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Discovery of 5-substituted pyrrolo[2,3-*d*]pyrimidine antifolates as dual acting inhibitors of glycinamide ribonucleotide formyltransferase and 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase in *de novo* purine nucleotide biosynthesis: implications of inhibiting 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase to AMPK activation and anti-tumor activity

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Abstract

We synthesized 5-substituted pyrrolo[2,3-*d*]pyrimidine antifolates (compounds **5–10**) with 1 to 6 bridge carbons and a benzo ring in the side chain as antitumor agents. Compound **8** with a 4-carbon bridge was the most active analog and potently inhibited proliferation of folate receptor (FR) α -expressing Chinese hamster ovary and KB human tumor cells. Growth inhibition was reversed completely or in part by excess folic acid, indicating that FR α is involved in cellular uptake, and resulted in S-phase accumulation and apoptosis. Anti-proliferative effects of compound **8** toward KB cells were protected by excess adenosine but not thymidine, establishing *de novo* purine nucleotide biosynthesis as the targeted pathway. However, 5-aminoimidazole-4-carboxamide (AICA) protection was incomplete, suggesting inhibition of both AICA ribonucleotide formyltransferase (AICARFTase) and glycinamide ribonucleotide formyltransferase (GARFTase). Inhibition of GARFTase and AICARFTase by compound **8** was confirmed by cellular metabolic assays and resulted in ATP pool depletion. To our knowledge,

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this is the first example of an antifolate that acts as a dual inhibitor of GARFTase and AICARFTase as its principal mechanism of action.

INTRODUCTION

The antifolates are a class of anti-proliferatives widely recognized for their inhibition of folate metabolism.^{1,2} Major antifolate enzyme targets include thymidylate synthase (TS) and dihydrofolate reductase (DHFR). Inhibition of these enzymes suppresses *de novo* nucleotide biosynthesis, resulting in an imbalance of purine and pyrimidine precursors, rendering cells incapable of undergoing accurate DNA replication, ultimately resulting in cell death. Clinically relevant TS and DHFR inhibitors, typified by pemetrexed (PMX), and methotrexate (MTX) and pralatrexate (Figure 1), respectively, continue to play important roles in treating hematologic malignancies and solid tumors.^{1,2}

Antifolates targeting *de novo* purine nucleotide biosynthesis were also described and include lometrexol [(6*R*),5,10-dideazatetrahydrofolate; LMTX] (Figure 1), (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-carbonyl]amino]pentanedioic acid (LY309887)^{3,4} and (2*S*)-2-[[5-[2-[(6*S*)-2-amino-4-oxo-1,6,7,8-tetrahydropyrimido[5,4-*b*ate][1,4]thiazin-6-yl]ethyl]thiophene-2-carbonyl]amino]pentanedioic acid (AG2034).⁵ All these drugs inhibit the first folate-dependent step in purine nucleotide biosynthesis, catalyzed by β -glycinamide ribonucleotide (GAR) formyltransferase (GARFTase), and have progressed to clinical trials.⁶⁻⁸ However, their toxicities were dose-limiting, most likely a consequence of their cellular uptake and metabolism to polyglutamates in normal tissues.

The reduced folate carrier (RFC) is one of three principal mechanisms of (anti)folate uptake into mammalian cells.⁹ Cellular requirements for folate cofactors for DNA replication provide a plausible explanation for the high levels of RFC in most tumors. However, demands for folates, and consequently RFC, are also shared by normal tissues, such that RFC may not be the optimal mechanism for tumor-selective uptake of cytotoxic folate analogs. Other cellular uptake mechanisms, notably the proton-coupled folate transporter (PCFT) and folate receptors (FRs) α and β , are also expressed in tumors, while showing more restricted expression in normal tissues.¹⁰ Further, PCFT is a proton symporter such that in the acidic microenvironment generated by glycolytic tumors, membrane transport and selective tumor targeting by this mechanism is enhanced.^{11,12} For FR α , the apical spatial orientation in normal epithelial tissues is often disrupted in tumors such that its basolateral membrane expression in tumors results in exposure to the circulation.¹⁰ These features provide compelling rationale for developing folate-based therapeutics which target PCFT and FR α for cancer therapy. Examples of FR α -targeted therapies include a monoclonal antibody, Farletuzumab (Morphotech)¹³, a cytotoxic folate conjugate, Vintafolide (EC145; Endocyte),¹⁴ and a classical antifolate which is selectively transported into cells by FRs over RFC and inhibits *de novo* thymidylate biosynthesis, ONX0801.¹⁵

PMX is a 5-substituted pyrrolo[2,3-*d*]pyrimidine antifolate with a 2-carbon bridge linked to a *p*-aminobenzoyl glutamate. PMX is a good transport substrate for RFC and among the best PCFT substrates,^{9,11,12} yet it is a modest substrate for FRs.¹⁶⁻²⁰ We previously described 6-substituted pyrrolo[2,3-*d*]pyrimidine benzoyl antifolates related to PMX (compounds **1-4**) (Figure 2).^{16,21} Whereas the 6-regioisomer of PMX was pharmacologically inert, the 3- and 4-bridge carbon analogs of this series (compounds **1** and **2**, respectively) were selectively transported by FR α and PCFT over RFC and were potently inhibitory toward FR α - and PCFT-expressing tumor cells.^{16,21}

PMX was originally described as a “multi-targeted” antifolate with a primary inhibition at TS and secondarily at GARFTase and DHFR.²² However, recent studies suggested that although TS was the primary cellular target of PMX, 5-aminoimidazole-4-carboxamide (AICA) ribonucleotide (ZMP) formyltransferase (AICARFTase) could be a pharmacologically important target in the presence of excess thymidine (circumvents TS) at sufficiently high PMX concentrations.^{23,24} Hence, in the presence of thymidine, inhibition of proliferation of CCRF-CEM leukemia cells and solid tumor cell lines was circumvented by hypoxanthine but only partially by AICA.^{23,24} Further, PMX resulted in marked accumulations of ZMP, the AICARFTase substrate. By contrast, the 6-substituted pyrrolo[2,3-*d*]pyrimidine analogs **1** and **2** are *exclusively* GARFTase inhibitors whose inhibitory effects are *completely* circumvented by AICA.^{16,21}

The accumulation of ZMP in PMX-treated cells is intriguing as ZMP is an AMP mimetic that activates AMP-activated protein kinase (AMPK).²⁵ AMPK negatively regulates mTOR, a critical pro-survival pathway that is activated in many tumor cells along with PI3K/AKT, secondary to loss or mutation of PTEN.^{26–28} This may provide a possible explanation for the growth inhibitory effects of PMX in the presence of thymidine, as purine nucleotides are not depleted.^{23,24} However, this has not been directly tested, as no AICARFTase-targeted drugs without TS inhibition have been described.

In this report, we synthesized and systematically characterized the *in vitro* anti-proliferative activities and cellular mechanisms of novel 5-substituted pyrrolo[2,3-*d*]pyrimidine antifolates (including PMX) with a benzoyl ring in the side chain and one-to-six bridge carbons (compounds **5–10**) closely related to the 6-substituted pyrrolo[2,3-*d*]pyrimidine analogs **1–4**^{16,21} (Figure 2). We reasoned that the 5-substituted analogs might inhibit AICARFTase, analogous to PMX, albeit without TS inhibition. Further, if this was accompanied by FR α and/or PCFT transport selectivity over RFC, this would be especially appealing for targeting tumors that express high levels of these systems.

We describe herein the impact of these structural modifications on (i) transporter specificity and (ii) the targeted pathway (TS versus *de novo* purine nucleotide biosynthesis), including (iii) the extent of cellular GARFTase and AICARFTase inhibition. Our results document an emerging structure-activity relationship (SAR) for the pyrrolo[2,3-*d*]pyrimidine antifolates, accompanying translocation of the 6-pyrrolo[2,3-*d*]pyrimidine substituent to the regio 5-position, including sustained FR α cellular uptake, accompanying restoration of RFC and loss of PCFT transport activities. The 4-carbon bridge analog of this series (compound **8**) is unique in that it shows dual inhibition of both AICARFTase and GARFTase exclusive of TS in tumor cells, resulting in depletion of purine nucleotides. Finally, our results directly examine the possibility of whether AMPK activation secondary to AICARFTase inhibition contributes to decreased proliferation of KB human tumor cells.

CHEMISTRY

Compounds **5–10** (*n* = 1–6) were obtained from a modified synthesis of PMX^{29,30} by using an α -bromo aldehyde condensation with 2,6-diamino-4-oxo-pyrimidine **15** as the key step (Scheme 1). Thus, a Heck coupling reaction³¹ of aryl iodide **11** with commercially available unsaturated alcohols **12a–f** in DMF at 70° C affords the unsaturated, coupled alcohols that rearrange to the vinyl alcohols and tautomerise to afford the aldehydes **13a–f** (yields: 83–92%), as reported by Larock et al.³¹ α -Bromination of **13a–f** with 5,5-dibromo-2,2-dimethyl-4,6-dioxo-1,3-dioxane³² at room temperature afforded the corresponding α -bromo aldehydes **14a–f** (yields: 74–78%). The 5-substituted pyrrolo[2,3-*d*]pyrimidines **16a–f** were synthesized by condensation of **14a–f** with **15** at 45 °C in the presence of sodium acetate (yields: 52–70%). Subsequent hydrolysis with 3N NaOH, followed by coupling with diethyl

L-glutamate using *N*-methyl morpholine and 2,4-dimethoxy-6-chlorotriazine as the activating agents, afforded the diesters **17a–f** (yields: 51–78% over two steps). Final saponification of the diesters with 1N NaOH provided the target compounds **5–10** (yields: 88–97%).

BIOLOGICAL EVALUATION

Transporter-selectivity for inhibition of cell proliferation by 5- and 6-substituted pyrrolo[2,3-*d*]pyrimidine regioisomers

Our initial goal was to better define the SARs for membrane transport of pyrrolo[2,3-*d*]pyrimidine antifolates relating to the impact of 5- versus 6-substitutions on the pyrrole ring, based on the established antitumor activities of the 6-substituted pyrrolo[2,3-*d*]pyrimidine-substituted compounds **1** and **2**, with 3- and 4-bridge carbons, respectively,^{16,21} and the preclinical and clinical efficacy of PMX,²² a 5-substituted pyrrolo[2,3-*d*]pyrimidine with a 2-carbon bridge (Figure 2). In our previous studies,^{16,21} compounds **1** and **2** were efficiently internalized by tumor cells via FR α and PCFT but not by RFC, whereas PMX was a substrate by all three systems.^{16–21}

Transporter specificities are exemplified by patterns of inhibition of cell proliferation toward isogenic Chinese hamster ovary (CHO) cell models, engineered to express human RFC (PC43-10), PCFT (R2/PCFT4), or FR α (RT16),^{16–20} treated with compounds **1–4** (Table 1). Results are compared to those for RFC-, PCFT-, and FR-null MTXRIIOua^R2–4 (R2) CHO cells. As previously reported,^{16,21} compounds **1–4** inhibited proliferation of FR α -expressing RT16 and R2/PCFT4 cells in the order of potency **1** > **2** > **3** > **4**. However, compounds **1–4** were largely inactive toward RFC-expressing PC43-10 and R2 cells. Analogous results were obtained with KB nasopharyngeal carcinoma cells which express elevated FR α , along with modest levels of RFC and PCFT.¹⁷ The antiproliferative effects of compounds **1–4** toward the FR α -expressing cell lines were abolished in the presence of excess (200 nM) folic acid, establishing that cellular uptake of these analogs is mediated primarily via FR α .¹⁶ (Table 1).

