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Synthesis and biological activity of 6-substituted pyrrolo[2,3-*d*]pyrimidine thienoyl regioisomers as inhibitors of *de novo* purine biosynthesis with selectivity for cellular uptake by high affinity folate receptors and the proton-coupled folate transporter over the reduced folate carrier

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Abstract

We reported the selective transport of classical 2-amino-4-oxo-6-substituted pyrrolo[2,3-*d*]pyrimidines with a thienoyl-for-benzoyl-substituted side chain and a 3- (**3a**) and 4-carbon (**3b**) bridge. Compound **3a** was more potent than **3b** against tumor cells; While **3b** was completely selective for transport by folate receptors (FRs) and the proton-coupled folate transporter (PCFT) over reduced folate carrier (RFC), **3a** was not. To determine if decreasing the distance between the bicyclic scaffold and *L*-glutamate in **3b** would preserve transport selectivity and potency against human tumor cells, **3b** regioisomers with [1,3] (**7** and **8**) and [1,2] (**4**, **5** and **6**) substitutions on the thienoyl ring, and with acetylenic insertions in the 4-atom bridge, were synthesized and evaluated. Compounds **7** and **8** were potent nanomolar inhibitors of KB and IGROV1 human tumor cells with complete selectivity for FR α and PCFT over RFC.

INTRODUCTION

Membrane transport of antifolate therapeutics such as methotrexate MTX is integral to chemotherapy efficacy in treating a variety of malignancies and non-malignant diseases.¹ The major membrane transporters include the reduced folate carrier (RFC), the proton-coupled folate transporter (PCFT) and the high affinity folate receptors (FRs) α and β .^{1–4} While all systems transport folate cofactors and classical antifolates such as MTX, raltitrexed (RTX) and pemetrexed (PMX), they differ in terms of mechanism and substrate

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Supporting Information Available: Colony formation assay data and elemental analysis are available free of charge via the internet at <http://pubs.acs.org>.

specificities. Whereas both RFC and PCFT are integral membrane proteins that act as facilitative transporters, FRs are glycosyl phosphatidylinositol-modified proteins that mediate cellular uptake of (anti)folates by receptor-mediated endocytosis.

The major folate transporters also differ in terms of their tissue distributions. For instance, RFC is ubiquitously expressed in tumors and tissues and is the primary uptake mechanism for folate cofactors.¹ FRs are expressed abundantly in certain malignancies, most notably as the FR α isoform in ovarian carcinomas, and in a limited number of normal epithelial tissues such as renal tubules.² Major sites of PCFT expression include the upper small intestine (e.g., jejunum), and the liver and kidney.^{3,5,6} In solid tumors such as hepatomas, ovarian carcinomas, and non-small cell lung carcinomas, PCFT is highly expressed.⁷ PCFT exhibits an acidic pH optimum^{4,6}, which is compatible with the low pH microenvironments of the small intestine and many solid tumors. While PCFT is modestly expressed in most other normal tissues, for those in which PCFT is expressed they are unlikely to present the low pH conditions optimal for membrane transport by this mechanism.³

Membrane transport of classical antifolates by RFC in itself would likely result in limited drug selectivity toward tumors over normal proliferative tissues such as bone marrow since these normal tissues all express RFC. The concept of selectively targeting FR-expressing tumors with folate-based antitumor agents has been tested. Examples of folate-based therapeutics include **1** [(2R)-2-((4R)-4-carboxy-5-(4-((2-(hydroxymethyl)-4-oxo-4,6,7,8-tetrahydro-3H-cyclopenta[g]quinazolin-6-yl)(prop-2-yn-1-yl)amino)phenyl)-5-oxopentanamido)pentanedioic acid] [ONX0801 (BGC945)], a small molecule antifolate licensed by Onyx Pharmaceuticals which is selectively transported by FRs and inhibits thymidylate synthase⁸, and vincalkebostatin-23-oic acid, O4-deacetyl-, 2-[(2-mercaptoethoxy)carbonyl]hydrazide, disulfide with N-[4-[(2-amino-1, 4-dihydro-4-oxo-6pteridiny]methyl)amino]benzoyl]-L-(γ -glutamyl-L- γ -aspartyl-L- γ -aspartyl-L-cysteine **2** [EC-145 (Endocyte)], a desacetylvinblastine monohydrazide-folic acid complex.⁹ Compound **1** is in clinical trials in the UK. A randomized Phase II trial is near completion in which **2** with doxorubicin was compared to doxorubicin alone for the treatment of platinum-resistant ovarian cancer (ClinicalTrials.gov Identifier: NCT00722592).

We recently identified 6-substituted pyrrolo- and thieno[2,3-*d*]pyrimidine antifolates with selective membrane transport by PCFT and/or FRs over RFC, thus selectively inhibiting proliferation of cells expressing these systems including human tumors.^{7,10–14} FR targeting to tumors is based on patterns of expression basolateral membrane localization unique to tumor cells. In terms of PCFT, the notion of using this transport mechanism to selectively deliver chemotherapy drugs is appealing given the low pH microenvironments of many solid tumors which approximate the pH optimum for PCFT.^{15–17} The pyrrolo[2,3-*d*]pyrimidine thienoyl analogs **3a** and **3b** (Figure 1) with 3- and 4-carbons, respectively, in the bridge between the pyrrolo[2,3-*d*]pyrimidine ring and the thiophene ring were the most potent of this series against cells expressing FRs and/or PCFT.^{12,13} Cytotoxicity was attributed to the potent inhibition of β -glycinamide ribonucleotide (GAR) formyltransferase (GARFTase), which catalyzes the first folate-dependent reaction in *de novo* purine nucleotide biosynthesis.

Our finding that compound **3a** was more potent than **3b** toward FR and PCFT-expressing cells suggested that the distance between the bicyclic scaffold and the *L*-glutamate may be a critical determinant of drug activity.¹³ However, in engineered hamster cells, transport selectivity for FR α and PCFT over RFC with compound **3a** was less complete than for **3b**. We hypothesized that structurally engineered substitutions on the thiophene ring of **3a** would afford distances between the bicyclic scaffold and the *L*-glutamate midway between those for the 3-atom bridge **3a** and the 4-atom bridge **3b**. We predicted that this would

preserve the potency of **3a** along with the selectivity for FR α and PCFT as in **3b**. We synthesized **3b** regioisomers, differing in positions of substitution of the 4 atom carbon bridge and the *L*-glutamic acid on the thiophene ring (compounds **4–8**), and in the presence of a linear, shorter acetylenic linkage (compounds **9–13** and **20**) in the carbon bridge region (Figure 1). These modifications decreased the distances between the bicyclic pyrrolo[2,3-*d*]pyrimidine and the *L*-glutamate moieties, in order to systematically determine their impact on drug selectivity for FRs and PCFT over RFC, and on potency against human tumor cells. In this report, we describe the synthesis and biologic properties of compounds **4–13** (Figure 1), and **20** (Scheme 3).

CHEMISTRY

The synthesis of target compounds **4–8** (Scheme 1) started from the reported intermediate **16**.¹² A Sonogashira coupling of **16** with various bromo-substituted thiophene carboxylates **15a–e** (Scheme 2) afforded **17a–e** in 50–56% yield. Subsequent hydrogenation and saponification of **17a–e** afforded target compounds **4–8**. Direct hydrolysis of intermediates **17a–e** provided conformationally restricted analogs **9–13**. Compounds **15a–e** (Scheme 2) required in Scheme 1 were synthesized by a peptide coupling of the commercially available bromo-substituted thiophene carboxylic acids **14a–e** (Scheme 2) with *L*-glutamate diethyl ester hydrochloride in 89–91% yield.

Compound **19** (Scheme 3) was previously reported as a precursor to **3b** (Figure 1).¹² For comparison to **3b**, it was also of interest to synthesize and evaluate the classical analog **20** derived from **19**. Synthesis of **20** from **19** is shown in Scheme 3.

BIOLOGICAL EVALUATION AND DISCUSSION

6-Substituted pyrrolo[2,3-*d*]pyrimidine thienoyl regioisomers as inhibitors of cell proliferation

The distance between the bicyclic scaffold and *L*-glutamate portions of a series of pyrrolo- and thieno[2,3-*d*]pyrimidine antifolates seems to dictate both selectivity for cellular uptake by FRs and PCFT over RFC, as well as cytotoxic potencies against tumor cells.^{10–14} Thus, decreasing the bridge lengths from 8-carbons to 4-carbons was accompanied by substantially increased antiproliferative activities, reflecting transport by PCFT and/or FRs and inhibition of intracellular GARFTase. Decreasing the bridge length from 4- to 3-carbons further increased drug potencies. However, for the pyrrolo[2,3-*d*]pyrimidine thienoyl antifolate series, this appeared to be accompanied by some loss of absolute transporter specificity for FRs and/or PCFT over RFC.¹³ Finally, for all series, analogs with bridge lengths less than 3-carbons showed substantially decreased drug activities.

In the present study, we used the pyrrolo[2,3-*d*]pyrimidine thienoyl platform with a 4-carbon bridge. We systematically assessed the effects on cell proliferation for an expanded series of pyrrolo[2,3-*d*]pyrimidine thienoyl regioisomers of **3b** with a 4-carbon bridge and thienoyl ring substitutions, 2',3' (**4**), 4',5' (**5**), 3',4' (**6**), 3',5' (**7**), and 2',4' (**8**) (Figure 1), as an alternative approach for decreasing the distance between the bicyclic pyrrolo[2,3-*d*]pyrimidine and the *L*-glutamate portions. Hence, in analogs **4–6**, the [1,2] substitution pattern on the thiophene forces the bicyclic scaffold and *L*-glutamate closer together than in the parent 3-atom bridge compound **3b** which includes a [1,3] pattern on the thiophene ring. Compounds **7** and **8**, also [1,3] substitutions on the thiophene ring, have an intervening smaller carbon compared to the larger sulfur in **3b**. Further, the C-C-C (112°) bond angle of both **7** and **8** is larger than the C-S-C (91.8°) bond angle of **3b**, and the lengths of the C-S (1.71 Å) and C-C (1.38 Å) bonds are also different (Figure 2, MOE 2008.10)¹⁸. Molecular modeling (Figure 2, MOE 2008.10) shows that the distances from the α -carbon of the bridge

to the amide carbonyl are shorter in compounds **7** and **8** (5.10 Å and 5.11 Å, respectively) than that of **3b** (5.33 Å). The distances from the bicyclic scaffold to the *L*-glutamate for compounds **7** and **8** are designed to be intermediate between those for **3b** and **3a**.

