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Structural and Enzymatic Analysis of Tumor-Targeted Antifolates That Inhibit Glycinamide Ribonucleotide Formyltransferase

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Abstract

Pemetrexed and methotrexate are antifolates used for cancer chemotherapy and inflammatory diseases. These agents have toxic side effects resulting, in part, from nonspecific cellular transport by the reduced folate carrier (RFC), a ubiquitously expressed facilitative transporter. We previously described 2-amino-4-oxo-6-substituted pyrrolo[2,3-*d*]pyrimidine antifolates with modifications of the side chain linker and aromatic ring that are poor substrates for RFC but are efficiently transported via folate receptors (FRs) and the proton-coupled folate transporter (PCFT). These targeted antifolates are cytotoxic *in vitro* toward FR- and PCFT-expressing tumor cells and *in vivo* with human tumor xenografts in immune-compromised mice, reflecting selective cellular uptake. Antitumor efficacy is due to inhibition of glycinamide ribonucleotide (GAR) formyltransferase (GARFTase) activity in *de novo* synthesis of purine nucleotides. This study used purified human GARFTase (formyltransferase domain) to assess *in vitro* inhibition by eight novel

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.6b00412. Fit data for K_i calculation, crystallographic data and refinement statistics, ligand interaction images with contact tables and $2F_o - F_c$ ligand electron density maps for all eight AGF compounds with human GARFTase, comparison of AGF linker binding modes, images describing interactions of β -GAR with human GARFTase, and proposed models for drug design (PDF)

Accession Codes

Atomic coordinates for the seven ternary complexes of human GARFTase have been deposited in the Protein Data Bank as entries 4PN4, 4X72, 4X73, 4X74, 4X75, 4X76, 4X77, and 4X78.

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thieno- and pyrrolo[2,3-*d*]pyrimidine antifolates. Seven analogues (AGF23, AGF71, AGF94, AGF117, AGF118, AGF145, and AGF147) inhibited GARFTase with K_i values in the low- to mid-nanomolar concentration range, whereas AGF50 inhibited GARFTase with micromolar potency similar to that of PMX. On the basis of crystal structures of ternary complexes with GARFTase, β -GAR, and the monoglutamyl antifolates, differences in inhibitory potencies correlated well with antifolate binding and the positions of the terminal carboxylates. Our data provide a mechanistic basis for differences in inhibitory potencies between these novel antifolates and a framework for future structure-based drug design. These analogues could be more efficacious than clinically used antifolates, reflecting their selective cellular uptake by FRs and PCFT and potent GARFTase inhibition.

Graphical Abstract

Antifolate drugs are analogues of the essential vitamin folic acid that are used clinically to treat cancers and inflammatory diseases. The first antifolate, aminopterin, was found to cause remission of childhood leukemia.^{1,2} However, because of its potential for substantial toxicity, aminopterin is rarely used today. Rather, an N10-methylated version of aminopterin, methotrexate, continues to see widespread use in the treatment of an array of malignant and nonmalignant conditions, including acute lymphoblastic leukemia, osteosarcoma, psoriasis, and rheumatoid arthritis.^{3,4} Other clinically used antifolates include raltitrexed, used for treating colorectal cancer,⁵ and pemetrexed (PMX), used throughout the world for treating malignant pleural mesothelioma⁶ and non-squamous non-small cell lung cancer.^{7,8}

Aminopterin and methotrexate both inhibit dihydrofolate reductase, which catalyzes the reduction of dihydrofolate to tetrahydrofolate, required to mediate transfer of one-carbon units (i.e., formyl, methylene, and methyl) leading to synthesis of purines, thymidylate, serine, and methionine.^{3,4} To decrease toxicity, methotrexate is co-administered with leucovorin, a folate analogue that is metabolized to tetrahydrofolate in the absence of dihydrofolate reductase.^{3,4,9} Raltitrexed and PMX primarily inhibit thymidylate synthase, which converts deoxyuridylate to thymidylate.^{10,11} In spite of their demonstrated clinical efficacy, none of these agents are selectively targeted to the diseased tissue, resulting in substantial dose-limiting toxicity reflecting toxic effects toward normal tissues.

