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Novel 5-Substituted Pyrrolo[2,3-*d*]pyrimidines as Dual Inhibitors of Glycinamide Ribonucleotide Formyltransferase and 5-Aminoimidazole-4-carboxamide Ribonucleotide Formyltransferase and as Potential Antitumor Agents

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Abstract

A new series of 5-substituted thiophenyl pyrrolo[2,3-*d*]pyrimidines **6–11** with varying chain lengths ($n = 1–6$) were designed and synthesized as hybrids of the clinically used anticancer drug pemetrexed (PMX) and our 6-substituted thiophenyl pyrrolo[2,3-*d*]pyrimidines **2c** and **2d** with folate receptor (FR) α and proton-coupled folate transporter (PCFT) uptake specificity over the reduced folate carrier (RFC) and inhibition of de novo purine nucleotide biosynthesis at glycinamide ribonucleotide formyltransferase (GARFTase). Compounds **6–11** inhibited KB human tumor cells in the order **9** = **10** > **8** > **7** > **6** = **11**. Compounds **8–10** were variously transported by FR α , PCFT, and RFC and, unlike PMX, inhibited de novo purine nucleotide rather than thymidylate biosynthesis. The antiproliferative effects of **8** and **9** appeared to be due to their dual inhibitions of both GARFTase and 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase. Our studies identify a unique structure–activity relationship for transport and dual target inhibition.

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Notes

The authors declare no competing financial interest.

Supporting Information

Elemental analyses and summary of previously reported SARs. This material is available free of charge via the Internet at <http://pubs.acs.org>.

INTRODUCTION

Antifolates are a class of antiproliferative agents widely used as anticancer agents.^{1,2} Antifolate drugs target enzymes involved in folate-dependent metabolic transformations, including thymidylate synthase (TS) and dihydrofolate reductase (DHFR). DHFR inhibitors, such as methotrexate (MTX) and pralatrexate (PDX), and TS inhibitors, such as raltitrexed (RTX) and pemetrexed (PMX) (Figure 1), continue to play important roles in treating hematologic malignancies and solid tumors.^{1–3} Antipurine antifolates, including lometrexol [(6*R*)-5,10-dideazatetrahydrofolate, LMTX] (Figure 1), (2*S*)-2-(((5-(2-((6*S*)-2-amino-4-oxo-1,6,7,8-tetrahydropyrimido[5,4-*b*]-[1,4]thiazin-6-yl)ethyl)thiophene-2-yl)carbonyl)amino)-pentanedioic acid (AG2034) (Figure 1), and (2*S*)-2-(((5-(2-((6*R*)-2-amino-4-oxo-5,6,7,8-tetrahydro-1 *H*-pyrido[2,3-*d*]-pyrimidin-6-yl)ethyl)thiophene-2-yl)carbonyl)amino)-pentanedioic acid (LY309887), were identified as inhibitors of de novo purine nucleotide biosynthesis at the first folate-dependent step catalyzed by β -glycinamide ribonucleotide formyltransferase (GARFTase) and progressed to clinical trials.^{4–7} However, a major drawback of these agents involves their dose-limiting toxicities. Indeed, for all classical antifolate drugs, clinical applications are often limited by toxicities, most likely a consequence of their cellular uptake in normal tissues, as well as in tumors. For LMTX, toxicity was obviated by coadministration of folic acid.⁶

There are three principal mechanisms of cellular uptake of (anti)folates. The reduced folate carrier (RFC) is an anion antiporter which is ubiquitously expressed in tissues and tumors.⁸ Other cellular uptake mechanisms include folate receptors (FRs) α and β and the proton-coupled folate transporter (PCFT).^{18–11} FR α is abundantly expressed on the membranes in certain malignancies such ovarian cancer where it is exposed to the circulation, whereas in normal epithelial tissues (e.g., renal tubules) FR α is inaccessible to the circulation.¹⁰ This provides a compelling rationale for antitumor therapeutics which selectively target FR α .^{12,13} PCFT is a proton symporter that is expressed in a range of solid tumors, including ovarian cancer and non-small-cell lung cancer,¹⁴ and is highly active at acidic pH values characterizing the tumor microenvironment.^{8,9} Although certain normal tissues also express PCFT, including the proximal small intestine, liver, and kidney,⁹ with the exception of the proximal small intestine, the microenvironments of most normal tissues are not conducive to high levels of PCFT transport.¹¹ Thus, PCFT would afford a novel mechanism for selective tumor targeting, reflecting high levels of tumor expression and transport at pH values less than 7.^{8,9}

An assortment of FR α -targeted therapeutics have been developed and include a monoclonal antibody [farletuzumab (Morphotech)],¹⁵ cytotoxic folate conjugates [e.g., vintafolide (EC145; Endocyte)],^{16,17} and small-molecule antifolates that are selectively transported into cells by FRs over RFC and inhibit de novo thymidylate [*N*-[4-[2-propyn-1-yl][(6*S*)-4,6,7,8-tetrahydro-2-(hydroxymethyl)-4-oxo-3*H*-cyclopenta[*g*]-quinazolin-6-yl]amino]benzoyl]-L- γ -glutamyl-D-glutamic acid (ONX0801)]¹⁸ or purine nucleotide (compounds **1a**, **1b**, **2c**, and **2d**; Figure 2) biosynthesis.^{19–21} Interestingly, compounds **1a**, **1b**, **2c**, and **2d** are also potent substrates for PCFT.^{14,19–24}

PMX is a classical 5-substituted pyrrolo[2,3-*d*]pyrimidine antifolate with a two-carbon bridge linked to a (*p*-aminobenzoyl)-L-glutamate³ (Figure 1). PMX is a good RFC substrate and is among the best PCFT substrates;⁸ at least some uptake of PMX by FRs has been reported.^{19–21,25} PMX was originally described as a “multitargeted antifolate” with a primary inhibition at TS and secondary inhibition at GARFTase.^{3,26} More recent studies suggested that 5-aminoimidazole-4-carboxamide (AICA) ribonucleotide (ZMP) formyltransferase (AICARFTase) is also a pharmacologically important target of PMX.^{27,28}

While the 6-substituted pyrrolo[2,3-*d*]pyrimidine analogue of PMX was pharmacologically inert, compounds **1a** and **1b**, novel 6-substituted pyrrolo[2,3-*d*]pyrimidine benzoyl antifolates related to PMX with three and four carbons in the bridge region (Figure 2), showed highly selective cellular uptake by FR α and PCFT over RFC, resulting in potent inhibition of cell proliferation, including that of human tumor cells.^{19,24} Antiproliferative effects were maintained for compounds **2c** and **2d**, including thienoyl-for-benzoyl replacements and three and four bridge carbons, respectively (Figure 2), with growth inhibitions at low to subnanomolar concentrations toward both FR- and PCFT-expressing human tumors.^{14,20–24} Compounds **1a**, **1b**, **2c**, and **2d** with three or four carbons in the bridge region were identified as selective inhibitors of GARFTase rather than AICARFTase or TS.^{14,20–24}

We continue to systematically explore the structure–activity relationships (SARs) for the pyrrolo[2,3-*d*]pyrimidine scaffold in an effort to optimize the structural requirements for tumor-targeted drug uptake and inhibition of intracellular enzyme targets. For instance, replacement of the pyrrolo[2,3-*d*]pyrimidine moiety of **1a** and **1b** with a thieno[2,3-*d*]pyrimidine (**3a** and **3b**)²⁵ or replacement of the benzoyl ring with an alkyl side chain (**4a–4d**)²⁹ (Figure 2) abolished PCFT transport while preserving substantial FR uptake and GARFTase inhibition, resulting in antiproliferative activity. Similarly, replacement of the terminal L-glutamate of compound **2c** by amino acids with variations in the length of the side chain (aspartate, adipate) or loss of α - or γ -COOH groups preserved FR binding and uptake but abolished PCFT transport.³⁰ In recent studies which compared the biological properties of 5-substituted pyrrolo[2,3-*d*]pyrimidine benzoyl analogues with a one-to six-carbon bridge, including PMX (**5a–5f**, Figure 2), with those of the corresponding 6-substituted compounds, RFC and FR transport were substantially preserved for the 5-substituted series, although PCFT transport decreased with increasing bridge lengths beyond two carbons.³¹ Interestingly, bridge lengths greater than two carbons (as in PMX) afforded active compounds that were exclusively inhibitors of de novo purine nucleotide biosynthesis rather than TS. For the three- and four-carbon analogues **5c** and **5d**, we documented that

both GARFTase and AICARFTase were intracellular enzyme targets, resulting in a depletion of purine nucleotides.³¹

As an extension of these findings, in the present study we synthesized 5-substituted pyrrolo[2,3-*d*]pyrimidine thienoyl analogues related to **2c** and **2d** with one to six carbon atoms in the bridge region (**6–11**) (Figure 3) and assessed the biological impacts, including specificities for transport by FR α and PCFT, versus RFC, and inhibition of intracellular enzyme targets. Analogous to the benzoyl analogues of this series,³¹ several of the 5-substituted thienoyl analogues were substrates for RFC transport, as well as for PCFT and FR α , although the impacts of bridge length were different. In addition, the thienoyl analogues were not inhibitors of TS, but rather they were inhibitors of de novo purine nucleotide biosynthesis, with the three- and four-carbon analogues (compounds **8** and **9**), like their benzoyl counterparts (**5c** and **5d**), acting as dual inhibitors of both AICARFTase and GARFTase. This dual activity attacks tumor cells at two separate sites in the purine biosynthetic pathway and could overcome tumor resistance due to lack of sensitivity at a single intracellular target. This would provide potential utility of these compounds for drug-sensitive as well as drug-resistant tumors, including tumors resistant to PMX. As there are no other dual-acting inhibitors of GARFTase and AICARFTase, our findings identify a unique structure–activity relationship involving the three- and four-carbon atom chains at the 5-position of the pyrrolo[2,3-*d*]pyrimidine scaffold that could be therapeutically exploited.

