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Tumor Targeting with Novel Pyridyl 6-Substituted Pyrrolo[2,3-*d*]Pyrimidine Antifolates via Cellular Uptake by Folate Receptor α and the Proton-Coupled Folate Transporter and Inhibition of *De novo* Purine Nucleotide Biosynthesis

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Abstract

Tumor-targeted specificities of 6-substituted pyrrolo-[2,3-*d*]pyrimidine analogues of **1**, where the phenyl side-chain is replaced by 3',6' (**5**, **8**), 2',5' (**6**, **9**), and 2',6' (**7**, **10**) pyridyls, were analyzed. Proliferation inhibition of isogenic Chinese hamster ovary (CHO) cells expressing folate receptors (FRs) α and β were in rank order, **6** > **9** > **5** > **7** > **8**, with **10** showing no activity, and **6** > **9** > **5** > **8**, with **10** and **7** being inactive, respectively. Antiproliferative effects toward FR α - and FR β -expressing cells were reflected in competitive binding with [³H]folic acid. Only compound **6** was active against proton-coupled folate receptor (PCFT)-expressing CHO cells (~4-fold more potent than **1**) and inhibited [³H]methotrexate uptake by PCFT. In KB and IGROV1 tumor cells, **6** showed <1 nM IC₅₀, ~2–3-fold more potent than **1**. Compound **6** inhibited glycylamide ribonucleotide formyltransferase in *de novo* purine biosynthesis and showed potent *in vivo* efficacy toward subcutaneous IGROV1 tumor xenografts in SCID mice.

Graphical Abstract

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Supporting Information

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Elemental analysis and copies of ¹H NMR spectra of final compounds (PDF)

Molecular formula strings (CSV)

Notes

The authors declare no competing financial interest.

INTRODUCTION

The antifolates, typified by methotrexate (MTX), pemetrexed (PMX), pralatrexate (PTX), and raltitrexed (RTX) (Figure 1), are important drugs for treating cancer.¹ Integral to the antitumor efficacies of these agents is their cellular uptake by the facilitative transporters, the reduced folate carrier (RFC) and proton-coupled folate transporter (PCFT), and folate receptors (FRs) α and β , which internalize folates and antifolates by receptor-mediated endocytosis.^{2–5}

RFC is an anion antiporter that functions optimally at neutral pH and is ubiquitously expressed in tissues and tumors.^{3,4} PCFT is a proton symporter that shows more limited expression in normal tissues than RFC but is highly expressed in the duodenum and jejunum, as well as in liver and kidney.^{2,4,6} PCFT is abundantly expressed in human tumors^{7–10} and shows high levels of transport at acidic pHs, including those typically associated with the tumor micro-environment.^{2,11} As PCFT transport is limited at neutral pH for most substrates, other than for the upper gastrointestinal tract, PCFT transport should be minimal in most normal tissues.^{2,11} FRs are expressed in certain cancers, including FR α in epithelial ovarian cancer (EOC) and nonsmall cell lung cancer, and FR β in myeloid leukemias.¹² While FRs are also expressed in normal tissues such as renal tubules (i.e., FR α) and thymus (FR β), these are either inaccessible to the circulation (FR α) or are nonfunctional (FR β).¹² Notably, FR β is also expressed in tumor-associated macrophages.¹³

Reflecting tumor-specific patterns of expression and function of FRs and PCFT, there is substantial interest in the notion of targeting cytotoxic agents to tumors via their selective cellular uptake by these systems over RFC.^{2,14} For instance, cytotoxic folate conjugates (e.g., vintafolide,¹⁵ EC1456^{16,17}) and small-molecule inhibitors (e.g., ONX0801)¹⁸ with FR-specificities were reported to selectively target FR α -expressing tumors including EOC. Vintafolide, EC1456, and ONX0801 were tested in phase I clinical trials.

We discovered a series of novel cytotoxic 6-substituted pyrrolo[2,3-*d*]pyrimidine compounds with selective cellular uptake by FRs and PCFT over RFC.^{7,19–23} The most active compounds inhibited proliferation of FR- and PCFT-expressing cells *in vitro* and contained four- and three-carbon bridges and isosteric side-chain phenyl (**1** and **2**, respectively) or thienyl (**3** and **4**, respectively) side chains (Figure 2). The analogues with the three-carbon bridge were more potent than those with the four-carbon bridge.²³ Further, analogues with a thienyl side chain (**3** and **4**) were 4–5-fold more potent than the corresponding phenyl compounds (**1** and **2**) (Figure 2). All these compounds were potent inhibitors of tumor cell proliferation *in vitro* and *in vivo*, reflecting their targeting of *de novo* purine nucleotide

biosynthesis at the first folate-dependent step, catalyzed by β -glycinamide ribonucleotide (GAR) formyltransferase (GARFTase), resulting in ATP depletion.^{7,20–23}

Whereas earlier iterations of GARFTase inhibitors have been reported, typified by Lometrexol [(6R)5,10-dideazatetrahydrofolate; LMTX],²⁴ these were excellent substrates for RFC and thus were nonselective for tumor cells over normal tissues. Indeed, the toxicity encountered in an early phase I clinical trial with LMTX²⁵ was likely in part due to its transport into normal tissues by RFC and subsequent metabolism to polyglutamates.

Nitrogen-containing heteroaromatic rings are ubiquitously present in drug-like molecules, and 59% of US FDA-approved small-molecule pharmaceuticals contain at least one nitrogen heterocycle.²⁶ Substituting a CH group with an N atom can lead to improvements in functional activities such as biochemical and cellular potencies, and increase target selectivities irrespective of whether the binding pose of the ligand in the target is known (i.e., FRs and GARFTase) or unknown (RFC and PCFT).²⁷ In addition, N-substitution redistributes the electron density in the aromatic ring, and introduces a dipole moment, hydrogen bond capability, and polarity into the molecule. Each of the modified properties could influence the biological activity.

In this report, we performed a systematic N atom scan (N-scan) by exchanging the trivalent CH groups in the phenyl side chain of the ligand **1** with trivalent N atoms, one at a time, to determine its optimal placement for the desired pharmacological effects associated with selective FR and PCFT cellular uptake, and for GARFTase inhibition, resulting in potent antitumor activity.

We describe herein the synthesis and biological activities of a novel series of compounds with a four-carbon alkyl bridge (**5**, **6**, and **7**) and pyridyl-for-phenyl side-chain replacements (Figure 3). We also characterize a series of closely related analogues (**8**, **9**, and **10**) with a conformational restriction in the four-carbon bridge, resulting from a linear acetylenic linkage (Figure 3). Molecular modeling was used to rationalize the differences in functional activity where the crystal structures of the transporter (FR α)²⁸ and the target enzyme (GARFTase)²⁹ are available.

CHEMISTRY

Peptide coupling of the commercially available halogenated pyridine carboxylic acids **18–20** (Scheme 1) with L-glutamate diethyl ester hydrochloride gave **21–23**, respectively. Compounds **12**, **13**, and **14** were obtained by Sonogashira coupling of the reported intermediate **11**²² with bromides **21–23**, respectively, in the presence of Pd(PPh₄)₃, CuI, and triethylamine. Hydrogenation of **12–14** to **15–17**, respectively, and saponification of **15–17** afforded target compounds **5**, **6**, and **7**, respectively. Direct saponification of alkynes **12–14** afforded target compounds **8**, **9**, and **10**, respectively.

BIOLOGICAL EVALUATION AND DISCUSSION

As part of our systematic structure–activity relationship (SAR) study for tumor-targeted antitumor compounds with specificities for FRs and PCFT over RFC, we synthesized a

series of 6-substituted pyrrolo[2,3-*d*]pyrimidine analogues of **1** with four carbon-saturated (**5–7**) and linear acetylenic (**8–10**) linkers and replacements of the side-chain phenyl moiety with 3',6' (**5** and **8**), 2',5' (**6** and **9**), and 2',6' (**7** and **10**) pyridyls.

We initially tested this series for their effects on cell proliferation with a panel of isogenic Chinese hamster ovary (CHO) sublines engineered to individually express human FR α (RT16), FR β (D4), RFC (PC43–10), or PCFT (R2/PCFT4).^{20,30,31} All of these CHO transfectants were derived from a RFC-, FR-, and PCFT-null MTXR11Oua^R2–4 CHO subline (hereafter, designated R2).³² Results in the CHO cells were compared to those for KB and NCI-IGROV1 human tumor cells that express FR α , along with RFC and PCFT.^{8,20,29}

Growth inhibition was measured in the continuous presence of the drugs up to 1000 nM, using a fluorescence-based assay. With the PC43–10 and R2/PCFT4 CHO cell lines, results were compared to those for R2 cells, and to R2 cells that were “mock” transfected with empty pcDNA3.1 plasmid [R2(VC)]. For the FR-expressing CHO sublines (RT16, D4), controls involved incubations with 200 nM folic acid so as to block FR-mediated drug uptake. Results with the novel analogues were also compared to those for the CHO sublines treated with standard antifolate inhibitors, including MTX, PTX, PMX, and RTX, that are transported by FRs, as well as PCFT and RFC.^{1,4}

All of the 6-substituted pyrrolo[2,3-*d*]pyrimidine analogues including **1** were inactive toward human RFC-expressing PC43–10 cells up to 1000 nM drug (Table 1). With FR α -expressing RT16 CHO cells, IC₅₀s ranged from ~1 to 186 nM and were in rank order, **6** > **9** ~ **1** > **5** >> **7** > **8** > **10**. Results with FR β -expressing D4 cells generally paralleled those in RT16 cells although there were quantitative differences between FR α - and FR β -expressing cells for **5**, **9**, and **7**. For **5** and **9**, these differences were statistically significant ($p < 0.05$). For both FR α and FR β targeting, drug effects were nearly completely reversed by 200 nM folic acid.

