., Littleton, CO).

Transport Assays. To assay hPCFT transport in assorted solid tumor cell lines, uptake of 0.5 μM [3H]MTX was measured in cell monolayers in 60-mm dishes over 5 min at 37°C. The transport buffer was MES-buffered saline (20 mM MES, 140 mM NaCl, 5 mM KCl, 2 mM MgCl2, and 5 mM glucose) at pH 5.5 (Zhao et al., 2004b). Some transport experiments were performed with 20 mM folic acid to exclude cellular uptake by FRs.

The pH-dependent uptake of [3H]C1, [3H]C2, [3H]PMX, or [3H]MTX (0.5 μM) in the HeLa sublines was assayed at 37°C in cell monolayers over 2 min at 37°C in 2 ml of “anion-free” HEPES-sucrose-Mg2+ buffer (20 mM HEPES and 235 mM sucrose, pH adjusted to 7.14 with MgO) (AFB) (Wong et al., 1997), HEPES-buffered saline (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 2 mM MgCl2, and 5 mM glucose), pH 6.8), or in MES-buffered saline, pH 5.5. Transport fluxes were stopped by aspirating the buffer and quenching with excess (>5 ml) ice-cold Dulbecco’s phosphate-buffered saline (DPBS), followed by 3 washes with DPBS. Cellular proteins were solubilized with 0.5 N NaOH and quantified with the Folin phenol reagent (Lowry et al., 1951). Drug uptake was expressed as picomoles per milligram of protein, calculated from measurements of radioactivity with a scintillation counter and the protein contents of the cells homogenates.

To measure PCFT transport kinetics (Kt and Vmax) for [3H]C2 in engineered R11-11-PCFT4 HeLa cells, cells were grown in spinner
flasks, collected by centrifugation, washed with DPBS, and sus- 
spected (at 1.5 × 10^5 cells) in 2 ml of MES-buffered saline at pH 5.5 
containing ^[3]H]C2 substrate concentrations ranging from 0.04 to 5 
µM. K_s and V_max values were determined from Lineweaver-Burk 
plots. Kinetic constants for C2 were compared with those previously 
published for R1-11-PCFT4 cells for C1 and PMX (Kugel Desmoulin 
et al., 2011).

**Real-Time RT-PCR Analysis of RFC, Frα, and PCFT Trans- 
scripts.** RNAs were prepared from WT, R5, and R5-RFC2 HeLa cells 
using TRizol reagent (Invitrogen). cDNAs were synthesized using 
random hexamers, RNase inhibitors, and MMLV reverse transcriptase 
and purified with the QIAquick PCR Purification Kit (QIAGEN, 
Valencia, CA). Quantitative real-time RT-PCR was performed on a 
Roche LightCycler 1.2 (Roche Diagnostics, Indianapolis, IN) 
with gene-specific primers and FastStart DNA Master SYBR Green I 
Reaction Mix (Roche Diagnostics) (Ge et al., 2007). Primers are listed 
in Supplemental Table 3S. Transcript levels for hRFC were normal- 
zized to those for glyceraldehyde-3-phosphate dehydrogenase. Exter-
nal 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.

**Cell Proliferation Assays.** For proliferation assays, WT and the 
R5 HeLa sublines were cultured in folate-free RPMI 1640 medium, 
pH 7.2, supplemented with 10% diazylated fetal bovine serum, 2 mM 
l-glutamine, and 100 units/ml penicillin/100 µg/ml streptomycin 
(hereafter referred to as “complete folate-free RPMI 1640 medium”), 
containing 25 nM 5-CHO-THF, for at least 2 weeks before the ex-
periment. For drug inhibition assays, the cells were plated in 96-well 
culture dishes (2500 cells/well; 200 µl/well) in the above medium 
with a range of drug concentrations; cells were incubated for up to 
96 h at 37°C in a CO_2 incubator. Numbers of cells were assayed 
with Cell Titer Blue cell viability assays (Promega, Madison, WI) with a 
fluorescent plate reader for determining IC_{50} values (drug concen-
trations that result in 50% loss of cell growth). To test the impact of 
extracellular folates on the collateral sensitivities of C1 and C2 in 
the HeLa sublines, some growth inhibition experiments included 
increasing concentrations (25, 100, and 1000 nM) of 5-CHO-THF.