5-Substituted pyrrolo[2,3-*d*]pyrimidine analogs with a side-chain benzoyl group (analogs of compounds **1–4**) and bridge lengths from 1 to 6 carbons (compounds **5–10**) were synthesized (Scheme 1) and tested for their antiproliferative activities toward the CHO and human tumor cell line models (Table 1). The 5-pyrrolo[2,3-*d*]pyrimidine analog with a 2-carbon bridge (compound **6**) is PMX. 5-Pyrrole substitution restored RFC-directed antiproliferative activity, as reflected in dramatically increased anti-proliferative effects of this series toward PC43-10 cells compared to RFC-, PCFT-, and FR-null R2 cells, in the order of potency (numbers of bridge carbons noted in parentheses), **6** (2C) > **8** (4C) ~ **7** (3C) > **9** (5C) >> **5** (1C) ~ **10** (6C). For PCFT-expressing R2/PCFT4 CHO cells, with the exception of compound **6**, anti-proliferative effects for the 5-substituted analogs were attenuated compared to the 6-substituted analogs (e.g., for direct comparisons between paired 5- and 6-substituted analogs, compare IC₅₀s for compounds **7–10** to **1–4**, respectively, in Table 1).

With the FR α -expressing sublines, potencies were also reduced for the 5-substituted compounds, compared to 6-substituted analogs. These differences ranged from slight (compare **2** to **8** and **3** to **9**) to substantial (compare **1** to **7**) (Table 1). For both series, the 6-carbon bridge analogs (compounds **4** and **10**) were inactive. In contrast to results for PCFT- and RFC-expressing cells, the impacts of bridge lengths were distinctly different, with antiproliferative activities toward FR α -expressing RT16 CHO cells in rank order, **8** (4C) > **6** (2C) > **9** (5C) > **7** (3C) >> **5** (1C) ~ **10** (6C). An analogous pattern of sensitivities was obtained with KB human tumor cells, and for both the KB and RT16 cell lines, inhibitory effects were substantially reversed by 200 nM folic acid, confirming FR α as an important uptake mechanism for these compounds.

To complement results showing anti-proliferative effects of the most potent 5-substituted analog **8** toward PC43-10 CHO cells expressing human RFC, we measured competitive inhibitions of [³H]MTX (0.5 μM) transport in this model (Figure 3A). MTX is a well-characterized surrogate substrate for RFC and the extent of [³H]MTX transport inhibition by competitive unlabeled (anti)folate ligands correlates with their membrane transport by RFC.⁹ For our study, we compared inhibitory effects of 10 μM of compound **8**, to those for the corresponding 6-substituted compound **2**, and to PMX and MTX. Transporter-null R2 cells were used to as a measure of baseline [³H]MTX uptake. Whereas **2** was a poor inhibitor of [³H]MTX uptake (< 25% inhibition), 10 μM compound **8** was a reasonably good inhibitor (> 90% inhibition), with an inhibitory potency approximating that for PMX and net transport only slightly greater than the very low levels recorded for R2 cells. Clearly, unlike the 6-substituted pyrrolo[2,3-*d*]pyrimidine analogs, the 5-substituted compounds lack specificity for FRα and/or PCFT over RFC.

Compound **8** showed anti-proliferative effects toward FRα-expressing RT16 CHO cells and KB human tumor cells (Table 1). To directly demonstrate drug binding to FRα, a prerequisite of cellular uptake by this endocytotic mechanism, we used competitive binding assays with [³H]folic acid with RT16 cells, treated with unlabeled ligands which compete for FRα binding^{16–20} (Figure 3B). Relative FRα binding affinities for the 5-(compound **8**) versus 6-substituted (compound **2**) analogs paralleled their anti-proliferative activities. However, this correlation did not quite extend to PMX (compound **6**) or to MTX, indicating that multiple factors in addition to FRα binding affinities contribute to net growth inhibition of these FRα-expressing cells for the antifolates.

Inhibition of *de novo* purine nucleotide biosynthesis by both 5- and 6-pyrrolo[2,3-*d*]pyrimidine compounds leads to a cytotoxic response via purine nucleotide depletion

De novo purine nucleotide biosynthesis includes 10 reactions by which phosphoribosyl pyrophosphate (PRPP) is converted into inosine monophosphate (IMP), the precursor of AMP and GMP (Figure 4). There are two folate-dependent enzymes in the pathway which are possible targets for folate-based therapies including GARFTase (catalyzes steps 2, 3 and 5) and AICARFTase (catalyzes steps 9 and 10). Previous studies established that GARFTase was the intracellular enzyme target for LMTX^{3,33} and for compounds **1** and **2**.¹⁶ For PMX, TS is the primary intracellular target, although modest inhibitions of GARFTase and DHFR were also reported.²² Most recently, AICARFTase was implicated as a potentially important secondary enzyme target for PMX (in the presence of excess thymidine to circumvent TS inhibition) by nucleoside/AICA protection experiments and metabolic assays.^{23,24}

To identify the targeted pathway for 6-substituted compounds **1** and **2**, we previously used nucleoside protection experiments with adenosine (60 μM) and thymidine (10 μM), to distinguish *de novo* purine nucleotide from thymidylate biosynthesis, respectively.^{16–21,33} To further identify the likely folate metabolizing enzyme targets in purine nucleotide biosynthesis (GARFTase versus AICARFTase), 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^{16–21,33} (Figure 4).

We used this approach for KB cells treated with compounds **7–9**, with results compared to those for compound **2** and PMX (compound **6**) (Table 1; Figure 5 shows the nucleoside/AICA protection results for PMX and for compound **2**, compared to those for compound **8**). With compound **2**, both adenosine and AICA were completely protective, establishing *de novo* purine biosynthesis and GARFTase as the principal cellular targets.^{16,21} With PMX, thymidine, adenosine, and AICA were all partially protective, albeit to different extents.

Combined thymidine and adenosine completely protected KB cells from the growth inhibitory effects of PMX (not shown; Table 1). The growth inhibitory effects of the 5-substituted compounds **7–9** with KB cells were unaffected by excess thymidine but were completely reversed in the presence of adenosine alone, indicating that exclusively *de novo* purine nucleotide synthesis was being targeted (rather than combined thymidylate and purine nucleotide synthesis as for PMX). Whereas AICA alone completely protected KB cells from the inhibitory effects of compound **9**, AICA alone was only partially protective with compounds **7** and **8**. Thus, inhibition of purine nucleotide biosynthesis by compounds **7** and **8** is likely a composite effect of dual inhibitions involving both GARFTase and AICARFTase. These results strongly imply that shifting the substituent from the 6- to the 5-position on the pyrrolo[2,3-*d*]pyrimidine ring system results in an altered inhibition of the targeted enzymes.

Decreased purine nucleotide biosynthesis, resulting from inhibition of folate-dependent enzymes can be monitored by following changes in ATP pools, a surrogate measure of total purine nucleotides (Figure 6). KB tumor cells were treated for 24 h with the novel 5-substituted analog **8**, or the corresponding 6-substituted regioisomer compound **2**. An additional incubation was performed with PMX. ATP pools were extracted and measured by an HPLC ion-pairing method.^{21,34} In KB cells following a 24 h incubation, compound **8**, like **2**, potentially depleted ATP pools (>90%), whereas PMX was less effective (~60% inhibition).

Inhibition of *de novo* purine nucleotide biosynthesis by compound **8 results in cytotoxicity and apoptosis**

To confirm that depletion of purine nucleotides by compound **8** results in cytotoxicity toward KB cells, we performed colony-forming assays. In these experiments, KB tumor cells were treated with various concentrations (0.5–10 μ M) for 48 h, then washed and plated without drug. Colonies were enumerated after 10 days (Figure 7A). Following drug exposures, inhibitory effects of compound **8** were irreversible with complete loss of colony formation at the highest drug concentrations, thereby establishing compound **8** as cytotoxic.

Programmed cell death type I (mitochondrial pathway) requires ATP to form the death complex with cytochrome C and caspase 9 and to carry out other energy-requiring processes for enzymatic degradation and modification of cellular components.³⁵ Previous studies suggested that the 6-substituted pyrrolo[2,3-*d*]pyrimidine antipurine antifolate **1** was capable of inducing apoptosis, albeit less than for daunorubicin over 24 h.¹⁶ Additional experiments were performed with KB cells treated with compound **8** to assess its effects on cell cycle progression and induction of apoptosis, with comparisons to PMX and compound **2**. Cells were exposed to 1 μ M drugs for 48 or 96 h. Cultures were then divided, with one fraction fixed and stained with propidium iodide (PI) for flow cytometry analysis of cell cycle distribution (Figure 7B). An additional fraction was assayed for apoptosis by flow cytometry with annexin V-fluorescein isothiocyanate (FITC) /7-amino actinomycin D (AAD) staining (Figure 7C).

At 48 h, minimal effects on the cell cycle were observed, except for PMX, which induced a G₁/G₀-phase arrest relative to the untreated (DMSO) control (Supplement, Figure 1S). At 96 h, all compounds tested increased the percentages of cells in S-phase (albeit to different extents), while reducing those in the G₁/G₀- and G₂/M-phases of the cell cycle (Figure 7B). A prominent increase in the sub-G₁ population at 96 h was also seen (Figure 7B; most notable for compounds **2** and **8**), indicating apoptosis was induced. Annexin V-FITC/7-AAD staining was used to further monitor induction of apoptosis. For compounds **2** and **8**, as well as PMX, positive annexin V-FITC staining increased over time (results for 96 h are shown

in Figure 7C; additional data at 48 h of drug exposure are shown in the Supplement, Figure 1S), with distinct increases in both early (annexin-V-FITC^{high}/7-AAD^{low}) and late (annexin-V-FITC^{high}/7-AAD^{high}) apoptotic fractions, compared those for the untreated control (i.e., the fractions of cells in early apoptosis were 33.8% and 0.49% for compound **8**-treated and control cells, respectively, whereas the fractions in late apoptosis at 96 h were 26.7% and 2.1%, respectively). Nearly identical results were obtained with compound **2**, although for PMX, the distribution between the early and late apoptotic fractions was somewhat different. Thus, the 5- and 6-substituted pyrrolo[2,3-*d*]pyrimidines **2** and **8** can both induce apoptosis, resulting in cell death in spite of depleted ATP pools.

Dual Inhibition of GARFTase and AICARFTase by 5-substituted pyrrolo[2,3-*d*]pyrimidine antifolates and downstream effects of accumulated ZMP on AMPK

Experiments were performed to measure cellular GARFTase activity in KB tumor cells treated with the 6- (compound **2**) and 5-substituted (compound **8** and PMX) pyrrolo[2,3-*d*]pyrimidines. 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.^{16–21,33}

Cells were treated with trichloroacetic acid (TCA) and following deproteinization, acid-soluble metabolites were ether-extracted and fractionated by ion-exchange chromatography, permitting isolation and quantitation of [¹⁴C]formyl GAR. Both 5- and 6-substituted analogs showed a dose-dependent decrease in the formation of [¹⁴C]formyl GAR. However, compound **2** was more potent than either PMX or compound **8**, with an IC₅₀ for this compound exceeding those for the 5-substituted compounds by 2- and 5-fold, respectively (Figure 8).

The anti-proliferative effects of PMX independent of its impact on TS were previously attributed to its inhibition of AICARFTase in addition to GARFTase, as reflected in incomplete protection by AICA in the presence of thymidine, and the accumulation of the AICARFTase substrate ZMP in tumor cell lines treated with PMX, as measured by anion-exchange HPLC analysis.^{23,24} ZMP accumulation showed both dose- and time-dependence for PMX and resulted in activation of AMPK (via its role as an AMP mimetic) and suppression of mTOR.