Compounds **9–13** and **20** are analogs of **3b–8** and contain a single conformational restriction in the 4-carbon atom bridge, in the form of a linear acetylenic linkage instead of the saturated C-C linkage of **3b–8**. This also provides some conformational rigidity in the four-carbon atom chain and decreases the length of the carbon bridge (3.52 Å), compared to the saturated analogs (3.87 Å) **3b–8**.

Compounds **4–13** and **20** were initially screened for growth inhibition in a series of cell lines with defined expression patterns of the major (anti)folate transporters for comparison with compound **3b**. The experimental models were a panel of isogenic Chinese hamster ovary (CHO) sublines derived from RFC-, FR- and PCFT-null MTXR^{II}Oua^R2–4 CHO cells engineered to express FR α (RT16),¹⁰ human RFC (PC43-10)¹⁸, or human PCFT (R2/hPCFT4)^{5,11}. Additional growth inhibition studies were performed with KB and IGROV1 human tumor cells that express FR α , RFC, and PCFT. We previously showed that during cell outgrowth, the pH of the culture media decreased from pH 7.2 to ~6.8¹⁴ such that all 3 transporters would be active in drug transport.

For measurements of inhibitory effects on cell proliferation, the cell lines were cultured with the antifolates over a range of concentrations, as described in the Experimental Section. With RT16 CHO, KB and IGROV1 cells, FR α -mediated drug uptake was established with a parallel culture treated with the cytotoxic drugs and excess (200 nM) folic acid to block FRs. For the PC43-10 and R2/hPCFT4 CHO sublines, results were compared to those for parental R2 cells or to vector control R2 cells transfected with empty pcDNA3.1 vector [designated R2(VC)]. Patterns of drug sensitivity or resistance for the novel pyrrolo[2,3-*d*]pyrimidine analogs were compared to those for compounds **3a** and **3b**, and to those previously reported for classic antifolates (e.g., MTX, PMX, LMTX, RTX).^{10–14} Results of the cell proliferation assays are summarized in Table 1.

MTX, PMX, RTX, and LMTX were all active (albeit variably) toward RFC-, PCFT-, or FR α -expressing CHO cells. The 6-substituted pyrrolo[2,3-*d*]pyrimidine thienoyl analogs with *contiguous* thiophene substitutions [2'3' (**4** and **10**); 4'5' (**5** and **11**); 3'4' (**6** and **12**)] were *completely inactive* toward the RFC- and PCFT-expressing CHO sublines. Compounds **7**, **8**, **13**, and **20** were likewise inert toward PC43-10 cells, although both compounds **7** and **8** (but not conformationally rigid **13** and **20**) inhibited proliferation of R2/hPCFT4 cells (IC₅₀s of 41.5 and 63.8 nM for **7** and **8**, respectively) essentially equivalent to compound **3b** (IC₅₀ of 43.4 nM). This establishes cellular uptake of compounds **7** and **8** by PCFT. Drug sensitivities for **7** and **8** with R2/hPCFT4 cells exceeded those for MTX and RTX, and were comparable to those for LMTX. However, **7** and **8** were less potent than PMX (~3- and ~5-fold, respectively) and **3a** (~12- and ~19-fold, respectively). None of the 6-substituted pyrrolo[2,3-*d*]pyrimidine regioisomers (**3b**, **4–13** and **20**) had any impact on proliferation of R2 or R2(VC) cells.

For FR α -expressing RT16 cells, compounds **4**, **5**, **10**, and **11** were all inert at drug concentrations up to 1000 nM. Compounds **6**, **7**, **8**, **12**, **13**, and **20**, like **3b**, all showed some level of antiproliferative activity toward RT16 cells. The active drugs followed an order of potency, **7** ~ **8** ~ **3b** > **12** ~ **13** > **20** >> **6**. FR mediated growth inhibitions for the most active analogs (i.e., **3b**, **7**, and **8**) exceeded those for standard antifolates (Table 1) and for all the active analogs, inhibitions were abolished with 100-fold excess folic acid.

KB and IGROV1 human tumor cells express all three transport systems⁷ and in these models, **7** and **8** were the most potent drugs in our series (Table 1). Inhibitory activities for **7**

and **8** were similar to those for **3a** and **3b** in both KB and IGROV1 cells. In KB cells, compounds **4**, **5**, and **6** were all substantially less active than **7** and **8**, in order of decreasing potency, **6** >> **5** ~ **4**. For the active analogs, growth inhibition was again reversed by excess folic acid, establishing that like compound **3b**, FR α was the major mechanism of uptake in these cells despite the presence of RFC and PCFT. Thus, for the **7** and **8** regioisomers of **3b**, we confirmed our hypothesis that by varying the distance between the bicyclic scaffold and the *L*-glutamate we preserved both the absolute selectivity of **3b** and the high antiproliferative potencies of **3a** and **3b**.

Interestingly, none of the acetylenic compounds (**9–13** and **20**) were inhibitory toward FR α -expressing human KB cells, in striking contrast to the results with RT16 CHO cells with compounds **12**, **13**, and **20**. This suggests that for **12**, **13**, and **20**, there must be differences in cellular metabolism, drug binding to intracellular targets, and/or substrate activity for folylpolyglutamate synthetase between the human and hamster cells. When several of these analogs (**3b**, **6**, **12**, **13**, and **20**, compared to **7** and **8**) were tested in IGROV1 cells, analogous results to those with KB cells were obtained (Table 1).

Additional experiments used colony-forming assays with KB tumor cells continuously exposed to compounds **3b**, **7**, or **8** for up to 14 days, after which colonies were stained with 1% methylene blue and counted. Compounds **7** and **8** inhibited KB cell colony formation (Supplement, Figure 1S) with similar IC₅₀s (0.39 and 0.16 nM, respectively) to that for compound **3b** (IC₅₀ = 0.20 nM) and substantially lower than that for MTX (IC₅₀ = 8.5 nM).

These results establish that (i) none of the regioisomers **3b**, **4–13**, and **20** are RFC substrates. (ii) For the saturated bridge analogs (**3b**, **4–8**), a [1,3] relationship between the carbon bridge domain and the *L*-glutamate moiety is an important determinant of transport substrate activity with FR α or PCFT. (iii) Compounds **7** and **8**, like **3b**, were substantially less inhibitory toward PCFT-expressing cells than compound **3a**. However, this difference was much reduced in human tumor cells for which FR α was the primary mode of drug delivery. The results with compounds **4**, **5**, and **6** and FR α -expressing cells suggest that decreasing distance between the bicyclic ring and the *L*-glutamate beyond a point has adverse effects on drug activity, analogous to those resulting from decreasing bridge lengths to less than 3-atoms. (iv) None of the conformationally restricted acetylenic analogs (**9–13** and **20**) were substrates for PCFT. (v) For this series, the [1,3] acetylenic analogs (**12**, **13**, and **20**) are substrates for FR α , although other factors unrelated to membrane transport seem likely to account for the marked differences in their antiproliferative activities between the human and hamster sublines.

Impact of elevated leucovorin and nucleosides on anti-tumor activity of compounds **7** and **8**

Since our proliferation assays with FR-expressing cells routinely use limiting (2 nM) concentrations of extracellular leucovorin [(6R,S)5-formyl tetrahydrofolate, a chemically stable reduced folate] (LCV), additional experiments were performed with KB cells to assess the impact of elevated (50 nM) LCV [for which the active (6S) stereoisomer approximates the concentration of 5-methyl tetrahydrofolate in serum] on the growth inhibitory effects of the most potent analogs (compounds **7** and **8**) of our series compared to compound **3b** (Figure 3). In KB cells, the impact of 50 nM LCV was uneven and ranged from ~7-fold for **3b** to ~20-fold for **7** and >38-fold for **8**. For the data in Figure 3, IC₅₀s in the presence of 50 nM LCV were 2.2 nM for **3b**, 3.4 nM for **7** and >20 nM for **8**. Thus, both compounds **3b** and **7** maintained substantial antitumor potencies even in the presence of elevated physiologic levels of reduced folate.

As previously reported for **3b**,¹² growth inhibitory effects of **7** and **8** toward KB (Figure 4) and IGROV1 cells (not shown) were completely reversed when drug exposures were performed in the presence of adenosine (60 μ M) but not thymidine (10 μ M). This established that the effects of **7** and **8** on cell proliferation were the consequence of inhibiting the *de novo* purine nucleotide biosynthetic pathway. For both **7** and **8**, 5-amino-4-imidazolcarboxamide (AICA) (320 μ M) was also protective. Since AICA is metabolized to AICA ribonucleotide (AICAR), the substrate for AICAR formyltransferase (AICARFTase; the 2nd folate-dependent reaction after GARFTase),^{10,19} **7** and **8** must derive their growth inhibitory effects by inhibiting GARFTase. This was further tested below.

Cellular pharmacology of cytotoxic 6-substituted pyrrolo[2,3-*d*]pyrimidine thienoyl regioisomers

Results of our cell proliferation assays in engineered CHO and human tumor cells suggested that **7** and **8** are *bona fide* transport substrates for FR α and PCFT. From results of nucleoside and AICA protection studies, **7** and **8**, like **3b**, following internalization, inhibit *de novo* purine nucleotide biosynthesis, apparently at the level of GARFTase. Interestingly, whereas the acetylenic bridge analogs **12**, **13** and **20** inhibited proliferation of FR α -expressing RT16 CHO cells, these compounds were *inactive* toward human tumor cells.

Using inhibition of human tumor cell proliferation as a primary metric, we selected compounds **3b** and **4–8** for testing FR α binding, reflected in competitive inhibition of [³H]folic acid binding to FR α in RT16 CHO cells. For these experiments, RT16 cells were washed with acid buffered saline (pH 3.5), treated (at neutral pH) with 50 nM [³H]folic acid, along with a range of concentrations of the pyrrolo[2,3-*d*]pyrimidines **3b** and **4–8**, folic acid, LMTX, PMX, or MTX. After additional washes (neutral pH), cells were solubilized (in 0.5 N NaOH). FR α -bound [³H]folic acid was measured, normalized to cellular protein, and relative inhibitor affinities were calculated as inverse molar ratios of the concentrations of unlabeled ligands which decreased [³H]folic acid binding by 50%.