With PCFT-expressing R2/PCFT4 cells, **6** was inhibitory (IC₅₀ ~ 58 nM, or ~4-fold more potent than the phenyl analogue **1**), whereas **5**, **7–10** were inactive. Proliferation of R2 and R2(VC) cells (devoid of any transporter) was not significantly inhibited by any of the pyrrolo[2,3-*d*]pyrimidine analogues up to 1000 nM.

In contrast to the targeted 6-substituted pyrrolo[2,3-*d*]pyrimidine analogues, the classical antifolate drugs MTX, PMX, RTX, and PTX all inhibited proliferation of PC43–10, R2/PCFT4, RT16, and D4 cells (i.e., these agents were nonselective for FRs and PCFT over RFC) with variations in sensitivities reflecting their substrate specificities.

With **1** and **5–7**, relative drug efficacies toward the CHO cells were mostly recapitulated with KB human tumor cells characterized by highly elevated FR α along with PCFT;^{8,20,29} however, the acetylenic analogues **8–10** were all inert toward the human tumor cells. With KB cells, **6** showed an impressive IC₅₀ < 1 nM which was reversed by excess folic acid (200 nM) up to ~100 nM but was slightly less effective at higher drug concentrations; an IC₅₀ of ~368 nM was calculated in the presence of 200 nM folic acid (Table 1). Thus, **6** inhibits KB cells at lower drug concentrations mediated primarily by FR α ; however, at higher drug

concentrations, PCFT uptake likely predominates. As noted above, **1**, **5**, and **7** are poor substrates for PCFT and folic acid was completely protective up to 1000 nM drug (Table 1).

We also compared the antiproliferative activities of **1** and **6** toward NCI-IGROV1 EOC cells, characterized by ~20% of the level of FR α in KB cells, accompanying similar levels of PCFT.^{8,29} The inhibitory effect of **6** toward NCI-IGROV1 cells exceeded that for **1** by ~2-fold [IC₅₀s of 0.68 ($\pm$ 0.10) nM and 1.12 ($\pm$ 0.13) nM, respectively] (p < 0.05). IC₅₀s in the presence of 200 nM folic acid were >1000 nM for **1** and 846 ($\pm$ 54) nM for **6**, establishing a predominant role for FR α uptake for both these agents with NCI-IGROV1 cells.

Transport Parameters for Compounds **1**, **5**–**7**.

Compounds **1**, **5**–**7** all exhibited FR-targeted activity toward FR α - and - β -expressing cells, including the engineered CHO sublines and KB human tumor cells, which was substantially reversed by 200 nM folic acid (Table 1).

We used competitive binding assays with [³H]folic acid (50 nM) and nonradioactive competitive ligands (1–1000 nM), including unlabeled folic acid, MTX, and **5**–**7**, as functional measures of FR α - and FR β -mediated drug uptake.^{20,22,23,31,33,34} Results were expressed as relative binding affinities, calculated as the inverse molar ratios of the concentrations of unlabeled competitive ligands required to decrease FR-bound [³H]folic acid by 50%. By this sensitive assay, for both FR α and FR β , binding affinities for **1**, **5**, and **6** were similar and slightly less than the affinity for folic acid (although this was statistically significant only for FR β) (Figure 4). There were no obvious direct correlations between relative binding affinities and growth inhibitions by **1**, **5**, and **6** toward RT16 (FR α) and D4 (FR β) cells. However, the binding affinities of **7** to both FR α and FR β were sharply reduced, accompanying its substantially decreased inhibition of cell proliferation at all but the highest drug concentrations (Table 1).

As **6** is an inhibitor of PCFT-expressing R2/PCFT4 CHO cells (Table 1), we measured competitive inhibition of [³H]MTX (0.5 μ M) uptake into R2/PCFT4 cells at pH 5.5 (the optimal pH for PCFT transport) and at pH 6.8 (approximates the pH of the tumor microenvironment) at 37 °C for 2 min, to assess **6** binding to the carrier. [³H]MTX uptake was measured without additions, or in the presence of 1 or 10 μ M **1**, **6**, PMX (among the best substrates reported for PCFT⁶) or PT523 [N α -(4-amino-4-deoxypteroyl)-N δ -hemipthaloyl-L-ornithine]³⁵ (an excellent RFC substrate without PCFT substrate activity^{6,22}) (Figure 1). PT523 had no effect on [³H]MTX uptake by PCFT at either pH. PMX, **1** and **6** inhibited transport at pH 5.5 at 1 μ M (85%, 50% and 70%, respectively) and 10 μ M (90% for all compounds) (Figure 5); at pH 6.8, this decreased for PMX (to ~40% at 1 μ M and ~75% at 10 μ M), **1** (0% at 1 μ M and 35% at 10 μ M) and **6** (~10% at 1 μ M and ~40% at 10 μ M). To further compare the binding affinity of **1** and **6** to PCFT compared to PMX, we measured [³H]MTX (0.5 μ M) uptake at pH 5.5 with R2/hPCFT4 cells over a range of inhibitor concentrations for Dixon analysis and calculation of K_i values. The calculated K_i values (mean $\pm$ SE) were as follows; **1**, 0.46 ($\pm$ 0.05) μ M; **6**, 0.332 $\pm$ 0.114 μ M; and PMX, 0.259 $\pm$ 0.118 μ M.

Identification of *De Novo* Purine Nucleotide Biosynthesis as the Targeted Pathway and GARFTase as the Intracellular Enzyme Target for 5–7.

Compound 1 was previously identified as a potent inhibitor of GARFTase in *de novo* purine nucleotide biosynthesis such that its antiproliferative effects were associated with depletion of purines.²⁰

To assess the impact of the pyridyl pyrrolo[2,3-*d*]pyrimidine antifolates 5–7 toward *de novo* nucleotide (purine nucleotide versus thymidylate) biosynthesis compared to 1, we measured antiproliferative effects of these agents toward KB cells in the presence of thymidine (10 μ M) or adenosine (60 μ M).^{20,22,23,31,33,34} Figure 6 shows results for 1 and 6, with results for these compounds along with those for 5 and 7 summarized in Table 1. Thymidine treatment had no impact in reversing the antiproliferative activity of any of the analogues; however, adenosine completely reversed drug effects up to 1000 nM drug. This strongly suggests that 5–7, like 1,²⁰ are principally inhibitors of *de novo* purine nucleotide biosynthesis (Figure 6). To distinguish the two folate-dependent reactions in the purine biosynthetic pathway [GARFTase and 5-aminoimidazole-4-carboxamide (AICA) ribonucleotide formyltransferase (AICARFTase)] as possible intracellular targets, cells were treated with the drugs in the presence of 320 μ M AICA which provides substrate for AICARFTase, thus circumventing the inhibitory effects of GARFTase.^{20,22,23,31,33,34} 34 AICA was just as effective as adenosine in reversing the inhibitory effects of the 6-substituted pyrrolo[2,3-*d*]pyrimidine pyridyl analogues (Figure 6). This identifies GARFTase as the likely intracellular target of this series.

We used an *in situ* GARFTase metabolic assay to measure GARFTase activity in intact KB cells treated with a range of inhibitor concentrations to confirm inhibition of intracellular GARFTase by the potent inhibitory 6-substituted pyrrolo[2,3-*d*]pyrimidine pyridyl analogues 5 and 6, compared to 1. KB human tumor cells were incubated with [¹⁴C]glycine with 1, 5, or 6, using conditions and drug concentrations approximating those used for the cell proliferation experiments (i.e., Figure 6 and Table 1). In this assay, the GARFTase product [¹⁴C]formyl GAR accumulates in the presence of azaserine and can be isolated by ion-exchange chromatography, quantified and levels normalized to cellular protein.^{20,22,23,31,33,34} Figure 7 shows the effects of the various inhibitory 6-substituted pyrrolo[2,3-*d*]pyrimidine analogues on the accumulation of [¹⁴C]formyl GAR. For 1, 5, and 6, the IC₅₀s for GARFTase inhibition were 5.6, 4.8, and 1.8 nM, respectively.

Molecular Modeling of 1, 5, and 6 with FR α and GARFTase.

To rationalize the changes in pharmacologic activity for the *para*-substituted phenyl side-chain analogue 1 (1',4') versus the *para*-substituted pyridyl side-chain analogues 6 (2',5') and 5 (3',6'), we performed molecular modeling studies with FR α and GARFTase (formyltransferase domain).

Molecular modeling was performed using LeadIT 2.1.6³⁶ for binding the active analogues in the folate binding cleft of FR α (PDB 5IZQ)²⁸ (Figure 8). The energy-minimized crystallized ligand AGF183²⁸ was redocked with an RMSD of 0.84 for the best scored pose, thus validating the docking process. The docked poses of 1 (Figures 8A,B), 5 (Figure 8A), and 6

(Figure 8B) maintain key protein interactions with the bicyclic scaffolds and benzoyl L-glutamate tail moieties, as seen for the crystal structure ligand **AGF183**²⁸ (not shown for clarity).