To validate the findings from our AICA protection experiments with compound **8** (Figure 6), and to explore whether AICARFTase is a *bona fide* target for this novel 5-substituted pyrrolo[2,3-*d*]pyrimidine, analogous experiments were performed to measure accumulation of ZMP in KB cells treated with 1 μM of compound **8**, as a measure of AICARFTase inhibition. Parallel assays in KB cells treated with PMX were performed. ZMP accumulated in the presence of PMX and compound **8** in a linear fashion for up to 48 h (not shown), with PMX showing approximately 2-fold increased levels over compound **8** (Figure 9A).

To verify the impact of ZMP accumulation on AMPK phosphorylation in KB cells treated with compound **8** or with PMX, western blot analysis was performed of total and phosphorylated AMPK in KB cells treated with these antifolates for 48 h, compared to levels of phospho-AMPK resulting from treatments with established AMPK activators including AICA, AICA ribonucleoside (AICAR), and metformin²⁵ (Figure 9B). Although KB cells are LKB1-null (the principal upstream kinase responsible for phosphorylating AMPK), AMPK is still activated upon treatment with known AMPK activators (Figure 9B). While AMPK is activated by PMX, compound **8** was not significantly activating despite the accumulation of ZMP. Interestingly, neither AICA (not shown) nor metformin (Supplement, Figure 2S) at concentrations found to activate AMPK in Figure 9B (1 and 10 mM,

respectively) were particularly growth inhibitory toward KB cells (20% and 32% inhibition, respectively).

Molecular modeling: docking studies of compound **8** with GARFTase and AICARFTase

Based on the cellular metabolic data that compound **8** inhibits both GARFTase and AICARFTase in KB cells (Figures 8 and 9, respectively), molecular modeling studies were performed. Molecular modeling of compound **8** used LeadIT 1.3.0 and was visualized using MOE 2011.10. Redocking the native crystal structure ligands 10-CF₃CO-DDACTHF³⁶ (Figure 10) for human GARFTase (PDB ID: 1NJS) and BW2315, **18**³⁷ (Figure 10) for AICARFTase (PDB ID: 1PL0) into their respective crystal structures afforded docked poses with RMSD ~ 1 Å.

Figure 11 shows the docked pose of compound **8** in the human GARFTase active site. 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 three parts: the pteridine binding cleft, the benzoylglutamate region, and the formyl transfer region. The docked pose shows the pyrrolo[2,3-*d*]pyrimidine scaffold of **8** to be buried deep in the active site and to occupy the same location as the diaminopyrimidone ring in the native crystal structure ligand (10-CF₃CO-DDACTHF). This orientation of the scaffold permits the 2-amino moiety to form hydrogen bonds to the backbone of Glu141 and Ser93. The N1 nitrogen interacts with the backbone of Leu92 to form a hydrogen bond. The 4-oxo moiety forms a hydrogen bond with Asp144 and forms water-mediated hydrogen bonds with Asp142 and Ala140 (not shown).

The molecule is oriented in a manner which aids the N7 nitrogen to form a hydrogen bond with Arg90. The pyrrolo[2,3-*d*]pyrimidine scaffold resides in a hydrophobic pocket formed by Leu85, Ile91 (not shown), Leu92, Val97, and the folate binding loop residues 141–146. The flexible 4-atom side chain helps to orient the benzoyl moiety of compound **8** into the benzoylglutamate region of the protein. The amide NH of the glutamate side chain forms a hydrogen bond with Met89. The α-carboxylic acid of the glutamate side chain interacts with Arg64 and additionally interacts with the backbone of Ile91. The γ-carboxylic acid can form a water-mediated hydrogen bond with Arg90. The interaction of the flexible glutamate side chain is very similar to the interaction network observed for the glutamate side chain of 10-CF₃CO-DDACTHF.

Figure 12 shows the overlay of the docked poses of **8** (blue) with the crystal structure ligand **18** (red) (a potent inhibitor) in human AICARFTase (PDB ID: 1PL0). The pyrrolo[2,3-*d*]pyrimidine scaffold of **8** occupies the same location as the dihydroquinazoline scaffold of **18**. Analogous to **18**, the 2-NH₂ and N3 nitrogens of **8** interact with Asp546, while the 4-oxo moiety forms a hydrogen bond with the side chain of Asn547. The N7-nitrogen of **8** forms a hydrogen bond with the backbone of Met312. The pyrrolo[2,3-*d*]pyrimidine scaffold of **8** forms hydrophobic interactions with Met312, Phe315, Ile452, Pro543 and Phe544. The aryl glutamate section of **8** is oriented similar to the phenyl glutamate side chain of **18**.

These docking results predict that **8** should bind and inhibit the two folate-dependent purine biosynthetic enzymes (GARFTase and AICARFTase) and are entirely consistent with the results of our *in situ* metabolic assays (Figures 8 and 9).

DISCUSSION

The development of rationally designed cytotoxic antifolates remains an area of significant interest to anticancer drug development, as exemplified by the relatively recent approval of PMX³⁸ and pralatrexate³⁹ for cancer, more than 50 years since the introduction of MTX.¹

Unfortunately, the ubiquitous presence of RFC may contribute to untoward toxicities of these classical antifolates toward normal tissues. Based on this, growing attention has focused on developing novel folate-based compounds with tumor targeting premised on transport selectivity for FRs or PCFT over RFC,^{12,14–21} a pursuit that until recently^{40,41} continued to be challenged by the lack of structural information on the major folate transport systems.

The antitumor efficacy for the 5-substituted 2-carbon bridge pyrrolo[2,3-*d*]pyrimidine PMX reflects its cellular uptake by all the major folate transport systems.^{17–21} While the corresponding 6-substituted analog of PMX was pharmacologically inert, with increasing bridge lengths the 6-substituted pyrrolo[2,3-*d*]pyrimidines were surprisingly potent inhibitors of cell proliferation, although membrane transport by RFC was lost.^{16,21} The 6-pyrrolo[2,3-*d*]pyrimidine benzoyl side-chain analogs with 3- and 4-bridge carbons (compounds **1** and **2**, respectively), were the most active of this series, reflecting their FR and PCFT cellular uptake, with little to no transport by RFC, and their potent inhibitions of GARFTase, the first folate-dependent step in *de novo* purine nucleotide biosynthesis leading to the synthesis of IMP.^{16,21} Inhibition of GARFTase resulted in decreased *de novo* synthesis of purine nucleotides and ATP depletion, leading to cytotoxicity and apoptosis.^{16,21}

In this report, our goal was to further establish a comprehensive SAR for the 5- versus 6-substituted pyrrolo[2,3-*d*]pyrimidine ring system in relation to folate transporter specificity and inhibition of intracellular targets, based in part on findings of transporter promiscuity for PMX,^{17–21} and reports that PMX may in part derive its antitumor effects by inhibiting AICARTase, resulting in accumulation of ZMP and activation of AMPK in addition to inhibiting TS.^{23,24} Compared to the 6-substituted analogs, we found that the 5-substituted pyrrolo[2,3-*d*]pyrimidine compounds in general: (i) were better substrates for RFC with the highest antiproliferative activities for compounds with 2–4 bridge carbons independent of the nature of the side chain ring system; (ii) were poorer substrates for PCFT with the exception of the 2-carbon bridge compound, PMX (**6**); and (iii) preserved FR α cellular uptake, with the 4-carbon bridge analog **8** showing the greatest FR α potency in this expanded 5-substituted series of analogs without TS inhibitory activity.

Compound **8** was cytotoxic, as reflected in loss of clonogenicity in KB cells, and effected S-phase accumulation and apoptosis over 96 h. Like its 6-substituted counterpart compound **1**,²¹ compound **8** resulted in ATP depletion, although this appeared to be only partly due to inhibition of GARFTase. Rather, a unique feature of the 5-substituted analog **8** relates to its dual inhibition of both the GARFTase and AICARFTase reactions in *de novo* purine nucleotide biosynthesis. While PMX also inhibited GARFTase, the finding that the AICARFTase substrate ZMP accumulates in the presence of this drug suggests that GARFTase inhibition must be incomplete and that AICARFTase must also be a target as previously described,^{23,24} although for PMX, total purine nucleotides, as reflected in ATP pools, were significantly preserved. Compound **8**, also inhibited AICARFTase, as reflected in incomplete protection in cell proliferation assays by AICA and demonstrated accumulation of the AICARFTase substrate ZMP, albeit to a lesser extent than for a comparable concentration of PMX. ATP pools were significantly depleted (~90%) with compound **8**.

Previous studies suggested that AMPK activation secondary to ZMP accumulation and AICARFTase inhibition may result in inhibition of mTOR and contribute to the antitumor effects of high levels of PMX in the presence of thymidine.^{23,24} In the present study, we used KB tumor cells treated with PMX or with compound **8**. In this LKB1-deficient tumor cell line treated with PMX or compound **8**, ZMP accumulation was accompanied by AMPK

activation in the presence of PMX but not compound **8**. AMPK activation was also seen upon treatment with AICA, AICAR, or metformin. However, ZMP accumulation and AMPK activation did not appear likely to contribute to the anti-proliferative effects of PMX in KB cells, as neither AICA nor metformin (both of which activated AMPK more than PMX) potently inhibited cell proliferation. The antiproliferative and cytotoxic effects of compound **8** toward KB cells must derive from a depletion of ATP pools, secondary to inhibition of both GARFTase and AICARFTase.

In summary, a series of classical 5-substituted pyrrolo[2,3-*d*]pyrimidine antifolates **5–10** was designed and synthesized as a hybrid of PMX and 6-substituted pyrrolo[2,3-*d*]pyrimidines **1–4**. Compound **8**, the lead compound of this series, has an IC₅₀ of 12.7 nM against KB tumor cells in culture. Rather than only targeting GARFTase, as with **1** and **2**, both GARFTase and AICARFTase were identified as the intracellular targets of **8**. Further, based on patterns of inhibition of cell proliferation toward isogenic CHO cells expressing FR α or RFC, compound **8** appears to be transported by both FR α and RFC. The unique dual GARFTase and AICARFTase inhibition of compound **8** makes it an excellent lead compound for further study, including the design of additional antitumor analogs to optimize cellular folate uptake selectivity by FRs and inhibition of folate metabolizing enzymes, leading to a new generation of potent tumor-targeted agents. Further, the dual enzyme inhibition results in potent inhibition of purine nucleotide biosynthesis and could preserve antitumor activity in the event that tumors become resistant due to alterations in one or the other targeted enzyme. Such dual targeted inhibitors would seem to have substantial advantages over currently used agents that principally target only one intracellular enzyme target.

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. Nuclear magnetic resonance spectra for proton (¹H NMR) 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 ppm (parts per million) relative to tetramethylsilane as an internal standard: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad singlet. 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 (Supplement, Table 1S).