In these experiments, compounds **7** and **8** were all high affinity ligands (relative affinities $\geq$ 1) for FR α , essentially equivalent to **3b** and folic acid, and substantially better than LMTX (Figure 5). Like MTX, compounds **4–6** did not bind to FR α in this assay, in spite of detectable (albeit lower) levels of FR α -directed activity in our cell proliferation experiments (with KB cells; Table 1). The detectable antiproliferative activities with **4**, **5**, and **6** in KB cells (but not RT16 CHO cells) likely reflect markedly elevated levels of FR α in this human tumor cell line, as previously reported.¹⁰

Since compounds **7** and **8** inhibited proliferation of R2/hPCFT4 cells, these analogs were tested as competitive inhibitors of PCFT transport of [³H]MTX in R2/hPCFT4 cells. Experiments were performed at pH 5.5, approximating the pH optimum for PCFT, and at pH 6.8, which is commonly achieved during cell outgrowth.¹⁴ K_i s were calculated over a range of inhibitor concentrations from Dixon plots, and results were compared to those for **3b** and PMX (Table 2). At pH 5.5, K_i values for **3b**, **7**, and **8** were all similar (within a factor of $\sim$ 2) and approached the K_i for PMX. At pH 6.8, K_i s for PMX and **3b** were likewise similar ($\sim$ 27- and 31-fold higher, respectively, than the values at pH 5.5). In comparison, K_i s for **7** and **8** were further increased at pH 6.8 ($\sim$ 70-fold greater) over the K_i s recorded at pH 5.5.

Collectively, these experiments confirm the high affinity binding for the [1,3] thienoyl regioisomers **3b**, **7**, and **8** to FR α and PCFT, suggested from their potent anti-proliferative and cytotoxic effects toward FR α - and PCFT-expressing CHO and human tumor cells. While compounds **7** and **8**, like **3b**, are potent inhibitors of PCFT at pH 5.5, binding of **7** and **8** to PCFT (as reflected in K_i values) appears to be more sensitive to pH than for either **3b** or PMX.

Results of nucleoside/AICA protection experiments with KB (Figure 4) and IGROV1 tumor cells (not shown) suggested that GARFTase in the *de novo* purine nucleotide biosynthetic pathway was the primary intracellular target of **7** and **8**, analogous to **3b**¹², thus explaining the potent anti-proliferative activities of these agents. To confirm GARFTase inhibition with compounds **7** and **8**, we used an *in situ* GARFTase activity assay in which intact cells were incubated with the antifolate drugs along with [¹⁴C]glycine as a radiotracer. In the presence of azaserine, [¹⁴C]glycine is incorporated into GAR, accumulating [¹⁴C]formyl GAR which can be isolated by ion exchange chromatography and radioactivity determined.^{10–13,19}

Data are shown in Figure 6 for the effects of compounds **3b**, **7**, and **8** on *in situ* GARFTase activity in IGROV1 cells, although completely analogous results were obtained with KB cells (not shown). In this assay, **3b**, **7**, and **8**, like LMTX, were all potent inhibitors at low nanomolar concentrations.

Conclusions

A lack of selectivity for folate transporters is a major cause of toxicity and chemotherapy failure with clinically used antifolates. We previously reported potent inhibition of tumor cell proliferation by a classical 2-amino-4-oxo-6-substituted pyrrolo[2,3-*d*]pyrimidine with a thienoyl-for-benzoyl-substituted side chain and a 4-carbon bridge (compound **3b**).^{7,12} Compound **3b** was selective for transport by FRs and PCFT over RFC and inhibited GARFTase in *de novo* purine biosynthesis.^{7,12} An analog of this series with a 3-carbon bridge (compound **3a**) was even more active, suggesting that the distance between the bicyclic scaffold and *L*-glutamate could be an important determinant of drug activity, although this may be partly at the expense of absolute transport selectivity.¹³ Accordingly, we pursued other approaches for altering this feature, without appreciably impacting drug potency toward PCFT- and FR α -expressing cells while preserving selectivity for transport by non-RFC uptake mechanisms over RFC.

We now describe the synthesis and biological characterization of regioisomers of **3b** (compounds **4–8**), differing in positions of thienoyl ring substitution so as to vary the distance and orientation between the bicyclic scaffold and the *L*-glutamate. Additional conformationally restricted analogs of **3b** and **4–8** (designated **20** and **9–13**, respectively) were also studied in which a linear acetylenic linkage was inserted into the bridge region. Of this expanded series, analogs with a saturated bridge and with a [1,3] substitution pattern on the thienoyl ring (compounds **3b**, **7**, and **8**) were the best inhibitors of KB and IGROV1 human tumor cell growth and clonogenicity. This was associated with a potent inhibition of GARFTase in *de novo* purine nucleotide biosynthesis. Whereas compounds **3b**, **7** and **8** all showed FR α - and PCFT-mediated effects on cell proliferation, neither **3b** nor compounds **4–8** showed activity toward CHO cells expressing only RFC. Further, none of the conformationally restricted linear acetylenic bridge analogs **9–13** and **20** inhibited human tumor cell growth, although the [1,3] thiophene-substituted compounds **12**, **13** and **20** were distinctly (and selectively) inhibitory toward FR α -expressing RT16 CHO cells. This further supports our conclusion that only the [1,3] substituted thiophene is amenable to high affinity FR α binding and cellular uptake, and that species differences in intracellular drug metabolism and/or target enzyme binding must account for the lack of activity of these acetylenic analogs with human tumor cells.

Thus, our present and published results establish that the pyrrolo[2,3-*d*]pyrimidine scaffold, along with a thienoyl side chain, is particularly amenable to FR and PCFT transport in the absence of RFC transport, and to potent GARFTase inhibition. Our new finding that [1,3] substituted regioisomers of this series are preferred over the [1,2] analogs is consistent with the notion of an optimal distance between the bicyclic ring system and *L*-glutamate moiety

for maximal anti-proliferative effects. However, among [1,3] compounds **3b**, **7** and **8**, or even including **3a**, there was no consistent relationship between relative drug sensitivities toward FR α - and PCFT-expressing cells and these intramolecular distances. Indeed, compounds **7** and **8** showed *in vitro* potencies that were generally comparable to those for compound **3b** or even **3a**, although this was cell line-dependent. This may reflect differential impacts of extra- and/or intracellular reduced folates on efficacies of the various analogs, combined with cell-specific differences in folate transport (i.e., RFC levels) or metabolism, and, for PCFT, differences in relative binding affinities between drugs at pH ~6.8.

Of course, the most distinguishing feature of the three active regioisomers, **3b**, **7**, and **8**, is their complete lack of RFC-mediated growth inhibition (presumably reflecting their lack of uptake) which is key to their potential therapeutic utility, as this would obviate toxicity to normal susceptible tissues for which drug transport by FRs or PCFT should be nominal. Clearly, the profound selectivity of these novel 6 substituted pyrrolo[2,3-*d*]pyrimidine [1,3] thienoyl antifolates for FRs and PCFT over RFC offers a paradigm for selective tumor targeting, based on established patterns of FR and PCFT expression in human tumors⁷ and optimal PCFT membrane transport under acidic conditions characteristic of the solid tumor microenvironment.

EXPERIMENTAL SECTION

All evaporations were carried out in vacuum with a rotary evaporator. Analytical samples were dried in vacuo (0.2 mmHg) in a CHEM-DRY drying apparatus over P₂O₅ at 60 °C. Melting points were determined on a MEL-TEMP II melting point apparatus with FLUKE 51 K/J electronic thermometer and are uncorrected. Nuclear magnetic resonance spectra for proton (¹H NMR) were recorded on Bruker Avance II 400 (400 MHz) and 500 (500 MHz) spectrometer. The chemical shift values are expressed in ppm (parts per million) relative to tetramethylsilane as an internal standard: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad singlet. Thin-layer chromatography (TLC) was performed on Whatman Sil G/UV254 silica gel plates with a fluorescent indicator, and the spots were visualized under 254 and 366 nm illumination. Proportions of solvents used for TLC are by volume. Column chromatography was performed on a 230–400 mesh silica gel (Fisher, Somerville, NJ) column. The amount (weight) of silica gel for column chromatography was in the range of 50–100 times the amount (weight) of the crude compounds being separated. Columns were dry-packed unless specified otherwise. Elemental analyses were performed by Atlantic Microlab, Inc., Norcross, GA. Element compositions are within $\pm 0.4\%$ of the calculated values. Fractional moles of water or organic solvents frequently found in some analytical samples of antifolates could not be prevented despite 24–48 h of drying in vacuo and were confirmed where possible by their presence in the ¹H NMR spectra. Elemental analysis was used to determine the purity of the final compounds **4–13**. All solvents and chemicals were purchased from Aldrich Chemical Co. and Fisher Scientific and were used as received. Purity of the final compounds **4–13** were >95% and were determined by elemental (C, H, N, S) analysis.

General procedure for the synthesis of compounds 15a–e

To a 250 mL round-bottomed flask, was added a mixture of bromo-substituted thiophene-carboxylic acid **14a–e** (1.04 g, 5 mmol), *N*-methylmorpholine (909 mg, 9 mmol), 2-chloro-4,6-dimethoxy-1,3,5-triazine (1.58 g, 9 mmol) and anhydrous DMF (20 mL). The mixture was stirred for 1.5 h at room temperature. *N*-methylmorpholine (909 mg, 9 mmol) and *L*-glutamic acid diethylester hydrochloride (1.8 g, 7.5 mmol) were added to the flask, and the reaction mixture was then stirred at room temperature for 12 h. After evaporation of solvent under reduced pressure, MeOH (20 mL) was added followed by silica gel (2.5 g). The resulting plug was loaded on to a silica gel column (2.5 $\times$ 12 cm) and eluted with 50%

EtOAc in hexane. Fractions with desired R_f (TLC) were pooled and evaporated to afford **15a–e** as colorless liquid.

(S)-2-[(2-Bromo-thiophene-3-carbonyl)-amino]-pentanedioic acid diethyl ester (15a)—Compound **15a** was prepared using the general method described for the preparation of **15a–e**, from 2-bromo-thiophene-3-carboxylic acid, **14a** (1.04 g, 5 mmol) to give 1.76 g (90%) of **15a** as a colorless liquid. TLC R_f 0.44 (hexane/EtOAc 1:1); ^1H NMR (CDCl_3 - d_1): δ 1.24–1.35 (m, 6H, $\text{COOCH}_2\text{CH}_3$), 2.09–2.56 (m, 4H, β - CH_2 , γ - CH_2), 4.10–4.17 (m, 2H, $\text{COOCH}_2\text{CH}_3$), 4.23–4.29 (m, 2H, $\text{COOCH}_2\text{CH}_3$), 4.78–4.86 (m, 1H, α -CH), 7.20–7.22 (d, $J = 7.2$ Hz, 1H, CONH, exch), 7.26–7.27 (d, $J = 5.6$ Hz, 1H, Ar), 7.36–7.37 (d, $J = 5.6$ Hz, 1H, Ar).