The pyrrolo[2,3-*d*]pyrimidine scaffolds are stacked between the side chains of Tyr85, Tyr60, and Trp171, similar to the bicyclic scaffold of **AGF183**.²⁸ The 2-NH₂ forms a hydrogen bond with the side chain carboxylate of Asp81. The 4-oxo moiety forms hydrogen bonds with the side chain –NH₂ of Arg103. The L-glutamate side chains of all the three ligands are oriented similar to the corresponding L-glutamate in **AGF183**.²⁸ The α -carboxylic acids form a network of hydrogen bonds involving the backbone NH of Gly137 and Trp138, and the pyrrole NH of Trp140, whereas the γ -carboxylic acids form an ionic bond with the side chain of Lys136, and interact via hydrogen bonds with Trp102 (except the γ -carboxylic acid of compound **5**). The bridge atoms of **1**, **5** and **6** form hydrophobic interactions with Tyr60, Phe62, Trp102, and His135. A comparison of the docked pose of compound **1** with compounds **5** (Figure 8A) and **6** (Figure 8B) in the crystal structure pose of **AGF183**²⁸ indicates that the four-atom bridges of **1**, **5**, and **6** are localized within a similar region as the 3-atom bridge of **AGF183**. For the latter, the extension of the bridge seems to better utilize the hydrophobic character of the bridge-binding region of FR α for improved interactions. Consistent with this notion, the docking scores were maintained for phenyl-to-pyridyl side chain replacements with values of –54.56 kJ/mol for **5** and –59.44 kJ/mol for **6**, compared to –55.40 kJ/mol for **1**.

Our X-ray crystal structure of human GARFTase bound to **AGF150**²⁹ (PDB 4ZZ1) was used to perform molecular modeling studies in LeadIT 2.1.6.³⁶ The energy-minimized crystallized ligand **AGF150** was redocked with an RMSD of 1.22 Å for the best scored pose, validating the docking process. Figure 9 shows the docked lead compound **1** in the GARFTase active site, along with the docked poses of **5** (Figure 9A) and **6** (Figure 9B). The pyrrolo[2,3-*d*]pyrimidine scaffolds of **1**, **5**, and **6** bind in the region occupied by the bicyclic scaffold of **AGF150** in the GARFTase structure (**AGF150** is not shown for clarity). Hydrogen bonds between the N1 nitrogen and the backbone amide –NH– of Leu899, 2-NH₂ and the backbone carbonyls of Leu899, N3 and the backbone carbonyl of Ala947, and 4-oxo and the backbone amide –NH– of Asp951, stabilize the bicyclic scaffold. The pyrrolo[2,3-*d*]pyrimidine scaffold additionally forms hydrophobic interactions with Ile898 and Leu899, CH– π interactions with Ile898 (compounds **1**, **5** and **7** in Figure 9A,B) and Val950 (compound **6** in Figure 9B), and a hydrogen bond network with the folate binding loop residues 948–951 via a bridging water molecule. The amide –NH– of the L-glutamate forms a hydrogen bond with the carbonyl of Met896. The L-glutamate moiety is oriented with the α -carboxylate, forming ionic and hydrogen bonds with Arg871, hydrogen bonds with the backbone NH of Ile898, and a hydrogen bond with the side chain of Arg897 via a bridging water molecule. The γ -carboxylates form hydrogen bonds with the conserved water molecules nearby. Additionally, the 2',5'-pyridyl ring of **6** forms CH– π interactions with the β -CH₂ of Ser925, and the amide carbonyl of its L-glutamate side chain forms hydrogen bonds with the neighboring water molecules (Figure 9B). A comparison of the docked poses of lead compound **1** and compounds **5** and **6** with the crystal structure pose of **AGF150**²⁹ indicates that the four-atom bridges (of **1**, **5**, and **6**) are accommodated within a similar

region as the three-atom bridge (of **AGF150**). An extension of the bridge could possibly better utilize the hydrophobic nature of the bridge binding region of the GARFTase binding pocket for improved interactions. Consistent with the inhibition results in Figure 7, **6** showed the highest docked score (-70 kJ/mol), whereas compounds **5** and **1** gave similar values (-58.65 and -58.38 kJ/mol, respectively).

The docked scores for FR α and GARFTase validate the FR α binding and GARFTase inhibition results of pyridyl-for-phenyl replacement for **1**, resulting in **5** and **6**. In **6** with the N atom placed ortho to the L-glutamate, the binding affinity to FRs was found to be similar to **1** both in direct binding assays and molecular modeling studies. The slightly superior *in situ* GARFTase inhibitory activity for **6** can be attributed to the additional electrostatic interactions of the pyridyl ring with Ser925 and the adjacent water molecule, predicted in the GARFTase binding site (Figure 9B). Because the binding poses of folate ligands in PCFT have not been identified, based on the cell proliferation data (Table 1) and the results of direct transport competition (Figure 5), we postulate that similar favorable interactions must be responsible for the enhanced binding of **6** to PCFT. In **5** where the CH-to-N replacement is meta to the L-glutamate, targeting FRs and GARFTase was found to be similar to **1** both by direct assays and molecular modeling studies. As such, the reason for loss in activity against KB cells compared to **1** is unclear.

As expected, pyridyl-for-phenyl replacement led to changes in functional activities, depending on the position of the pyridyl N atom. In **6**, placing the N atom ortho to the L-glutamate, led to improved biochemical potency (~ 3 -fold improvement in IC_{50} s for *in situ* GARFTase inhibition), cellular potency (~ 2 – 3 -fold improvement in IGROV1 and KB cell growth inhibition), and target selectivity (~ 5 -fold, ~ 10 -fold, and ~ 4 -fold increased potency for FR α , FR β , and PCFT, respectively), accompanying a lack of inhibition toward RFC-expressing PC43–10 cells. A N-for-CH substitution introduced weak hydrogen bond capability and CH- π interactions into the molecule, while only slightly changing the molecular weight (MW = +1 Da).

***In Vivo* Antitumor Efficacy of **6** with IGROV1 EOC Xenografts.**

Based on the promising *in vitro* efficacy results with **6** toward a number of cell line models including NCI-IGROV1 EOC cells, we performed an *in vivo* drug efficacy trial with **6** compared to PMX (Alimta). The trial used 10 week old female SCID mice (Charles River) implanted with subcutaneous NCI-IGROV1 EOC cells. Mice were fed *ad libitum* a folate-deficient diet so as to decrease serum folate concentrations to those reported in humans.³⁷ A control cohort of mice was fed standard folate-replete diet.

For the trial, the mice in each (folate-deficient and standard diet) group were pooled and implanted subcutaneously with NCI-IGROV1 tumor fragments. Mice were nonselectively randomized into control and treatment groups (5 mice/group). Compound **6** and PMX were administered at doses slightly below their respective maximum tolerated doses. For **6**, a Q2Dx8 schedule of drug administration was used, with intravenous drug administration (150 mg/kg/inj; 1200 mg/kg total) beginning on day 3. PMX was administered intravenously beginning on day 3 on a Q4DX4 schedule (8.1 mg/kg/inj; total dose of 32.4 mg/kg). Mice

were weighed daily and tumors were measured twice per week. For mice on the folate-deficient diet, antitumor activities, as reflected in tumor growth delay (T-C to reach 1000 mg in days), were 45 days for compound **6** and 0 days for PMX (Figure 10). Remarkably, a palpable tumor was undetected for up to 49 days in mice treated with compound **6**. Treatment with **6** was well tolerated, as reflected in a modest weight loss (5% at the nadir) compared with PMX (14% weight loss at the nadir). Weight losses were reversible upon conclusion of therapy. For mice on the standard (high folate) diet, antitumor activity for **6** was abolished.

CONCLUSIONS

In this report, we continued our systematic study of the SAR for the 6-substituted pyrrolo[2,3-*d*]pyrimidine series of novel compounds as tumor-targeted drugs based on their selective membrane transport and inhibition of cellular folate-dependent enzyme targets and pathways. We synthesized and tested a series of analogues closely related to **1**, a previously described 6-substituted pyrrolo[2,3-*d*]pyrimidine compound with a four-carbon linker and benzoyl side chain,^{20,21} characterized by cellular uptake by FRs and PCFT, resulting in inhibition of the GARFTase reaction and cell proliferation. The test compounds maintained the four-carbon bridge in **1** but included side-chain 3',6' (**5**), 2',5' (**6**), or 2',6' (**7**) pyridyls in place of the phenyl moiety. An additional series of compounds based on **5**, **6**, and **7** (**8**, **9**, and **10**, respectively) with a conformationally restricted linear acetylenic linker was also tested to assess the impact of increased rigidity in the bridge region on inhibitory potency.

Our results documented a dramatically increased drug efficacy for the 2',5' pyridyl compound **6** over **1**, reflected in 4–10-fold increased activities toward isogenic engineered CHO cell lines expressing exclusively FRs or PCFT, and no activity for either compound toward RFC-expressing CHO cells. This indicates absolute selectivity for FR and/or PCFT over RFC with the potential of tumor-selective activity. For both FR- and PCFT-expressing cells, the 2',5' regioisomer **6** was substantially more potent than either **5** or **7**, with modestly increased potency toward FR β over FR α . The 2',5' acetylenic analogue **9** was likewise active toward FR α -expressing CHO cells (albeit ~4-fold less than **6**), indicating that rigidity in the bridge region is detrimental to activity. Activities for the 3',6' (**8**) and 2',6' (**10**) regioisomers were dramatically reduced. This suggests that the location of the pyridine N in the side chain dictates biological activity.