Ethyl 4-(3-oxopropyl)benzoate (13a)

To a solution of ethyl 4-iodobenzoate (**11**) (5 mmol, 1.38 g) in 20 mL anhydrous DMF was added prop-2-en-1-ol **12a** (6 mmol, 348 mg), LiCl (5 mmol, 210 mg), LiOAc (12.5 mmol, 850 mg), Bu₄NCl (2.5 mmol, 840 mg), Pd(OAc)₂ (0.3 mmol, 60 mg) and the mixture was stirred at 70 °C for 3 hours. TLC (hexane/EtOAc, 3:1) showed the disappearance of the starting material (*R_f* = 0.70) and formation of one major spot at *R_f* = 0.60. To the reaction mixture cooled to room temperature was added ethyl acetate (30 mL). The resulting solution was extracted with H₂O (10 mL × 3) and dried over Na₂SO₄. After evaporation of solvent, the residue was loaded on a silica gel column (4 × 20 cm) and flash-chromatographed with hexane/EtOAc (2:1) and the desired fractions were pooled. After evaporation of solvent the residue was dried in vacuo using P₂O₅ to afford **13a** 0.83 g, yield 83% as colorless liquid, *R_f* = 0.60 (hexane/EtOAc, 3:1). ¹H NMR (500M, CDCl₃) δ 1.38 (t, 3 H, CH₃, *J* = 7.0 Hz), 2.81 (t, 2 H, CH₂, *J* = 7.5 Hz), 3.01 (t, 2 H, CH₂, *J* = 7.5 Hz), 4.36 (q, 2 H, CH₂, *J* = 7.0 Hz), 7.26 (d, 2 H, 2 CH, *J* = 4.0 Hz), 7.97 (d, 2 H, 2 CH, *J* = 4.3 Hz), 9.82 (t, 1 H, CHO, *J* = 1.5 Hz).

Ethyl 4-(4-oxobutyl)benzoate (13b)

Compound **13b** was synthesized as described for **13a**: yield 88% as colorless liquid, *R_f* = 0.62 (hexane/EtOAc, 3:1). ¹H NMR (500M, CDCl₃) δ 1.39 (t, 3 H, CH₃, *J* = 7.0 Hz), 1.95–2.01 (m, 2 H, CH₂), 2.46 (t, 2 H, CH₂, *J* = 7.5 Hz), 2.71 (t, 2 H, CH₂, *J* = 7.5 Hz), 4.37 (q, 2 H, CH₂, *J* = 7.0 Hz), 7.24 (d, 2 H, 2 CH, *J* = 4.3 Hz), 7.97 (d, 2 H, 2 CH, *J* = 4.3 Hz), 9.76 (t, 1 H, CHO, *J* = 1.5 Hz).

Ethyl 4-(5-oxopentyl)benzoate (13c)

Compound **13c** was synthesized as described for **13a**: yield 84% as colorless liquid, *R_f* = 0.62 (hexane/EtOAc, 3:1). ¹H NMR (500M, CDCl₃) δ 1.38 (t, 3 H, CH₃, *J* = 7.0 Hz), 1.66–1.70 (m, 4 H, 2 CH₂), 2.46 (t, 2 H, CH₂, *J* = 7.0 Hz), 2.69 (t, 2 H, CH₂, *J* = 7.0 Hz), 4.36 (q, 2 H, CH₂, *J* = 7.0 Hz), 7.23 (d, 2 H, 2 CH, *J* = 4.0 Hz), 7.96 (d, 2 H, 2 CH, *J* = 4.0 Hz), 9.75 (t, 1 H, CHO, *J* = 1.5 Hz).

Ethyl 4-(6-oxohexyl)benzoate (13d)

Compound **13d** was synthesized as described for **13a**: yield 86% as colorless liquid, *R_f* = 0.62 (hexane/EtOAc, 3:1). ¹H NMR (500M, CDCl₃) δ 1.25–1.27 (m, 2 H, CH₂), 1.38 (t, 3 H, CH₃, *J* = 7.0 Hz), 1.64–1.68 (m, 4 H, 2 CH₂), 2.42 (t, 2 H, CH₂, *J* = 7.5 Hz), 2.66 (t, 2 H, CH₂, *J* = 7.5 Hz), 4.36 (q, 2 H, CH₂, *J* = 7.0 Hz), 7.23 (d, 2 H, 2 CH, *J* = 4.3 Hz), 7.96 (d, 2 H, 2 CH, *J* = 4.3 Hz), 9.75 (t, 1 H, CHO, *J* = 2.0 Hz).

Ethyl 4-(7-oxoheptyl)benzoate (13e)

Compound **13e** was synthesized as described for **13a**: yield 92% as colorless liquid, *R_f* = 0.63 (hexane/EtOAc, 3:1). ¹H NMR (500M, CDCl₃) δ 1.34–1.36 (m, 4 H, 2 CH₂), 1.38 (t, 3 H, CH₃, *J* = 7.0 Hz), 1.58–1.65 (m, 4 H, 2 CH₂), 2.42 (t, 2 H, CH₂, *J* = 7.5 Hz), 2.66 (t, 2 H, CH₂, *J* = 7.5 Hz), 4.36 (q, 2 H, CH₂, *J* = 7.0 Hz), 7.23 (d, 2 H, 2 CH, *J* = 4.3 Hz), 7.95 (d, 2 H, 2 CH, *J* = 4.3 Hz), 9.75 (t, 1 H, CHO, *J* = 2.0 Hz).

Ethyl 4-(8-oxooctyl)benzoate (13f)

Compound **13f** was synthesized as described for **13a**: yield 87% as colorless liquid, *R_f* = 0.63 (hexane/EtOAc, 3:1). ¹H NMR (500M, CDCl₃) δ 1.28–1.34 (m, 6 H, 3 CH₂), 1.38 (t, 3 H, CH₃, *J* = 7.0 Hz), 1.60–1.63 (m, 4 H, 2 CH₂), 2.42 (t, 2 H, CH₂, *J* = 7.5 Hz), 2.64 (t, 2 H, CH₂, *J* = 7.5 Hz), 4.36 (q, 2 H, CH₂, *J* = 7.0 Hz), 7.23 (d, 2 H, 2 CH, *J* = 4.3 Hz), 7.95 (d, 2 H, 2 CH, *J* = 4.3 Hz), 9.75 (t, 1 H, CHO, *J* = 2.0 Hz).

Ethyl 4-(3-bromo-4-oxobutyl)benzoate (14b)

To a solution of aldehyde **13b** (1 mmol, 220 mg) in 5 mL anhydrous Et₂O was added 5,5-dibromo-2,2-dimethyl-4,6-dioxo-1,3-dioxane (0.5 mmol, 150 mg), 2N HCl in Et₂O solution (0.1 mmol, 50 μ L) and the mixture was stirred at room temperature for 24 hours. TLC (hexane/EtOAc, 3:1) showed the disappearance of the starting material (R_f = 0.62) and formation of one major spot at R_f = 0.50. The reaction solution was washed with 5% NaHCO₃ solution and extracted with H₂O (10 mL $\times$ 3) and dried over Na₂SO₄. After evaporation of solvent, the residue was loaded on a silica gel column (4 $\times$ 20 cm) and flash-chromatographed with hexane/EtOAc (2:1) and the desired fractions were pooled. After evaporation of solvent the residue was dried in vacuo using P₂O₅ to afford **14b** 235 mg : yield 78% as colorless oil, R_f = 0.50 (hexane/EtOAc, 3:1). ¹H NMR (500M, CDCl₃) δ 1.39 (t, 3 H, CH₃, J = 7.0 Hz), 2.19–2.27 (m, 1 H, CH₂), 2.34–2.42 (m, 1 H, CH₂), 2.79–2.85 (m, 1 H, CH₂), 2.91–2.97 (m, 1 H, CH₂), 4.16–4.18 (m, 1 H, CHBr), 4.37 (q, 2 H, CH₂, J = 7.0 Hz), 7.28 (d, 2 H, 2 CH, J = 4.3 Hz), 7.99 (d, 2 H, 2 CH, J = 4.3 Hz), 9.46 (d, 1 H, CHO, J = 1.0 Hz).

Ethyl 4-(2-bromo-3-oxopropyl)benzoate (14a)

Compound **14a** was synthesized as described for **14b**. **14a** is not stable. Use directly to next step without purification.

Ethyl 4-(4-bromo-5-oxopentyl)benzoate (14c)

Compound **14c** was synthesized as described for **14b**: yield 77% as colorless oil, R_f = 0.52 (hexane/EtOAc, 3:1). ¹H NMR (500M, CDCl₃) δ 1.39 (t, 3 H, CH₃, J = 7.0 Hz), 1.73–1.82 (m, 2 H, CH₂), 1.86–1.96 (m, 1 H, CH₂), 2.03–2.10 (m, 1 H, CH₂), 2.71–2.75 (m, 2 H, CH₂), 4.21–4.25 (m, 1 H, CHBr), 4.37 (q, 2 H, CH₂, J = 7.0 Hz), 7.24 (d, 2 H, 2 CH, J = 4.3 Hz), 7.97 (d, 2 H, 2 CH, J = 4.3 Hz), 9.42 (d, 1 H, CHO, J = 1.3 Hz).

Ethyl 4-(5-bromo-6-oxohexyl)benzoate (14d)

Compound **14d** was synthesized as described for **14b**: yield 75% as colorless oil, R_f = 0.52 (hexane/EtOAc, 3:1). ¹H NMR (400M, CDCl₃) δ 1.39 (t, 3 H, CH₃, J = 7.0 Hz), 1.63–1.75 (m, 4 H, 2 CH₂), 1.87–1.99 (m, 1 H, CH₂), 2.03–2.12 (m, 1 H, CH₂), 2.69 (t, 2 H, CH₂, J = 7.0 Hz), 4.19–4.22 (m, 1 H, CHBr), 4.36 (q, 2 H, CH₂, J = 7.0 Hz), 7.23 (d, 2 H, 2 CH, J = 4.0 Hz), 7.96 (d, 2 H, 2 CH, J = 4.0 Hz), 9.42 (d, 1 H, CHO, J = 1.2 Hz).

Ethyl 4-(6-bromo-7-oxoheptyl)benzoate (14e)

Compound **14e** was synthesized as described for **14b**: yield 77% as colorless oil, R_f = 0.54 (hexane/EtOAc, 3:1). ¹H NMR (500M, CDCl₃) δ 1.30–1.40 (m, 2 H, CH₂), 1.38 (t, 3 H, CH₃, J = 7.0 Hz), 1.62–1.70 (m, 4 H, 2 CH₂), 1.86–1.94 (m, 1 H, CH₂), 2.00–2.07 (m, 1 H, CH₂), 2.66 (t, 2 H, CH₂, J = 7.5 Hz), 4.18–4.22 (m, 1 H, CHBr), 4.36 (q, 2 H, CH₂, J = 7.0 Hz), 7.23 (d, 2 H, 2 CH, J = 4.3 Hz), 7.95 (d, 2 H, 2 CH, J = 4.0 Hz), 9.42 (d, 1 H, CHO, J = 1.5 Hz).

Ethyl 4-(7-bromo-8-oxooctyl)benzoate (14f)

Compound **14f** was synthesized as described for **14b**: yield 74% as colorless oil, R_f = 0.56 (hexane/EtOAc, 3:1). ¹H NMR (500M, CDCl₃) δ 1.30–1.40 (m, 4 H, 2 CH₂), 1.38 (t, 3 H, CH₃, J = 7.0 Hz), 1.62–1.64 (m, 4 H, 2 CH₂), 1.86–1.94 (m, 1 H, CH₂), 2.00–2.07 (m, 1 H, CH₂), 2.65 (t, 2 H, CH₂, J = 7.5 Hz), 4.19–4.22 (m, 1 H, CHBr), 4.36 (q, 2 H, CH₂, J = 7.0 Hz), 7.23 (d, 2 H, 2 CH, J = 4.0 Hz), 7.95 (d, 2 H, 2 CH, J = 4.0 Hz), 9.42 (d, 1 H, CHO, J = 1.5 Hz).