(S)-2-[(3-Bromo-thiophene-2-carbonyl)-amino]-pentanedioic acid diethyl ester (15b)—Compound **15b** was prepared using the general method described for the preparation of **15a–e**, from 3-bromo-thiophene-2-carboxylic acid, **14b** (1.04 g, 5 mmol) to give 1.8 g (91%) of **15b** as a colorless liquid. TLC R_f 0.45 (hexane/EtOAc 1:1); ^1H NMR (CDCl_3 - d_1): δ 1.24–1.36 (m, 6H, $\text{COOCH}_2\text{CH}_3$), 2.13–2.55 (m, 4H, β - CH_2 , γ - CH_2), 4.10–4.16 (m, 2H, $\text{COOCH}_2\text{CH}_3$), 4.25–4.30 (m, 2H, $\text{COOCH}_2\text{CH}_3$), 4.83–4.88 (m, 1H, α -CH), 7.07–7.08 (d, $J = 5.6$ Hz, 1H, Ar), 7.48–7.49 (d, $J = 5.6$ Hz, 1H, Ar), 7.73–7.75 (d, $J = 7.2$ Hz, 1H, CONH, exch).

(S)-2-[(4-Bromo-thiophene-3-carbonyl)-amino]-pentanedioic acid diethyl ester (15c)—Compound **15c** was prepared using the general method described for the preparation of **15a–e**, from 4-bromo-thiophene-3-carboxylic acid, **14c** (1.04 g, 5 mmol) to give 1.74 g (89%) of **15c** as a colorless liquid. TLC R_f 0.45 (hexane/EtOAc 1:1); ^1H NMR (CDCl_3 - d_1): δ 1.24–1.35 (m, 6H, $\text{COOCH}_2\text{CH}_3$), 2.07–2.56 (m, 4H, β - CH_2 , γ - CH_2), 4.11–4.17 (m, 2H, $\text{COOCH}_2\text{CH}_3$), 4.23–4.30 (m, 2H, $\text{COOCH}_2\text{CH}_3$), 4.78–4.87 (m, 1H, α -CH), 7.35–7.36 (d, $J = 3.6$ Hz, 1H, Ar), 7.36–7.38 (d, $J = 7.2$ Hz, 1H, CONH, exch), 8.07–8.08 (d, $J = 3.6$ Hz, 1H, Ar).

(S)-2-[(4-Bromo-thiophene-2-carbonyl)-amino]-pentanedioic acid diethyl ester (15d)—Compound **15d** was prepared using the general method described for the preparation of **15a–e**, from 4-bromo-thiophene-2-carboxylic acid, **14d** (1.04 g, 5 mmol) to give 1.8 g (91%) of **15d** as a colorless liquid. TLC R_f 0.44 (hexane/EtOAc 1:1); ^1H NMR (CDCl_3 - d_1): δ 1.25–1.34 (m, 6H, $\text{COOCH}_2\text{CH}_3$), 2.13–2.51 (m, 4H, β - CH_2 , γ - CH_2), 4.14–4.17 (m, 2H, $\text{COOCH}_2\text{CH}_3$), 4.23–4.28 (m, 2H, $\text{COOCH}_2\text{CH}_3$), 4.71–4.76 (m, 1H, α -CH), 7.04–7.06 (d, $J = 7.2$ Hz, 1H, CONH, exch), 7.42–7.43 (d, $J = 1.6$ Hz, 1H, Ar), 7.16–7.47 (d, $J = 1.6$ Hz, 1H, Ar).

(S)-2-[(5-Bromo-thiophene-3-carbonyl)-amino]-pentanedioic acid diethyl ester (15e)—Compound **15e** was prepared using the general method described for the preparation of **15a–e**, from 5-bromo-thiophene-3-carboxylic acid, **14e** (1.04 g, 5 mmol) to give 1.76 g (90%) of **15e** as a colorless liquid. TLC R_f 0.45 (hexane/EtOAc 1:1); ^1H NMR (CDCl_3 - d_1): δ 1.25–1.34 (m, 6H, $\text{COOCH}_2\text{CH}_3$), 2.12–2.53 (m, 4H, β - CH_2 , γ - CH_2), 4.12–4.18 (m, 2H, $\text{COOCH}_2\text{CH}_3$), 4.23–4.28 (m, 2H, $\text{COOCH}_2\text{CH}_3$), 4.70–4.75 (m, 1H, α -CH), 6.96–6.98 (d, $J = 7.2$ Hz, 1H, CONH, exch), 7.40–7.41 (d, $J = 1.6$ Hz, 1H, Ar), 7.83–7.84 (d, $J = 1.6$ Hz, 1H, Ar).

General procedure for the synthesis of compounds **17a–e**

To a 250-mL round-bottomed flask, equipped with a magnetic stirrer and gas inlet, was added a mixture of tetrakis(triphenylphosphine)palladium(0) (277 mg, 0.24 mmol), triethylamine (1.52 g, 15 mmol), **15a–e** (882 mg, 2.25 mmol) and anhydrous DMF (20 mL).

To the stirred mixture, under N₂, were added copper(I) iodide (46 mg, 0.24 mmol) and 2-amino-6-but-3-ynyl-3,7-dihydro-pyrrolo[2,3-*d*]pyrimidin-4-one, **16** (303 mg, 1.5 mmol). The reaction mixture was stirred at room temperature overnight (17–18 h). Silica gel (0.5 g) was then added, and the solvent was evaporated under reduced pressure. The resulting plug was loaded on to a silica gel column (1.5 × 12 cm) and eluted with CHCl₃ followed by 3% MeOH in CHCl₃ and then 5% MeOH in CHCl₃. Fractions with desired *R_f* (TLC) were pooled and evaporated to afford **17a–e**.

(S)-2-((2-[4-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-*d*]pyrimidin-6-yl)-but-1-ynyl]-thiophene-3-carbonyl)-amino)-pentanedioic acid diethyl ester (17a)—

Compound **17a** was prepared using the general method described for the preparation of **17a–e**, from **16** (303 mg, 1.5 mmol) and **12a** (882 mg, 2.25 mmol) to give 412 mg (53%) of **17a** as a brown powder. mp 81–82 °C; TLC *R_f* 0.53 (CHCl₃/MeOH 5:1); ¹H NMR (DMSO-*d*₆): δ 1.16–1.22 (m, 6H, COOCH₂CH₃), 1.92–2.14 (m, 2H, β-CH₂), 2.43–2.46 (t, *J* = 8 Hz, 2H, γ-CH₂), 2.80 (m, 4H, CH₂CH₂), 4.01–4.52 (q, *J* = 7 Hz, 2H, COOCH₂CH₃), 4.10–4.16 (m, 2H, COOCH₂CH₃), 4.43–4.48 (m, 1H, α-CH), 5.99 (s, 1H, C5-CH), 6.01 (s, 2H, 2-NH₂, exch), 7.35–7.36 (d, *J* = 5.5 Hz, 1H, Ar), 7.53–7.54 (d, *J* = 5.5 Hz, 1H, Ar), 8.34–8.36 (d, *J* = 7.5 Hz, 1H, CONH, exch), 10.16 (s, 1H, 3-NH, exch), 10.88 (s, 1H, 7-NH, exch).

(S)-2-((3-[4-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-*d*]pyrimidin-6-yl)-but-1-ynyl]-thiophene-2-carbonyl)-amino)-pentanedioic acid diethyl ester (17b)—

Compound **17b** was prepared using the general method described for the preparation of **17a–e**, from **16** (303 mg, 1.5 mmol) and **15b** (882 mg, 2.25 mmol) to give 423 mg (55%) of **17b** as a brown powder. mp 77–78 °C; TLC *R_f* 0.52 (CHCl₃/MeOH 5:1); ¹H NMR (DMSO-*d*₆): δ 1.16–1.22 (m, 6H, COOCH₂CH₃), 1.92–2.18 (m, 2H, β-CH₂), 2.39–2.43 (m, 2H, γ-CH₂), 2.84 (m, 4H, CH₂CH₂), 3.99–4.03 (q, *J* = 7 Hz, 2H, COOCH₂CH₃), 4.14–4.18 (q, *J* = 7 Hz, 2H, COOCH₂CH₃), 4.53–4.57 (m, 1H, α-CH), 6.00 (s, 1H, C5-CH), 6.01 (s, 2H, 2-NH₂, exch), 7.16–7.17 (d, *J* = 5.0 Hz, 1H, Ar), 7.81–7.82 (d, *J* = 5.0 Hz, 1H, Ar), 8.19–8.20 (d, *J* = 7.5 Hz, 1H, CONH, exch), 10.16 (s, 1H, 3-NH, exch), 10.91 (s, 1H, 7-NH, exch).

(S)-2-((4-[4-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-*d*]pyrimidin-6-yl)-but-1-ynyl]-thiophene-3-carbonyl)-amino)-pentanedioic acid diethyl ester (17c)—

Compound **17c** was prepared using the general method described for the preparation of **17a–e**, from **16** (303 mg, 1.5 mmol) and **15c** (882 mg, 2.25 mmol) to give 433 mg (56%) of **17c** as a brown powder. mp 80–81 °C; TLC *R_f* 0.53 (CHCl₃/MeOH 5:1); ¹H NMR (DMSO-*d*₆): δ 1.16–1.22 (m, 6H, COOCH₂CH₃), 1.92–2.14 (m, 2H, β-CH₂), 2.42–2.46 (m, 2H, γ-CH₂), 2.69–2.78 (m, 4H, CH₂CH₂), 4.01–4.05 (q, *J* = 7 Hz, 2H, COOCH₂CH₃), 4.11–4.16 (m, 2H, COOCH₂CH₃), 4.45–4.50 (m, 1H, α-CH), 5.99 (s, 1H, C5-CH), 6.00 (s, 2H, 2-NH₂, exch), 7.16–7.17 (d, *J* = 3.5 Hz, 1H, Ar), 8.08–8.09 (d, *J* = 3.5 Hz, 1H, Ar), 8.40–8.41 (d, *J* = 7.5 Hz, 1H, CONH, exch), 10.16 (s, 1H, 3-NH, exch), 10.88 (s, 1H, 7-NH, exch).

(S)-2-((4-[4-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-*d*]pyrimidin-6-yl)-but-1-ynyl]-thiophene-2-carbonyl)-amino)-pentanedioic acid diethyl ester (17d)—

Compound **17d** was prepared using the general method described for the preparation of **17a–e**, from **16** (303 mg, 1.5 mmol) and **15d** (882 mg, 2.25 mmol) to give 406 mg (53%) of **17d** as a brown powder. mp 79–80 °C; TLC *R_f* 0.53 (CHCl₃/MeOH 5:1); ¹H NMR (DMSO-*d*₆): δ 1.16–1.22 (m, 6H, COOCH₂CH₃), 1.92–2.12 (m, 2H, β-CH₂), 2.42–2.45 (t, *J* = 7.5 Hz, 2H, γ-CH₂), 2.71–2.78 (m, 4H, CH₂CH₂), 4.03–4.07 (q, *J* = 7 Hz, 2H, COOCH₂CH₃), 4.09–4.13 (q, *J* = 6.5 Hz, 2H, COOCH₂CH₃), 4.36–4.40 (m, 1H, α-CH), 6.00 (s, 1H, C5-CH), 6.02 (s, 2H, 2-NH₂, exch), 7.87–7.88 (m, 2H, Ar), 8.76–8.77 (d, *J* = 7.5 Hz, 1H, CONH, exch), 10.17 (s, 1H, 3-NH, exch), 10.90 (s, 1H, 7-NH, exch).