CHEMISTRY

Compound **6** ($n = 1$) was synthesized as previously reported³² and is included for comparison. Similarly, compounds **7–11** ($n = 2–6$) were obtained from a modified synthesis^{33,34} using α -bromo aldehydes **14a–e** and condensation with 2,4-diamino-6-oxopyrimidine (**15**) as the key step outlined in Scheme 1. α -Bromination of the aldehydes **12a–e** with 5,5-dibromo-2,2-dimethyl-4,6-dioxo-1,3-dioxane (**13**) at room temperature afforded the corresponding α -bromo aldehydes **14a–e** (yields 60–68%).³⁵ The 5-substituted pyrrolo[2,3-*d*]pyrimidines **16a–e** were synthesized by condensation of **14a–e** with **15** at 45 °C in the presence of sodium acetate. Subsequent hydrolysis with 3 N NaOH, followed by coupling with diethyl L-glutamate using *N*-methylmorpholine and 6-chloro-2,4-dimethoxytriazine as the activating agents, afforded the diesters **17a–e** (yields 52–72% over two steps). Final saponification of the diesters with 1 N NaOH gave the target 5-substituted pyrrolo[2,3-*d*]pyrimidines **7–11** (yields 89–96%).

BIOLOGICAL EVALUATION

Transport Specificities of 5- and 6-Substituted Pyrrolo[2,3-*d*]pyrimidine Antifolates as Determinants of Antiproliferative Activities

Our goal was to identify the SARs for cellular uptake and intracellular enzyme targeting for the novel 5-substituted pyrrolo[2,3-*d*]pyrimidine thienoyl antifolates **6–11** with one to six bridge carbons, analogous to the corresponding 6-substituted analogues (**2a–2f**), including the most potent compounds (**2c** and **2d**) of this series.

As in our previous studies,^{19–21,25,29,31} transport specificities were established by measuring inhibition of cell proliferation using a novel cohort of isogenic Chinese hamster ovary (CHO) cell lines,^{19,25,36} with stable expression of human RFC (PC43-10), PCFT (R2/PCFT4), and FR α (RT16)^{19,25,36} (Table 1). For the RFC- and PCFT-expressing cells, results were compared to those for RFC-, PCFT-, and FR-null MTXRIOua^{R2–4} (R2) cells,³⁷ whereas results for FR α -expressing cells were compared to those for a parallel incubation in the presence of excess folic acid (200 nM) as a negative control.

The patterns of growth inhibition were distinct for the 5- and 6-substituted analogues. With the exception of **2c**, the 6-substituted analogues with one to six bridge carbons were poor inhibitors of RFC-expressing PC43-10 cells (compared to R2 cells). For **2c**, the modest growth inhibition seen in the PC43-10 and R2 sublines appeared to be nonspecific. Conversely, with the 5-substituted analogues, inhibition of PC43-10 was substantially restored in the order **7** (2C) = **9** (4C) > **8** (3C) > **10** (5C) > **6** (1C) = **11** (6C) and was abolished in the R2 cells (Table 1).

As previously reported,^{14,20–23} the 6-substituted pyrrolo[2,3-*d*]pyrimidine thienoyl side chain analogues with three (**2c**) and four (**2d**) bridge carbons were uniquely potent inhibitors of proliferation for both R2/PCFT4 and RT16 cells, although inhibitory activity fell dramatically with decreasing (**2b**, **2a**) or increasing (**2e**, **2f**) bridge lengths (Table 1). Conversely, antiproliferative activities toward R2/PCFT4 and RT16 cells fell dramatically for the 5-substituted pyrrolo[2,3-*d*]pyrimidine antifolates **8** (three carbons) and **9** (four carbons), although inhibition was partly restored for the five-carbon analogue **10** toward R2/PCFT4 cells. For R2/PCFT4 cells (PCFT), inhibition potency followed the sequence **10** (5C) = **7** (2C) > **9** (4C) > **8** (3C) > **6** (1C) = **11** (6C), and for RT16 (FR α), the order of potency was **10** (5C) = **9** (4C) > **8** (3C) > **7** (2C) > **6** (1C) = **11** (6C). Growth inhibition in RT16 cells was abolished in the presence of 200 nM folic acid (not shown).

The 5-substituted benzoyl analogues previously described³¹ show an SAR distinct from that of the 5-substituted thienoyl compounds **6–11** in relation to inhibition of cell proliferation via RFC [PMX (2C) = **5c** (3C) = **5d** (4C) > **5e** (5C) > **5a** (1C) = **5f** (6C)], FR α [**5d** (4C) > PMX (2C) > **5e** (5C) > **5c** (3C) > **5a** (1C) = **5f** (6C)] and PCFT [PMX (2C) > **5c** (3C) > **5a** (1C) = **5d** (4C) = **5e** (5C) = **5f** (6C)]. Of particular interest is the finding that the 5-substituted thienoyl analogue **10** (five carbons) showed a potency (IC₅₀ = 57.4 ± 21.4 nM) comparable to that of the 6-substituted analogue **2d** (four carbons) (IC₅₀ = 43.4 ± 4.1 nM) against PCFT-expressing R2/PCFT4 cells, in contrast to the corresponding 5-carbon benzoyl analogue **5e**, which was inactive (IC₅₀ > 1000 nM)³¹ (Table 1). This establishes a significant SAR for 5-substituted pyrrolo[2,3-*d*]pyrimidines with a thienoyl side chain for targeting PCFT-expressing cells. Given the acidic microenvironment of many solid tumors and their high-level PCFT expression,⁹ combined with efficient PCFT function at low pH at which RFC is relatively inactive,^{8,9,11} compound **10** would be uniquely selective toward solid tumors.

Results essentially identical to those with RT16 cells were obtained with KB human tumor cells which express very high levels of FR α , along with modest levels of RFC and PCFT¹⁴ (Table 1). The most potent 5-substituted compounds, **9** (four carbons) and **10** (five carbons),

gave IC₅₀ values that were 120- and 13.1-fold increased, respectively, compared to the corresponding 6-substituted compounds (**2d** and **2e**, respectively) against KB cells. Antiproliferative effects of the active compounds toward KB cells were abolished by excess (200 nM) folic acid (not shown), establishing FR-mediated uptake as the predominant mechanism of drug accumulation in spite of the presence of all three transporters.

5-Substituted Pyrrolo[2,3-*d*]pyrimidine Thienoyl Antifolates Are Dual Inhibitors of de Novo Purine Nucleotide Biosynthesis at Both GARFTase and AICARFTase

De novo purine nucleotide biosynthesis includes two folate-dependent steps, GARFTase and AICARFTase. Compounds **2c** and **2d**, both 6-substituted pyrrolo[2,3-*d*]pyrimidine analogues, are potent inhibitors of GARFTase,^{20,21} whereas PMX inhibits TS as its primary intracellular target,²⁶ although there are secondary inhibitions of GARFTase and AICARFTase which can be important at higher drug concentrations in the presence of excess thymidine or with increased levels of TS accompanying PMX resistance.^{26–28} Our recent study established dual GARFTase–AICARFTase inhibition for the 5-substituted pyrrolo[2,3-*d*]pyrimidine benzoyl antifolates with a three- or four-carbon bridge, **5c** and **5d**, respectively.³¹

To identify the targeted pathway for the active 5-substituted pyrrolo[2,3-*d*]pyrimidine analogues with an isosteric thienoylfor-benzoyl replacement (Table 1), we treated KB cells with compound **8**, **9**, or **10** without additions, or in the presence of adenosine (60 μM) or thymidine (10 μM), to distinguish de novo purine nucleotide from thymidylate biosynthesis, respectively.^{19–21,25,29,31} Unlike PMX, another 5-substituted pyrrolo[2,3-*d*]pyrimidine analogue, the inhibitory effects of **8–10**, like those for **2c** and **2d** previously,^{20,21} were all abolished by adenosine alone, but not by thymidine (Table 1 and Figure 4). This identifies de novo purine nucleotide biosynthesis as the targeted pathway. To confirm this, for compound **9** we measured ATP pools in KB cells treated with drug for 24 h, with results compared to those for **2d** and PMX (Figure 5). Compounds **2d** and **9** depleted ATP pools (>95%), consistent with the results of the protection experiments. As previously reported, PMX reduced ATP pools by only ~60%.³¹

To further identify the probable target enzyme(s) in de novo purine biosynthesis (GARFTase versus AICARFTase), KB cells were treated with the antifolates in the presence of AICA (320 μM), which is metabolized to AICA ribonucleotide (ZMP; the substrate for AICARFTase), thus bypassing the step catalyzed by GARFTase.^{19–21,29,30,32} Like **2d**, AICA completely protected KB cells from the antiproliferative effects of **10** (Table 1). However, AICA only slightly protected KB cells from the effects of **8** and **9** (Table 1 and Figure 4). These results establish that the growth inhibition of KB cells and inhibition of de novo purine biosynthesis by **8** and **9** are not due exclusively to GARFTase inhibition (as for **2d** and **10**) but rather are due at least partly to inhibition of AICARFTase as well.³¹

Experiments were performed to directly measure cellular GARFTase activity in KB tumor cells treated with **9** as a prototype; the results are compared to those for **2d** and PMX. For this purpose, we used an in situ activity assay for GARFTase which measures incorporation of [¹⁴C]glycine into [¹⁴C] formyl GAR in the presence of azaserine over 16 h.^{19–21,29,30,32}

Both 5-substituted (PMX, **9**) and 6-substituted (**2d**) analogues showed a dose-dependent decrease in the formation of [^{14}C] formyl GAR, with the effects of **2d** showing far greater potency than those for either **9** or PMX (Figure 6).