**Accumulation of [H]5-CHO-THF.** WT and R5 HeLa sublines 
were cultured in complete folate-free RPMI 1640 medium supple-
mented with 0.06 mM adenosine and 0.01 mM thymidine for 5 days 
before the experiment. Adenosine and thymidine were added to 
circuit folate requirements and to maintain cell viability in the 
absence of exogenous folates. Cells were treated with 0, 25, 100, 
and 1000 nM [H]5-CHO-THF (because [H](6S)-5-CHO-THF was 
diluted with nonradioactive (6R,S)-5-CHO-THF for these experiments, 
the actual concentration of (6S) stereoisomer was 12.5, 50, and 
500 nM, respectively) for 4 days, followed by three washes with ice-cold 
DPBS. A control without added [H]5-CHO-THF (including 0.06 mM 
adenosine and 0.01 mM thymidine) was incubated in parallel. Cel-

ular proteins were solubilized with 0.5 N NaOH and quantified 
using the Folin phenol reagent (Lowry et al., 1951). Total cellular 
[H]5-CHO-THF accumulations were expressed as picomoles per 
milligram of protein, calculated from direct measurements of radio-
activity and protein contents of cell homogenates. Because [H](6S)- 
5-CHO-THF was diluted with unlabeled (6R,S)-5-CHO-THF (see 
above), for purposes of calculating intracellular folate metabolites 
only the unlabeled (6S) isomer was considered. To measure the 
impact of the PCFT-targeted therapeutics C1 and C2 on [H]5-CHO-
THF accumulations, some experiments analyzed the uptake of 25 
µM [H]5-CHO-THF in the presence of increasing concentrations 
(0–1000 nM) of compounds C1 and C2 in complete folate-free RPMI 
1640 medium supplemented with 0.06 mM adenosine.

**Measurement of C1 and C2 Polyglutamylation.** WT and R5 
HeLa cells were depleted of folates by growth for 2 weeks in complete 
folate-free RPMI 1640 medium plus 25 nM 5-CHO-THF. Cells were 
plated in medium without nucleosides, supplemented with 25 nM 
5-CHO-THF, and allowed to adhere overnight. Cells were washed 
with DPBS and incubated in the same medium, with 25 mM 
PIPES/25 mM HEPES, pH 6.8, 0.06 mM adenosine, and 1 µM 
([H]C1 or [H]C2). After 16 h, cells were washed three times with 
ice-cold DPBS and scraped into 5 ml of DPBS, pelleted (1500 rpm), 
and flash-frozen. Polyglutamyl and unmetabolized drug forms were 
extracted and levels were quantified by HPLC (Kugel Desmoulin 
et al., 2011). Cellular proteins were quantified with the Folin phenol 
reagent (Lowry et al., 1951). Polyglutamyl and parent drug forms 
were normalized to cellular proteins and are expressed as picomoles 
per milligram of cell protein.

**In Vivo Efficacy Study of C1 and C2 in WT and R5 HeLa 
Xenografts.** Cultured WT and R5 HeLa cells were implanted subcu-

taneously (~10^3 cells/flank) to establish a solid tumor xenograft 
model (passage 0) in female ICR SCID mice (National Institutes of 
Health DCT/DTDP Animal Production Program, Frederick, MD). The 
mice were supplied food and water ad libitum. Study mice were 
maintained on a folate-deficient diet (TD.00434; Harlan Teklad, 
Madison, WI) starting 18 days before tumor implant to ensure that 
serum folate levels would approximate those of humans before the 
start of therapy (Wang et al., 2010, 2011; Kugel Desmoulin et 
et al., 2011). This design is analogous to those previously published 
by others (Alati et al., 1996; Gibbs et al., 2005).