(S)-2-((5-[4-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-6-yl)-but-1-ynyl]-thiophene-3-carbonyl)-amino)-pentanedioic acid diethyl ester (17e)—

Compound **17e** was prepared using the general method described for the preparation of **17a–e**, from **16** (303 mg, 1.5 mmol) and **15e** (882 mg, 2.25 mmol) to give 387 mg (50%) of **17e** as a brown powder. mp 81–82 °C; TLC R_f 0.53 (CHCl₃/MeOH 5:1); ¹H NMR (DMSO-*d*₆): δ 1.16–1.22 (m, 6H, COOCH₂CH₃), 1.92–2.12 (m, 2H, β-CH₂), 2.41–2.44 (t, *J* = 7.5 Hz, 2H, γ-CH₂), 2.78 (m, 4H, CH₂CH₂), 4.03–4.07 (q, *J* = 7 Hz, 2H, COOCH₂CH₃), 4.09–4.13 (m, 2H, COOCH₂CH₃), 4.36–4.41 (m, 1H, α-CH), 6.00 (s, 1H, C5-CH), 6.01 (s, 2H, 2-NH₂, exch), 7.58–7.59 (d, *J* = 1.5 Hz, 1H, Ar), 8.12–8.13 (d, *J* = 1.5 Hz, 1H, Ar), 8.56–8.58 (d, *J* = 7.5 Hz, 1H, CONH, exch), 10.16 (s, 1H, 3-NH, exch), 10.88 (s, 1H, 7-NH, exch).

General procedure for the synthesis of compounds 18a–e

To a Parr hydrogenation flask was added **17a–e** (200 mg, 0.39 mmol), 10% palladium on activated carbon (100 mg), and MeOH (50 mL). Hydrogenation was carried out at 55 psi of H₂ for 4 h. The reaction mixture was filtered through Celite, washed with MeOH (100 mL) and concentrated under reduced pressure to give **18a–e**.

(S)-2-((2-[4-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-6-yl)-butyl]-thiophene-3-carbonyl)-amino)-pentanedioic acid diethyl ester (18a)—

Compound **18a** was prepared using the general method described for the preparation of **18a–e**, from **17a** (200 mg, 0.39 mmol) to give 191 mg (95%) of **18a** as a light yellow powder. mp 81–82 °C; TLC R_f 0.54 (CHCl₃/MeOH 5:1); ¹H NMR (DMSO-*d*₆): δ 1.15–1.17 (m, 6H, COOCH₂CH₃), 1.61 (m, 4H, CH₂CH₂), 1.91–2.12 (m, 2H, β-CH₂), 2.41–2.44 (t, *J* = 7.0 Hz, 2H, γ-CH₂), 2.51–2.53 (m, 4H, CH₂CH₂), 4.03–4.07 (q, *J* = 7 Hz, 2H, COOCH₂CH₃), 4.08–4.13 (m, 2H, COOCH₂CH₃), 4.35–4.40 (m, 1H, α-CH), 5.96 (s, 3H, C5-CH, 2-NH₂, exch), 7.35 (s, 2H, Ar), 8.40–8.42 (d, *J* = 7.5 Hz, 1H, CONH, exch), 10.99 (s, 1H, 3-NH, exch), 11.35 (s, 1H, 7-NH, exch).

(S)-2-((3 [4-(2 Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-6-yl)-butyl]-thiophene-2-carbonyl)-amino)-pentanedioic acid diethyl ester (18b)—

Compound **18b** was prepared using the general method described for the preparation of **18a–e**, from **17b** (200 mg, 0.39 mmol) to give 187 mg (93%) of **18b** as a light yellow powder. mp 80–81 °C; TLC R_f 0.53 (CHCl₃/MeOH 5:1); ¹H NMR (DMSO-*d*₆): δ 1.15–1.20 (m, 6H, COOCH₂CH₃), 1.57 (m, 4H, CH₂CH₂), 1.93–2.12 (m, 2H, β-CH₂), 2.40–2.43 (t, *J* = 8.0 Hz, 2H, γ-CH₂), 2.51 (m, 2H, CH₂), 2.87 (m, 2H, CH₂), 4.03–4.07 (q, *J* = 7.0 Hz, 2H, COOCH₂CH₃), 4.08–4.14 (m, 2H, COOCH₂CH₃), 4.34–4.39 (m, 1H, α CH), 5.96 (m, 3H, C5-CH, 2-NH₂, exch), 7.02–7.03 (d, *J* = 5.0 Hz, 1H, Ar), 7.59–7.60 (d, *J* = 5.0 Hz, 1H, Ar), 8.39–8.40 (d, *J* = 7.5 Hz, 1H, CONH, exch), 11.09 (s, 1H, 3 NH, exch), 11.41 (s, 1H, 7-NH, exch).

(S)-2-((4-[4-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-6-yl)-butyl]-thiophene-3-carbonyl)-amino)-pentanedioic acid diethyl ester (18c)—

Compound **18c** was prepared using the general method described for the preparation of **18a–e**, from **17c** (200 mg, 0.39 mmol) to give 193 mg (96%) of **18c** as a light yellow powder. mp 79–80 °C; TLC R_f 0.54 (CHCl₃/MeOH 5:1); ¹H NMR (DMSO-*d*₆): δ 1.17–1.18 (m, 6H, COOCH₂CH₃), 1.56 (m, 4H, CH₂CH₂), 1.91–2.11 (m, 2H, β-CH₂), 2.43–2.46 (t, *J* = 7.5 Hz, 2H, γ-CH₂), 2.46–2.49 (m, 2H, CH₂), 2.75–2.77 (m, 2H, CH₂), 4.03–4.08 (q, *J* = 7.0 Hz, 2H, COOCH₂CH₃), 4.08–4.13 (m, 2H, COOCH₂CH₃), 4.35–4.39 (m, 1H, α CH), 5.83 (s, 1H, C5-CH), 5.98 (s, 2H, 2-NH₂, exch), 7.20–7.21 (d, *J* = 3.5 Hz, 1H, Ar), 7.92–7.93 (d, *J* = 3.5 Hz, 1H, Ar), 8.53–8.54 (d, *J* = 7.5 Hz, 1H, CONH, exch), 10.13 (s, 1H, 3-NH, exch), 10.78 (s, 1H, 7-NH, exch).

(S)-2-((4-[4-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-6-yl)-butyl]-thiophene-2-carbonyl)-amino)-pentanedioic acid diethyl ester (18d)—

Compound **18d** was prepared using the general method described for the preparation of **18a–e**, from **17d** (200 mg, 0.39 mmol) to give 185 mg (92%) of **18d** as a light yellow powder. mp 81–82 °C; TLC R_f 0.55 (CHCl₃/MeOH 5:1); ¹H NMR (DMSO-*d*₆): δ 1.17–1.19 (m, 6H, COOCH₂CH₃), 1.62 (m, 4H, CH₂CH₂), 1.95–2.12 (m, 2H, β-CH₂), 2.41–2.45 (t, *J* = 7.5 Hz, 2H, γ-CH₂), 2.54–2.62 (m, 4H, CH₂CH₂), 4.03–4.07 (q, *J* = 7.0 Hz, 2H, COOCH₂CH₃), 4.09–4.13 (m, 2H, COOCH₂CH₃), 4.36–4.40 (m, 1H, α-CH), 5.99 (s, 3H, C5 CH, 2-NH₂, exch), 7.41 (s, 1H, Ar), 7.76 (s, 1H, Ar), 8.67–8.68 (d, *J* = 7.5 Hz, 1H, CONH, exch), 11.00 (s, 1H, 3-NH, exch), 11.39 (s, 1H, 7-NH, exch).

(S)-2-((5-[4-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-6-yl)-butyl]-thiophene-3-carbonyl)-amino)-pentanedioic acid diethyl ester (18e)—

Compound **18e** was prepared using the general method described for the preparation of **18a–e**, from **17e** (200 mg, 0.39 mmol) to give 183 mg (91%) of **18e** as a light yellow powder. mp 82–83 °C; TLC R_f 0.54 (CHCl₃/MeOH 5:1); ¹H NMR (DMSO-*d*₆): δ 1.15–1.18 (m, 6H, COOCH₂CH₃), 1.64 (m, 4H, CH₂CH₂), 1.93–2.12 (m, 2H, β-CH₂), 2.41–2.44 (t, *J* = 7.5 Hz, 2H, γ CH₂), 2.55 (m, 2H, CH₂), 2.81 (m, 2H, CH₂), 4.03–4.07 (q, *J* = 7.0 Hz, 2H, COOCH₂CH₃), 4.08–4.12 (q, *J* = 7.0 Hz, 2H, COOCH₂CH₃), 4.36–4.40 (m, 1H, α CH), 5.96 (s, 3H, C5-CH, 2-NH₂, exch), 7.26 (s, 1H, Ar), 7.98 (s, 1H, Ar), 8.46–8.48 (d, *J* = 8.0 Hz, 1H, CONH, exch), 10.76 (s, 1H, 3-NH, exch), 11.22 (s, 1H, 7-NH, exch).

General procedure for the synthesis of compounds 4–13

To a solution of **18a–e** (100 mg, 0.19 mmol) or **17a–e** (100 mg, 0.19 mmol) in 10 mL of MeOH/CHCl₃ (1:1) or MeOH/CH₂Cl₂ (1:1) was added 1 N NaOH (5 mL) and the mixture was stirred under N₂ at room temperature for 16 h. TLC showed the disappearance of the starting material and one major spot at the origin. The reaction mixture was evaporated to dryness under reduced pressure. The residue was dissolved in water (10 mL), the resulting solution was cooled in an ice bath, and the pH was adjusted to 3–4 with dropwise addition of 1 N HCl. The resulting suspension was frozen in a dry ice-acetone bath, thawed to 4–5 °C in the refrigerator, and filtered. The residue was washed with a small amount of cold water and dried in vacuum using P₂O₅ to afford **4–13**.