To confirm inhibition of AICARFTase by **9**, as indicated by our protection experiments with AICA, we directly measured levels of ZMP, the AICARFTase substrate, in cells treated for 48 h with 1 μM **9** or PMX.^{27,28,31} Nucleotides were extracted and analyzed by anion-exchange HPLC. Both **9** and PMX treatments increased ZMP levels in KB cells over the very low levels in untreated cells (~3- and ~10-fold, respectively) (Figure 7). These results confirm that **9** is a bona fide inhibitor of AICARFTase, albeit apparently less potent than PMX. As has been demonstrated earlier, PMX primarily inhibits TS and the pharmacologic impact of its inhibition of AICARFTase would manifest primarily in the presence of excess thymidine, which circumvents TS inhibition by PMX.^{27,28}

Molecular Modeling and Computational Studies

The X-ray crystal structures of human GARFTase (at 1.60 Å resolution; PDB ID 4EW2),³⁸ AICARFTase (at 2.55 Å resolution; PDB ID 1P4R),³⁹ and FR α (at 2.8 Å resolution; PDB ID 4LRH)⁴⁰ were obtained from the Protein Data Bank. The GARFTase crystal structure contains human GARFTase complexed with *N*-({4-[(1*S*)-4-(2,4-diamino-6-oxo-1,6-dihydropyrimidin-5-yl)-1-(methylsulfanyl)butyl]phenyl}carbonyl)-L-glutamic acid (**18**, 10*S*-methylthio-DDACTHF), the AICARFTase crystal structure contains human AICARFTase with the sulfamido-bridged *N*-[(*S*)-(4-{[(2-amino-4-hydroxy-quinazolin-6-yl)dihydroxy- λ -4-sulfanyl]amino}phenyl)-hydroxymethyl]-L-glutamic acid (**19**, BW1540), and FR α was cocrystallized with folic acid. Docking studies were performed using LeadIT 2.1.⁴¹ Compounds **6–11** were all docked into the crystal structures of GARFTase, AICARFTase, and FR α . Compound **9** is illustrated as a representative example in Figures 8 – 10. Prior to docking **9**, LeadIT 2.1 was validated by redocking the native crystal structure ligands for GARFTase, AICARFTase, and FR α .

Figure 8 shows the docked pose of **9** in the human GARFTase active site (PDB ID 4EW2³⁸). The cofactor binding pocket of GARFTase is located at the interface between the N-terminal mononucleotide binding domain and the C-terminal half of the structure. The binding site for the folate cofactor moiety consists of the pteridine binding cleft, the benzoylglutamate region, and the formyl transfer region.⁴² The docked pose shows the pyrrolo[2,3-*d*]pyrimidine scaffold of **9** as buried deep in the active site and oriented similar to the diaminopyrimidone ring of the native crystal structure ligand **18**. Hydrogen bonds could be formed between the 2-amino moiety of **9** and the backbone carbonyls of Leu92 and Glu141 and between the N3 nitrogen and the backbone of Ala140 (not labeled). Additionally, the pyrrole N7 nitrogen forms a hydrogen bond with the backbone of Arg90. Several hydrophobic residues (Leu85, Ile91, Leu92, Val97, and the folate binding loop residues 141–146) flank the pocket which holds the pyrrolo[2,3-*d*]pyrimidine scaffold. The flexible four-atom bridge helps to orient the side chain thiophene ring into a hydrophobic cleft consisting of Phe88 (not labeled), Met89, Arg90, Ile91, and Val143. The α -carboxylic acid of the glutamate interacts with Arg64. The γ -carboxylic acid interacts with Arg90 and the backbone carbonyl of Met89. The interaction of the flexible glutamate side chain is very

similar to the interaction network observed for the glutamate side chain of **18**. The docking score of **9** was -43.93 kJ/mol, somewhat better than -40.81 kJ/mol for the standard **18** and -36.97 kJ/mol for PMX.

Figure 9 shows the docked pose of **9** in the AICARFTase active site. Hydrogen bonds could be formed between the 2-NH₂ and N3 moieties of **9** and Asp546 and between the 4-oxomoiety of **9** and the side chain NH₂ of Asn547. The pyrrolo[2,3-*d*]pyrimidine scaffold of **9** overlaps with the 5,8-dideazafolate scaffold of the native crystal structure ligand **19** (not shown) and is in contact with the hydrophobic side chains of Met312, Ile452 (not shown), Pro543, Phe544, and Phe315. The pyrrole NH could form a hydrogen bond with the backbone carbonyl of Met312. This hydrogen bond is not observed with the 5,8-dideazafolate scaffold of **19**. The four-carbon bridge at the 5-position of compound **9** aids in positioning the glutamate moiety in an orientation similar to that observed with the corresponding glutamate moiety of **19**. The extensive electropositive surface surrounding the active site³⁹ could presumably account for the binding stabilization of the negatively charged glutamate side chain. The docking score of **9** was -42.46 kJ/mol, compared to the docking score of -47.00 kJ/mol for **19** and -39.55 kJ/mol for PMX.

Figure 10 shows the docked pose of **9** in the human FR α (PDB ID 4LRH, 2.8 Å)⁴⁰ active site. Given the modest resolution of the crystal structure, interactions described by the docking studies could be considered as suggestive of the most probable interactions of **9** in the pocket. In its docked pose, the pyrrolo[2,3-*d*]pyrimidine scaffold of **9** binds in the region occupied by the pteridine scaffold of the native ligand, folic acid (not shown). The N1 and 2-NH₂ moieties of **9** interact with the side chain carboxylic acid of Asp81. Additional hydrogen bonds observed between the side chain hydroxyl of Ser174 and N3 of folic acid are not seen in the docked pose of **9**. The pyrrolo[2,3-*d*]pyrimidine is stabilized by interactions with Tyr60 and Trp171. The pyrrole NH of **9** does not interact with the binding pocket. The 5-position four-carbon chain in **9** optimally orients the glutamate tail in the pocket to enable interactions similar to that observed with the glutamate tail of folic acid. Interactions between the pyrrolo[2,3-*d*]pyrimidine scaffold with Asp81 and His135 and the interactions of the glutamate tail appear to provide the bonds that anchor compounds **6–11** in the docking studies. The α -carboxylic acid of **9** could form hydrogen bonds with the backbone NH of Gly137 and Trp138, while the γ -carboxylic acid could interact with the backbone NH of Lys136 and the side chain NH of Trp102. The hydrophobic four-carbon bridge of **9** affords interactions in the binding pocket with surrounding hydrophobic amino acids such as Tyr60, Phe62 (not labeled), Trp102 (not labeled), Trp134 (not labeled), and His135. Thus, the chain length at the 5-position would enable the glutamate side chain to reach appropriate amino acids at the entrance of the binding pocket, thus dictating the binding orientation and thereby affecting the nature of the bonds formed. While shorter chain lengths cause a shift of the pyrrolo[2,3-*d*]pyrimidine scaffold in the pocket to maintain interactions of the glutamate chain, longer chain lengths in the docked structures of **10** and **11** cause the side chain thiophene ring to shift away from the benzoyl binding site of folic acid (not shown), affecting its interactions in the pocket. The optimal chain length at the 5-position was predicted from molecular modeling to be four carbons (compound **9**). The docking score of **9** was -36.94 kJ/mol compared to -48.63 kJ/mol for folic acid.

Thus, molecular modeling suggested that our hybrid-designed PMX homologues, particularly compound **9**, should afford FR α binding. The docking results with GARFTase and AICARFTase suggest that **9** should bind and inhibit both folate-dependent purine biosynthetic enzymes with good-to-excellent potencies, consistent with the in vitro results.

The inhibitory activities of **8** and **9** on cell proliferation are a result of their binding and internalization via FR α and, once internalized, their inhibition of both GARFTase and AICARFTase. With the recent availability of X-ray crystal structures of FR α ⁴⁰ and FR β ⁴³ and the known structures of GARFTase³⁸ and AICARFTase,³⁹ it was of interest to compare the docked conformations of **9** in their energy-minimized conformations with both FRs and with GARFTase and AICARFTase. A comparison of the docked poses of **9** (Figure 11) shows that it needs to adopt significantly different conformations to bind to FR α and its target enzymes, likely dictated by the constraints of the respective binding pockets (molecular modeling of **9** with FR β is also shown in Figure 11 and is similar to that with FR α). Furthermore, the curved docked poses of **9** (Figure 11) vary from the relatively linear energy-minimized conformation, implying that flexibility of the molecule through 180° is critical for its biological activity. Further inspection reveals differences in the orientation of the linker atoms, the thiophene ring, and the glutamate tail, suggesting that subtle differences involving these moieties could influence binding affinities of **9** and other compounds in this series to FRs and to intracellular enzyme targets and could provide the structural basis for antitumor selectivity and potency.