For the efficacy trial, the experimental animals were pooled, 
divided into groups (4 mice/group), and implanted bilaterally subcu-
taneously with 30- to 60-mg tumor fragments (from passage 3) using a 
12-gauge trocar. Chemotherapy began on day 3 after tumor implanta-
tion, when the number of cells was between 10^7 and 10^8 cells 
(below the limit of palpation). An organic solvent (ethanol, 5% v/v), 
carrier (TWEEN 80, 1% v/v), and sodium bicarbonate (0.5% v/v) were 
used to effect solubilization of C1 and C2. Injection volumes were 0.2 
ml i.v. Mice were sacrificed when cumulative tumor burdens 
reached 1500 mg. Methods of protocol design, drug treatments, 
toxicity evaluation, and data analysis were described previously 
(Corbett et al., 1997, 1998; Polin et al., 1997, 2011). Experimental 
parameters as qualitative and quantitative end points to assess 
antitumor activities include T/C as a percentage (see legend to 
Table 1 for further details) 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., 1000 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 tumor cell 
kill (log_{10} cell kill total (gross) = (T – C)/(3.32)(Td), 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 exponen-
tial growth (100–800 mg)). The day of tumor implant was day 0. 
For comparison of antitumor activities with standard agents or be-
tween tumors, log_{10} kill values were converted to an arbitrary activity 
rating (Corbett et al., 1997). With the exception of the xenograft 
model, these methods are essentially identical to those described 
previously (Wang et al., 2010, 2011; Kugel Desmoulin et al., 2011).

**Statistical Analysis.** Statistical analyses were performed with 
GraphPad Instat 4.0.

**Results**

**Expression and Function of hRFC and hPCFT in 
Human Solid Tumor Cell Lines.** We previously measured 
substantial levels of hPCFT transcripts by real-time RT-PCR 
in 52 of 53 human solid tumor cell lines of different origins 
(e.g., breast, prostate, ovarian, and others) (Kugel Desmoulin 
et al., 2011). As an extension of this work, we measured levels 
of hPCFT proteins for 10 of the tumor cell lines (HepG2, 
Hep3B, H596, CRL5810, H2595, HCT15, Caco-2, DU145, 
MDA-MB 321, and SK-MEL5) (Supplemental Table 2S). We 
also analyzed four HeLa sublines, including WT and hRFC-
null R5 cells, both of which express hPCFT, and two HeLa sublines derived from hRFC- and hPCFT-null R1-11 cells including R1-11-mock (hereafter, R1-11) and R1-11-PCFT4 (R1-11 cells stably transfectted with hPCFT) cells. For each cell line, plasma membrane proteins were separated by SDS-PAGE and immunoblotted with hPCFT-specific antibodies. Levels of heterogeneously glycosylated hPCFT proteins detected by Western blotting (Fig. 1A) paralleled those of hPCFT transcripts (Kugel Desmoulin et al., 2011). hPCFT proteins were detected in all tumor cell lines except for the parental R1-11 cells.

Functional validation of hPCFT expression in the solid tumor cell lines was established by transport assays with [³H]MTX uptake at pH 5.5 was detected in cell lines expressing hPCFT protein with the highest levels in Hep3B and Caco-2, accompanying elevated PCFT protein levels.

**hPCFT Transport of [³H]C2 in R1-11-PCFT4 Cells.** We found previously that the novel 6-substituted pyrrolo[2,3-d]pyrimidine thienyl antifolates C1 and C2 (Fig. 2A) were potent (nanomolar) inhibitors of proliferation in cells engineered to express hPCFT in the absence of hRFC or FRs (Wang et al., 2010, 2011; Kugel Desmoulin et al., 2011), suggesting that C1 and C2 are substrates for hPCFT-mediated cellular uptake. In engineered cell lines, C1 and C2 seemed to be poorly transported by hRFC (Wang et al., 2010, 2011; Kugel Desmoulin et al., 2011). Both analogs induced current at −90 mV and pH 5.5 in Xenopus laevis oocytes microinjected with hPCFT cRNAs, and both were competitive inhibitors of [³H]MTX transport in hPCFT transfectants from pH 5.5 to pH 6.8 (Kugel Desmoulin et al., 2011; Wang et al., 2011). Transport of [³H]C1 by hPCFT was directly demonstrated in R1-11-PCFT4 cells (Kugel Desmoulin et al., 2011).