Ethyl 4-(2-(2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)ethyl)benzoate (16b)

To a solution of 2,6-diamino-6,4-oxopyrimidine **15** (151 mg, 1.2 mmol) and sodium acetate (180 mg, 2.2 mmol) in water (3 mL) and methanol (3 mL) was added α -bromo aldehyde **14b** (330 mg, 1.1 mmol). The reaction mixture was stirred at 45 °C for 3 hours. TLC showed the disappearance of starting materials and the formation of one major spot at R_f = 0.38 (CHCl₃/MeOH, 5:1). After evaporation of solvent, CH₃OH (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 CHCl₃ followed by 10% MeOH in CHCl₃ and then 15% MeOH in CHCl₃. Fractions showing R_f = 0.38 were pooled and evaporated to afford **16b** 230 mg, yield 70% as a pink solid: mp > 250 °C decomposed. ¹H NMR (500M, DMSO-*d*₆) δ 1.30 (t, 3 H, CH₃, J = 7.5 Hz), 2.84 (t, 2 H, CH₂, J = 7.0 Hz), 2.99 (t, 2 H, CH₂, J = 7.0 Hz), 4.29 (q, 2 H, CH₂, J = 7.5 Hz), 6.03 (s, 2 H, 2-NH₂), 6.29 (d, 1 H, CH, J = 1.0 Hz), 7.33 (d, 2 H, CH, J = 4.0 Hz), 7.85 (d, 2 H, CH, J = 4.0 Hz), 10.21 (s, 1 H, 3-NH), 10.60 (s, 1H, 7-NH).

Ethyl 4-((2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)methyl)benzoate (16a)

Compound **16a** was synthesized as described for **16b**: yield 35% over two steps as a yellow solid: mp > 232 °C decomposed, R_f = 0.42 (CHCl₃/MeOH, 5:1). ¹H NMR (500M, DMSO-*d*₆) δ 1.29 (t, 3 H, CH₃, J = 7.5 Hz), 4.00 (s, 2 H, CH₂), 4.28 (q, 2 H, CH₂, J = 7.5 Hz), 6.00 (s, 2 H, 2-NH₂), 6.34 (d, 1 H, CH, J = 1.0 Hz), 7.41 (d, 2 H, CH, J = 4.0 Hz), 7.83 (d, 2 H, CH, J = 4.0 Hz), 10.12 (s, 1 H, 3-NH), 10.74 (s, 1H, 7-NH).

Ethyl 4-(3-(2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)propyl)benzoate (16c)

Compound **16c** was synthesized as described for **16b**: yield 68% as a purple solid: mp > 264 °C decomposed, R_f = 0.42 (CHCl₃/MeOH, 5:1). ¹H NMR (500M, DMSO-*d*₆) δ 1.30 (t, 3 H, CH₃, J = 7.5 Hz), 1.89–1.95 (m, 2 H, CH₂), 2.57 (t, 2 H, CH₂, J = 7.5 Hz), 2.66 (t, 2 H, CH₂, J = 7.5 Hz), 4.29 (q, 2 H, CH₂, J = 7.5 Hz), 5.96 (s, 2 H, 2-NH₂), 6.35 (d, 1 H, CH, J = 1.0 Hz), 7.34 (d, 2 H, CH, J = 4.0 Hz), 7.86 (d, 2 H, CH, J = 4.0 Hz), 10.08 (s, 1 H, 3-NH), 10.63 (s, 1H, 7-NH).

Ethyl 4-(4-(2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)butyl)benzoate (16d)

Compound **16d** was synthesized as described for **16b**: yield 53% as a blue solid: mp > 256 °C decomposed, R_f = 0.44 (CHCl₃/MeOH, 5:1). ¹H NMR (500M, DMSO-*d*₆) δ 1.30 (t, 3 H, CH₃, J = 7.5 Hz), 1.56–1.62 (m, 4 H, 2 CH₂), 2.58 (t, 2 H, CH₂, J = 7.5 Hz), 2.65 (t, 2 H, CH₂, J = 7.5 Hz), 4.29 (q, 2 H, CH₂, J = 7.5 Hz), 5.94 (s, 2 H, 2-NH₂), 6.31 (d, 1 H, CH, J = 1.0 Hz), 7.33 (d, 2 H, CH, J = 4.0 Hz), 7.85 (d, 2 H, CH, J = 4.0 Hz), 10.08 (s, 1 H, 3-NH), 10.59 (s, 1H, 7-NH).

Ethyl 4-(5-(2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)pentyl)benzoate (16e)

Compound **16e** was synthesized as described for **16b**: yield 60% as a pink solid: mp > 212 °C decomposed, R_f = 0.45 (CHCl₃/MeOH, 5:1). ¹H NMR (500M, DMSO-*d*₆) δ 1.29–1.32 (m, 5 H, CH₃, CH₂), 1.58–1.62 (m, 4 H, 2 CH₂), 2.53 (t, 2 H, CH₂, J = 7.5 Hz), 2.63 (t, 2 H, CH₂, J = 7.5 Hz), 4.29 (q, 2 H, CH₂, J = 7.5 Hz), 5.94 (s, 2 H, 2-NH₂), 6.30 (d, 1 H, CH, J = 1.0 Hz), 7.33 (d, 2 H, CH, J = 4.0 Hz), 7.85 (d, 2 H, CH, J = 4.0 Hz), 10.08 (s, 1 H, 3-NH), 10.58 (s, 1H, 7-NH).

Ethyl 4-(6-(2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)hexyl)benzoate (16f)

Compound **16f** was synthesized as described for **16b**: yield 52% as a pink solid: mp > 208 °C decomposed, R_f = 0.45 (CHCl₃/MeOH, 5:1). ¹H NMR (500M, DMSO-*d*₆) δ 1.27–1.33 (m, 7 H, CH₃, 2 CH₂), 1.53–1.60 (m, 4 H, 2 CH₂), 2.53 (t, 2 H, CH₂, J = 7.5 Hz), 2.62 (t, 2 H, CH₂, J = 7.5 Hz), 4.29 (q, 2 H, CH₂, J = 7.5 Hz), 5.94 (s, 2 H, 2-NH₂), 6.30 (d, 1 H, CH, J = 1.0 Hz), 7.33 (d, 2 H, CH, J = 4.0 Hz), 7.86 (d, 2 H, CH, J = 4.0 Hz), 10.06 (s, 1 H, 3-NH), 10.57 (s, 1H, 7-NH).

(S)-diethyl 2-(4-((2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)methyl)benzamido)pentanedioate (17a)

To a suspension of **16a** (100 mg, 0.35 mmol) in 10 mL CH₃OH was added 3 N NaOH (10 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 3 to 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 (10 mL) was added 6-chloro-2,4-dimethoxy-1,3,5-triazine (72 mg, 0.42 mmol) and *N*-methylmorpholine (43 mg, 0.42 mmol). After the mixture was stirred at r.t. for 2 h, *N*-methylmorpholine (43 mg, 0.42 mmol) and dimethyl *L*-glutamate hydrochloride (126 mg, 0.53 mmol) were added all at once. The mixture was stirred at r.t. for 4 h. TLC showed the formation of one major spot at R_f = 0.38 (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 = 0.38 were pooled and evaporated to dryness to afford **17a** 100 mg, yield 61% as a yellow syrup. ¹H NMR (400M, DMSO-*d*₆) δ 1.14–1.19 (m, 6 H, 2 CH₃), 1.88–2.15 (m, 2 H, CH₂), 2.42–2.45 (m, 2 H, CH₂), 3.98 (s, 2 H, CH₂), 4.03–4.12 (m, 4 H, 2 CH₂), 4.36–4.46 (m, 1 H, CH), 6.00 (s, 2 H, 2-NH₂), 6.32 (d, 1 H, CH, J = 1.0 Hz), 7.37 (d, 2 H, C₆H₄, J = 4.0 Hz), 7.74 (d, 2 H, C₆H₄, J = 4.0 Hz), 8.60 (d, 1 H, CONH, J = 4.0 Hz), 10.13 (s, 1 H, 3-NH), 10.74 (s, 1H, 7-NH).

(S)-diethyl 2-(4-(2-(2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)ethyl)benzamido)pentanedioate (17b)

Compound **17b** was synthesized as described for **17a**: yield 51% as a colorless syrup, R_f = 0.40 (CHCl₃/MeOH, 5:1). ¹H NMR (500M, DMSO-*d*₆) δ 1.15–1.20 (m, 6 H, 2 CH₃), 1.97–2.03 (m, 1 H, CH₂), 2.06–2.13 (m, 1 H, CH₂), 2.42–2.45 (m, 2 H, CH₂), 2.85 (t, 2 H, CH₂, J = 7.0 Hz), 2.97 (t, 2 H, CH₂, J = 7.0 Hz), 4.02–4.13 (m, 4 H, 2 CH₂), 4.40–4.44 (m, 1 H, CH), 5.99 (s, 2 H, 2-NH₂), 6.30 (d, 1 H, CH, J = 1.0 Hz), 7.29 (d, 2 H, C₆H₄, J = 4.0 Hz), 7.77 (d, 2 H, C₆H₄, J = 4.0 Hz), 8.61 (d, 1 H, CONH, J = 4.0 Hz), 10.13 (s, 1 H, 3-NH), 10.60 (s, 1H, 7-NH).

(S)-diethyl 2-(4-(3-(2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)propyl)benzamido)pentanedioate (17c)

Compound **17c** was synthesized as described for **17a**: yield 65% as a colorless syrup, R_f = 0.40 (CHCl₃/MeOH, 5:1). ¹H NMR (DMSO-*d*₆) δ 1.15–1.20 (m, 6 H, 2 CH₃), 1.91–1.94 (m, 2 H, CH₂), 1.97–2.03 (m, 1 H, CH₂), 2.06–2.13 (m, 1 H, CH₂), 2.42–2.45 (m, 2 H, CH₂), 2.58 (t, 2 H, CH₂, J = 7.0 Hz), 2.64 (t, 2 H, CH₂, J = 7.0 Hz), 4.02–4.13 (m, 4 H, 2 CH₂),

4.42–4.45 (m, 1 H, CH), 5.95 (s, 2 H, 2-NH₂), 6.35 (d, 1 H, CH, *J* = 1.0 Hz), 7.29 (d, 2 H, C₆H₄, *J* = 4.0 Hz), 7.79 (d, 2 H, C₆H₄, *J* = 4.0 Hz), 8.63 (d, 1 H, CONH, *J* = 4.0 Hz), 10.08 (s, 1 H, 3-NH), 10.62 (s, 1H, 7-NH).