Differences in FR-targeted activities for **5** and **6** were modestly reflected in binding affinities toward FR α and FR β with the engineered CHO sublines exclusively expressing these receptors in the absence other transporters, although the affinity for **7** was increased for FR α -expressing RT16 cells accompanying increased antiproliferative activity over FR β -expressing cells. For **6**, PCFT-targeted activity was accompanied by binding to human PCFT, as reflected in competitive inhibition approximating that for PMX, among the most active substrates for PCFT.^{4,6}

We extended our studies to human tumor cells including KB nasopharyngeal carcinoma and NCI-IGROV1 EOC cells which express both FR α and PCFT.^{8,20,29} For both KB and NCI-IGROV1 cells, **6** was highly active, with potencies exceeding those measured for **1** by

~2–3-fold. Interestingly, the acetylenic analogues including **9** were inactive toward KB human tumor cells, suggesting that factors unrelated to FR-mediated internalization must be responsible. A similar discrepant activity pattern with CHO versus KB tumor cells was previously reported for a series of pyrrolo[2,3-*d*]pyrimidine acetylenic analogues related to **3**.³⁸

For NCI-IGROV1 cells, antitumor efficacy of **6** was confirmed *in vivo* with tumor xenografts in SCID mice. Notably **6** had excellent activity and PMX was inactive toward NCIIGROV1 tumors *in vivo*, although weight loss accompanying drug treatment for PMX exceeded that for **6**. The inactivity of PMX, at least in part, likely reflects increased circulating thymidine in mice.³⁹ Antitumor activity of this series was due to inhibition of *de novo* purine nucleotide biosynthesis and GARFTase as the enzyme target, as reflected in adenosine/AICA protection from inhibition of cellular proliferation. Further, by *in situ* GARFTase assays with [¹⁴C]glycine, the potency for **6** exceeded that for **5** and **1**. Consistent with these results were the predicted binding affinities by molecular modeling of this series in human GARFTase.²⁹

In conclusion, we established that 2',5'-pyridyl-for-phenyl substitution in the side-chain of the 6-substituted 4-atom bridged pyrrolo[2,3-*d*]pyrimidine scaffold resulted in pronounced increases in drug efficacy, a result not seen with the 3',6' and 2',6' pyridyl regioisomers. Enhanced antiproliferative activity for the lead compound **6** over other analogues predominantly reflects increased PCFT transport and/or GARFTase inhibition with modest differences in FR binding. Reflecting its selective cellular uptake via these non-RFC processes and inhibition of *de novo* purine biosynthesis, **6** showed remarkably potent antitumor efficacy and would be expected to possess significantly lower toxicities toward normal tissues.

EXPERIMENTAL PROCEDURES

All evaporations were carried out with a rotary evaporator in vacuum. CHEM-DRY drying apparatus in vacuo (0.2 mmHg) over P₂O₅ at 60 °C was used to dry the analytical samples. MEL-TEMP II melting point apparatus with uncorrected FLUKE 51 K/J electronic thermometer was used to determine melting points. Bruker Avance II 400 (400 MHz) and 500 (500 MHz) spectrometers were used to record the Nuclear magnetic resonance spectra for proton (¹H NMR). Tetramethylsilane is the internal standard used to relatively express the chemical shift values in ppm (parts per million): s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad singlet. Whatman Sil G/UV254 silica gel plates were used to perform thin-layer chromatography (TLC) with a fluorescent indicator to visualize the spots under 254 and 366 nm illuminations. Proportions of TLC solvents are by volume. A 230–400 mesh silica gel (Fisher, Somerville, NJ) column was used for column chromatography. The amount (weight) of silica gel used for column chromatography was 50–100 times the amount (weight) of the crude compounds needed to be separated. Dry-packed columns were used unless specified otherwise. Compounds were sent to Atlantic Microlab, Inc., Norcross, GA for elemental analyses (C, H, N) to determine the purities (including the final compounds). Element compositions are evaluated to be within ±0.4% of the calculated values. Frequently found fractional moles of water or organic solvents in some analytical

antifolates samples could not be removed despite drying for 24–48 h in vacuo; however, these were confirmed, when possible, by their presence in the ^1H NMR spectra. All chemicals and solvents were purchased from Fisher Scientific and Aldrich Chemical Co. and used as received. Purities of the final compounds **4–13**, determined by elemental analysis, were >95%.

General Procedure for the Synthesis of Compounds **5–10**.

To a solution of **12–17** (100 mg, 0.19 mmol) in MeOH (10 mL) was added 1 N NaOH (5 mL) and the mixture was stirred under N_2 at room temperature for 16 h. TLC showed the disappearance of the starting material (R_f 0.54, $\text{CHCl}_3/\text{MeOH}$ 5:1) and one major spot at the origin. The reaction mixture was evaporated to dryness under reduced pressure. The residue was dissolved in water (10 mL), the resulting solution was cooled in an ice bath, and the pH was adjusted to 3–4 with dropwise addition of 1 N HCl. The resulting suspension was frozen in a dry ice-acetone bath, thawed to 4–5 °C in the refrigerator, and filtered. The residue was washed with a small amount of cold water and dried in vacuum using P_2O_5 to afford **5–10**.

(6-(4-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-6-yl)butyl)nicotinoyl)-L-glutamic Acid (5).—Compound **5** was prepared using the general method described for the preparation of **5–10**, from **15** (100 mg, 0.19 mmol) to give 82 mg (95%) of **5** as a light yellow powder. mp 115–116 °C; ^1H NMR ($\text{DMSO}-d_6$): δ 1.54–1.71 (m, 4H, CH_2CH_2), 1.90–2.12 (m, 2H, $\beta\text{-CH}_2$), 2.33–2.37 (t, J = 7.6 Hz, 2H, $\gamma\text{-CH}_2$), 2.51 (m, 2H, CH_2), 2.77–2.81 (m, 2H, CH_2), 4.37–4.42 (m, 1H, $\alpha\text{-CH}$), 5.83 (s, 1H, C5-CH), 5.95 (s, 2H, 2- NH_2 , exch), 7.34–7.36 (d, J = 8.4 Hz, 1H, Ar), 8.10–8.12 (d, J = 8.4 Hz, 1H, Ar), 8.72–8.74 (d, J = 7.2 Hz, 1H, CONH, exch), 8.91 (s, 1H, Ar), 10.11 (s, 1H, 3-NH, exch), 10.78 (s, 1H, 7-NH, exch), 12.44 (br, 2H, COOH, exch). Anal. Calcd for $(\text{C}_{21}\text{H}_{24}\text{N}_6\text{O}_6 \cdot 0.7 \text{H}_2\text{O})$ C, H, N.

(5-(4-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-6-yl)butyl)picolinoyl)-L-glutamic Acid (6).—Compound **6** was prepared using the general method described for the preparation of **5–10**, from **16** (100 mg, 0.19 mmol) to give 82 mg (95%) of **6** as a light yellow powder. mp 156–157 °C; ^1H NMR ($\text{DMSO}-d_6$): δ 1.56 (m, 4H, CH_2CH_2), 1.97–2.14 (m, 2H, $\beta\text{-CH}_2$), 2.25–2.29 (t, J = 7.6 Hz, 2H, $\gamma\text{-CH}_2$), 2.51 (m, 2H, CH_2), 2.68–2.71 (m, 2H, CH_2), 4.41–4.46 (m, 1H, $\alpha\text{-CH}$), 5.85 (s, 1H, C5-CH), 5.95 (s, 2H, 2- NH_2 , exch), 7.80–7.82 (m, 1H, Ar), 7.92–7.94 (d, J = 7.98 Hz, 1H, Ar), 8.49 (s, 1H, Ar), 8.75–8.77 (d, J = 8.0 Hz, 1H, CONH, exch), 10.12 (s, 1H, 3-NH, exch), 10.78 (s, 1H, 7-NH, exch), 12.59 (br, 2H, COOH, exch). Anal. Calcd for $(\text{C}_{21}\text{H}_{24}\text{N}_6\text{O}_6 \cdot 1.2 \text{H}_2\text{O})$ C, H, N.

(6-(4-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-6-yl)butyl)picolinoyl)-L-glutamic Acid (7).—Compound **7** was prepared using the general method described for the preparation of **5–10**, from **17** (100 mg, 0.19 mmol) to give 82 mg (95%) of **7** as a light yellow powder. mp 121–122 °C; ^1H NMR ($\text{DMSO}-d_6$): δ 1.62–1.79 (m, 4H, CH_2CH_2), 1.99–2.20 (m, 2H, $\beta\text{-CH}_2$), 2.29–2.32 (t, J = 7.5 Hz, 2H, $\gamma\text{-CH}_2$), 2.53–2.56 (t, J = 12.0 Hz, 2H, CH_2), 2.84–2.87 (t, J = 7.5 Hz, 2H, CH_2), 4.48–4.53 (m, 1H, $\alpha\text{-CH}$), 5.88 (s, 1H, C5-CH), 5.96 (s, 2H, 2- NH_2 , exch), 7.48–7.50 (dd, J_1 = 1.0 Hz, J_2 = 7.5 Hz, 1H, Ar), 7.84–7.92 (m, 2H, Ar), 8.71–8.73 (d, J = 8.5 Hz, 1H, CONH, exch),

10.14 (s, 1H, 3-NH, exch), 10.80 (s, 1H, 7-NH, exch), 12.51 (br, 2H, COOH, exch). Anal. Calcd for (C₂₁H₂₄N₆O₆ · 1.0 H₂O) C, H, N.

(6-(4-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-6-yl)but-1-yn-1-yl)nicotinoyl)-L-glutamic Acid (8).—Compound **8** was prepared using the general method described for the preparation of **5–10**, from **12** (100 mg, 0.20 mmol) to give 84 mg (95%) of **8** as a light yellow powder. mp 155–156 °C; ¹H NMR (DMSO-*d*₆): δ 1.94–2.07 (m, 2H, β-CH₂), 2.33–2.37 (m, 2H, γ-CH₂), 2.66 (m, 2H, CH₂), 2.94 (m, 2H, CH₂), 4.40 (m, 1H, α-CH), 6.00 (s, 3H, C5-CH, 2-NH₂, exch), 7.52–7.54 (d, *J* = 8.0 Hz, 1H, Ar), 8.16–8.19 (d, *J* = 8.0 Hz, 1H, CONH, exch), 8.94–9.02 (m, 2H, Ar), 10.16 (s, 1H, 3-NH, exch), 10.89 (s, 1H, 7-NH, exch), 12.55 (br, 2H, COOH, exch). Anal. Calcd for (C₂₁H₂₀N₆O₆ · 0.8 H₂O) C, H, N.