To confirm hPCFT transport of C2, R1-11-PCFT4 cells were incubated with [³H]C2 (0.5 μM, 5 min, 37°C) at pH 5.5 and 6.8 in the presence and absence of unlabeled C1 (10 μM) as a competitive inhibitor. Transport was detected at a 4-fold higher level at pH 5.5 than at pH 6.8, and at both pHs uptake was substantially inhibited in the presence of unlabeled C1 (Fig. 2B). These results establish that C2, like its 4-carbon chain homolog, C1, is a bona fide substrate for membrane transport by hPCFT. Transport kinetic parameters for C2 with R1-11-PCFT4 cells at pH 5.5 are summarized in Supplemental Table 4S. Kᵢ and Vₘₐₓ values for C2 were similar to those previously reported for C1 and PMX (Kugel Desmoulin et al., 2011).

**Transport and Membrane Expression of hPCFT and hRFC in WT and R5 HeLa Sublines.** Although C1 and C2 are not RFC transport substrates (Wang et al., 2010, 2011; Kugel Desmoulin et al., 2011), RFC levels could nonetheless have a marked impact on the antiproliferative effects of these agents, via expansion or contraction of intracellular THF cofactor pools. Thus, RFC levels or ratios of PCFT to RFC transport might effectively predict the antitumor potencies of these prototypical PCFT-targeted antifolates. This result could be further affected by varying concentrations of extracellular THF cofactors.

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**Fig. 1.** PCFT expression and function in human solid tumor cell lines. A, plasma membrane preparations were isolated as described under Materials and Methods. Membrane proteins (25 μg) from human tumor cell lines were electrophoresed on a 7.5% denaturing polyacrylamide gel and immunoblotted with hPCFT antibody. Na⁺/K⁺-ATPase protein levels were used as loading controls. hPCFT migrates as a broadly banding species because of its heterogeneous glycosylation. The banding in the lane for the R1-11 HeLa cells is nonspecific. The major nonspecific band appearing in all lanes is labeled NS. B, uptake of 0.5 μM [³H]MTX was measured at 37°C for 5 min in cell monolayers at pH 5.5, as described under Materials and Methods. Internalized [³H]MTX was normalized to total protein. Results are shown for mean values ± S.E. for three to four independent experiments. The characteristics of the 14 tumor cell lines are summarized in Supplemental Table 2S.
A, structures of 6-substituted pyrrolo[2,3-d]pyrimidine thienoyl antifolates C1 and C2 (Wang et al., 2010, 2011) are shown. B, uptake of [3H]C2 (0.5 μM) was measured at 37°C for 5 min at pH 5.5 and 6.8 in the presence or absence of unlabeled C1 (10 μM). Internalized [3H]C2 was normalized to total protein. Details are provided under Materials and Methods. Results are shown for mean values ± S.E. for three independent experiments.

To explore this concept, we used WT and R5 HeLa cells, which express comparable levels of hPCFT with or without hRFC (Figs. 1 and 3A; Supplemental Fig. 1SA). R5 cells are resistant to MTX (Fig. 5A) because of a genomic deletion that results in loss of hRFC (Zhao et al., 2004a). MTX uptake in R5 cells in AFB at pH 7.2 was decreased ~3-fold compared with that in WT cells (Fig. 3C), consistent with the loss of hRFC. Ratios of MTX transport at pH 5.5 (PCFT) to pH 7.2 (RFC) for R5 and WT cells were ~11 and ~3, respectively (Fig. 3D). Transport was also measured for [3H]C1 and [3H]C2 (both at 0.5 μM, 2 min, 37°C) and for [3H]PMX at pH 5.5 and pH 7.2. Results were compared with those for [3H]MTX (Fig. 3, B and C). For all compounds and both cell lines, transport by hPCFT over hRFC predominated, because uptake showed the characteristic pH dependence for PCFT with the highest levels at pH 5.5. Although there were slight differences in initial rates of uptake of the various analogs at pH 5.5, these were not statistically significant. Furthermore, there were no obvious differences in membrane transport of the individual analogs between WT and R5 HeLa cell lines.