(S)-2-((2-[4-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-6-yl)-butyl]-thiophene-3-carbonyl)-amino)-pentanedioic acid (4)—

Compound **4** was prepared using the general method described for the preparation of **4–13**, from **15a** (100 mg, 0.19 mmol) to give 83 mg (95%) of **4** as a light yellow powder. mp 176–177 °C; ¹H NMR (DMSO-*d*₆): δ 1.61 (m, 4H, CH₂CH₂), 1.87–2.10 (m, 2H, β-CH₂), 2.34–2.37 (t, *J* = 7.5 Hz, 2H, γ CH₂), 2.47 (m, 2H, CH₂), 3.09–3.11 (m, 2H, CH₂), 4.32–4.37 (m, 1H, α CH), 5.86 (s, 1H, C5-CH), 5.98 (s, 2H, 2-NH₂, exch), 7.33–7.34 (d, *J* = 5.0 Hz, 1H, Ar), 7.35–7.36 (d, *J* = 5.0 Hz, 1H, Ar), 8.28–8.29 (d, *J* = 7.5 Hz, 1H, CONH, exch), 10.16 (s, 1H, 3-NH, exch), 10.80 (s, 1H, 7-NH, exch), 12.36 (br, 2H, COOH, exch). Anal. (C₂₀H₂₃N₅O₆S × 1.85 H₂O) C, H, N, S.

(S)-2-((3-[4-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-6-yl)-butyl]-thiophene-2-carbonyl)-amino)-pentanedioic acid (5)—

Compound **5** was prepared using the general method described for the preparation of **4–13**, from **18b** (100 mg, 0.19 mmol) to give 83 mg (95%) of **5** as a light yellow powder. mp 159–160 °C; ¹H NMR (DMSO-*d*₆): δ 1.56 (m, 4H, CH₂CH₂), 1.88–2.11 (m, 2H, β-CH₂), 2.33–2.36 (t, *J* = 7.5 Hz, 2H, γ CH₂), 2.48 (m, 2H, CH₂), 2.86–2.88 (m, 2H, CH₂), 4.31–4.36 (m, 1H, α CH), 5.85 (s, 1H, C5-CH), 5.98 (s, 2H, 2-NH₂, exch), 7.01–7.02 (d, *J* = 5.0 Hz, 1H, Ar), 7.58–7.59 (d, *J* = 5.0 Hz, 1H, Ar), 8.24–8.26 (d, *J* = 7.5 Hz, 1H, CONH, exch), 10.16 (s, 1H, 3-NH, exch),

10.79 (s, 1H, 7-NH, exch), 12.38 (br, 2H, COOH, exch). Anal. ($C_{20}H_{23}N_5O_6S \times 1.0 H_2O$) C, H, N, S.

(S)-2-((4-[4-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-6-yl)-butyl]-thiophene-3-carbonyl)-amino)-pentanedioic acid (6)—Compound **6** was prepared using the general method described for the preparation of **4–13**, from **18c** (100 mg, 0.19 mmol) to give 83 mg (95%) of **6** as a light yellow powder. mp 195–196 °C; 1H NMR (DMSO- d_6): δ 1.56 (m, 4H, CH_2CH_2), 1.87–2.10 (m, 2H, β - CH_2), 2.35–2.38 (t, J = 8.0 Hz, 2H, γ CH_2), 2.47–2.48 (m, 2H, CH_2), 2.75–2.81 (m, 2H, CH_2), 4.32–4.36 (m, 1H, α CH), 5.85 (s, 1H, C5-CH), 5.96 (s, 2H, 2-NH₂, exch), 7.19–7.20 (d, J = 3.0 Hz, 1H, Ar), 7.92–7.93 (d, J = 3.0 Hz, 1H, Ar), 8.40–8.41 (d, J = 8.0 Hz, 1H, CONH, exch), 10.14 (s, 1H, 3-NH, exch), 10.78 (s, 1H, 7-NH, exch), 12.31 (br, 2H, COOH, exch). Anal. ($C_{20}H_{23}N_5O_6S \times 0.4 CHCl_3$) C, H, N, S.

(S)-2-((4-[4-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-6-yl)-butyl]-thiophene-2-carbonyl)-amino)-pentanedioic acid (7)—Compound **7** was prepared using the general method described for the preparation of **4–13**, from **18d** (100 mg, 0.19 mmol) to give 83 mg (95%) of **7** as a light yellow powder. mp 174–175 °C; 1H NMR (DMSO- d_6): δ 1.61 (m, 4H, CH_2CH_2), 1.88–2.11 (m, 2H, β - CH_2), 2.33–2.36 (t, J = 7.5 Hz, 2H, γ - CH_2), 2.56 (m, 4H, CH_2CH_2), 4.32–4.37 (m, 1H, α -CH), 5.88 (s, 1H, C5-CH), 5.96 (s, 2H, 2-NH₂, exch), 7.39 (s, 1H, Ar), 7.75 (s, 1H, Ar), 8.54–8.55 (d, J = 8.0 Hz, 1H, CONH, exch), 10.13 (s, 1H, 3-NH, exch), 10.81 (s, 1H, 7-NH, exch), 12.45 (br, 2H, COOH, exch). Anal. ($C_{20}H_{23}N_5O_6S \times 0.31 CHCl_3$) C, H, N, S.

(S)-2-((5-[4-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-6-yl)-butyl]-thiophene-3-carbonyl)-amino)-pentanedioic acid (8)—Compound **8** was prepared using the general method described for the preparation of **4–13**, from **18e** (100 mg, 0.19 mmol) to give 83 mg (95%) of **8** as a light yellow powder. mp 175–176 °C; 1H NMR (DMSO- d_6): δ 1.64 (m, 4H, CH_2CH_2), 1.87–2.10 (m, 2H, β - CH_2), 2.32–2.35 (t, J = 7.5 Hz, 2H, γ - CH_2), 2.55 (m, 2H, CH_2), 2.80–2.82 (m, 2H, CH_2), 4.33–4.37 (m, 1H, α CH), 5.88 (s, 1H, C5-CH), 5.96 (s, 2H, 2-NH₂, exch), 7.27 (s, 1H, Ar), 7.96 (s, 1H, Ar), 8.31–8.33 (d, J = 8.0 Hz, 1H, CONH, exch), 10.12 (s, 1H, 3-NH, exch), 10.80 (s, 1H, 7-NH, exch), 12.43 (br, 2H, COOH, exch). Anal. ($C_{20}H_{23}N_5O_6S \times 1.5 H_2O$) C, H, N, S.

(S)-2-((2-[4-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-6-yl)-butyl]-thiophene-3-carbonyl)-amino)-pentanedioic acid (9)—Compound **9** was prepared using the general method described for the preparation of **4–13**, from **17a** (100 mg, 0.19 mmol, dissolved in 10 mL of MeOH/ CH_3COOH (9:1)) to give 84 mg (95%) of **9** as a light yellow powder. mp 196–197 °C; 1H NMR (DMSO- d_6): δ 1.91–2.13 (m, 2H, β - CH_2), 2.34–2.37 (t, J = 7 Hz, 2H, γ - CH_2), 2.81 (m, 4H, CH_2CH_2), 4.41–4.45 (m, 1H, α -CH), 6.01 (s, 3H, C5 CH, 2-NH₂, exch), 7.37–7.38 (d, J = 5.5 Hz, 1H, Ar), 7.53–7.54 (d, J = 5.5 Hz, 1H, Ar), 8.25–8.26 (d, J = 7.5 Hz, 1H, CONH, exch), 10.19 (s, 1H, 3-NH, exch), 10.88 (s, 1H, 7-NH, exch), 12.47 (br, 2H, COOH, exch). Anal. ($C_{20}H_{19}N_5O_6S \times 1.0 CH_3COOH$) C, H, N, S.

(S)-2-((3-[4-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-6-yl)-butyl]-thiophene-3-carbonyl)-amino)-pentanedioic acid (10)—Compound **10** was prepared using the general method described for the preparation of **4–13**, from **17b** (100 mg, 0.19 mmol) to give 83 mg (94%) of **10** as a light yellow powder. mp 179–180 °C; 1H NMR (DMSO- d_6): δ 1.96–2.18 (m, 2H, β - CH_2), 2.31–2.34 (t, J = 7.5 Hz, 2H, γ - CH_2), 2.84 (m, 4H, CH_2CH_2), 4.41–4.53 (m, 1H, α -CH), 6.01 (s, 3H, C5-CH, 2-NH₂, exch), 7.16–7.17 (d, J = 5.0 Hz, 1H, Ar), 7.80–7.81 (d, J = 5.0 Hz, 1H, Ar), 8.22–8.23 (d, J = 7.5 Hz, 1H, CONH,

exch), 10.20 (s, 1H, 3-NH, exch), 10.89 (s, 1H, 7-NH, exch), 12.30 (br, 2H, COOH, exch). Anal. ($C_{20}H_{19}N_5O_6S \times 0.3 CHCl_3$) C, H, N, S.

(S)-2-((4-[4-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-6-yl)-butyl]-thiophene-3-carbonyl)-amino)-pentanedioic acid (11)—Compound **11** was prepared using the general method described for the preparation of **4–13**, from **17c** (100 mg, 0.19 mmol) to give 84 mg (95%) of **11** as a light yellow powder. mp 211–212 °C; 1H NMR (DMSO- d_6): δ 1.92–2.14 (m, 2H, β -CH $_2$), 2.33–2.37 (t, J = 8.0 Hz, 2H, γ -CH $_2$), 2.70–2.80 (m, 4H, CH $_2$ CH $_2$), 4.45–4.50 (m, 1H, α -CH), 6.00 (s, 3H, C5-CH, 2-NH $_2$, exch), 7.74–7.75 (d, J = 3.0 Hz, 1H, Ar), 8.10–8.11 (d, J = 3.0 Hz, 1H, Ar), 8.30–8.32 (d, J = 8.0 Hz, 1H, CONH, exch), 10.17 (s, 1H, 3-NH, exch), 10.86 (s, 1H, 7-NH, exch), 12.44 (br, 2H, COOH, exch). Anal. ($C_{20}H_{19}N_5O_6S \times 0.3 CHCl_3$) C, H, N, S.

(S)-2-((4-[4-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-6-yl)-but-1-ynyl]-thiophene-2-carbonyl)-amino)-pentanedioic acid (12)—Compound **12** was prepared using the general method described for the preparation of **4–13**, from **17d** (100 mg, 0.19 mmol) to give 85 mg (96%) of **12** as a light yellow powder. mp 195–196 °C; 1H NMR (DMSO- d_6): δ 1.91–2.11 (m, 2H, β -CH $_2$), 2.34–2.37 (t, J = 7.5 Hz, 2H, γ -CH $_2$), 2.71–2.79 (m, 4H, CH $_2$ CH $_2$), 4.32–4.36 (m, 1H, α -CH), 6.01 (s, 3H, C5-CH, 2-NH $_2$, exch), 7.86–7.88 (m, 2H, Ar), 8.64–8.65 (d, J = 7.5 Hz, 1H, CONH, exch), 10.16 (s, 1H, 3-NH, exch), 10.88 (s, 1H, 7-NH, exch), 12.46 (br, 2H, COOH, exch). Anal. ($C_{20}H_{19}N_5O_6S \times 0.37 CH_2Cl_2$) C, H, N, S.