(5-(4-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-6-yl)but-1-yn-1-yl)picolinoyl)-L-glutamic Acid (9).—Compound **9** was prepared using the general method described for the preparation of **5–10**, from **13** (100 mg, 0.20 mmol) to give 84 mg (95%) of **9** as a light yellow powder. mp 192–193 °C; ¹H NMR (DMSO-*d*₆): δ 2.02–2.11 (m, 2H, β-CH₂), 2.27 (m, 2H, γ-CH₂), 2.80 (m, 4H, CH₂CH₂), 4.44 (m, 1H, α-CH), 6.01 (s, 3H, C5-CH, 2-NH₂, exch), 7.97 (s, 2H, Ar), 8.62 (s, 1H, Ar), 8.84 (s, 1H, CONH, exch), 10.16 (s, 1H, 3-NH, exch), 10.89 (s, 1H, 7-NH, exch), 12.57 (br, 2H, COOH, exch). Anal. Calcd for (C₂₁H₂₀N₆O₆ · 1.5 H₂O, 0.1 CHCl₃) C, H, N, Cl.

(6-(4-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-6-yl)but-1-yn-1-yl)picolinoyl)-L-glutamic Acid (10).—Compound **10** was prepared using the general method described for the preparation of **5–10**, from **14** (100 mg, 0.20 mmol) to give 84 mg (95%) of **10** as a light yellow powder. mp 161–162 °C; ¹H NMR (DMSO-*d*₆): δ 2.01–2.20 (m, 2H, β-CH₂), 2.28–2.31 (t, *J* = 7.5 Hz, 2H, γ-CH₂), 2.79–2.86 (m, 4H, CH₂CH₂), 4.48–4.51 (m, 1H, α-CH), 6.00 (s, 1H, C5-CH), 6.04 (s, 2H, 2-NH₂, exch), 7.64–7.66 (dd, *J*₁ = 2.5 Hz, *J*₂ = 8.5 Hz, 1H, Ar), 7.97–8.00 (m, 2H, Ar), 8.70–8.72 (d, *J* = 8.5 Hz, 1H, CONH, exch), 10.17 (s, 1H, 7-NH, exch), 10.91 (s, 1H, 7-NH, exch), 12.53 (br, 2H, COOH, exch). Anal. Calcd for (C₂₁H₂₀N₆O₆ · 1.5 H₂O) C, H, N.

General Procedure for the Synthesis of Compounds 12–14.

To a 250 mL rbf, equipped with a magnetic stir-bar and gas inlet, a mixture of tetrakis (triphenylphosphine)-palladium(0) (277 mg, 0.24 mmol), triethylamine (1.52 g, 15 mmol), **21–23** (882 mg, 2.25 mmol), and anhydrous DMF (20 mL) was added. Under N₂, copper(I) iodide (61 mg, 0.32 mmol) and **11** (404 mg, 2 mmol) were added to the stirred mixture which was then stirred overnight (17–18 h) at room temperature. To the reaction mixture was added silica gel (1 g), and the solvent was evaporated at reduced pressure. The resulting plug was loaded on to a silica gel column (2 × 12 cm) and eluted with CHCl₃ followed by 3% MeOH in CHCl₃ and then 5% MeOH in CHCl₃. Fractions with desired *R_f* (TLC) were pooled and evaporated to afford **12–14**.

Diethyl (6-(4-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]pyrimidin-6-yl)but-1-yn-1-yl)nicotinoyl)-L-glutamate (12).—Compound **12** was prepared using the

general method described for the preparation of **12–14**, from **11** (404 mg, 2 mmol) and (6-iodonicotinoyl)-L-glutamic acid, **21** (1.16 g, 3 mmol) to give 559 mg (55%) of **12** as a brown powder. mp 81–82 °C; TLC R_f 0.53 (CHCl₃/MeOH 5:1); ¹H NMR (DMSO-*d*₆): δ 1.15–1.18 (m, 6H, COOCH₂CH₃), 1.97–2.10 (m, 2H, β -CH₂), 2.42–2.46 (m, 2H, γ -CH₂), 2.80 (m, 4H, CH₂CH₂), 4.01–4.13 (m, 4H, COOCH₂CH₃), 4.44 (m, 1H, α -CH), 6.01 (s, 3H, C5-CH, 2-NH₂, exch), 7.53–7.55 (d, J = 8.0 Hz, 1H, Ar), 8.16–8.18 (d, J = 8.0 Hz, 1H, CONH, exch), 8.93–8.97 (m, 2H, Ar), 10.16 (s, 1H, 3-NH, exch), 10.90 (s, 1H, 7-NH, exch).

Diethyl (5-(4-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]-pyrimidin-6-yl)but-1-yn-1-yl)picolinoyl)-L-glutamate (13).—Compound **13** was prepared using the general method described for the preparation of **12–14**, from **11** (404 mg, 2 mmol) and (5-iodopicolinoyl)-L-glutamic acid, **22** (1.16 g, 3 mmol) to give 579 mg (57%) of **13** as a brown powder. mp 79–80 °C; TLC R_f 0.53 (CHCl₃/MeOH 5:1); ¹H NMR (DMSO-*d*₆): δ 1.12–1.18 (m, 6H, COOCH₂CH₃), 2.03–2.17 (m, 2H, β -CH₂), 2.33–2.38 (m, 2H, γ -CH₂), 2.79 (m, 4H, CH₂CH₂), 3.97–4.13 (m, 4H, COOCH₂CH₃), 4.46–4.52 (m, 1H, α -CH), 6.01 (s, 3H, C5-CH, 2-NH₂, exch), 7.96 (s, 2H, Ar), 8.62 (s, 1H, Ar), 8.98–9.00 (d, J = 7.6 Hz, 1H, CONH, exch), 10.15 (s, 1H, 3-NH, exch), 10.89 (s, 1H, 7-NH, exch).

Diethyl (6-(4-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]-pyrimidin-6-yl)but-1-yn-1-yl)picolinoyl)-L-glutamate (14).—Compound **14** was prepared using the general method described for the preparation of **12–14**, from **11** (404 mg, 2 mmol) and (6-iodopicolinoyl)-L-glutamic acid, **23** (1.16 g, 3 mmol) to give 548 mg (54%) of **14** as a brown powder. mp 80–81 °C; TLC R_f 0.52 (CHCl₃/MeOH 5:1); ¹H NMR (DMSO-*d*₆): δ 1.14–1.21 (m, 6H, COOCH₂CH₃), 2.07–2.22 (m, 2H, β -CH₂), 2.36–2.39 (t, J = 8.0 Hz, 2H, γ -CH₂), 2.80–2.85 (m, 4H, CH₂CH₂), 4.00–4.05 (q, J = 7.0 Hz, 2H, COOCH₂CH₃), 4.10–4.16 (m, 2H, COOCH₂CH₃), 4.52–4.56 (m, 1H, α -CH), 6.01 (s, 2H, 2-NH₂, exch), 6.04 (s, 1H, C5-CH), 7.65–7.67 (m, 1H, Ar), 7.95–8.0 (m, 2H, Ar), 8.83–8.85 (d, J = 8.0 Hz, 1H, CONH, exch), 10.16 (s, 1H, 3-NH, exch), 10.91 (s, 1H, 7-NH, exch).

General Procedure for the Synthesis of Compounds 15–17.

To a Parr flask was added **12–14** (200 mg, 0.39 mmol), 10% palladium on activated carbon (100 mg), and MeOH (50 mL). Hydrogenation was carried out at 55 psi of H₂ for 4 h. The reaction mixture was filtered through Celite, washed with MeOH (100 mL) and concentrated under reduced pressure to give **15–17**.

Diethyl (6-(4-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]-pyrimidin-6-yl)butyl)nicotinoyl)-L-glutamate (15).—Compound **15** was prepared using the general method described for the preparation of **15–17**, from **12** (200 mg, 0.40 mmol) to give 195 mg (95%) of **15** as a light yellow powder. mp 81–82 °C; TLC R_f 0.54 (CHCl₃/MeOH 5:1); ¹H NMR (DMSO-*d*₆): δ 1.17–1.20 (m, 6H, COOCH₂CH₃), 1.60 (m, 2H, CH₂), 1.71 (m, 2H, CH₂), 2.01–2.11 (m, β -CH₂), 2.44–2.47 (m, 2H, γ -CH₂), 2.53 (m, 2H, CH₂), 2.86–2.89 (m, 2H, CH₂), 4.03–4.13 (m, 4H, COOCH₂CH₃), 4.46 (m, 1H, α -CH), 5.91 (s, 1H, C5-CH), 6.46 (s, 2H, 2-NH₂, exch), 7.55 (d, J = 8.0 Hz, 1H, Ar), 8.31 (d, J = 8.0 Hz, 1H, CONH, exch), 8.99 (m, 2H, Ar), 10.57 (s, 1H, 3-NH, exch), 11.07 (s, 1H, 7-NH, exch).