The lack of tumor selectivity for the clinically useful antifolates, in part, reflects their cellular uptake by the reduced folate carrier (RFC), a ubiquitously expressed facilitative carrier.¹² Folate receptors (FRs) can also mediate uptake of folates and cytotoxic antifolates into mammalian cells via endocytosis.¹³ FRs are glycerolphosphatidylinositol-linked proteins and are expressed on plasma membranes of a limited number of normal tissues with presentation most common on the apical surface of polarized epithelial cells, which renders them generally inaccessible to circulating antifolates following intravenous administration.^{13–15} Conversely, FRs are expressed at high levels in tumors of epithelial origin (e.g., ovarian, non-small cell lung cancer, and uterine cancers) as FR α ,¹³ and in certain hematologic malignancies (e.g., acute myeloid leukemia),¹⁶ activated macrophages associated with tumors,¹⁷ and sites of inflammation (e.g., rheumatoid arthritis)¹⁸ as FR β . The proton-coupled folate transporter (PCFT) is a proton–folate symporter that serves as the primary means of dietary folate absorption in the upper gastrointestinal tract.¹⁹ PCFT expressed in tumors functions in the transport of folates and antifolates at acidic pHs characterizing the tumor microenvironment.^{20,21}

We previously discovered a series of novel cytotoxic antifolates (AGF23, AGF50, AGF71, AGF94, AGF117, AGF118, AGF145, and AG147) that are selectively targeted to cells expressing FR α , FR β , and PCFT, including tumor cells.^{22–28} These compounds are 6-substituted pyrrolo- and thieno[2,3-*d*]pyrimidines structurally similar to PMX, a 5-substituted pyrrolo[2,3-*d*]pyrimidine antifolate. Unlike PMX, these novel targeted antifolates are principally inhibitors of *de novo* purine nucleotide biosynthesis at glycineamide ribonucleotide (GAR) formyltransferase (GARFTase), which catalyzes the transfer of the formyl group from 10-formyl-tetrahydrofolate (10-fTHF) to β -GAR to produce formyl-GAR.^{22–28} While antifolates targeting GARFTase were previously described, including lometrexol, LY309887,²⁹ and AG2034,³⁰ clinical progression was limited by their toxicity,^{31–33} likely at least in part because of their lack of tumor selectivity and cellular uptake by RFC.

It is well established that most clinically used antifolates require polyglutamylation via folylpoly- γ -glutamate synthetase (FPGS) to improve their inhibitory effects on their target enzymes. For example, PMX increased its level of inhibition of thymidylate synthase by up to 100-fold following its polyglutamylation.³⁴ In addition, polyglutamylation is required for cellular retention of cytotoxic antifolates.³⁵ Tumor cell resistance to antifolates is known to occur in cases of low or decreased FPGS activity.³⁵ However, antifolates with sufficiently potent folate-metabolizing enzyme inhibition can exert biological effects independent of polyglutamylation and thus could be useful against antifolate resistant tumors with low or decreased FPGS activity.

In this study, we determined the relative inhibition of human GARFTase by eight novel tumor-targeted 6-substituted pyrrolo[2,3-*d*]pyrimidine antifolates (AGF23, AGF50, AGF71, AGF94, AGF117, AGF118, AGF145, and AG147;^{22–28} as their monoglutamates) to improve our understanding of key structural determinants involved in GARFTase inhibition and to aid in the design of future generations of GARFTase inhibitors. To correlate antifolate structural features with variations in inhibitor potencies, reflected in inhibition constant (K_i) values, molecular details of interactions of the drugs with GARFTase were determined from

crystallographic structures of the formyltransferase domain of human GARFTase complexed with the β -GAR substrate. Collectively, our results provide a rational basis for understanding the *in vitro* inhibition profiles of a series of novel FR- and PCFT-targeted antifolates as their monoglutamates and serve as a valuable guide for developing future generations of analogues with substantially enhanced therapeutic potentials.