(S)-diethyl 2-(4-(4-(2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)butyl)benzamido)pentanedioate (17d)

Compound **17d** was synthesized as described for **17a**: yield 70% as a light blue syrup, *R_f* = 0.40 (CHCl₃/MeOH, 5:1). ¹H NMR (500M, DMSO-*d*₆) δ 1.15–1.20 (m, 6 H, 2 CH₃), 1.55–1.64 (m, 4 H, 2 CH₂), 1.97–2.03 (m, 1 H, CH₂), 2.06–2.13 (m, 1 H, CH₂), 2.42–2.45 (m, 2 H, CH₂), 2.58 (t, 2 H, CH₂, *J* = 7.5 Hz), 2.65 (t, 2 H, CH₂, *J* = 7.5 Hz), 4.02–4.13 (m, 4 H, 2 CH₂), 4.42–4.45 (m, 1 H, CH), 5.94 (s, 2 H, 2-NH₂), 6.31 (d, 1 H, CH, *J* = 1.0 Hz), 7.28 (d, 2 H, C₆H₄, *J* = 4.0 Hz), 7.77 (d, 2 H, C₆H₄, *J* = 4.0 Hz), 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-(4-(5-(2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)pentyl)benzamido)pentanedioate (17e)

Compound **17e** was synthesized as described for **17a**: yield 67% as a light blue syrup, *R_f* = 0.40 (CHCl₃/MeOH, 5:1). ¹H NMR (500M, DMSO-*d*₆) δ 1.15–1.20 (m, 6 H, 2 CH₃), 1.29–1.33 (m, 2 H, CH₂), 1.55–1.62 (m, 4 H, 2 CH₂), 1.97–2.03 (m, 1 H, CH₂), 2.06–2.13 (m, 1 H, CH₂), 2.42–2.45 (m, 2 H, CH₂), 2.51–2.53 (m, 2 H, CH₂), 2.62 (t, 2 H, CH₂, *J* = 7.5 Hz), 4.02–4.12 (m, 4 H, 2 CH₂), 4.41–4.45 (m, 1 H, CH), 5.94 (s, 2 H, 2-NH₂), 6.31 (d, 1 H, CH, *J* = 1.0 Hz), 7.28 (d, 2 H, C₆H₄, *J* = 4.0 Hz), 7.78 (d, 2 H, C₆H₄, *J* = 4.0 Hz), 8.62 (d, 1 H, CONH, *J* = 4.0 Hz), 10.07 (s, 1 H, 3-NH), 10.58 (s, 1H, 7-NH).

(S)-diethyl 2-(4-(6-(2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)hexyl)benzamido)pentanedioate (17f)

Compound **17f** was synthesized as described for **17a**: yield 78% as a colorless syrup, *R_f* = 0.42 (CHCl₃/MeOH, 5:1). ¹H NMR (500M, DMSO-*d*₆) δ 1.15–1.20 (m, 6 H, 2 CH₃), 1.27–1.34 (m, 4 H, 2 CH₂), 1.53–1.62 (m, 4 H, 2 CH₂), 1.97–2.03 (m, 1 H, CH₂), 2.06–2.13 (m, 1 H, CH₂), 2.42–2.45 (m, 2 H, CH₂), 2.51–2.53 (m, 2 H, CH₂), 2.61 (t, 2 H, CH₂, *J* = 7.5 Hz), 4.02–4.12 (m, 4 H, 2 CH₂), 4.41–4.44 (m, 1 H, CH), 5.93 (s, 2 H, 2-NH₂), 6.30 (d, 1 H, CH, *J* = 1.0 Hz), 7.28 (d, 2 H, C₆H₄, *J* = 4.0 Hz), 7.78 (d, 2 H, C₆H₄, *J* = 4.0 Hz), 8.62 (d, 1 H, CONH, *J* = 4.0 Hz), 10.06 (s, 1 H, 3-NH), 10.57 (s, 1H, 7-NH).

(S)-2-(4-((2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)methyl)benzamido)pentanedioic acid (5)

To **17a** (75 mg, 0.26 mmol) was added 1 N NaOH (3 mL). The resulting mixture was stirred at r.t. 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 3 to 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 94% as a yellow powder: mp > 264 °C decomposed, *R_f* = 0.05 (CHCl₃/MeOH, 5:1). ¹H NMR (400M, DMSO-*d*₆) δ 1.88–2.15 (m, 2 H, CH₂), 2.42–2.45 (m, 2 H, CH₂), 3.98 (s, 2 H, CH₂), 4.37–4.39 (m, 1 H, CH), 6.00 (s, 2 H, 2-NH₂), 6.32 (s, 1 H, CH), 7.37 (d, 2 H, C₆H₄, *J* = 4.0 Hz), 7.75 (d, 2 H, C₆H₄, *J* = 4.0 Hz), 8.47 (d, 1 H, CONH, *J* = 4.0 Hz), 10.13 (s, 1 H, 3-NH), 10.73 (s, 1H, 7-NH). Anal. (C₁₉H₁₉N₅O₆ · 1.5 H₂O).

(S)-2-(4-(2-(2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)ethyl)benzamido)pentanedioic acid (6)

Compound **6** was synthesized as described for **5**: yield 96% as a pink powder: mp > 250 °C decomposed, identical in all respects (NMR, mp) reported by the method of Taylor et al.,⁴² R_f = 0.07 (CHCl₃/MeOH, 5:1). ¹H NMR (400M, DMSO-*d*₆) δ 1.97–2.03 (m, 1 H, CH₂), 2.06–2.13 (m, 1 H, CH₂), 2.42–2.45 (m, 2 H, CH₂), 2.85 (t, 2 H, CH₂, J = 7.0 Hz), 2.97 (t, 2 H, CH₂, J = 7.0 Hz), 4.36–4.40 (m, 1 H, CH), 6.00 (s, 2 H, 2-NH₂), 6.30 (s, 1 H, CH), 7.28 (d, 2 H, C₆H₄, J = 4.0 Hz), 7.78 (d, 2 H, C₆H₄, J = 4.0 Hz), 8.51 (d, 1 H, CONH, J = 4.0 Hz), 10.15 (s, 1 H, 3-NH), 10.61 (s, 1H, 7-NH). Anal. (C₂₀H₂₁N₅O₆ · 1.2 H₂O).

(S)-2-(4-(3-(2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)propyl)benzamido)pentanedioic acid (7)

Compound **7** was synthesized as described for **5**: yield 97% as a pale blue powder: mp > 189 °C decomposed, R_f = 0.07 (CHCl₃/MeOH, 5:1). ¹H NMR (500M, DMSO-*d*₆) δ 1.90–1.94 (m, 2 H, CH₂), 1.94–2.00 (m, 1 H, CH₂), 2.06–2.13 (m, 1 H, CH₂), 2.33–2.36 (m, 2 H, CH₂), 2.58 (t, 2 H, CH₂, J = 7.0 Hz), 2.64 (t, 2 H, CH₂, J = 7.0 Hz), 4.36–4.40 (m, 1 H, CH), 5.96 (s, 2 H, 2-NH₂), 6.35 (d, 1 H, CH, J = 1.0 Hz), 7.29 (d, 2 H, C₆H₄, J = 4.0 Hz), 7.79 (d, 2 H, C₆H₄, J = 4.0 Hz), 8.51 (d, 1 H, CONH, J = 4.0 Hz), 10.08 (s, 1 H, 3-NH), 10.62 (s, 1H, 7-NH). Anal. (C₂₁H₂₃N₅O₆ · 0.70 H₂O).

(S)-2-(4-(4-(2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)butyl)benzamido)pentanedioic acid (8)

Compound **8** was synthesized as described for **5**: yield 88% as a blue powder: mp > 153 °C decomposed, R_f = 0.07 (CHCl₃/MeOH, 5:1). ¹H NMR (500M, DMSO-*d*₆) δ 1.55–1.63 (m, 4 H, 2 CH₂), 1.92–2.00 (m, 1 H, CH₂), 2.03–2.13 (m, 1 H, CH₂), 2.34 (t, 2 H, CH₂, J = 7.5 Hz), 2.58 (t, 2 H, CH₂, J = 7.5 Hz), 2.63 (t, 2 H, CH₂, J = 7.5 Hz), 4.37–4.39 (m, 1 H, CH), 5.95 (s, 2 H, 2-NH₂), 6.31 (d, 1 H, CH, J = 1.0 Hz), 7.28 (d, 2 H, C₆H₄, J = 4.0 Hz), 7.78 (d, 2 H, C₆H₄, J = 4.0 Hz), 8.49 (d, 1 H, CONH, J = 4.0 Hz), 10.09 (s, 1 H, 3-NH), 10.58 (s, 1H, 7-NH). Anal. (C₂₂H₂₅N₅O₆ · 1.2 H₂O).

(S)-2-(4-(5-(2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)pentyl)benzamido)pentanedioic acid (9)

Compound **9** was synthesized as described for **5**: yield 93% as a blue powder: mp > 167 °C decomposed, R_f = 0.08 (CHCl₃/MeOH, 5:1). ¹H NMR (500M, DMSO-*d*₆) δ 1.29–1.32 (m, 4 H, CH₂), 1.58–1.62 (m, 4 H, 2 CH₂), 1.92–2.00 (m, 1 H, CH₂), 2.03–2.13 (m, 1 H, CH₂), 2.34 (t, 2 H, CH₂, J = 7.5 Hz), 2.53–2.55 (m, 2 H, CH₂), 2.62 (t, 2 H, CH₂, J = 7.5 Hz), 4.37–4.40 (m, 1 H, CH), 5.94 (s, 2 H, 2-NH₂), 6.31 (d, 1 H, CH, J = 1.0 Hz), 7.28 (d, 2 H, C₆H₄, J = 4.0 Hz), 7.78 (d, 2 H, C₆H₄, J = 4.0 Hz), 8.50 (d, 1 H, CONH, J = 4.0 Hz), 10.08 (s, 1 H, 3-NH), 10.58 (s, 1H, 7-NH). Anal. (C₂₃H₂₇N₅O₆ · 1.0 H₂O).

(S)-2-(4-(6-(2-amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-5-yl)hexyl)benzamido)pentanedioic acid (10)

Compound **10** was synthesized as described for **5**: yield 93% as a blue powder: mp > 178 °C decomposed, R_f = 0.10 (CHCl₃/MeOH, 5:1). ¹H NMR (500M, DMSO-*d*₆) δ 1.27–1.32 (m, 4 H, 2 CH₂), 1.53–1.62 (m, 4 H, 2 CH₂), 1.92–2.00 (m, 1 H, CH₂), 2.03–2.13 (m, 1 H, CH₂), 2.34 (t, 2 H, CH₂, J = 7.5 Hz), 2.52–2.54 (m, 2 H, CH₂), 2.60 (t, 2 H, CH₂, J = 7.5 Hz), 4.36–4.41 (m, 1 H, CH), 5.94 (s, 2 H, 2-NH₂), 6.31 (d, 1 H, CH, J = 1.0 Hz), 7.28 (d, 2 H, C₆H₄, J = 4.0 Hz), 7.78 (d, 2 H, C₆H₄, J = 4.0 Hz), 8.50 (d, 1 H, CONH, J = 4.0 Hz), 10.07 (s, 1 H, 3-NH), 10.57 (s, 1H, 7-NH). Anal. (C₂₄H₂₉N₅O₆ · 1.77CH₃OH · 0.1HCl).

Molecular modeling and computational studies

The X-ray crystal structures of human GARFTase at 1.98 Å resolution (PDB ID: 1NJS)³⁶ and human AICARFTase at 2.55 Å resolution (PDB ID: 1P4R)³⁷ were obtained from the protein database. The GARFTase crystal structure contains human GARFTase complexed with the hydrolyzed form of 10-trifluoroacetyl-5,10-dideaza-acyclic-5,6,7,8-tetrahydrofolic acid (10-CF₃CO-DDACTHF) while the AICARFTase crystal structure contains human AICARFTase complexed with the sulfamido-bridged 5,8-dideazafolate analog BW1540.

Docking studies were performed using LeadIT 1.3.⁴³ The protonation state of the proteins and the ligands were calculated using the default settings. Water molecules in the active site 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 2010.10⁴⁴ and energy minimized using the MMFF94X forcefield to a constant of 0.05 kcal/mol. Triangle matching was used as the placement method and the docked poses were scored using default settings. The docked poses were exported and visualized in MOE.

Molecules used for the docking experiments were constructed in MOE 2010.11 and minimized using the MMFF94x forcefield to a constant of 0.05 kcal/mol. In order to validate the docking software for docking the proposed compounds, the native ligands 10-CF₃CO-DDACTHF for GARFTase and BW1540 for AICARFTase, were built using the molecule builder function in MOE, energy minimized and docked, as described above. RMSD of the docked poses were calculated using an SVL code obtained from the MOE website (www.chemcomp.com) and compared to the conformation of the crystal structure ligands.