**Impact of hRFC and Extracellular Folate on Antitumor Activities of C1 and C2.** (6S)-5-CHO-THF is poorly transported by PCFT at neutral pH and is less effective in supporting proliferation of PCFT-expressing cells (without RFC) than RFC-expressing cells (without PCFT) (Zhao et al., 2008). Thus, loss of hRFC in R5 cells would be predicted to contract intracellular pools of reduced folates derived from 5-CHO-THF, compared with those in WT HeLa cells (Zhao et al., 2004b; Chattopadhyay et al., 2006). This effect may be exacerbated in the presence of high-affinity hPCFT-selective substrates, which could further restrict the modest levels of THF cofactor uptake via PCFT through direct competition.

To examine these possibilities, folate-depleted WT, R5, and R5-RFC2 cells were cultured in 25, 100, or 1000 nM [3H]-5-CHO-THF (corresponding to 12.5, 50, or 500 nM concentrations of the active (6S) stereoisomer) for 96 h to determine cellular accumulations of [3H]THF metabolites. During sustained culture, the media pH decreased to ~6.8 (Kugel Destr-
moulin et al., 2010) and was accompanied by dose-dependent accumulations of \(^{3}H\)5-CHO-THF (Fig. 4A). At 25 nM \(^{3}H\)5-CHO-THF, R5 cells experienced a 31.6% decreased net accumulation of tritiated folates compared with that in WT cells (p < 0.05) and a 49.6% decrease compared with that in R5-RFC2 cells (p < 0.05). Whereas this difference between WT and R5 cells decreased at 100 and 1000 nM \(^{3}H\)5-CHO-THF, statistically significant differences in \(^{3}H\)5-CHO-THF accumulations were preserved at these concentrations between R5 and R5-RFC2 cells (Fig. 4A).

We measured proliferation of WT and R5 HeLa cells grown in 25 nM 5-CHO-THF in the presence of a range of concentrations (0–1000 nM) of the PCFT-selective antifolates C1 and C2 for comparison with MTX, lometrexol (LMX), raltrexed (RTX), and PMX, classic antifolates that are transported by both RFC and PCFT (Goldman et al., 2010; Kugel Desmoulin et al., 2010, 2011) and with N\(^{1-}\)(4-amino-4-deoxypropyl)-N\(^{6}\)-hemiphthaloyl-L-ornithine (PT523), which is transported by RFC but not by PCFT (Zhao and Goldman, 2007; Kugel Desmoulin et al., 2010, 2011) (Fig. 5A; Supplemental Table S5). Similar to published results (Zhao et al., 2004c), R5 (and R5-mock transfected) cells were substantially resistant to PT523 compared with WT (> 213-fold) and R5-RFC2 (> 303-fold) cells. Furthermore, R5 cells were resistant (5- to 14-fold) to MTX, LMX, and RTX compared with WT HeLa cells. For PMX, IC\(_{50}\) values for WT and R5 cells were modestly different (IC\(_{50}\) values of 48.3 and 66.1 nM, respectively). It is noteworthy that hRFC-deficient R5 cells were substantially more sensitive to the PCFT-specific antifolates C1 and C2 than were WT cells (3.6- and 3.2-fold, respectively) and R5-RFC2 transfected cells (3.6- and 8.3-fold, respectively). Although differences in growth inhibitions between R5 and WT cells for C1 and C2 were preserved when the extracellular 5-CHO-THF was increased to 100 nM (4.3- and 15-fold, respectively), the effects of both drugs were effectively abolished when the 5-CHO-THF concentration was increased to 1000 nM (Fig. 5, B and C).

Because C1 and C2 are high-affinity substrates for PCFT, we hypothesized that these drugs compete with \(^{3}H\)5-CHO-THF for PCFT uptake, leading to a more severe contraction of the cellular folate pool in R5 cells compared with WT cells than in their absence. Indeed, both C1 and C2 effected a striking dose-dependent decrease in net accumulations of \(^{3}H\)5-CHO-THF, which were greater in hRFC-null R5 cells than in WT HeLa cells. At 1000 nM C1, levels of \(^{3}H\)5-CHO-THF accumulation in R5 and WT HeLa cells were 52.9 and 72.9%, respectively, of levels without drug; for C2, the corresponding values were 52.7 and 71.1%, respectively (Fig. 4, B and C).