CONCLUSIONS

In this study, a new series of 5-substituted side chain thiophenyl pyrrolo[2,3-*d*]pyrimidines **6–11** with varying chain lengths ($n = 1–6$) were designed and synthesized as hybrid molecules of the clinically used anticancer drug PMX and our previously reported FR- and PCFT-selective 6-substituted thiophenyl pyrrolo[2,3-*d*]pyrimidines typified by **2c** and **2d**.^{20,21} The 6-substituted analogues were previously found to exhibit specificities for cellular uptake by FRs and PCFT over RFC with optimal activity for analogues with three or four bridge carbons. By contrast, the 5-substituted series described herein showed restored antiproliferative effects toward RFC-expressing cells which were greatest for the two- and four-carbon bridge analogues. These were dramatically reduced toward FR α - and PCFT-expressing cells, with maximal effects for the four- and five-carbon compounds with FR α and for the two- and five-carbon compounds with PCFT. Although a similar pattern of transporter specificity was seen with the 5-substituted benzoyl series (**5a–5f**), the impact of bridge length was somewhat different.³¹

In tumor cells, uptake of the 6-substituted pyrrolo[2,3-*d*]pyrimidine benzoyl and thienoyl antifolates **1a–1b** and **2c–2d**, respectively, resulted in potent inhibition of de novo purine nucleotide biosynthesis at the step catalyzed by GARF-Tase.^{14,20–24} The 5-substituted analogues, compounds **8–10**, were also inhibitors of purine nucleotide biosynthesis. Compounds **8** and **9**, with RFC-, FR α -, and PCFT-directed activity, were dual inhibitors of GARFTase as well as AICARFTase, as reflected in a lack of protection in cell proliferation assays by AICA and, as demonstrated for compound **9**, cellular accumulation of the AICARFTase substrate ZMP. Parallel findings were reported for **5c** and **5d**, 5-substituted

benzoyl pyrrolo[2,3-*d*]pyrimidine analogues closely related to PMX but with three- and four-carbon bridges, respectively.³¹ For compounds **5d**³¹ and **9**, the net effect of GARFTase and AICARFTase inhibition was a nearly complete ablation of ATP pools.

From docking studies of **9** with both GARFTase and AICARFTase, we report for the first time that the conformations for drug binding to GARFTase and AICARFTase are similar and are diametrically opposite the similar conformations required for binding to both FRs. This indicates that conformational flexibility is essential for this molecule to be transported and to inhibit the purine nucleotide biosynthesis enzymes required for antitumor activity.

The notion of dual inhibition of both GARFTase and AICARFTase, previously by **5c** and **5d**³¹ and now by both **8** and **9**, is particularly appealing since this strategy could preserve antitumor activity in the event that tumor cells become resistant due to mutation of one of these targets. Clearly, this unique feature makes these compounds excellent leads for further study, including the design of additional analogues to better optimize cellular folate uptake by non-RFC transport mechanisms to enhance their tumor-targeting capability and to decrease toxicity.

EXPERIMENTAL SECTION

All evaporations were carried out in vacuo with a rotary evaporator. Analytical samples were dried in vacuo (0.2 mmHg) in a CHEM-DRY drying apparatus over P₂O₅ at 80 °C. Melting points were determined on a MEL-TEMP II melting point apparatus with a FLUKE 51 K/J electronic thermometer and are uncorrected. Proton nuclear magnetic resonance (¹H NMR) spectra were recorded on either a Bruker WH-400 (400 MHz) spectrometer or a Bruker WH-500 (500 MHz) spectrometer. The chemical shift values are expressed in parts per million relative to tetramethylsilane as an internal standard: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad. Mass spectra were recorded on a VG-7070 double-focusing mass spectrometer or in a LKB-9000 instrument in the electron ionization (EI) mode. Chemical names follow IUPAC nomenclature. 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 365 nm illumination. All analytical samples were homogeneous on TLC in three different solvent systems. Proportions of solvents used for TLC are by volume. Column chromatography was performed on a 230–400 mesh silica gel (Fisher, Somerville, NJ) column. Elemental analyses were performed by Atlantic Microlab, Inc., Norcross, GA. Element compositions are within 0.4% of the calculated values. Fractional moles of water frequently found in the analytical sample of antifolates could not be prevented in spite of 24–48 h of drying in vacuo and was confirmed where possible by the presence in the ¹H NMR spectra. All solvents and chemicals were purchased from Aldrich Chemical Co. or Fisher Scientific and were used as received. For all the compounds submitted for biological evaluation, elemental analysis (C, H, N) was performed to confirm >95% purity.

Methyl 5-(3-Bromo-4-oxobutyl)thiophene-2-carboxylate (14a)—To a solution of aldehyde **12a** (2.9 mmol, 540 mg) in 5 mL of anhydrous Et₂O were added **13** (1.45 mmol, 435 mg) and a 2 N HCl in Et₂O solution (0.29 mmol, 150 μ L), and the mixture was stirred at

room temperature for 24 h. TLC (hexane/EtOAc, 3:1) showed the disappearance of the starting material and formation of one major spot at $R_f = 0.43$. The reaction solution was washed with 5% NaHCO_3 solution, extracted with H_2O (10 mL $\times$ 3), and dried over Na_2SO_4 . After evaporation of solvent, the residue was loaded onto a silica gel column (4 $\times$ 20 cm) and flash-chromatographed with hexane/EtOAc (5:1), and the desired fractions were pooled. After evaporation of solvent, the residue was dried in vacuo using P_2O_5 to afford **14a**: 440 mg, yield 65% as yellow oil; $R_f = 0.43$ (hexane/EtOAc, 3:1); ^1H NMR (CDCl_3) δ 2.88–2.89 (m, 2 H, CH_2), 2.95–2.96 (m, 2 H, CH_2), 3.87 (s, 3 H, CH_3), 4.23–4.26 (m, 1 H, CHBr), 6.86–6.87 (d, 1 H, 1 CH, $J = 2.0$ Hz), 7.64–7.65 (d, 1 H, CH, $J = 2.0$ Hz), 9.48–9.49 (d, 1 H, CHO, $J = 1.0$ Hz).

Methyl 5-(4-Bromo-5-oxopentyl)thiophene-2-carboxylate (14b)—Compound **14b** was synthesized as described for **14a**: yield 68% as yellow oil; $R_f = 0.45$ (hexane/EtOAc, 3:1); ^1H NMR (CDCl_3) δ 1.71–1.72 (m, 2 H, CH_2), 1.93–2.00 (m, 1 H, CH_2), 2.08–2.13 (m, 1 H, CH_2), 2.85–2.90 (m, 2 H, CH_2), 3.86 (s, 3 H, CH_3), 4.23–4.25 (m, 1 H, CHBr), 6.79–6.80 (d, 1 H, 1 CH, $J = 2.0$ Hz), 7.62–7.63 (d, 1 H, CH, $J = 2.0$ Hz), 9.44–9.45 (d, 1 H, CHO, $J = 1.0$ Hz).

Methyl 5-(5-Bromo-6-oxohexyl)thiophene-2-carboxylate (14c)—Compound **14c** was synthesized as described for **14a**. Compound **14c** was not stable and was used directly in the next step without purification.

Methyl 5-(6-Bromo-7-oxoheptyl)thiophene-2-carboxylate (14d)—Compound **14d** was synthesized as described for **14a**: yield 60% as yellow oil; $R_f = 0.47$ (hexane/EtOAc, 3:1); ^1H NMR (CDCl_3) δ 1.37–1.42 (m, 2 H, CH_2), 1.56–1.59 (m, 4 H, 2 CH_2), 2.04–2.08 (m, 2 H, CH_2), 2.82–2.85 (t, 2 H, CH_2 , $J = 7.5$ Hz), 3.85 (s, 3 H, CH_3), 4.19–4.22 (m, 1 H, CHBr), 6.77–6.78 (d, 1 H, 1 CH, $J = 2.0$ Hz), 7.62–7.63 (d, 1 H, CH, $J = 2.0$ Hz), 9.42–9.43 (d, 1 H, CHO, $J = 1.0$ Hz).

Methyl 5-(7-Bromo-8-oxooctyl)thiophene-2-carboxylate (14e)—Compound **14e** was synthesized as described for **14a**: yield 65% as yellow oil; $R_f = 0.50$ (hexane/EtOAc, 3:1); ^1H NMR (CDCl_3) δ 1.34–1.39 (m, 4 H, 2 CH_2), 1.57–1.61 (m, 4 H, 2 CH_2), 2.00–2.06 (m, 2 H, CH_2), 2.81–2.84 (t, 2 H, CH_2 , $J = 7.5$ Hz), 3.86 (s, 3 H, CH_3), 4.19–4.26 (m, 1 H, CHBr), 6.77–6.78 (d, 1 H, 1 CH, $J = 2.0$ Hz), 7.62–7.63 (d, 1 H, CH, $J = 2.0$ Hz), 9.42–9.43 (d, 1 H, CHO, $J = 1.0$ Hz).

Methyl 5-(2-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)ethyl)thiophene-2-carboxylate (16a)—To a solution of 2,4-diamino-6-oxopyrimidine (**15**) (171 mg, 1.36 mmol) and sodium acetate (223 mg, 2.72 mmol) in water (3 mL) and methanol (3 mL) was added α -bromo aldehyde **14a** (360 mg, 1.36 mmol). The reaction mixture was stirred at 45 °C for 3 h. TLC showed the disappearance of starting materials and the formation of one major spot at $R_f = 0.40$ ($\text{CHCl}_3/\text{MeOH}$, 5:1). After evaporation of solvent, CH_3OH (10 mL) was added followed by silica gel (3 g). Evaporation of the solvent afforded a plug, which was loaded onto a silica gel column (3.5 cm $\times$ 15 cm) and eluted initially with CHCl_3 followed by 10% MeOH in CHCl_3 and then 15% MeOH in

CHCl₃. Fractions showing $R_f = 0.40$ were pooled and evaporated to afford **16a**: 245 mg, yield 50% as a yellow solid; mp 185–186 °C dec; $R_f = 0.40$ (CHCl₃/MeOH, 5:1); ¹H NMR (DMSO-*d*₆) δ 2.88–2.91 (t, 2 H, CH₂, $J = 7.5$ Hz), 3.20–3.23 (t, 2 H, CH₂, $J = 7.5$ Hz), 3.77 (s, 3 H, CH₃), 5.99 (s, 2 H, 2-NH₂), 6.35–6.36 (d, 1 H, CH, $J = 1.0$ Hz), 6.90–6.91 (d, 1 H, CH, $J = 1.8$ Hz), 7.60–7.61 (d, 1 H, CH, $J = 1.8$ Hz), 10.15 (s, 1 H, 3-NH), 10.64 (s, 1H, 7-NH).