The best docked pose for 10-CF₃CO-DDACTHF in the human GARFTase crystal structure had an RMSD of 1.0368 Å and the best docked pose for BW1540 had an RMSD of 1.0995 Å in the AICARFTase crystal structure. Thus LeadIT 1.3. was validated for our docking purposes in GARFTase and AICARFTase.

Reagents for biological studies

[3', 5', 7-³H]MTX (20 Ci/mmol), [3', 5', 7, 9-³H] folic acid (25 Ci/mmol), and [¹⁴C(U)]-glycine (87mCi/mmol) were purchased from Moravek Biochemicals (Brea, CA). Unlabeled folic acid was purchased from the 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); LMTX (5,10-dideaza-5,6,7,8-tetrahydrofolate), Eli Lilly and Co. (Indianapolis, IN); and PMX [N-{4-[2-(2-amino-3,4-dihydro-4-oxo-7H-pyrrolo[2,3-d]pyrimidin-5-yl)ethyl]benzoyl}-L-glutamic acid] (Alimta) (LC Laboratories, Woburn, MA). Other chemicals were obtained from commercial sources in the highest available purity.

Cell culture

MTXR2IOua^{R2-4} (referred to as R2) is a RFC-, PCFT- and FR α -null Chinese hamster ovary (CHO) cell line that was a gift from Dr. Wayne Flintoff (University of Western Ontario).⁴⁵ 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).^{16-21,46} 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 (FF-RPMI) (Invitrogen) with dialyzed fetal bovine serum (DFBS) (Invitrogen), supplemented with 5% penicillin-streptomycin and *L*-glutamate. FR α -expressing human KB nasopharyngeal carcinoma cells were continuously maintained in complete FF-RPMI with 10% fetal bovine serum and 5% penicillin-streptomycin and *L*-glutamate.

Cell proliferation assays were performed exactly as previously described.^{16–20} Cell lines were treated with a range of concentrations of standard or novel antifolates (0–1 μ M), metformin (0–10 mM) or AICA (0–2 mM) in a 96 well plate with in FF-RPMI complete with DFBS, and 2 nM (RT-16, KB, IGROV1) 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, media was removed and Cell Titer-blue™ fluorescent reagent (Promega) was added. Relative cell numbers were determined by relative 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.

In order 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 AICA hydrochloride (320 μ M), and results compared to incubations in parallel without nucleoside/AICA or drug additions.

Colony-forming assays were performed with compound **8** to verify a cytotoxic (as opposed to cytostatic) response. KB cells (~500 cells) were plated in 60 mm dishes in FF-RPMI/DFBS supplemented with 2 nM LCV for a 24 h incubation period at 37° C in the presence of 5% CO₂. Concentrations from 0–10 μ M of compound **8** were added to media for an additional 24 h, after which the media was aspirated and replaced with complete FF-RPMI supplemented with 2 nM LCV for 8–10 days. To visualize colonies, the cells were washed (2x) with room temperature Dulbecco's phosphate-buffered saline (PBS) (Sigma), followed by a wash of 5% trichloroacetic acid (TCA). The TCA was removed, 10 mM borate buffer (pH 8.8) was added, followed by a 30 min incubation with 1% methylene blue in borate buffer at room temperature. The cells were washed three times with borate buffer and the stained colonies were counted.

Competitive MTX transport assays

To determine if novel antifolates bound to RFC, reflecting membrane transport by this mechanism, competition with [³H]MTX for cellular uptake was measured in RFC-expressing PC43-10 CHO cells.^{16,19} PC43-10 cells (~1.5×10⁶ cells) were seeded in 60 mm dishes. After 48 h, cells were washed with PBS and uptake of [³H]MTX (0.5 μ M) was measured in Hank's Balanced Salts Solution for 2 min at 37° C. Ice-cold PBS was used to quench the reactions. Cells were washed with ice-cold PBS (3X), then solubilized with 0.5N NaOH. The cellular homogenates were assayed for radioactivity with a liquid scintillation counter; protein concentrations were measured with Folin-phenol reagent.⁴⁷ Levels of drug uptake were expressed as pmol/mg cell protein.

FR α binding assay

To determine the binding affinity of novel antifolates to FR α , FR α -expressing RT16 CHO cells ($\sim 1.5 \times 10^6$ cells) were seeded in a monolayer with complete α -MEM media. Cells were washed with ice-cold PBS, followed by ice-cold acetate buffer (10 mM sodium acetate, 150 mM NaCl, pH 3.5) to release FR α -bound folates, then neutralized with ice-cold Hepes-buffered saline (HBS) (20 mM Hepes, 140 mM NaCl, 5 mM KCl, 2mM MgCl₂, 5 mM glucose, pH 7.4). Cells were then incubated at 0°C for 15 min in a cocktail containing [3', 5', 7', 9 ³H]folic acid, in the absence or presence of unlabeled folic acid or novel antifolate compounds over a range of concentrations. Cells were washed with ice-cold HBS and solubilized with 0.5 N NaOH. The cell homogenates were collected and analyzed for radioactivity using a liquid scintillation counter. Protein concentrations were measured with Folin-phenol reagent.⁴⁷ Bound [³H]folic acid was expressed as pmol/mg protein. Using Sigma plot (v. 12) software, dose-response curves were created and IC_{50s} were calculated. Relative binding affinities were calculated as the inverse molar ratios of unlabeled ligands required to inhibit [³H]folic acid by 50%, as determined graphically with Graphpad (v. 6.0) software. By definition, the relative affinity of folic acid is 1.

HPLC detection of ATP

Intracellular ATP concentrations were determined using nucleotide extractions and an HPLC method, exactly as previously described.^{21,34} 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 of PMX, or compounds **2** or **8** 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.²¹

Apoptosis and cell cycle analyses

Apoptosis and cell cycle analyses were performed through the Microscopy, Imaging, and Cytometry Resources Core of the Karmanos Cancer Institute. KB cells were treated with 1 μ M of PMX, or compounds **2** or **8**, for 48 and 96 h in FF-RPMI media complete with 10% DFBS and 5% penicillin-streptomycin and 2 nM LCV. Cells were harvested by trypsinization and washed with cold PBS. Cells were equally divided into two pools and either assayed for cell cycle distribution or for apoptosis by flow cytometry using the BD FACSCanto™ II flow cytometer operated with BD FACSDiva™ software (v6.0) (BD Biosciences). For cell cycle analysis, cells were fixed with ice-cold ethanol (added drop-wise) and then stained with 50 μ g/mL PI and 100 μ g/mL RNase (type I-A) (Sigma) in PBS for at least 20 min at room temperature in the dark prior to analysis by flow cytometry with a minimum of 10,000 events (cells) analyzed. Apoptosis was measured using the annexin V-FITC/7-AAD kit, as specified by the manufacturer (Beckman Coulter) with a minimum of 20,000 events (cells) analyzed for early apoptosis (annexin-V-FITC^{high}/7-AAD^{low}; Q3) and late apoptosis/necrosis (annexin-V-FITC^{high}/7-AAD^{high}; Q2); viable cells are shown in Q4 (annexin-V-FITC^{low}/7-AAD^{low}). All data were analyzed with FlowJo (v7.6.1) software (Tree Star, Inc.). The Watson Pragmatic model was used to analyze cell cycle distributions of G₁/G₀-, S-, and G₂/M-phase cells and to estimate the sub-G₁ (late apoptotic cell) fraction.

In situ GARFTase inhibition assays

To measure the extent of intracellular GARFTase inhibition in the presence of antifolate inhibitors, we examined the metabolic incorporation of [¹⁴C(U)] glycine into [¹⁴C]formyl GAR in the presence of azaserine.^{16–21,33} KB cells were seeded in complete media (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 media

containing 2 nM LCV, with PMX, or with compounds **2** or **8** 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 allowed to incubate for 30 minutes. This was followed by the addition of *L*-glutamine (2 mM) and [14 C(U)]glycine (final specific activity 0.1 mCi/L). Cells were incubated 16 h. Cells were washed with ice-cold PBS, trypsinized and collected. Cells were treated with 5% 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 (Bio-rad) of AG1x8 (chloride form). The columns were washed with 10 mL 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 8 fractions. The eluate was measured for radioactivity and the percentages of radioactivity in the [14 C]formyl GAR and non-specific [14 C]fractions. The results were calculated as pmol [14 C]formyl GAR/mg protein. Data were graphed using Sigmaplot (v.12) and the IC₅₀s 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 based on those described by Rascanelli *et al.*^{23,24} Briefly, KB cells were treated with PMX or compound **8** for 48 h in FF-RPMI with 10% DFBS, 5% penicillin-streptomycin, 2 mM *L*-glutamine, and 2 nM LCV. Cells were washed with PBS, 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 $\times$) and fractionated by ion-exchange HPLC on a SAX 250 $\times$ 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 absorbances to a standard curve with commercial ZMP. AraCMP was used as an internal control.

Western blot analysis

KB cells were treated for 48 h with PMX or with compound **8** in the presence of thymidine (10 μ M). Controls include vehicle control (water or DMSO, appropriate; results were identical), or known activators of AMPK, including metformin (10 mM), AICA (1 mM), or AICA ribonucleoside (AICAR) (1 mM). Cells were washed, suspended in 10 mM Tris-HCl (pH 7.0) plus proteolytic and phosphatase inhibitors (Roche Applied Science), and disrupted by sonication. Samples were centrifuged (14,000 rpm, 10 min) and 90 μ g protein were fractionated by SDS polyacrylamide gel electrophoresis on Laemmli gradient gels (4–20%).⁴⁸ Proteins were electrotransferred to polyvinylidene difluoride membranes (Pierce).⁴⁹ Detection of immunoreactive AMPK was with rabbit total and phospho-AMPK antibodies (Cell Signaling) and IRDye800-conjugated secondary anti-rabbit antibody (LI-COR, Lincoln, NE) using an Odyssey® infrared imaging system (LI-COR). β -actin was detected with anti- β -actin mouse antibody (Sigma) and IRDye800-conjugated secondary anti-mouse antibody (LI-COR).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS USED

(IC₅₀)	50% inhibitory concentration
(AAD)	α -minimal essential medium (α -MEM) amino actinomycin D
(AICA)	5-aminoimidazole-4-carboxamide
(AICAR)	5-aminoimidazole-4-carboxamide ribonucleoside
(AICARFTase)	5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase
(AMPK)	AMP-activated protein kinase
(CHO)	Chinese hamster ovary
(DFBS)	dialyzed fetal bovine serum
(DHFR)	dihydrofolate reductase
(FITC)	fluorescein isothiocyanate
(FF-RPMI)	folate-free RPMI1640
(FR)	folate receptor
(GAR)	glycinamide ribonucleotide
(GARFTase)	glycinamide ribonucleotide formyltransferase
(HBS)	HEPES-buffered saline
(LCV)	leucovorin
(LMTX)	lometrexol
(MTX)	methotrexate
(PMX)	pemetrexed
(PBS)	phosphate-buffered saline
(PCFT)	proton-coupled folate transporter
(PI)	propidium iodine
(RTX)	raltitrexed
(RFC)	reduced folate carrier
(SAR)	structure-activity relationship
(TCA)	trichloroacetic acid
(TS)	thymidylate synthase
(ZMP)	5-aminoimidazole-4-carboxamide ribonucleotide

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Figure 1. Structures of classical antifolates including methotrexate (MTX), pemetrexed (PMX), raltitrexed (RTX), lometrexol (LMTX), and pralatrexate.