Collectively, these results establish that loss of hRFC contributes to a contraction of cellular folate pools, which is exacerbated in the presence of the PCFT-selective analogs C1 and C2. Of importance, decreased intracellular folates were accompanied by markedly increased antiproliferative effects of C1 and C2.

**Polyglutamylation of C1 and C2 in WT and R5 HeLa Cells.** Analogous to physiologic folates and other classic antifolate drugs such as MTX (Goldman and Matherly, 1985; Shane, 1989; Assaraf, 2007), C1 is metabolized to polyglutamates (PGs) (Kugel Desmoulin et al., 2011). Polyglutamylation of C2 has not been assessed previously. Because polyglutamylation of antifolate drugs by polyglutamate synthetase (PGS) can be regulated by elevated extra- and intracellular folates (Tse and Moran, 1998; Zhao et al., 2001), it seemed possible that the impact of hRFC and cellular THF cofactors on the antiproliferative effects of C1 and C2 may be partly explained in this manner.

To assess this possibility, WT and R5 HeLa cells were incubated with 1 \(\mu\)M \(^{3}H\)C1 or \(^{3}H\)C2 for 16 h at pH 6.8 in the presence of 25 nM 5-CHO-THF and 0.06 mM adenosine. Total cellular radiolabeled drug levels were quantified, and tritiated parent drug and PGs were extracted and analyzed. At least four polyglutamyl metabolites (PG\(_{2-5}\)) of \(^{3}H\)C1 and five metabolites of \(^{3}H\)C2 (PG\(_{2-6}\)) were resolved by HPLC. Migrations were compared with those for nonpolyglutamyl
C1 or C2 and with MTX and MTX PG standards. Furthermore, samples were treated in parallel with conjugase (Kugel Desmoulin et al., 2011), which reverted the majority of the polyglutamyl metabolites to the parental drugs (not shown). Results are summarized in Supplemental Fig. 2S. HPLC chromatograms for the radiolabeled drug forms in HeLa and R5 cells are shown in Supplemental Fig. 3S. For R5 and WT cells, there was a 7- to 8-fold greater accumulation of total and polyglutamyl [3H]C2 than for [3H]C1. WT and R5 cells accumulated similar levels of total C1 and C2 drug forms, although there were slight differences in relative accumulations of individual PGs between the cell lines. This difference was most obvious for the longest chain length PGs (PG₅ and PG₆) and was somewhat greater for C1 than for C2.

These results establish that 1) both C1 and C2 are excellent substrates for polyglutamylase under conditions that favor their membrane transport by PCFT, 2) net drug accumulation and polyglutamylation of C2 far exceeds that for C1, and 3) the presence or absence of functional RFC manifests at most a modest effect on net C1 and C2 polyglutamate synthesis.

**In Vivo Efficacy of C1 and C2 against WT and R5 HeLa Xenografts.** To extend our in vitro cell proliferation studies in vivo, we performed in vivo antitumor efficacy studies with 8-week-old female ICR SCID mice implanted with subcutaneous R5 or WT HeLa cells. Mice were maintained ad libitum on a folate-deficient diet, which decreased serum folates to levels approximating those seen in humans. For the efficacy trial, control and drug treatment groups were non-selectively randomized (four mice/group); C1 or C2 was administered intravenously (180 and 32 mg/kg per injection, respectively) on days 3, 7, 14, and 18 after implantation. As reported for other tumor models (Kugel Desmoulin et al., 2011; Wang et al., 2011), C1 and C2 showed substantial efficacies toward R5 and WT HeLa xenografts (Table 1). Both C1 and C2 showed greater efficacies toward R5 cells (T = 23 days, 3.3 gross log₁₀ kill for C1; T = 17.5 days, 2.5 gross log₁₀ kill for C2) than toward WT cells (T = 13 days, 1.9 gross log₁₀ kill for C1; T = 13 days, 1.9 gross log₁₀ kill for C2). Differences in C and log₁₀ kill results between R5 and WT HeLa cells for compound C1 were statistically significant (p = 0.014 and p = 0.0135, respectively). However, differences in these parameters for C2 did not quite reach statistical significance (p = 0.146). The treatment regimens with C1 and C2 were well tolerated with dose-limiting symptoms manifesting as reversible body weight loss.