Diethyl (5-(4-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]-pyrimidin-6-yl)butyl)picolinoyl)-L-glutamate (16).—Compound **16** was prepared using the general method described for the preparation of **15–17**, from **13** (200 mg, 0.40 mmol) to give 195 mg (95%) of **16** as a light yellow powder. mp 77–78 °C; TLC R_f 0.53 (CHCl₃/MeOH 5:1); ¹H NMR (DMSO-*d*₆): δ 1.10–1.19 (m, 6H, COOCH₂CH₃), 1.60 (m, 4H, CH₂CH₂), 2.03–2.17 (m, 2H, β-CH₂), 2.34–2.38 (t, J = 8.0 Hz, 2H, γ-CH₂), 2.52–2.54 (m, 2H, CH₂), 2.69–2.72 (m, 2H, CH₂), 3.97–4.13 (m, 4H, COOCH₂CH₃), 4.48–4.53 (m, 1H, α-CH), 5.92 (s, 1H, C5-CH), 6.53 (s, 2H, 2-NH₂, exch), 7.81–7.83 (dd, J_I = 2.0 Hz, J_I = 8.0 Hz, 1H, Ar), 7.92–7.94 (d, J = 8.0 Hz, 1H, Ar), 8.51 (s, 1H, Ar), 8.88–8.90 (d, J = 8.0 Hz, 1H, CONH, exch), 10.61 (s, 1H, 3-NH, exch), 11.09 (s, 1H, 7-NH, exch).

Diethyl (6-(4-(2-Amino-4-oxo-4,7-dihydro-3H-pyrrolo[2,3-d]-pyrimidin-6-yl)butyl)picolinoyl)-L-glutamate (17).—Compound **17** was prepared using the general method described for the preparation of **15–17**, from **14** (200 mg, 0.40 mmol) to give 195 mg (95%) of **17** as a light yellow powder. mp 80–81 °C; TLC R_f 0.54 (CHCl₃/MeOH 5:1); ¹H NMR (DMSO-*d*₆): δ 1.11–1.14 (m, 6H, COOCH₂CH₃), 1.62–1.79 (m, 4H, CH₂CH₂), 2.05–2.22 (m, 2H, β-CH₂), 2.37–2.40 (t, J = 8.0 Hz, 2H, γ-CH₂), 2.53–2.56 (t, J = 7.5 Hz, 2H, CH₂), 2.84–2.87 (t, J = 7.5 Hz, 2H, CH₂), 3.99–4.03 (q, J = 7.0 Hz, 2H, COOCH₂CH₃), 4.10–4.17 (m, 2H, COOCH₂CH₃), 4.52–4.57 (m, 1H, α-CH), 5.87 (s, 1H, C5-CH), 5.99 (s, 2H, 2-NH₂, exch), 7.49–7.50 (d, J = 7.5 Hz, 1H, Ar), 8.83–7.92 (m, 2H, Ar), 8.77–8.79 (d, J = 8.0 Hz, 1H, CONH, exch), 10.14 (s, 1H, 3-NH, exch), 10.81 (s, 1H, 7-NH, exch).

General Procedure for the Synthesis of Compounds 21–23.

To a 250 mL rbf was added a mixture of bromo-substituted pyridine carboxylic acids **18–20** (1.01 g, 4 mmol), *N*-methylmorpholine (485 mg, 4.8 mmol), 2-chloro-4,6-dimethoxy-1,3,5-triazine (843 mg, 4.8 mmol), and anhydrous DMF (10 mL). The reaction mixture was stirred for 1.5 h at room temperature after which, *N*-methylmorpholine (485 mg, 4.8 mmol) and L-glutamic acid diethyl ester hydrochloride (1.44 g, 6 mmol) were added to the flask. The reaction mixture was then stirred for 12 h at room temperature. After evaporating the solvent under reduced pressure, MeOH (20 mL), followed by silica gel was added (2.5 g) and the solvent evaporated. The resulting plug was loaded onto a silica gel column (2.5 cm × 12 cm) and eluted with 50% EtOAc in hexane. Fractions with the desired R_f (TLC) were pooled and evaporated to afford **21–23** as colorless liquids.

(6-Iodonicotinoyl)-L-glutamic Acid (21).—Compound **21** was prepared using the general method described for the preparation of **21–23**, from 6-iodonicotinic acid, **18** (1.01 g, 4 mmol), to give 1.59 g (90%) of **21** as a colorless liquid. TLC R_f = 0.44 (hexane/EtOAc, 1:1). ¹H NMR (DMSO-*d*₆): δ 1.14–1.2 (m, 6H, COOCH₂CH₃), 1.91–2.15 (m, 2H, β-CH₂), 2.41–2.46 (m, 2H, γ-CH₂), 4.02–4.17 (m, 2H, COOCH₂CH₃), 4.09–4.14 (m, 2H, COOCH₂CH₃), 4.43–4.47 (m, 1H, α-CH), 7.79–7.81 (d, J = 8.29 Hz, 1H, Ar) 8.14–8.16 (d, J = 8.28 Hz, 1 H, Ar), 8.29–8.30 (d, J = 7.26 Hz, 1 H, CONH, exch), 8.82 (s, 1 H, Ar).

(5-Iodopicolinoyl)-L-glutamic Acid (22).—Compound **22** was prepared using the general method described for the preparation of **21–23**, from 5-iodopicolinic acid, **19** (1.01 g, 4 mmol), to give 1.6 g (90%) of **22** as a colorless liquid. TLC R_f = 0.44 (hexane/EtOAc,

1:1). ^1H NMR (CDCl_3 - d_1): δ 1.22–1.34 (m, 6H, $\text{COOCH}_2\text{CH}_3$), 2.09–2.53 (m, 4H, β - CH_2 , γ - CH_2), 4.10–4.17 (m, 2H, $\text{COOCH}_2\text{CH}_3$), 4.23–4.29 (m, 2H, $\text{COOCH}_2\text{CH}_3$), 4.78–4.86 (m, 1H, α -CH), 7.96–7.98 (d, J = 8.31 Hz, 1 H, Ar), 8.27–8.29 (d, J = 8.40 Hz, 1 H, Ar), 8.83 (s, 1 H, Ar), 9.02–9.04 (d, J = 8.36 Hz, 1 H, CONH, exch).

(6-Iodopicolinoyl)-L-glutamic Acid (23).—Compound **23** was prepared using the general method described for the preparation of **21–23**, from 6-iodopicolinic acid, **20** (1.01 g, 4 mmol), to give 1.6 g (90%) of **23** as a colorless liquid. TLC R_f = 0.44 (hexane/EtOAc, 1:1). ^1H NMR ($\text{DMSO}-d_6$): δ 1.25–1.30 (m, 6H, $\text{COOCH}_2\text{CH}_3$), 2.09–2.53 (m, 4H, β - CH_2 , γ - CH_2), 4.10–4.17 (m, 2H, $\text{COOCH}_2\text{CH}_3$), 4.23–4.29 (m, 2H, $\text{COOCH}_2\text{CH}_3$), 4.79–4.84 (m, 1H, α -CH), 7.64–7.66 (d, J = 7.87 Hz, 1 H, Ar), 7.71–7.75 (t, J = 7.71, 7.71 Hz, 1 H, Ar), 8.14–8.16 (d, J = 7.51 Hz, 1 H, Ar), 8.28–8.30 (d, J = 8.13 Hz, 1 H, CONH, exch).

Molecular Modeling and Computational Studies.

The X-ray crystal structures of **AGF183** bound human $\text{FR}\alpha$ (PDB 5IZQ, 3.60 Å)²⁸ and **AGF150** bound human GARFTase (PDB 4ZZ1, 1.35 Å)²⁹ were obtained from the protein database. Ligand GARFTase and $\text{FR}\alpha$ docking studies were performed using LeadIT 2.1.6.³⁶ The protonation states of the proteins and the ligands were calculated using the default settings. In the ligand binding site, free rotation of water molecules (from the crystal structure ligand, defined by amino acids within 6.5 Å) was permitted. Docked ligands were sketched and energy-minimized using MOE 2016.08⁴⁰ and Amber10:EHT force field (limit of 0.1 kcal/mol), respectively. The triangle matching placement method was used to dock molecules using LeadIT 2.1.6 and scores were calculated using default settings. The docked poses were visualized using MOE 2016.08. The docking process was validated (using LeadIT 2.1.6) by redocking the energy minimized crystallized ligands (**AGF183** for $\text{FR}\alpha$ and **AGF150** for GARFTase). RMSD SVL code obtained from the ChemCompWeb site (www.chemcomp.com) was used to calculate the deviation of the best docked pose from the crystal structure conformation. The best scored pose of **AGF150** in human GARFTase had an RMSD of 1.22 Å and **AGF183** in $\text{FR}\alpha$ had an RMSD of 0.84 Å. LeadIT 2.1.6 was thus validated for our docking purposes in $\text{FR}\alpha$ and human GARFTase.

Reagents for Biological Studies.

[3',5',7,9- ^3H]Folic acid (25 Ci/mmol), [3',5',7- ^3H]MTX (20 Ci/mmol), and [$^{14}\text{C}(\text{U})$]glycine (87mCi/mmol) were purchased from Moravek Biochemicals (Brea, CA). Unlabeled folic acid was purchased from Sigma Chemical Co. (St. Louis, MO). Leucovorin [(6R,S) 5-formyl tetrahydrofolate] (LCV) was obtained from 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); 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), Eli Lilly and Co. (Indianapolis, IN); and PTX ((2S)-2-[[4-[(1RS)-1-[(2, 4-diaminopteridin-6-yl)methyl]but-3-ynyl]benzoyl]amino]-pentanedioic acid), Allos Therapeutics (Henderson, NV); and (PT523) [$N\alpha$ -(4-amino-4-

deoxypteroyl)-N δ -hemiphthaloyl-L-ornithine], Dr. Andre Rosowsky (Boston, MA). Other chemicals were obtained from commercial sources in the highest available purity.

Cell Lines and Assays of Antitumor Drug Activities.