Methyl 5-(3-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-*d*]-pyrimidin-5-yl)propyl)thiophene-2-carboxylate (16b)—Compound **16b** was synthesized as described for **16a**: yield 64% as a clear syrup; $R_f = 0.42$ (CHCl₃/MeOH, 5:1); ¹H NMR (DMSO-*d*₆) δ 1.94–2.00 (m, 2 H, CH₂), 2.59–2.62 (t, 2 H, CH₂, $J = 7.5$ Hz), 2.81–2.84 (t, 2 H, CH₂, $J = 7.5$ Hz), 3.79 (s, 3 H, CH₃), 5.97 (s, 2 H, 2-NH₂), 6.36–6.37 (d, 1 H, CH, $J = 1.0$ Hz), 6.97–6.98 (d, 1 H, CH, $J = 2.0$ Hz), 7.63–7.64 (d, 1 H, CH, $J = 2.0$ Hz), 10.10 (s, 1 H, 3-NH), 10.65 (s, 1H, 7-NH).

Methyl 5-(4-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-*d*]-pyrimidin-5-yl)butyl)thiophene-2-carboxylate (16c)—Compound **16c** was synthesized as described for **16a**: yield 46% as a light yellow solid; mp 150–151 °C dec; $R_f = 0.44$ (CHCl₃/MeOH, 5:1); ¹H NMR (DMSO-*d*₆) δ 1.62–1.64 (m, 4 H, 2 CH₂), 2.57–2.60 (t, 2 H, CH₂, $J = 7.5$ Hz), 2.83–2.86 (t, 2 H, CH₂, $J = 7.5$ Hz), 3.78 (s, 3 H, CH₃), 5.95 (s, 2 H, 2-NH₂), 6.33–6.34 (d, 1 H, CH, $J = 1.0$ Hz), 6.94–6.95 (d, 1 H, CH, $J = 1.8$ Hz), 7.62–7.63 (d, 1 H, CH, $J = 1.8$ Hz), 10.09 (s, 1 H, 3-NH), 10.60 (s, 1H, 7-NH).

Methyl 5-(5-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-*d*]-pyrimidin-5-yl)pentyl)thiophene-2-carboxylate (16d)—Compound **16d** was synthesized as described for **16a**: yield 47% as a pink solid; mp 139–140 °C dec; $R_f = 0.46$ (CHCl₃/MeOH, 5:1); ¹H NMR (DMSO-*d*₆) δ 1.32–1.34 (m, 2 H, CH₂), 1.58–1.66 (m, 4 H, 2 CH₂), 2.51–2.54 (m, 2 H, CH₂), 2.82–2.85 (t, 2 H, CH₂, $J = 7.5$ Hz), 3.78 (s, 3 H, CH₃), 5.94 (s, 2 H, 2-NH₂), 6.31–6.32 (d, 1 H, CH, $J = 1.0$ Hz), 6.94–6.95 (d, 1 H, CH, $J = 1.8$ Hz), 7.62–7.63 (d, 1 H, CH, $J = 1.8$ Hz), 10.08 (s, 1 H, 3-NH), 10.59 (s, 1H, 7-NH).

Methyl 5-(6-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-*d*]-pyrimidin-5-yl)hexyl)thiophene-2-carboxylate (16e)—Compound **16e** was synthesized as described for **16a**: yield 50% as a pink solid; mp 168–169 °C dec; $R_f = 0.45$ (CHCl₃/MeOH, 5:1); ¹H NMR (DMSO-*d*₆) δ 1.30–1.33 (m, 4 H, 2 CH₂), 1.55–1.64 (m, 4 H, 2 CH₂), 2.51–2.54 (m, 2 H, CH₂), 2.80–2.83 (t, 2 H, CH₂, $J = 7.5$ Hz), 3.78 (s, 3 H, CH₃), 5.94 (s, 2 H, 2-NH₂), 6.31–6.32 (d, 1 H, CH, $J = 1.0$ Hz), 6.94–6.95 (d, 1 H, CH, $J = 1.8$ Hz), 7.60–7.61 (d, 1 H, CH, $J = 1.8$ Hz), 10.07 (s, 1 H, 3-NH), 10.58 (s, 1H, 7-NH).

(S)-Diethyl 2-(5-(2-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo-[2,3-*d*]pyrimidin-5-yl)ethyl)thiophene-2-carboxamido)-pentanedioate (17a)—To a suspension of **16a** (75 mg, 0.26 mmol) in 3 mL of CH₃OH was added 3 N NaOH (3 mL). The resulting mixture was stirred under N₂ at 40–50 °C for 24 h. TLC indicated the disappearance of starting material and the formation of one major spot at the origin. The resulting solution was passed through Celite and washed with a minimum amount of CH₃OH. The combined

filtrate was evaporated under reduced pressure to dryness. To this residue was added distilled water (10 mL). The solution was cooled in an ice bath, and the pH was adjusted to 3–4 using 3 N HCl. The resulting suspension was chilled in a dry ice/acetone bath and thawed to 4 °C overnight in a refrigerator. The precipitate was filtered, washed with cold water, and dried in a desiccator under reduced pressure using P₂O₅ to a brown powder, which was used directly for the next step.

To a solution of this brown powder in anhydrous DMF (5 mL) were added 6-chloro-2,4-dimethoxy-1,3,5-triazine (55 mg, 0.31 mmol) and *N*-methylmorpholine (32 mg, 0.31 mmol). After the mixture was stirred at rt for 2 h, *N*-methylmorpholine (32 mg, 0.31 mmol) and dimethyl L-glutamate hydrochloride (75 mg, 0.31 mmol) were added all at once. The mixture was stirred at rt for 4 h. TLC showed the formation of one major spot at R_f = 0.42 (CHCl₃/MeOH, 5:1). The reaction mixture was evaporated to dryness under reduced pressure. The residue was dissolved in a minimum amount of CHCl₃/MeOH, 5:1, and chromatographed on a silica gel column (2 cm × 15 cm) with 4% MeOH in CHCl₃ as the eluent. Fractions that showed the desired single spot at R_f = 0.42 were pooled and evaporated to dryness to afford **17a**: 85 mg, yield 72% as a yellow syrup; R_f = 0.42 (CHCl₃/MeOH, 5:1); ¹H NMR (DMSO-*d*₆) δ 1.15–1.20 (m, 6 H, 2 CH₃), 1.92–1.99 (m, 1 H, CH₂), 2.05–2.12 (m, 1 H, CH₂), 2.40–2.43 (t, 2 H, CH₂, J = 7.5 Hz), 2.87–2.90 (t, 2 H, CH₂, J = 7.5 Hz), 3.15–3.18 (t, 2 H, CH₂, J = 7.5 Hz), 4.02–4.13 (m, 4 H, 2 CH₂), 4.35–4.39 (m, 1 H, CH), 5.98 (s, 2 H, 2-NH₂), 6.35–6.36 (d, 1 H, CH, J = 1.0 Hz), 6.84–6.85 (d, 1 H, CH, J = 2.0 Hz), 7.64–7.65 (d, 1 H, CH, J = 2.0 Hz), 8.55–8.56 (d, 1 H, CONH, J = 4.0 Hz), 10.14 (s, 1 H, 3-NH), 10.63 (s, 1H, 7-NH).

(S)-Diethyl 2-(5-(3-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo-[2,3-*d*]pyrimidin-5-yl)propyl)thiophene-2-carboxamido)-pentanedioate (17b)—Compound **17b** was synthesized as described for **17a**: yield 60% as a colorless syrup; R_f = 0.44 (CHCl₃/MeOH, 5:1); ¹H NMR (DMSO-*d*₆) δ 1.15–1.20 (m, 6 H, 2 CH₃), 1.92–1.99 (m, 3 H, CH₂), 2.05–2.12 (m, 1 H, CH₂), 2.40–2.43 (t, 2 H, CH₂, J = 7.5 Hz), 2.59–2.62 (t, 2 H, CH₂, J = 7.5 Hz), 2.77–2.80 (t, 2 H, CH₂, J = 7.5 Hz), 4.02–4.13 (m, 4 H, 2 CH₂), 4.35–4.39 (m, 1 H, CH), 5.97 (s, 2 H, 2-NH₂), 6.36–6.37 (d, 1 H, CH, J = 1.0 Hz), 6.89–6.90 (d, 1 H, CH, J = 2.0 Hz), 7.67–7.68 (d, 1 H, CH, J = 2.0 Hz), 8.60–8.61 (d, 1 H, CONH, J = 4.0 Hz), 10.10 (s, 1 H, 3-NH), 10.65 (s, 1H, 7-NH).

(S)-Diethyl 2-(5-(4-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo-[2,3-*d*]pyrimidin-5-yl)butyl)thiophene-2-carboxamido)-pentanedioate (17c)—Compound **17c** was synthesized as described for **17a**: yield 52% as a colorless syrup; R_f = 0.45 (CHCl₃/MeOH, 5:1); ¹H NMR (DMSO-*d*₆) δ 1.15–1.20 (m, 6 H, 2 CH₃), 1.58–1.66 (m, 4 H, 2 CH₂), 1.92–1.99 (m, 3 H, CH₂), 2.05–2.12 (m, 1 H, CH₂), 2.40–2.43 (t, 2 H, CH₂, J = 7.5 Hz), 2.57–2.60 (t, 2 H, CH₂, J = 7.5 Hz), 2.79–2.81 (t, 2 H, CH₂, J = 7.5 Hz), 4.02–4.12 (m, 4 H, 2 CH₂), 4.35–4.39 (m, 1 H, CH), 5.94 (s, 2 H, 2-NH₂), 6.33–6.34 (d, 1 H, CH, J = 1.0 Hz), 6.87–6.88 (d, 1 H, CH, J = 2.0 Hz), 7.66–7.67 (d, 1 H, CH, J = 2.0 Hz), 8.58–8.60 (d, 1 H, CONH, J = 4.0 Hz), 10.09 (s, 1 H, 3-NH), 10.60 (s, 1H, 7-NH).