Figure 2.

Design of 5-substituted pyrrolo[2,3-*d*]pyrimidines 5 and 7–10 ($n=1, 3–6$) based on structures of 6-substituted analogs 1–4 and pemetrexed.

Figure 3. Inhibition of RFC-mediated transport and relative FR α -binding affinities by 6- (compound 2) and 5- (compound 8) substituted pyrrolo[2,3-*d*]pyrimidine antifolates

Panel A: To determine if novel antifolates were able to bind human RFC and inhibit transport of [3 H]MTX, reflecting transport of the novel analogs by this mechanism, we used PC43-10 CHO cells ectopically expressing human RFC but not FRs or PCFT. Uptake of 0.5 μ M [3 H]MTX was measured in the absence or presence of 10 μ M of non-radioactive inhibitors, including compound (Cpd) 2 or 8, or the classical agents, PMX and MTX. Transport results are compared to those for RFC-null R2 cells. Panel B: To assess the relative binding affinities of the novel antifolates to FR α , we performed direct competitive binding assays using [3 H]folic acid (FA) in the FR α -engineered CHO cell line, RT16, in the presence or absence of various concentrations of unlabeled pyrrolo[2,3-*d*]pyrimidine compounds 2 or 8, or folic acid, PMX, or MTX.¹⁶ Binding affinities for FR α are expressed relative to that for folic acid. For both the transport and binding assays, experimental details are provided in the Experimental Section and results are presented as mean values $\pm$ standard errors from 3 experiments.

Figure 4. *De novo* purine nucleotide biosynthesis and relationship to AMPK

The *de novo* purine nucleotide biosynthetic pathway including the steps from phosphoribosyl pyrophosphate (PRPP) to IMP is shown. Reactions 1, 4, and 8 are catalyzed by glutamine phosphoribosylpyrophosphate amidotransferase, formylglycinamide ribonucleotide synthase, and adenylosuccinate lyase, respectively. Reactions 2, 3 and 5 are catalyzed by the trifunctional glycinamide ribonucleotide (GAR) formyltransferase (GARFTase) which contains GAR synthase (reaction 2), GAR formyltransferase (GARFTase; reaction 3) and 5-aminoimidazole ribonucleotide synthase (reaction 5) activities. Reactions 6 and 7 are catalyzed by the bifunctional phosphoribosylaminoimidazole carboxylase/phosphoribosylaminoimidazole succinocarboxamide synthetase enzyme, which contains carboxyaminoimidazole ribonucleotide synthase (reaction 6) and 5-aminoimidazole-4-(*N*-succinylcarboxamide) ribonucleotide synthase (reaction 7) activities. Reactions 9 and 10 are catalyzed by 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase (AICARFTase)/IMP cyclohydrolase that catalyzes the last two steps in the pathway for *de novo* synthesis of IMP. Folate-dependent reactions (reactions 3 and 9) in which 10-formyl tetrahydrofolate (THF) serves as the one-carbon donor are catalyzed by GARFTase and AICARFTase. 5-Aminoimidazole-4-carboxamide (AICA) and AICA ribonucleoside (AICAR) can be metabolized to 5-aminoimidazole-4-carboxamide ribonucleotide monophosphate (ZMP) by either adenine phosphoribosyl transferase (APRT) or adenosine kinase (AK), thus circumventing the reaction catalyzed by GARFTase. The activation of AMPK that results in inhibition of mTOR is also depicted. Abbreviations: AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside; FGAR, formyl glycinamide ribonucleotide; FAICAR, formyl 5-aminoimidazole-4-carboxamide ribonucleotide; MP, monophosphate.

Figure 5. Cell proliferation assays with protection by nucleosides including thymidine and adenosine, and by 5-aminoimidazole-4-carboxamide (AICA) to identify intracellular targets of PMX, compound 2 and compound 8

To identify the targeted pathways and the folate-dependent intracellular enzymes in KB cells treated with compound **8** (1–1000 nM), cell proliferation assays were performed in the presence of 10 μM thymidine, 60 μM adenosine, or 320 μM AICA. Results were normalized to those for untreated cells (no drug) and for compound **8**, and results are compared to those for PMX and compound **2**. Details are provided in the Experimental Section. Results shown are representative of triplicate experiments. Analogous protection experiments were performed for compounds **7** and **9** and the results are summarized in Table 1 along with those for compounds **2** and **8**.

Figure 6. Compounds 2 and 8 deplete ATP pools in KB cells

KB cells were treated with 1 μ M of the novel 5- and 6-substituted pyrrolo[2,3-*d*]pyrimidine antifolates **2** and **8**, or with PMX for 24 h. Nucleotides were extracted and analyzed by ion-pair HPLC. Experimental details are in the Experimental Section. Results are presented as mean values $\pm$ standard errors.

Figure 7. Inhibition of *de novo* purine biosynthesis by compound 8 results in cytotoxicity and apoptosis

Panel A. KB cells (1000 cells) were treated with compound **8** (0.5 to 10 μM) for 48 h, after which drug was removed and colonies were allowed to grow in drug-free medium over 10 days. Colonies were enumerated as described in the Experimental Methods. Results are presented as mean values $\pm$ standard errors from 3 experiments. Panel B: Cell cycle analysis. KB cells were treated with 1 μM antifolate (PMX, compound **2**, or compound **8**) for 96 h, washed, then fixed and stained with PI. Cell cycle distributions were analyzed by flow cytometry. The sub-G₁ fraction (white fill) increased from 3.5% in the absence of drugs upon treatment with PMX (17.3%) or compounds **2** (45.1%) or **8** (36.7%). For the non-sub-G₁ population, the cell cycle distributions [colors are green for G₁/G₀, yellow for S, and blue for G₂/M] are as follows: no additions, 67.4% G₁/G₀, 15.9% S, 13.2% G₂/M; PMX, 47.8% G₁/G₀, 27.5% S, 7.5% G₂/M; compound **2**, 22.3% G₁/G₀, 31.4% S, 1.2% G₂/M; and compound **8**, 12.9% G₁/G₀, 48.2% S, 2.3% G₂/M. Panel C: Analysis of apoptosis by annexin V-FITC/7-AAD staining and flow cytometry are shown for KB cells treated with PMX, or with compounds **2** or **8** for 96 h. Results are compared to those for controls treated with DMSO *in lieu* of drug. The percentages of cells in each quadrant [viable cells (annexin V-FITC^{low}/7-AAD^{low}; Q4), early apoptosis (annexin V-FITC^{high}/7-AAD^{low}; Q3), and late apoptosis/necrosis (annexin V-FITC^{high}/7-AAD^{high}; Q2)] are summarized.

Figure 8. *In situ* GARFTase assay

GARFTase activity and inhibition by 5- and 6-substituted pyrrolo[2,3-*d*]pyrimidine analogs, **2**, **8** and PMX were evaluated *in situ* with KB cells. Results are presented as mean values ± standard errors from 3 experiments. IC₅₀s are summarized in the table. Additional details are described in the Experimental Section.

Figure 9. Accumulation of ZMP by 5-substituted pyrrolo[2,3-*d*]pyrimidines PMX, and compound 8

Panel A: ZMP (AICA ribonucleotide) accumulation was measured in KB cells treated for 48 h with the 5-substituted pyrrolo[2,3-*d*]pyrimidine antifolates **8** and PMX. Methods for extracting nucleotide metabolites and for measuring ZMP levels are detailed in the Experimental Section. Results are shown as mean values $\pm$ standard errors for 3 experiments. Panel B: KB cells were treated at indicated for 48 h, followed by western blot analysis of phosphorylated AMPK. Blots were probed with antibodies to phospho- and total AMPK, and to β -actin. Details are included in the Experimental Section. Abbreviations: AICA, 5-aminoimidazole-4-carboxamide; AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside; NA, no additions.

Figure 10.

Structures of co-crystallized ligands 10-CF₃CO-DDACTHF (human GARFTase, PDB ID: 1NJS) and BW2315 18 (human AICARFTase, PDB ID: 1PL0)

Figure 11.
Stereoview overlay of the docked pose of compound 8 (blue) with 10-CF₃CO-DDACTHF (red) in human GARFTase (PDB ID: 1NJS).

Figure 12.

Stereoview overlay of the docked pose of compound 8 (blue) with 18 (red) in human AICARFTase (PDB ID: 1PL0).

Scheme 1.

Table 1

IC₅₀s (in nM) for 5- and 6-substituted pyrrolo[2,3-*d*]pyrimidine antifolates and classical antifolates in RFC-, PCFT-, and FR-expressing cell lines^a

Compound	Structure	hRFC		hFR α		hPCFT		hRFC/ FR α /hPCFT		
		PC43-10	R2	RT16	RT16 (+FA)	R2/hPCFT4	R2(VC)	KB	KB (+FA)	KB(+Thd/Ade/AICA)
1	6-pyrrolole/3C	648.6(38.1)	>1000	4.1(1.6)	>1000	23.0(3.3)	>1000	1.7(0.4)	>1000	Ade/AICA
2	6-pyrrolole/4C	>1000	>1000	6.3(1.6)	>1000	213(28)	>1000	1.9 (0.7)	>1000	Ade/AICA
3	6-pyrrolole/5C	>1000	>1000	54 (21)	>1000	>1000	>1000	13 (7.2)	>1000	Ade/AICA
4	6-pyrrolole/6C	>1000	>1000	162 (18)	>1000	>1000	>1000	23 (12)	>1000	Ade/AICA
5	5-pyrrolole/1C	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	--
6 (PMX)	5-pyrrolole/2C	30.6 (6.2)	>1000	18.2(3.8)	>1000	22.3(8.6)	>1000	9.94(3.11)	690 (310)	Thd/Ade
7	5-pyrrolole/3C	68.8(21.2)	>1000	72.0(27.1)	>1000	329(61)	>1000	49.5(13.2)	533(233)	Ade
8	5-pyrrolole/4C	56.6(5.8)	>1000	8.6(2.1)	>1000	840(90)	>1000	12.7(5.4)	700 (300)	Ade
9	5-pyrrolole/5C	196.4 (55.0)	>1000	33.5(2.5)	>1000	>1000	>1000	17.3(8.9)	898(102)	Ade/AICA
10	5-pyrrolole/6C	>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)	Thd/Ade
RTX	-	6.3(1.3)	>1000	15(5)	>1000	99.5(11.4)	>1000	5.9(2.2)	22(5)	Thd
LMTX	-	12(2.3)	>1000	12(8)	188(41)	38.0(5.3)	>1000	1.2(0.6)	31(7)	Ade/AICA

^aGrowth inhibition assays were performed for CHO sublines engineered to express human RFC (PC43-10), FR α (RT16), or PCFT (R2/PCFT4), for comparison with transporter-null R2, R2(VC) CHO cells, and for the KB human tumor sublines (express RFC, FR α , and PCFT). For the FR α 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 3–10 experiments (+/- standard errors in parentheses). Results are presented as IC₅₀ values, corresponding to the concentrations that inhibit growth by 50% relative to cells incubated without drug. Data for MTX, RTX, LMTX, and compounds 1, 2, 3, and 4 were previously published¹⁶ (structures for compounds 1–10 are in Figure 2). 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 in Figure 5. For the 5- and 6-pyrrolo-2,3-*d*pyrimidine compounds 1–10, the structural motifs are noted, i.e., the pyrrole substitution/bridge length. Experimental details are provided in the Experimental Section. Undefined abbreviations: Cpd, compound; Ade, adenosine; AICA, 5-aminoimidazole-4-carboxamide; Thd, thymidine; ND, not determined.