(S)-2-((5-[4-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-6-yl)-but-1-ynyl]-thiophene-3-carbonyl)-amino)-pentanedioic acid (13)—Compound **13** was prepared using the general method described for the preparation of **4–13**, from **17e** (100 mg, 0.19 mmol) to give 84 mg (95%) of **13** as a light yellow powder. mp 197–198 °C; 1H NMR (DMSO- d_6): δ 1.92–2.14 (m, 2H, β -CH $_2$), 2.33–2.37 (t, J = 8.0 Hz, 2H, γ -CH $_2$), 2.70–2.80 (m, 4H, CH $_2$ CH $_2$), 4.45–4.50 (m, 1H, α -CH), 6.00 (s, 3H, C5-CH, 2-NH $_2$, exch), 7.74–7.75 (d, J = 3.0 Hz, 1H, Ar), 8.10–8.11 (d, J = 3.0 Hz, 1H, Ar), 8.30–8.32 (d, J = 8.0 Hz, 1H, CONH, exch), 10.17 (s, 1H, 3-NH, exch), 10.86 (s, 1H, 7-NH, exch), 12.44 (br, 2H, COOH, exch). Anal. ($C_{20}H_{19}N_5O_6S \times 0.41 CH_2Cl_2$) C, H, N, S.

(S)-2-((5-[4-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-6-yl)-but-1-ynyl]-thiophene-2-carbonyl)-amino)-pentanedioic acid (20)—To a solution of **19** (50 mg, 0.1 mmol) in MeOH (10 mL) was added 1 N NaOH (5 mL) and the mixture was stirred under N $_2$ at room temperature for 16 h. TLC showed the disappearance of the starting material and one major spot at the origin. The reaction mixture was evaporated to dryness under reduced pressure. The residue was dissolved in water (10 mL), the resulting solution was cooled in an ice bath, and the pH was adjusted to 3–4 with dropwise addition of 1 N HCl. The resulting suspension was frozen in a dry ice-acetone bath, thawed to 4–5 °C in the refrigerator, and filtered. The residue was washed with a small amount of cold water and dried in vacuum using P $_2$ O $_5$ to afford 43 mg (95%) of **20** as a light yellow powder. mp 189–190 °C; 1H NMR (DMSO- d_6): δ 1.89–2.05 (m, 2H, β -CH $_2$), 2.30–2.34 (t, J = 7.6 Hz, 2H, γ -CH $_2$), 2.76 (m, 4H, CH $_2$ CH $_2$), 4.33 (m, 1H, α -CH), 5.92 (s, 2H, 2-NH $_2$, exch), 5.98–5.99 (d, J = 4 Hz, 1H, C5-CH), 7.19–7.20 (d, J = 4 Hz, 1H, Ar), 7.74–7.75 (d, J = 4 Hz, 1H, Ar), 8.69–8.71 (d, J = 8 Hz, 1H, CONH, exch), 10.15 (s, 1H, 3-NH, exch), 10.86 (s, 1H, 7-NH, exch), 12.47 (br, 2H, COOH, exch). HRMS calcd for $C_{20}H_{19}N_5O_6S$ (M+H) $^+$, 458.1134; found: 458.1155.

Reagents for biological studies

[3', 5', 7-³H]MTX (20 Ci/mmol) and [3', 5', 7, 9-³H] folic acid (25 Ci/mmol), [¹⁴C(U)] glycine (87mCi/mmol) were purchased from Moravek Biochemicals (Brea, CA). Unlabeled folic acid was purchased from Sigma Chemical Co. (St. Louis, MO). LCV [(6R,S) 5-formyl tetrahydrofolate] was provided by the Drug Development Branch, National Cancer Institute, Bethesda, MD. The sources of the classical antifolate drugs were as follows: MTX, Drug Development Branch, National Cancer Institute (Bethesda, MD); RTX [*N*-(5-[*N*-(3,4-dihydro-2-methyl-4-oxyquinazolin-6-ylmethyl)-*N*-methyl-amino]-2-thienoyl)-*L*-glutamic acid], AstraZeneca Pharmaceuticals (Macclesfield, Cheshire, England); and LMTX (5,10-dideaza 5,6,7,8-tetrahydrofolate) and PMX [*N*-(4-[2-(2-amino-3,4-dihydro-4-oxo-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl)-*L*-glutamic acid] (Alimta), Eli Lilly and Co. (Indianapolis, IN). Other chemicals were obtained from commercial sources in the highest available purity.

Cell lines and assays of antitumor drug activities

The origin of the engineered CHO sublines including RFC- and FR α -null MTXR11Oua^{R2-4} (R2), and RFC- (pC43-10), PCFT- (R2/PCFT4), or FR α - (RT16) expressing CHO sublines were previously described.^{10,11,14,18} Likewise, pDNA3.1 vector control CHO cells (R2/VC) were reported. The CHO cells were cultured in α -minimal essential medium (MEM) supplemented with 10% bovine calf serum (Invitrogen, Carlsbad, CA), 100 units/ml penicillin/100 μ g/ml streptomycin, and 2 mM *L*-glutamine at 37°C with 5% CO₂. All the R2 transfected cells [PC43-10, RT16, R2/hPCFT4, R2(VC)] were routinely cultured in α -MEM plus 1.5 mg/ml G418. Prior to the cytotoxicity assays (see below), RT16 cells were cultured in complete folate-free RPMI 1640 (without added folate) for three days. R2/hPCFT4 and R2(VC) cells were cultured in complete folate-free RPMI 1640 including dialyzed fetal bovine serum (Invitrogen) and 25 nM LCV plus 1.5 mg/ml G418. KB human nasopharyngeal carcinoma cells were purchased from the American Type Culture Collection (Manassas, VA) whereas IGROV1 ovarian carcinoma cells were a gift of Dr. Manohar Ratnam (University of Toledo). KB and IGROV1 cells were routinely cultured in folate-free RPMI 1640 medium, supplemented with 10% fetal bovine serum, penicillin-streptomycin solution, and 2 mM *L*-glutamine at 37°C with 5% CO₂.

For growth inhibition assays, cells (CHO, KB, or IGROV1) were plated in 96 well dishes (~2500–5000 cells/well, total volume of 200 μ l medium) with a range of antifolate concentrations. The medium was RPMI 1640 (contains 2.3 μ M folic acid) with 10% dialyzed fetal bovine serum and antibiotics for experiments with R2 and PC43-10 cells. For RT16, KB, and IGROV1 cells, cells were cultured in folate-free RPMI media with 10% dialyzed fetal bovine serum and antibiotics supplemented with 2 nM LCV and 2 mM *L*-glutamine. The requirement for FR-mediated drug uptake in these assays was established in parallel incubations including 200 nM folic acid. For R2/hPCFT4 cells, the medium was folate-free RPMI 1640 (pH 7.2) containing 25 nM LCV, supplemented with 10% dialyzed fetal bovine serum, antibiotics, and *L*-glutamine. Cells were routinely incubated for up to 96 h and metabolically active cells (a measure of cell viability) were assayed with CellTiter-blue Cell Viability Assay (Promega, Madison, WI), with fluorescence measured (590 nm emission, 560 nm excitation) using a fluorescence plate reader. Raw data were exported from Softmax Pro software to an Excel spreadsheet for analysis and determinations of IC₅₀s, corresponding to the drug concentrations that result in 50% loss of cell growth.

For some of the *in vitro* growth inhibition studies, the inhibitory effects of the antifolate inhibitors on *de novo* thymidylate biosynthesis (i.e., thymidylate synthase) and *de novo* purine biosynthesis (GARFTase and AICARFTase) were tested by co-incubations with thymidine (10 μ M) and adenosine (60 μ M), respectively. For *de novo* purine biosynthesis,

additional protection experiments used AICA (320 μ M) to distinguish inhibitory effects at GARFTase from those at AICARFTase.^{10–12}

For assays of clonogenicity (colony formation), KB cells (500 cells) were plated in folate-free RPMI1640 medium supplemented with 2 nM LCV, 10% dialyzed fetal bovine serum, penicillin-streptomycin solution, and 2 mM *L*-glutamine, in the presence of antifolate drugs. The dishes were incubated at 37°C with 5% CO₂ for 10–14 days. To stain the colonies for counting, the dishes were rinsed with Dulbecco's phosphate-buffered saline (DPBS), 5% trichloroacetic acid, then borate buffer (10 mM, pH 8.8), followed by 30 min incubation in 1% methylene blue in the borate buffer. The dishes were rinsed with the borate buffer and colonies were counted for calculating percent colony-forming efficiency normalized to control.

FR binding assay

Competitive inhibition of [³H]folic acid binding to FR α with RT16 CHO cells was used to assess relative binding affinities for assorted (anti)folate ligands.^{10–12} Briefly, RT16 cells ($\sim 1.6 \times 10^6$) were rinsed twice with DPBS, followed by two washes with an acidic buffer (10 mM sodium acetate, 150 mM NaCl, pH 3.5) to remove FR bound folates. Cells were washed twice with ice-cold HEPES-buffered saline (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 5 mM glucose, pH 7.4) (HBS), then incubated in HBS with [³H]folic acid (50 nM, specific activity 0.5 Ci/mmol) in the presence and absence of unlabeled folic acid or antifolate (over a range of concentrations) for 15 min at 0°C. The dishes were rinsed three times with ice-cold HBS, after which the cells were solubilized (0.5 N NaOH) and aliquots measured for radioactivity and protein contents. Protein concentrations were measured with Folin phenol reagent.²⁰ [³H]Folic acid bound to FR α was calculated as pmol/mg protein and relative binding affinities were calculated as the inverse molar ratios of unlabeled ligands required to inhibit [³H]folic acid binding by 50%. By definition, the relative affinity of folic acid is 1.