(S)-Diethyl 2-(5-(5-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo-[2,3-d]pyrimidin-5-yl)pentyl)thiophene-2-carboxamido)-pentanedioate (17d)—Compound **17d** was synthesized as described for **17a**: yield 65% as a colorless syrup, $R_f = 0.44$ (CHCl₃/MeOH, 5:1); ¹H NMR (DMSO-*d*₆) δ 1.15–1.20 (m, 6 H, 2 CH₃), 1.30–1.36 (m, 2 H, CH₂), 1.58–1.66 (m, 4 H, 2 CH₂), 1.92–1.99 (m, 3 H, CH₂), 2.05–2.12 (m, 1 H, CH₂), 2.40–2.43 (t, 2 H, CH₂, $J = 7.5$ Hz), 2.52–2.55 (t, 2 H, CH₂, $J = 7.5$ Hz), 2.78–2.81 (t, 2 H, CH₂, $J = 7.5$ Hz), 4.02–4.12 (m, 4 H, 2 CH₂), 4.35–4.39 (m, 1 H, CH), 5.94 (s, 2 H, 2-NH₂), 6.32–6.33 (d, 1 H, CH, $J = 1.0$ Hz), 6.87–6.88 (d, 1 H, CH, $J = 2.0$ Hz), 7.66–7.67 (d, 1 H, CH, $J = 2.0$ Hz), 8.59–8.61 (d, 1 H, CONH, $J = 4.0$ Hz), 10.08 (s, 1 H, 3-NH), 10.59 (s, 1H, 7-NH).

(S)-Diethyl 2-(5-(6-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo-[2,3-d]pyrimidin-5-yl)hexyl)thiophene-2-carboxamido)-pentanedioate (17e)—Compound **17e** was synthesized as described for **17a**: yield 61% as a colorless syrup; $R_f = 0.42$ (CHCl₃/MeOH, 5:1); ¹H NMR (DMSO-*d*₆) δ 1.15–1.20 (m, 6 H, 2 CH₃), 1.29–1.35 (m, 4 H, 2 CH₂), 1.54–1.63 (m, 4 H, 2 CH₂), 1.92–1.99 (m, 3 H, CH₂), 2.05–2.12 (m, 1 H, CH₂), 2.40–2.43 (t, 2 H, CH₂, $J = 7.5$ Hz), 2.52–2.54 (t, 2 H, CH₂, $J = 7.5$ Hz), 2.76–2.79 (t, 2 H, CH₂, $J = 7.5$ Hz), 4.02–4.12 (m, 4 H, 2 CH₂), 4.35–4.40 (m, 1 H, CH), 5.94 (s, 2 H, 2-NH₂), 6.31–6.32 (d, 1 H, CH, $J = 1.0$ Hz), 6.87–6.88 (d, 1 H, CH, $J = 2.0$ Hz), 7.67–7.68 (d, 1 H, CH, $J = 2.0$ Hz), 8.59–8.61 (d, 1 H, CONH, $J = 4.0$ Hz), 10.07 (s, 1 H, 3-NH), 10.58 (s, 1H, 7-NH).

(S)-2-(5-(2-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)ethyl)thiophene-2-carboxamido)pentanedioic Acid (7)—To **17a** (75 mg, 0.26 mmol) was added 1 N NaOH (3 mL). The resulting mixture was stirred at rt for 2 h. TLC indicated the disappearance of starting material and the formation of one major spot at the origin. The resulting solution was passed through Celite. The combined filtrate was evaporated under reduced pressure to dryness. To this residue was added distilled water (10 mL). The solution was cooled in an ice bath, and the pH was adjusted to 3–4 using 1 N HCl. The resulting suspension was chilled in a dry ice/acetone bath and thawed to 4 °C overnight in a refrigerator. The precipitate was filtered, washed with cold water, and dried in a desiccator under reduced pressure using P₂O₅: yield 90% as a pink powder; mp 181 °C dec; $R_f = 0.05$ (CHCl₃/MeOH, 5:1); ¹H NMR (DMSO-*d*₆) δ 1.88–1.93 (m, 1 H, CH₂), 2.02–2.08 (m, 1 H, CH₂), 2.31–2.34 (t, 2 H, CH₂, $J = 7.5$ Hz), 2.86–2.89 (t, 2 H, CH₂, $J = 7.5$ Hz), 3.14–3.17 (t, 2 H, CH₂, $J = 7.5$ Hz), 4.30–4.35 (m, 1 H, CH), 6.00 (s, 2 H, 2-NH₂), 6.35–6.36 (d, 1 H, CH, $J = 1.0$ Hz), 6.83–6.84 (d, 1 H, CH, $J = 2.0$ Hz), 7.64–7.65 (d, 1 H, CH, $J = 2.0$ Hz), 8.45–8.47 (d, 1 H, CONH, $J = 4.0$ Hz), 10.16 (s, 1 H, 3-NH), 10.64 (s, 1H, 7-NH). Anal. (C₁₈H₁₉N₅O₆S · 1.1H₂O) C, H, N, S.

(S)-2-(5-(3-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)propyl)thiophene-2-carboxamido)pentanedioic Acid (8)—Compound **8** was synthesized as described for **7**: yield 90% as a pale blue powder; mp 159 °C dec; $R_f = 0.06$ (CHCl₃/MeOH, 5:1); ¹H NMR (DMSO-*d*₆) δ 1.88–1.97 (m, 3 H, CH₂), 2.01–2.08 (m, 1 H, CH₂), 2.31–2.34 (t, 2 H, CH₂, $J = 7.5$ Hz), 2.59–2.62 (t, 2 H, CH₂, $J = 7.5$ Hz), 2.77–2.80 (t, 2 H, CH₂, $J = 7.5$ Hz), 4.30–4.35 (m, 1 H, CH), 5.97 (s, 2 H, 2-NH₂), 6.36–6.37 (d, 1 H, CH, $J = 1.0$ Hz), 6.88–6.89 (d, 1 H, CH, $J = 2.0$ Hz), 7.67–7.68 (d, 1 H, CH, $J = 2.0$ Hz),

8.46–8.48 (d, 1 H, CONH, $J = 4.0$ Hz), 10.11 (s, 1 H, 3-NH), 10.64 (s, 1H, 7-NH). Anal. ($C_{19}H_{21}N_5O_6S \cdot 0.95CH_3OH$) C, H, N, S.

(S)-2-(5-(4-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)butyl)thiophene-2-carboxamido)pentanedioic Acid (9)—Compound **9** was synthesized as described for **7**: yield 96% as a blue powder; mp 141 °C dec; $R_f = 0.06$ ($CHCl_3/MeOH$, 5:1); 1H NMR ($DMSO-d_6$) δ 1.59–1.66 (m, 4 H, 2 CH_2), 1.88–1.94 (m, 3 H, CH_2), 2.03–2.10 (m, 1 H, CH_2), 2.32–2.35 (t, 2 H, CH_2 , $J = 7.5$ Hz), 2.57–2.60 (t, 2 H, CH_2 , $J = 7.5$ Hz), 2.79–2.81 (t, 2 H, CH_2 , $J = 7.5$ Hz), 4.31–4.35 (m, 1 H, CH), 5.95 (s, 2 H, 2-NH₂), 6.33–6.34 (d, 1 H, CH, $J = 1.0$ Hz), 6.87–6.88 (d, 1 H, CH, $J = 2.0$ Hz), 7.66–7.67 (d, 1 H, CH, $J = 2.0$ Hz), 8.47–8.49 (d, 1 H, CONH, $J = 4.0$ Hz), 10.09 (s, 1 H, 3-NH), 10.60 (s, 1H, 7-NH). Anal. ($C_{20}H_{23}N_5O_6S \cdot 0.55CH_3OH$) C, H, N, S.

(S)-2-(5-(5-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)pentyl)thiophene-2-carboxamido)pentanedioic Acid (10)—Compound **10** was synthesized as described for **7**: yield 95% as a pale yellow powder; mp 158 °C dec; $R_f = 0.07$ ($CHCl_3/MeOH$, 5:1); 1H NMR ($DMSO-d_6$) δ 1.31–1.36 (m, 2 H, CH_2), 1.59–1.66 (m, 4 H, 2 CH_2), 1.88–1.94 (m, 3 H, CH_2), 2.03–2.10 (m, 1 H, CH_2), 2.32–2.35 (t, 2 H, CH_2 , $J = 7.5$ Hz), 2.52–2.55 (t, 2 H, CH_2 , $J = 7.5$ Hz), 2.77–2.80 (t, 2 H, CH_2 , $J = 7.5$ Hz), 4.31–4.35 (m, 1 H, CH), 5.94 (s, 2 H, 2-NH₂), 6.32–6.33 (d, 1 H, CH, $J = 1.0$ Hz), 6.87–6.88 (d, 1 H, CH, $J = 2.0$ Hz), 7.66–7.67 (d, 1 H, CH, $J = 2.0$ Hz), 8.48–8.50 (d, 1 H, CONH, $J = 4.0$ Hz), 10.08 (s, 1 H, 3-NH), 10.59 (s, 1H, 7-NH). Anal. ($C_{21}H_{25}N_5O_6S \cdot 0.70H_2O$) C, H, N, S.