The results of the in vivo efficacy trial provide proof-of-principle confirmation of our in vivo findings that the antitumor effects of both C1 and C2 are greater in hRFC-deficient R5 cells than in WT cells. Of interest, the impact of loss of hRFC seems to be somewhat greater with C1 than with C2 in vivo.

**Discussion**

In this study, we expanded on previous reports (Zhao and Goldman, 2007; Goldman et al., 2010; Kugel Desmoulin et al., 2010, 2011; Wang et al., 2010, 2011) that hPCFT may be
TABLE 1
Antitumor efficacy evaluation of C1 and C2 against early-stage human R5 and HeLa in female SCID mice

<table>
<thead>
<tr>
<th>Tumor and Agent</th>
<th>Total Dose (mg/kg)</th>
<th>Median (Range) Tumor Mass on Day 21 (mg)</th>
<th>T/C (%) Values at 1000 mg</th>
<th>Time (Range) to 1000 mg</th>
<th>T/C</th>
<th>Gross Log10 Kill</th>
<th>Activity Rating</th>
</tr>
</thead>
<tbody>
<tr>
<td>R5</td>
<td>No treatment</td>
<td>1054 (916–1372)</td>
<td>21 (20–22)</td>
<td>44 (39–49)</td>
<td>3</td>
<td>3.3</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>C1</td>
<td>720</td>
<td>0 (all zeros)</td>
<td>3</td>
<td>38.5 (36–42)</td>
<td>17.5</td>
<td>2.5</td>
</tr>
<tr>
<td>C2</td>
<td>128</td>
<td>32 (0–75)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HeLa</td>
<td>No treatment</td>
<td>1009 (847–1701)</td>
<td>21 (18–22)</td>
<td>34 (31–37)</td>
<td>13</td>
<td>1.9</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>C1</td>
<td>720</td>
<td>63 (0–247)</td>
<td></td>
<td>34 (30–40)</td>
<td>13</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>C2</td>
<td>128</td>
<td>69 (0–189)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

+++ , >2.8 log10 kill, highly active; ++ , 2.0 to 2.8; + , 1.3 to 1.9; and − , <0.7, inactive.

exploitable for cancer therapy, reflecting unique patterns of hPCFT expression and appreciable transport activity at pH values approximating the tumor microenvironment. We extended our previous findings establishing broad-ranging hPCFT gene expression in a large number of human tumor cell lines (Kugel Desmoulin et al., 2011) to include measurements of hPCFT protein and transport activity. Our results document the fact that hPCFT protein levels are abundantly expressed, accompanying substantial transport activities at pH 5.5.

We demonstrated that functional loss of hRFC in R5 cells caused a contraction of total cellular THF cofactor pools derived from 5-CHO-THF, which enhanced both C1 and C2 antitumor activities compared with those in WT cells. Of importance, the reduction in total cellular folate pools in R5 cells was exacerbated in the presence of C1 and C2, through direct competition at hPCFT, which further restricted cellular uptake of exogenous 5-CHO-THF. Efficacies of C1 and C2 toward R5 tumors were also increased compared with those toward WT tumors transplanted into SCID mice with serum folate concentrations approximating those achieved in humans, although this effect seemed to be somewhat greater with C1 than with C2 in vivo. We previously reported that the in vivo efficacy of C2 toward IGROV1 xenografts in SCID mice was less affected by serum folates than that for C1 (Wang et al., 2010, 2011).