The origins of the engineered CHO sublines including RFC- and FR α -null MTXRIIOua^{R2-4} (R2), and RFC- (PC43–10), PCFT- (R2/PCFT4), FR α - (RT16), and FR β (D4)-expressing CHO sublines were previously described.^{20,30–32} Likewise, pCDNA3.1 vector control CHO cells (R2/VC) were reported.²⁰ The CHO cells were cultured in α -minimal essential medium (MEM) supplemented with 10% bovine calf serum (Invitrogen, Carlsbad, CA), 100 units/ml penicillin/100 μ g/mL streptomycin, and 2 mM L-glutamine at 37 °C with 5% CO₂. The PC43–10, RT16, R2/PCFT4, and R2(VC) sublines were all routinely cultured in α -MEM plus 1 mg/mL G418. Prior to the cell proliferation assays (see below), RT16 cells were cultured in complete folate-free RPMI 1640 for 3 days. R2/PCFT4 and R2(VC) cells were cultured in complete folate-free RPMI 1640 including dialyzed fetal bovine serum (FBS) (Invitrogen) and 25 nM LCV with 1 mg/mL G418. KB human nasopharyngeal carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA). IGROV1 (NCI-IGROV1) (passage 5) epithelial ovarian cancer cells were obtained from the Division of Cancer Treatment and Diagnosis, National Cancer Institute (Frederick, MD). KB and NCI-IGROV1 cells were cultured in folate-free RPMI 1640 medium, supplemented with 10% FBS, penicillin-streptomycin solution, and 2 mM L-glutamine at 37 °C with 5% CO₂.

For growth inhibition assays, cells (CHO, KB and NCI-IGROV1) were plated in 96-well dishes (~2500–5000 cells/well, total volume of 200 μ L medium) with antifolate drugs over a range of concentrations.^{8,20,31} For experiments with RT16, D4, NCI-IGROV1, and KB cells, cells were cultured in folate-free RPMI 1640 media with 10% dialyzed FBS and antibiotics supplemented with 2 nM LCV and 2 mM L-glutamine. The requirement for FR-mediated drug uptake in these assays was established in parallel incubations, including 200 nM folic acid. With R2/PCFT4 cells, PC43–10, R2, and R2(VC) cells, the medium was folate-free RPMI 1640 (pH 7.2) containing 25 nM LCV, supplemented with 10% dialyzed FBS, antibiotics, and L-glutamine. Cells were incubated for up to 96 h and viable cells were assayed with CellTiter-blue cell viability assay (Promega, Madison, WI) and a fluorescence plate reader.^{20,22,23,29,31,34,38} Raw data were exported to an Excel spreadsheet for analysis and results plotted (Prism Graphpad 6.0) for determinations of IC₅₀s, corresponding to the drug concentrations that result in 50% losses of cell growth.

For some of the *in vitro* growth inhibition studies, the inhibitory effects of the inhibitors on *de novo* thymidylate biosynthesis (i.e., thymidylate synthase) and *de novo* purine nucleotide biosynthesis (GARFTase and AICARFTase) were tested by coincubations with thymidine (10 μ M) and adenosine (60 μ M), respectively.^{20,22,23,29,31,34,38} For *de novo* purine nucleotide biosynthesis, additional protection experiments used AICA (320 μ M) to distinguish inhibitory effects at GARFTase from those at AICARFTase.^{20,22,23,29,31,34,38}

FR Binding Assay.

Competitive inhibition of [³H]folic acid binding to FR α and FR β using RT16 and D4 CHO cells, respectively, was used to assess relative binding affinities for assorted (anti)folate

ligands.^{20,22,23,29,31,34,38} Cells were plated in 60 mm culture dishes 48 h prior to experiment. For the experiments, cells ($\sim 5 \times 10^6$) were rinsed twice with Dulbecco's phosphate-buffered saline (DPBS), followed by two washes with an acidic buffer (10 mM sodium acetate, 150 mM NaCl, pH 3.5) to remove FR-bound folates. Cells were washed twice with ice-cold HEPES-buffered saline (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 2 mM MgCl_2 , 5 mM glucose, pH 7.4) (HBS), then incubated in HBS with [^3H]folic acid (50 nM, specific activity 0.5 Ci/mmol) in the presence and absence of unlabeled folic acid or antifolate (over a range of concentrations) for 15 min at 0 °C. The dishes were rinsed three times with ice-cold HBS, after which the cells were solubilized (0.5 N NaOH) and aliquots measured for radioactivity and protein. Protein concentrations were measured with Folin phenol reagent.⁴¹ [^3H]Folic acid bound to FR α and FR β was calculated as pmol/mg protein and relative binding affinities were calculated as the inverse molar ratios of unlabeled ligands required to inhibit [^3H]folic acid binding by 50%. By definition, the relative affinity of folic acid was 1.

Transport Assays.

R2 and R2/PCFT4 sublines were routinely grown in suspension as spinner cultures at densities of $(2-5) \times 10^5$ cells/mL. Cells were collected by centrifugation, washed with DPBS, and the cell pellets ($\sim 2 \times 10^7$ cells) were suspended in transport buffer (2 mL) for cellular uptake assays. Uptake of 0.5 μM [^3H]MTX was assayed in cell suspensions over 2 min at 37 °C in HBS at pH 6.8, or in 4-morpholinopropane sulfonic (MES)-buffered saline (20 mM MES, 140 mM NaCl, 5 mM KCl, 2 mM MgCl_2 , and 5 mM glucose) at pH 5.5 in the presence or 1 or 10 μM inhibitor.²¹ At the end of the incubations, transport was quenched with ice-cold DPBS, cells were washed 3 times with ice-cold DPBS, and cellular proteins were solubilized with 0.5 N NaOH. Levels of drug uptake were expressed as pmol/mg protein, calculated from direct measurements of radioactivity and protein contents of cell homogenates. Radioactivity was measured with a scintillation counter, and proteins were quantified using Folinphenol reagent.⁴¹ Transport results were normalized to levels in untreated controls. For determining K_i values for **1** and **6** compared to PMX, transport was measured over 2 min with 0.5 μM [^3H]MTX and 0–5 μM of the unlabeled inhibitor. Data were analyzed by Dixon plots.

In Situ GARFT Enzyme Inhibition Assay.

Incorporation of [^{14}C]glycine into [^{14}C]formyl GAR, as an *in situ* measure of endogenous GARFTase activity, was measured.^{20,22,31,11–17} For these experiments, KB cells were seeded in 4 mL of complete folate-free RPMI 1640 (pH 7.4) plus 2 nM LCV in 60 mm dishes at a density of 2×10^6 cells per dish. On the next day, the medium was replaced with 2 mL of fresh complete folate-free RPMI 1640 plus 2 nM LCV (without supplementing glutamine). Azaserine (4 μM final concentration) was added in the presence and absence of the antifolate inhibitors. After 30 min, L-glutamine (final concentration, 2 mM) and [^{14}C]glycine (tracer amounts; final specific activity 0.1 mCi/L) were added. Incubations were at 37 °C for 15 h, at which time cells were washed (one-time) with ice-cold folate-free RPMI 1640 plus serum. Cell pellets were dissolved in 2 mL 5% trichloroacetic acid at 0 °C. Cell debris was removed by centrifugation (the cell protein contents in the pellets were measured following solubilization with 0.5 N NaOH⁴¹), and the supernatants were extracted twice with 2 mL of ice-cold ether. The aqueous layer was passed through a 1 cm column of AG1 $\times$ 8 (chloride

form), 100–200 mesh (Bio-Rad), washed with 10 mL of 0.5N formic acid and then 10 mL of 4N formic acid, and finally eluted with 8 mL 1 N HCl. The eluates were collected and determined for radioactivity. The accumulation of [¹⁴C]formyl GAR was calculated as pmol per mg protein over a range of inhibitor concentrations. IC₅₀s were calculated as the concentrations of inhibitors that resulted in a 50% decrease in [¹⁴C]formyl GAR synthesis.

***In Vivo* Efficacy Study with IGROV1 Human Ovarian Tumor Xenografts.**

The methods for protocol design, drug treatment, toxicity evaluation, data analysis, quantification of tumor cell death, tumor model systems, and the biological significance of the drug treatment results with transplantable tumors have been described previously.^{22,23,29,42–44} Cultured NCI-IGROV1 human ovarian tumor cells were implanted subcutaneously (5×10^6 cells/flank) to establish a solid tumor xenograft model in female NCR SCID mice (obtained from NCI Animal Production Program). For the efficacy study, mice were 10 weeks old on day 0 (tumor implant) with an average body weight of 20 g. Mice were supplied food and water *ad libitum*. Study mice were maintained on either a folate-deficient diet from Harlan-Teklad (TD.00434) or a folate-replete diet from Lab Diet (5021; autoclavable mouse breeder diet) starting 14 days before subcutaneous tumor implant to ensure serum folate levels would approximate those of humans. Folate serum levels were determined prior to tumor implant and post study via *Lactobacillus casei* bioassay.³⁷ The animals on both diets were respectively pooled and implanted bilaterally subcutaneously with 30 to 60 mg tumor fragments by a 12 gauge trocar, and again respectively pooled before unselective distribution to the various treatment and control groups. Chemotherapy began 3 days after tumor implantation, when the number of cells was relatively small (below the established limit of palpation of 63 mg). Tumors were measured with a caliper two to three times weekly (depending on the doubling time of the tumor). Mice were sacrificed when the cumulative tumor burden reached 1500 mg. Tumor weights were estimated from two-dimensional measurements [i.e., tumor mass (in mg) = $(a \times b^2)/2$, where a and b are the tumor length and width in mm, respectively]. For calculation of end points, both tumors on each mouse were added together, and the total mass per mouse was used. Tumor growth delay [$T - C$, where T is the median time in days required for the treatment group tumors to reach a predetermined size (e.g., 1000 mg), and C is the median time in days for the control group tumors to reach the same size; tumor-free survivors are excluded from these calculations] was used as a quantitative end-point to assess antitumor activity. Treatment (T) and control (C) groups were measured when the control groups reached 1000 mg in size (exponential growth phase). The median of each group was determined (including zeros) for calculating the T and C values.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS USED

AICA	5-aminoimidazole-4-carboxamide
AICARFTase	5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase
CHO	Chinese hamster ovary
FBS	fetal bovine serum
DPBS	Dulbecco's phosphate-buffered saline
EOC	epithelial ovarian cancer
FR	folate receptor
GAR	glycinamide ribonucleotide
GARFTase	glycinamide ribonucleotide formyltransferase
HBSS	Hank's balanced salts solution
HBS	HEPES-buffered saline
LCV	leucovorin
LMTX	Lometrexol
MTX	methotrexate
MEM	minimal essential media
PMX	Pemetrexed
PTX	Pralatrexate
PCFT	proton-coupled folate transporter
RTX	Raltitrexed
RFC	reduced folate carrier
SAR	structure–activity relationship

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Figure 1.
Structures of classical antifolates.