(S)-2-(5-(6-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)hexyl)thiophene-2-carboxamido)pentanedioic Acid (11)—Compound **11** was synthesized as described for **7**: yield 89% as a blue powder; mp 126 °C dec; $R_f = 0.07$ ($CHCl_3/MeOH$, 5:1); 1H NMR ($DMSO-d_6$) δ 1.29–1.35 (m, 4 H, 2 CH_2), 1.53–1.64 (m, 4 H, 2 CH_2), 1.88–1.94 (m, 3 H, CH_2), 2.03–2.10 (m, 1 H, CH_2), 2.32–2.35 (t, 2 H, CH_2 , $J = 7.5$ Hz), 2.52–2.55 (t, 2 H, CH_2 , $J = 7.5$ Hz), 2.76–2.79 (t, 2 H, CH_2 , $J = 7.5$ Hz), 4.31–4.35 (m, 1 H, CH), 5.94 (s, 2 H, 2-NH₂), 6.31–6.32 (d, 1 H, CH, $J = 1.0$ Hz), 6.87–6.88 (d, 1 H, CH, $J = 2.0$ Hz), 7.66–7.67 (d, 1 H, CH, $J = 2.0$ Hz), 8.48–8.50 (d, 1 H, CONH, $J = 4.0$ Hz), 10.07 (s, 1 H, 3-NH), 10.58 (s, 1H, 7-NH). Anal. ($C_{22}H_{27}N_5O_6S \cdot 0.25H_2O$) C, H, N, S.

Molecular Modeling and Computational Studies

The X-ray crystal structures of human GARFTase (PDB ID 4EW2)³⁸ at 1.60 Å resolution, human AICARFTase (PDB ID 1P4R)³⁹ at 2.55 Å resolution, human FR α (PDB ID 4LRH)⁴⁰ at 2.80 Å resolution, and human FR β (PDB ID 4KN0)⁴³ at 2.10 Å resolution were obtained from the Protein Data Bank. The GARFTase crystal structure contains human GARFTase complexed with *N*-({4-[(1*S*)-4-(2,4-diamino-6-oxo-1,6-dihydropyrimidin-5-yl)-1-(methylsulfanyl)butyl]phenyl}-carbonyl)-L-glutamic acid (10*S*-methylthio-DDACTHF) (**18**), and the AICARFTase crystal structure contains human AICARFTase complexed with the sulfamido-bridged 5,8-dideazafolate analogue BW1540 (**19**). The FR α crystal structure is cocrystallized with folic acid, while the FR β crystal structure is cocrystallized with MTX.

Docking studies were performed using LeadIT 2.1.⁴¹ The protonation states of the proteins and the ligands were calculated using the default settings. Water molecules in the active site, if any, were permitted to rotate freely. The active site was defined by a sphere of 6.5 Å from the native ligand in the crystal structure. Ligands for docking were prepared using MOE 2013.08⁴⁴ and then energy minimized using the MMF94X force field to a constant of 0.05 kcal/mol. Ligand binding for docking purposes in LeadIT 2.1 was driven by a hybrid enthalpy and entropy method using default settings. The docked compounds were scored using default settings. The maximum number of solutions per iteration and the maximum number of solutions per fragment were capped at 300. The total number of final poses per compound was restricted to 10. The docked poses were initially exported and visualized in MOE. Generation of images for publication purposes was performed using the software CCP4MG.⁴⁵

To validate the software for docking the proposed compounds, the native ligands **18** for GARFTase, **19** for AICARFTase, folic acid for FR α , and MTX for FR β were built using the molecule builder function in MOE, energy minimized, and docked, as described above. RMSDs of the docked poses were calculated in LeadIT using the crystal structure conformation of the ligands.

The best docked poses for **18** in human GARFTase, **19** in human AICARFTase, folic acid in FR α , and MTX in FR β showed RMSD values of 0.95, 1.0995, 0.63, and 0.75 Å, respectively, thereby validating LeadIT 2.1 for our docking purposes in these targets.

Reagents for Biological Studies

[U-¹⁴C]Glycine (87mCi/mmol) was purchased from Moravek Biochemicals (Brea, CA). Folic acid was purchased from the Sigma Chemical Co. (St. Louis, MO). LCV [(6*R,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-yl)methyl)-*N*-methylamino]-2-thienoyl)-L-glutamic acid], AstraZeneca Pharmaceuticals (Macclesfield, Cheshire, England); LMTX (5,10-dideaza-5,6,7,8-tetrahydrofolate), Eli Lilly and Co. (Indianapolis, IN); 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), LC Laboratories, Woburn, MA. Other chemicals were obtained from commercial sources in the highest available purity.

Cell Culture

MTXR2-4 (hereafter, referred to as R2) is an RFC-, PCFT-, and FR α -null Chinese hamster ovary (CHO) cell line that was a gift from Dr. Wayne Flintoff (University of Western Ontario).³⁷ From this parental cell line, RFC, PCFT, and FR α were transfected to give rise to isogenic CHO cell lines designated PC43-10 (expresses human RFC but not PCFT or FR α), R2/PCFT4 (expresses PCFT), and RT16 (expresses FR α).^{19,25,36} The CHO sublines were all maintained in α -minimal essential medium (α -MEM) supplemented with 5% penicillin-streptomycin solution, 2 mM L-glutamate, and 10% heat-treated bovine calf serum (Invitrogen). Transfected cell lines were cultured in the presence of 1.5 mg/mL G418.

CHO cell lines were cultured prior to cell viability experiments in folate-free RPMI (FFRPMI) (Invitrogen) with dialyzed fetal bovine serum (dFBS) (Invitrogen), supplemented with 5% penicillin–streptomycin and L-glutamate. FR α -expressing human KB nasopharyngeal carcinoma cells (ATCC, Manassas, VA) were continuously maintained in complete FF-RPMI with 10% fetal bovine serum and 5% penicillin–streptomycin and L-glutamate.

Cell proliferation assays were performed as previously described.^{19–21,25} Cell lines were treated with a range of concentrations of standard or novel antifolates (0–1000 nM) in a 96-well plate in FF-RPMI complete with dFBS, 2 nM (RT-16, KB) or 25 nM (R2/PCFT4) LCV, penicillin–streptomycin, and L-glutamate over a 96 h incubation period at 37 °C with 5% CO₂. To confirm FR α -mediated drug uptake and growth inhibition, 200 nM folic acid was added to the incubations with drug. For proliferation assays with PC43-10 cells, cells were cultured in standard RPMI1640 with dFBS, penicillin–streptomycin, and L-glutamate for 96 h. To quantitate viable cells, medium was removed and CellTiter-Blue reagent (Promega) was added. Relative cell numbers were determined by fluorescence units measured with a fluorescence plate reader (Molecular Devices) at 590 nm emission and 560 nm excitation with Softmax Pro plate reader software. IC₅₀ values, corresponding to the drug concentrations that resulted in 50% loss of cell growth, were calculated from dose–response curves using Sigma Plot (v.12) software.

To identify the targeted pathways/folate-dependent enzymes by the classic and novel pyrrolo[2,3-*d*]pyrimidine antifolates, proliferation assays were performed in the presence of adenosine (60 μ M), thymidine (10 μ M), or 5-aminoimidazole-4-carboxamide hydrochloride (320 μ M) and the results compared to those of incubations in parallel without nucleoside/AICA or drug additions.^{19–21,25,29,31}

HPLC Detection of ATP

Intracellular ATP concentrations were determined using nucleotide extractions and an HPLC method, exactly as previously described.^{21,31} Briefly, KB cells were seeded in a T75 flask with FF-RPMI with 10% DFBS, 5% penicillin–streptomycin, 2 mM L-glutamine, and 2 nM LCV. Cells were treated with 1 μ M PMX or compound **2d** or **9** for 24 h. After the incubations, cells were trypsinized and washed with cold PBS. Nucleotide pools were fractionated by reversed-phase HPLC on a C18 column for determination of ATP pools.⁴⁶

In Situ GARFTase Inhibition Assays

To measure the extent of intracellular GARFTase inhibition in the presence of antifolate inhibitors, we measured the metabolic incorporation of [U-¹⁴C]glycine into [¹⁴C] formyl GAR in the presence of azaserine.^{19–21,25,31} KB cells were seeded in complete medium (above) and allowed to adhere to the substratum for 24 h. Cells were then washed with FF-RPMI (L-glutamine-free) with 10% dFBS, 5% penicillin–streptomycin, and 2 nM LCV. Cells were incubated at 37 °C with 5% CO₂ for 1 h with folate- and L-glutamine-depleted medium containing 2 nM LCV, with PMX, or with the novel antifolate compounds over a range of concentrations. Control cells were treated with DMSO of equal volume. Azaserine (4 μ M final concentration) was added to the cells, and the cells were incubated for 30 min.

This was followed by the addition of L-glutamine (2 mM) and [U-¹⁴C]glycine (final specific activity 0.1 mCi/L). Cells were incubated for 16 h. Cells were washed with ice-cold DPBS, trypsinized, and collected. Cells were treated with 5% trichloroacetic acid (TCA) at 0 °C. Samples were centrifuged (4 °C, 14 000 rpm), and the protein precipitants were solubilized with 0.5 N NaOH for quantitation of protein concentrations with the Folin phenol protein method.⁴⁷ The TCA supernatants were extracted with cold ether. After evaporation of ether, the remaining aqueous layer was gravity filtered through a 1 cm chromatography column (Biorad) of AG1X8 (chloride form). The columns were washed with 10 mL of 0.5 N formic acid, followed by 4 N formic acid (10 mL), and finally eluted with 8 mL of 1 N HCl collected in eight fractions. The eluate was measured for radioactivity and the percentages of radioactivity in the [¹⁴C] formyl GAR and nonspecific [¹⁴C] fractions. The results were calculated as picomoles of [¹⁴C] formyl GAR per milligram of cell protein. Data were graphed using Sigmaplot (v.12), and the IC₅₀ values were calculated for the antifolate-treated samples, compared to the untreated controls.