Transport assays

For transport assays, R2/hPCFT4 CHO cells were grown as monolayers and used to seed spinner flasks. For experiments to determine the inhibitions of transport by antifolate substrates, cells were collected from spinners, washed with DPBS and resuspended in 2 ml of HBS, adjusted to pH 6.8, or 4-morpholinepropanesulfonic acid-buffered saline (20 mM MES, 140 mM NaCl, 5 mM KCl, 2 mM MgCl₂, and 5 mM glucose), adjusted to pH 5.5. For determinations of K_is, cells were incubated with 1 μ M [³H]MTX over 2 min at 37°C, in the presence and absence of a range of concentrations of unlabeled antifolates. Controls included a comparable amount of vehicle (DMSO to 0.5%). Uptakes of [³H]MTX were quenched with ice-cold DPBS. Cells were washed with ice-cold DPBS (3 $\times$) and solubilized with 0.5 N NaOH. Levels of intracellular radioactivity were expressed as pmol/mg protein, calculated from direct measurements of radioactivity and protein contents of cell homogenates. Protein concentrations were measured with Folin phenol reagent. K_is were calculated from Dixon plots and kinetic constants (K_t, V_{max}) for [³H]MTX uptake, calculated from Lineweaver Burk plots, as previously described.¹⁴

In situ GARFT enzyme inhibition assay

Incorporation of [¹⁴C]glycine into [¹⁴C]FGAR, as an *in situ* measure of endogenous GARFTase activity, was measured as described.^{10–12} For these experiments, IGROV1 cells were seeded in 4 mL of complete folate free RPMI 1640 plus 2 nM LCV in 60 mm dishes at a density of 2×10^6 cells per dish. On the next day, the medium was replaced with 2 mL of fresh complete folate free RPMI 1640 plus 2 nM LCV (without supplementing glutamine). Azaserine (4 μ M final concentration) was added in the presence and absence of the

antifolate inhibitors. After 30 min, *L*-glutamine (final concentration, 2 mM) and [^{14}C]glycine (tracer amounts; final specific activity 0.1 mCi/L) were added. Incubations were at 37°C for 15 h, at which time cells were washed (one-time) with ice-cold folate-free RPMI 1640 plus serum. Cell pellets were dissolved in 2 ml 5% trichloroacetic acid at 0°C. Cell debris was removed by centrifugation (the cell protein contents in the pellets were measured), and the supernatants were extracted twice with 2 mL of ice-cold ether. The aqueous layer was passed through a 1 cm column of AG1×8 (chloride form), 100–200 mesh (Bio-Rad), washed with 10 mL of 0.5N formic acid and then 10 mL of 4N formic acid, and finally eluted with 8 mL 1N HCl. The eluates were collected and determined for radioactivity. The accumulation of radioactive formyl GAR was calculated as pmol per mg protein over a range of inhibitor concentrations. IC₅₀s were calculated as the concentrations of inhibitors that resulted in a 50% decrease in [^{14}C]formyl GAR synthesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AICA	5-amino-4-imidazolecarboxamide
AICAR	5-amino-4-imidazolecarboxamide ribonucleotide
AICARFTase	5-amino-4-imidazolecarboxamide ribonucleotide formyltransferase
CHO	Chinese hamster ovary
DPBS	Dulbecco's phosphate-buffered saline
FR	folate receptor
GAR	glycinamide ribonucleotide
GARFTase	glycinamide ribonucleotide formyltransferase
HBSS	Hank's balanced salts solution
HBS	HEPES-buffered saline
IC₅₀	50% inhibitory concentration
LCV	leucovorin
LMTX	Lometrexol
MEM	minimal essential media
MTX	methotrexate
PMX	Pemetrexed
PCFT	proton-coupled folate transporter
RTX	Raltitrexed

RFC	reduced folate carrier
SCID	severe combined immunodeficient

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Figure 1.
Structures of 6-Substituted pyrrolo[2,3-*d*]pyrimidines 3a and 3b and the thienoyl regioisomers 4–13.

Figure 2. Molecular modeling of the thiophene ring (MOE 2008.10) and angles and distances of the regioisomers of 3b.

Figure 3. Growth inhibition of KB cells by the 6-Substituted pyrrolo[2,3-*d*]pyrimidine thienoyl regioisomers 3b, 7, and 8 in the presence of physiologic concentrations of reduced folate
Cell proliferation inhibition was measured over a range of concentrations of the antifolates in complete folate free RPMI1640 in the presence of LCV at 2 and 50 nM. Cell densities were measured as in the experimental with a fluorescence dye and a fluorescence plate reader. Results were normalized to cell density in the absence of drug. Results shown are representative data of experiments performed in duplicate.

Figure 4. Protection of KB cells from growth inhibition by the 6-Substituted pyrrolo[2,3-*d*]pyrimidine thienoyl regioisomers 7 and 8 in the presence of nucleosides and 5-amino-4-imidazole (AICA)

Proliferation inhibition was measured for KB cells over a range of concentrations of compounds 7 and 8, as shown, in complete folate-free RPMI1640 with 2 nM LCV in the absence of other additions (labeled “No additions”), or in the presence of 200 nM folic acid (labeled “Folate-added”), adenosine (60 μ M), thymidine (10 μ M), or AICA (320 μ M). Cell densities were measured as in the experimental with a fluorescence dye and a fluorescence plate reader. Results were normalized to cell densities in the absence of drug. Results shown are representative data of experiments performed in triplicate. Analogous results were obtained with IGROV1 cells (not shown).

Figure 5. Binding of 6-Substituted pyrrolo[2,3-*d*]pyrimidine thienoyl regioisomers (3b, 4–8) to FR α

Data are shown for the effects of the unlabeled ligands with FR α -expressing RT16 CHO cells. Relative binding affinities for assorted folate/antifolate substrates were determined over a range of ligand concentrations and were calculated as the inverse molar ratios of unlabeled ligands required to inhibit [^3H]folic acid binding by 50%. By definition, the relative affinity of folic acid is 1. Details for the binding assays are provided in the Experimental Section. Results are presented as average values and ranges from 2–3 experiments. Undefined abbreviations: FA, folic acid.

Figure 6. *In situ* GARFTase inhibition assay

For the *in situ* assays, incorporation of [^{14}C]glycine into [^{14}C]formyl GAR was measured in IGROV1 tumor cells cultured for 15 h in complete folate-free RPMI plus 2 nM LCV. Details are described in the Experimental Section. Results are presented as a percent of controls treated without drugs for IGROV1 cells treated with nM concentrations of **3b**, **7**, **8**, or LMTX. LMTX was not tested at 5 nM (labeled “ND” for not determined). Results are presented as average values plus/minus ranges. Mean IC_{50} s were calculated as 3.46 nM for **3b**, 1.79 nM for **7**, 2.03 nM for **8**, and 5.77 nM for LMTX.

Scheme 1.

^aReagents and conditions: (a) CuI , $\text{Pd(0)(PPh}_3)_4$, Et_3N , DMF, RT, 12 h; (b) i. 1N NaOH, MeOH, CHCl_3 , RT, 6 h; ii. 1N HCl; (c) 10% Pd/C, H_2 , 55 psi, 2 h.

Scheme 2.

^aReagents and conditions: (a) *N*-methylmorpholine, 2 chloro 4,6 dimethoxy 1,3,5-triazine, *L*-glutamate diethyl ester hydrochloride, DMF, RT, 12 h.

Scheme 3.

^aReagents and conditions: (a) i. 1N NaOH; ii. 1N HCl

IC₅₀s (in nM) for 6-substituted pyrrolo[2,3-*d*]pyrimidine thienoyl regioisomers 3b, 4–13, and 20, compared to IC₅₀s for 3a and classical antifolates in RFC, PCFT, and FR-expressing cell lines.

Table 1

Antifolate	RFC			hFR α		PCFT		RFC/FR α /PCFT		RFC/FR α /PCFT	
	PC43-10	R2	RT16	RT16	RT16 (+FA)	R2/hPCFT4	R2(VC)	KB	KB (+FA)	IGROV1	IGROV1 (+FA)
3a	101.0(16.6)	273.5(49.1)	0.31 (0.14)	>1000	>1000	3.34 (0.26)	288 (12)	0.26(0.03)	>1000	0.55(0.10)	>1000
3b	>1000	>1000	1.82(0.28)	>1000	>1000	43.4(4.1)	>1000	0.55(0.10)	>1000	0.97(0.12)	>1000
4	>1000	>1000	>1000	>1000	>1000	>1000	>1000	171(36)	>1000	ND	ND
5	>1000	>1000	>1000	>1000	>1000	>1000	>1000	267(33)	>1000	ND	ND
6	>1000	>1000	147(53)	>1000	>1000	>1000	>1000	35.3(4.4)	>1000	102.9(25.9)	>1000
7	>1000	>1000	2.54(0.52)	>1000	>1000	41.54 (13.09)	>1000	0.17(0.02)	>1000	0.48(0.08)	>1000
8	>1000	>1000	2.60(0.59)	>1000	>1000	63.82 (16.23)	>1000	0.27(0.07)	>1000	1.4 (0.5)	>1000
9	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	ND	ND
10	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	ND	ND
11	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	ND	ND
12	>1000	>1000	13.48(2.42)	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000
13	>1000	>1000	8.26 (1.86)	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000
20	>1000	>1000	12.07(5.02)	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000
MTX	12(1.1)	216(8.7)	114(31)	461(62)	120.5 (16.8)		>1000	6.0(0.6)	20(2.4)	21(3.4)	22(2.1)
PMX	138(13)	894(93)	42(9)	388(68)	13.2(2.4)	974.0(18.1)	68(12)	327(103)	102(25)	200(18)	20(4.3)
RTX	6.3(1.3)	>1000	15(5)	12(8)	38.0(5.3)		>1000	5.9(2.2)	22(5)	12.6(3.3)	16(6)
LMTX	12(2.3)	>1000					>1000	1.2(0.6)	31(7)	3.1(0.9)	

ND: Not determined

Growth inhibition assays were performed for CHO sublines engineered to express RFC (PC43-10), FR α (RT16), or PCFT (R2/hPCFT4), for comparison with transporter-null [R2, R2(VC)] CHO cells, and for the KB and IGROV1 tumor sublines (express RFC, FR α , and PCFT), as described in the Experimental Section. For the FR experiments, growth inhibition assays were performed in the presence and the absence of 200 nM folic acid (FA). The data shown are mean values from 2–10 experiments (plus/minus SEM in parentheses). Results are presented as IC₅₀ values (in nmolar concentrations), corresponding to the concentrations that inhibit growth by 50% relative to cells incubated without drug. Data for MTX, PMX, RTX, and LMTX were previously published.^{10–14}

Table 2

K_i values for PCFT substrates.

Substrate	Parameter	pH 5.5	pH 6.8
3b	K _i (micromolar)	0.23 ± 0.06 (n=7)	7.23 ± 2.23 (n=7)
7	K _i (micromolar)	0.39 ± 0.10 (n=4)	28.1 ± 5.3 (n=4)
8	K _i (micromolar)	0.42 ± 0.11 (n=4)	30.7 ± 8.5 (n=4)
PMX	K _i (micromolar)	0.094 ± 0.006 (n=8)	2.54 ± 0.31 (n=8)

K_i values for PCFT substrates were determined by Dixon plots with [³H]MTX as substrate and a range of inhibitor concentrations in R2/hPCFT4 cells. K_iS were calculated from the slopes of the Dixon plots and kinetic constants (K_t and V_{max}) for MTX. Results are presented as mean values ± standard errors from multiple experiments (numbers shown in parentheses).