Figure 2.
Structures of the 6-pyrrolo[2,3-*d*]pyrimidine phenyl (**1** and **2**) and thienyl (**3** and **4**) L-glutamates.

Figure 3.
Structures of the 6-pyrrolo[2,3-*d*]pyrimidine pyridyl (**5–10**) analogues in this study.

Figure 4. Binding of 6-substituted pyrrolo[2,3-*d*] pyrimidines **1**, **5**, **6**, and **7** to FR α and FR β , compared to folic acid and MTX. Data are shown for the effects of the unlabeled ligands with FR α -expressing RT16 CHO cells and FR β -expressing D4 CHO cells. Relative binding affinities for folate/antifolate substrates were determined over a range of ligand concentrations and were calculated as the inverse molar ratios of unlabeled ligands required to inhibit [3 H]folic acid binding by 50%. Results are presented as mean values plus/minus standard errors from 3 experiments. Undefined abbreviations: FA, folic acid. * Difference from folic acid is statistically significant, $p < 0.05$.

Figure 5.

Binding of 6-substituted pyrrolo[2,3-*d*]pyrimidine phenyl and pyridyl analogues **1** and **6** respectively, to PCFT in R2/PCFT4 cells. Data are shown for the effects of **1** or 10 μ M PMX, **1**, **6**, or PT523 on uptake of [3 H]MTX (0.5 μ M) at pH 5.5 and pH 6.8 over 5 min at 37 °C in R2/PCFT4 CHO cells. Experimental details are provided in the Experimental Procedures. Results are presented as mean values plus/minus standard errors for 3 experiments. Results are compared to normalized rates in the absence of any additions and to those for PCFT-null R2 cells without additions. Abbreviations: NA, no additions. The bars noted with asterisks were statistically different from the untreated control (NA) ($p < 0.05$) by paired *t* test analysis.

Figure 6.

Growth inhibition of KB cells by 6-substituted pyrrolo[2,3-*d*]pyrimidine phenyl and pyridyl analogues **1** and **6**, respectively, and protection by excess folic acid, nucleosides, or 5-aminoimidazole-4-carboxamide (AICA). KB cells were plated (4000 cells/well) in folate-free RPMI 1640 medium with 10% dialyzed serum, antibiotics, L-glutamine, and 2 nM LCV with a range of concentrations of **1** or **6** in the presence of folic acid (200 nM), adenosine (60 μ M) and/or thymidine (10 μ M), or in the presence of AICA (320 μ M). Cell proliferation was assayed with CellTiter-Blue with a fluorescent plate reader. These results, along with

those for **5** and **7**, are summarized in Table 1. The data shown are representative of those from triplicate experiments.

Figure 7.

In situ GARFTase assay in KB tumor cells treated with 6-substituted pyrrolo[2,3-*d*]pyrimidine analogues **1**, **5**, and **6**. KB cells were treated with drug for 15 h, followed by azaserine (4 μ M) and [14 C]glycine. 14 C-labeled metabolites were extracted and fractionated on ion-exchange columns, permitting quantitation of accumulated [14 C]formyl GAR. Results are expressed as a percent of vehicle control over a range of drug concentrations. Experimental details are provided in the Experimental Procedures. Results are presented as mean values $\pm$ standard errors from 3 experiments. IC_{50} values were as follows: 5.6 nM, **1**; 4.8 nM, **5**; and 1.8 nM **6**. For comparison, the IC_{50} s for GARFTase inhibition in KB cells by PMX and LMTX were previously reported as 30.0 nM and 14.0 nM, respectively.²⁰

Figure 8.

Molecular modeling studies with human FR α (PDB 5IZQ).²⁸ (A) Superimposition of the docked pose of **1** (light pink) and **5** (magenta). (B) Docked pose of **1** (light pink) and **6** (green). Modeled using LeadIT 2.1.6.³⁶

Figure 9.

Molecular modeling studies with human GARFTase (PDB 4ZZ1).²⁹ (A) Superimposition of docked pose of **1** (light pink) and **5** (magenta). (B) Docked pose of **1** (light pink) and **6** (green). Modeled using LeadIT 2.1.6.³⁶

Figure 10.

in vivo efficacy trial of **6** with NCI-IGROV1 EOC xenografts. Female ICR SCID mice (10 weeks old; 20 g average body weight) were maintained on a folate-deficient diet *ad libitum* for 14 d prior to subcutaneous tumor implant to decrease serum folate to a concentration approximating that in human serum. Human NCIIGROV1 tumors were implanted bilaterally and mice were non-selectively randomized into 5 mice/group. Compound **6** (150 mg/kg/inj; 1200 mg/kg total; Q2Dx8) and PMX (Alimta) (8.1 mg/kg/inj; 32.4 mg/kg total; Q4Dx4) [dissolved in 5% ethanol (v/v), 1% Tween-80 (v/v), 0.5% NaHCO₃] were administered intravenously (0.2 mL/injection). Mice were observed and weighed daily; tumors were measured twice per week. Abbreviation: Rx, treatment.

Scheme 1. Synthesis of Classical 2-Amino-4-oxo-6-substituted-pyrrolo[2,3-*d*]pyrimidines 5–10^a

^aConditions: (a) 21 or 22 or 23, CuI, Pd(0)(PPh₃)₄, Et₃N, DMF, RT, 12 h, 54–57%; (b) 5% Pd/C, H₂, 55 psi, 2 h, quantitative yield; (c) i. 1N NaOH, RT, 6 h; ii. 1 N HCl, 95%; (d) *N*-methylmorpholine, 2-chloro-4,6-dimethoxy-1,3,5-triazine, L-glutamate diethyl ester hydrochloride, DMF, RT, 12 h, 90%.

Table 1.

IC₅₀ (in nM) for 6-Substituted Pyrrolo[2,3-*d*]pyrimidine Antifolates 1, 5–10 and Classical Antifolates in RFC-, PCFT-, and FR-Expressing Cell Lines^a

antifolate	RFC		FR α		FR β		PCFT		RFC/FR α /PCFT	
	PC43-10	R2	RT16	RT16 (+FA)	D4	D4 (+FA)	R2/hPCFT4	R2(VC)	KB (+FA)	Ade/Thd/AICA
1	>1000	>1000	6.3(1.6)	>1000	5.6(1.2)	>1000	213 (28)	>1000	1.0(0.7)	Ade/AICA
5	>1000	>1000	15.02(3.42)	>1000	37.41(11.64)	>1000	>1000	>1000	17.36(8.75)	Ade/AICA
6	>1000	>1000	1.27(0.12)	687(312)	0.58(0.13)	471(335)	57.60(10.66)	>1000	0.37(0.08)	Ade/AICA
7	>1000	>1000	186 (57)	>1000	>1000	>1000	>1000	>1000	667(176)	Ade/AICA
8	>1000	>1000	449 (26)	>1000	839.10 (230)	>1000	>1000	>1000	>1000	ND
9	>1000	>1000	4.43 (1.60)	>1000	18.80 (0.5)	>1000	>1000	>1000	>1000	ND
10	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	ND
MTX	12(1.1)	114(31)	114(31)	216(8.7)	106(11)	211(43)	121 (17)	>1000	6.0(0.6)	Ade/Thd
PMX	138(13)	849(93)	42(9)	894(93)	60(8)	254(78)	13.2(2.4)	974 (18)	68(12)	Thd/Ade
RTX	6.3(1.3)	15(5)	15(5)	>1000	22(10)	746(138)	99.5(11.4)	>1000	5.9(2.2)	Thd
PTX	0.69(0.07)	819(94)	168(50)	>1000	ND	ND	57(12)	>1000	0.47(0.20)	Ade/Thd

^aGrowth inhibition assays were performed for CHO sublines engineered to express human RFC (PC43–10), FRs (RT16, D4), or PCFT (R2/PCFT4), for comparison with transporter-null [R2, R2(VC)] CHO cells, and for the KB human tumor subline (expresses human RFC, FR α , and PCFT).^{8,20,29} Experimental details are described in the Experimental Procedures. For the FR experiments, growth inhibition assays were performed in the presence and the absence of 200 nM folic acid (FA). The data shown are mean values from 3–10 experiments (plus/minus standard errors in parentheses). Results are presented as IC₅₀ values, corresponding to the concentrations that inhibit cell growth by 50% relative to cells incubated without drug. For KB cells, results are summarized for the protective effects of nucleoside additions, including adenosine (Ade) (60 μ M) or thymidine (Thd) (10 μ M), or 5-aminoimidazole-4-carboxamide (AICA) (320 μ M), as in Figure 6. Some of the data for MTX, PMX, RTX, and PTX were previously published.^{20,22,31} The structures of compounds **1**, **5–10** and the classical antifolates are in Figures 1, 2, and 3. ND, not determined.