HPLC Detection of ZMP Pools

Methods for detecting ZMP in KB cells treated with the antifolate drugs were as previously described.^{27,28,31} Briefly, KB cells were treated with PMX or compound **9** for 48 h in FF-RPMI with 10% dFBS, 5% penicillin–streptomycin, 2 mM L-glutamine, and 2 nM LCV. Cells were washed with DPBS and treated with ice-cold 5% TCA and the precipitated proteins collected by centrifugation (1600 rpm, 10 min). The soluble fractions were extracted with ether (2×) and fractionated by ion-exchange HPLC on an SAX 250 × 2 mm column (Phenomenex) with a linear gradient from 5 mM NH₄H₂PO₄ (pH 2.8) to 750 mM NH₄H₂PO₄ (pH 3.9) over 25 min at a flow rate of 0.2 mL/min. Absorbance was monitored at 280 nm, and the intracellular ZMP concentrations were established by fitting the peak absorbance to a standard curve with commercial ZMP. AraCMP was used as an internal control.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS USED

AICA	5-aminoimidazole-4-carboxamide
ZMP	5-aminoimidazole-4-carboxamide ribonucleotide
AICARFTase	5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase

CHO	Chinese hamster ovary
dFBS	dialyzed fetal bovine serum
DPBS	Dulbecco's phosphate-buffered saline
FF-RPMI	folate-free RPMI
FR	folate receptor
GAR	glycinamide ribonucleotide
GARFTase	glycinamide ribonucleotide formyl-transferase
HBSS	Hank's balanced salts solution
HBS	Hepesbuffered saline
LCV	leucovorin
LMTX	lometrexol
MTX	methotrexate
MEM	minimal essential medium
10S-methylthio-DDACTHF	<i>N</i> -({4-[(1S)-4-(2,4-diamino-6-oxo-1,6-dihydropyrimidin-5-yl)-1-(methylsulfonyl)butyl]phenyl}carbonyl)-L-glutamic acid
PMX	pemetrexed
PDX	pralatrexate
PCFT	proton-coupled folate transporter
RTX	raltitrexed
RFC	reduced folate carrier
SAR	structure–activity relationship
TS	thymidylate synthase
TCA	trichloroacetic acid

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Figure 1.
Structures of classical antifolates.

Figure 2.
Novel 5- and 6-substituted pyrrolo[2,3-d]pyrimidines **1**–**5**.

Figure 3.
Design of 5-substituted thienoyl pyrrolo[2,3-*d*]pyrimidines **6–11** ($n = 1–6$).

Figure 4.

Cell proliferation assays with protection by nucleosides, including thymidine and adenosine, and by 5-aminoimidazole-4-carboxamide (AICA) to identify intracellular targets of **2d** and **8–10**. To identify the targeted pathways and the folate-dependent intracellular enzymes in KB cells treated with **2d** and **8–10** (1–1000 nM), cell proliferation assays were performed in the presence of 10 μ M thymidine, 60 μ M adenosine, or 320 μ M AICA. Results were normalized to those for untreated cells (no drug). Details are provided in the Experimental Section. Results shown are representative of triplicate experiments. These results are summarized in Table 1.

Figure 5.

Effects of compounds **2d** and **9** on ATP pools, compared to PMX. KB cells were treated with 1 μ M **2d**, **9**, or PMX. After 24 h, the nucleotides were extracted and analyzed by ion pair HPLC. Experimental details are described in the Experimental Section. Results are presented as mean values $\pm$ standard errors. The results with PMX were previously reported.³¹

Figure 6.

In situ GARFTase assay and inhibition by 5- and 6-substituted pyrrolo[2,3-*d*]pyrimidine antifolates. In situ GARFTase activity and inhibition by the 5- and 6-substituted pyrrolo[2,3-*d*]pyrimidine analogues **2d**, **9**, and PMX were assessed with KB cells. Results are presented as mean values ± standard errors from three experiments. IC₅₀ values for GARFTase inhibition are summarized in the table. Additional details are described in the Experimental Section.

Figure 7.

Accumulation of ZMP in KB cells treated with **9** and PMX. ZMP (AICA ribonucleotide) accumulation was measured in KB cells treated for 48 h with 1 μ M **9** or PMX, compared to untreated KB cells. Detailed methods for extracting nucleotide metabolites and for measuring ZMP levels are described in the Experimental Section. Results are shown as mean values $\pm$ standard errors for three experiments.

Figure 8.

Docking of **9** in human GARFTase. The stereoview shows the docked pose of **9** (pink) in human GARFTase (PDB ID 4EW2³⁸). Amino acids that interact with **9** are shown as thick blue cylinders, while pocket amino acids are shown as thick green lines.

Figure 9.

Docking of **9** in AICARFTase. A stereoview of the docked pose of **9** (pink) in AICARFTase is shown (PDB ID 1P4R³⁹). Amino acids that interact with **9** are shown as thick blue cylinders, while pocket amino acids are shown as thin blue lines.

Figure 10.

Docking of **9** in FR α . A stereoview is shown for the docked pose of **9** (pink) in FR α (PDB ID 4LRH).⁴⁰ Amino acids that interact with **9** are shown as thick blue cylinders, while pocket amino acids are shown as thin green lines.

Figure 11.

A stereoview is shown for the superimposition of the energy minimum conformation of **9** (blue) on its docked conformations in FR α (green), FR β (brown), GARFTase (red), and AICARFTase (gold).

Scheme 1.

Table 1

IC₅₀ Values (nM) for Inhibition of Proliferation of CHO and KB Sublines^a

compd	structure	RFC		FR α		PCFT		RFC/FR α /PCFT	
		PC43-10	R2	RT16	R2/PCFT4	R2(VC)	KB	KB (+Thd/Ade/AICA)	
2a	6-pyr/1C/thieno	>1000	>1000	122(86)	291(71)	>1000	41.9(24)	Ade/AICA	
2b	6-pyr/2C/thieno	>1000	>1000	38.9(10.2)	135(18)	>1000	3.22(0.50)	Ade/AICA	
2c	6-pyr/3C/thieno	101.0(16.6)	273.5(49.1)	0.31(0.14)	3.34(0.26)	288(12)	0.26(0.03)	Ade/AICA	
2d	6-pyr/4C/thieno	>1000	>1000	1.82(0.28)	43.4(4.1)	>1000	0.55(0.10)	Ade/AICA	
2e	6-pyr/5C/thieno	>1000	>1000	4.53(1.60)	102.4(17.9)	>1000	3.17(0.32)	Ade/AICA	
2f	6-pyr/6C/thieno	>1000	>1000	137(40)	>1000	>1000	231(88)	Ade/AICA	
6	5-pyr/1C/thieno	>1000	>1000	>1000	>1000	>1000	>1000		
7	5-pyr/2C/thieno	59.8(11.1)	>1000	550(50)	80.2(5.5)	>1000	875(125)		
8	5-pyr/3C/thieno	116.0(22.5)	>1000	109(44)	312(90)	>1000	211(58)	Ade	
9	5-pyr/4C/thieno	38.3(6.6)	>1000	49.3(11.5)	141(40)	>1000	66.0(14.4)	Ade	
10	5-pyr/5C/thieno	243.2(49.9)	>1000	45.5(22.8)	57.4(21.4)	>1000	41.7(10.1)	Ade/AICA	
11	5-pyr/6C/thieno	>1000	>1000	>1000	>1000	>1000	>1000		
MTX		12(1.1)	216(8.7)	114(31)	120.5(16.8)	>1000	6.0(0.6)	Thd/Ade	
PMX	5-pyr/2C/benzoyl	138(13)	42(9)	42(9)	13.2(2.4)	974(18)	68(12)	Thd/Ade	
RTX		6.3(1.3)	>1000	15(5)	99.5(11.4)	>1000	5.9(2.2)	Thd	
LMTX		12(2.3)	>1000	12(8)	38.0(5.3)	>1000	1.2(0.6)	Ade/AICA	

^a Growth inhibition assays were performed for CHO sublines engineered to express human RFC (PC43-10), FR α (RT16), or PCFT (R2/PCFT4), 19,25,36 for comparison with transporter-null [R2, R2(VC)] CHO cells, and for the KB human tumor subline (expresses RFC, FR α , and PCFT).¹⁴ For the FR α experiments, growth inhibition assays were performed in the presence and the absence of 200 nM folic acid. Data shown are mean values from 3–10 experiments ($\pm$ standard errors in parentheses). Results are presented as IC₅₀ values, corresponding to the nanomolar concentrations that inhibit growth by 50% relative to cells incubated without drug. Data for MTX, RTX, LMTX, and compounds 2a–2f were previously published.^{20,21} Structures for compounds 2a–2f and 6–11 are given in Figures 2 and 3, respectively. For KB cells, data are shown for the protective effects of nucleoside additions, including adenosine (60 μ M) or thymidine (10 μ M), or 5-aminoimidazole-4-carboxamide (320 μ M). Examples of these data are given in Figure 4. For the 5- and 6-pyrrolo[2,3-d]pyrimidine compounds, the structural motifs are noted, i.e., the pyrrole substitution/bridge length/aromatic B-ring. Experimental details are provided in the Experimental Section. Undefined abbreviations: Ade, adenosine; AICA, 5-aminoimidazole-4-carboxamide; Thd, thymidine.