There is ample precedent for an impact of folate pools on antifolate drug efficacy. Indeed, this is the premise of leucovorin rescue from MTX toxicity (Matherly et al., 1987) and of low-dose folic acid protection from LMX toxicity in vivo (Roberts et al., 2000), whereby elevated extra- and intracellular folates compete at multiple levels to reverse drug activity. In vitro studies have extended these findings to both classic (PMX and LMX) and nonclassic (trimethrextate) antifolates, which inhibit a range of cellular targets (Tse and Moran, 1998; Zhao et al., 2001; Goldman et al., 2010). Furthermore, antifolate drug activities are enhanced by decreased intracellular folates resulting from impaired efflux of folic acid (Assaraf and Goldman, 1997) or enhanced folic acid influx by a mutant RFC (Tse and Moran, 1998; Tse et al., 1998).

Regardless of the underlying mechanism and intracellular drug target involved, markedly decreased total intracellular THF pools can result in collateral sensitivities to antifolates, often in the face of substantially decreased levels of drug uptake (Zhao et al., 2000b, 2001, 2004c; Chattopadhyay et al., 2006). The effects of folate levels on antifolate activities could reflect inhibitory effects on drug polyglutamylation (with consequent impact on drug retention and inhibition of folate-dependent enzyme targets) as a result of competitive feedback inhibition at FPGs by high levels of THF cofactor PGs (Nimec and Galivan, 1983; Shane, 1989; Tse and Moran, 1998; Zhao et al., 2001, 2004b) or increased FPGs activity levels in response to decreased extra- and intracellular folates (Gates et al., 1996). Furthermore, direct competitive interactions between polyglutamyl folates and antifolates may substantially interfere with drug binding and inhibition at their enzyme targets, as documented for MTX (Mathery et al., 1983). Changes in drug efflux are also possible, because ABC transporter (ABCG2, ABCC1) levels and/or intracellular distributions have been described in response to folate deprivation (Ifergan et al., 2005).

In the present study, we found that C1 and C2 membrane transport by PCFT was virtually identical between the hRFC-deficient R5 and WT HeLa cell lines. Total and polyglutamyl accumulations of C1 and C2 during sustained drug exposures were similar between WT and R5 cells, although there were slight differences in distributions of C1 and C2 polyglutamates between the lines. This difference was most pronounced for the longest chain length PGs (PG6 and PG8) and seemed to be somewhat greater for C1 than C2. By analogy with other classic antifolates (Mendelsohn et al., 1999; Shih and Thornton, 1999), increased accumulation of long-chain polyglutamyl forms of C1 and C2 in the R5 subline might result in enhanced inhibition of intracellular glycinamide ribonucleotide formyltransferase and de novo purine nucleotide biosynthesis (Wang et al., 2010, 2011; Kugel Desmoulin et al., 2011), thus further exacerbating the impact
of reduced cellular folate pools in directly competing for binding with this enzyme target.

Our results provide proof-of-principle evidence that hRFC levels and function are critical determinants of antitumor activities and in vivo efficacies of PCFT-targeted antifolates that are not themselves RFC substrates. This lack of RFC transport confers tumor selectivity and decreased toxicity to normal tissues for this novel class of agents. Tumor selectivity would be enhanced by substantial levels of hPCFT protein in solid tumors and by acidic pH values characterizing the tumor microenvironment, which favor PCFT over RFC transport (Kugel Desmoulin et al., 2011). Conversely, at neutral pH values characterizing most normal tissues, RFC transport of reduced folates would be increased, resulting in elevated levels of THF cofactors within cells, which further protect from untoward drug effects. Of course, as suggested in this report, in tumors with sufficiently high hRFC, uptake of THF cofactors by this process will probably occur, even at somewhat acidic pH values. Accordingly, any decrease in hRFC function would serve to augment the inherent anti-tumor selectivities and increase sensitivities to PCFT-selective antifolates.

Acknowledgments

We thank Dr. I. David Goldman for his generous gifts of the R5 and R1-11 HeLa cell lines. We thank Kelly Haagenson for editorial assistance.

Authorship Contributions

*Participated in research design: Kugel Desmoulin, Wang, Polin, Hou, Gangjee, and Matherly.*


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*Wrote or contributed to the writing of the manuscript: Kugel Desmoulin, Polin, Hou, Gangjee, and Matherly.*

References


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