MATERIALS AND METHODS

Materials

Syntheses of the antifolates were performed as previously described.^{22–27} 10-Formyl-5,8-dideaza-tetrahydrofolate (10-CHODDF) was a gift from R. Moran (Virginia Commonwealth University, Richmond, VA). α,β -GAR was synthesized in the METACyt Biochemical Analysis Center at Indiana University under the direction of T. S. Widlanski. PMX was a gift from F. Zhang (Indiana University).

Protein Cloning, Expression, and Purification

Two constructs were used to express the formyltransferase domain of human GARFTase in different experiments. A codon-optimized human GARFTase construct with an N-terminal hexahistidine tag (His-GARFTase, plasmid pSD002) was generated via total gene synthesis using the DNA Works web server for oligonucleotide selection.³⁶ Oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville, IA), and the resulting cDNA amplicon was ligated into pHisparallel³⁷ digested with NcoI and NotI using T4 DNA ligase (New England Biolabs, Ipswich, MA). A second human GARFTase construct with a C-terminal hexahistidine tag (GARFTase-His, plasmid pSD009) for use in crystallographic studies was generated via amplification from the pSD002 template. The resulting amplicon, which included sequences encoding a C-terminal hexahistidine tag as part of the antisense primer design, was ligated via isothermal assembly into the NdeI and XhoI sites of pHisparallel.³⁸ All constructs were confirmed by DNA sequencing.

For expression of His-GARFTase or GARFTase-His, the plasmids were transformed into Rosetta (DE3)pLysS cells and grown in the presence of 34 μ g/mL chloramphenicol and 100 μ g/mL ampicillin. LB cultures (1 L) were inoculated with 15 mL of a 100 mL overnight culture and grown at 37 °C and 230 rpm until the OD₆₀₀ reached 0.6. Expression was induced via the addition of 0.5 mM isopropyl β -D-1-thiogalactopyranoside, and cells were incubated overnight at 20 °C and 230 rpm. Cultures were pelleted and resuspended in 40 mL of buffer containing 25 mM Tris-HCl (pH 8), 300 mM NaCl, 5 mM β -mercaptoethanol (β -ME), 10 mM CaCl₂, and 10 mM MgCl₂ prior to cell lysis using a pressurized cell disruptor. Lysates were loaded onto Ni-NTA resin (Qiagen, Valencia, CA). The Ni-NTA column was washed with 8 column volumes of 25 mM Tris-HCl (pH 8), 300 mM NaCl, 10% glycerol, and 5 mM β -ME, and GARFTase protein was eluted with a 0 to 75% gradient of 25 mM Tris-HCl (pH 8), 300 mM NaCl, 10% glycerol, 300 mM imidazole, and 5 mM β -ME over 10 column volumes. Final purification was conducted via size exclusion chromatography on a Superdex 75 16/60 (GE Healthcare) column using 25 mM Tris-HCl (pH 8), 10 mM β -ME, and 300 mM NaCl. To prevent a loss of enzymatic activity, His-GARFTase was stored at 90 μ M in 20% glycerol at –80 °C.

GAR Transformylase Inhibition Assay

GARFTase activity was determined by measuring the rate of 5,8-dideaza-tetrahydrofolate production in the presence of varying concentrations of antifolate inhibitors using an established spectrophotometric protocol.³⁹ A 150 μ L total volume containing 30 μ M α,β -GAR, 5.4 μ M 10-CHODDF and antifolate (stock solution dissolved in DMSO) in 0.1 M HEPES (pH 7.5) was preincubated at 37 °C in a UV transparent 96-well plate (Costar). To initiate the assay, 150 μ L of 20 nM His-GARFTase or buffer was added, and the plate was shaken for 5 s. Absorbance changes at 295 nm were recorded at 15 s intervals over 20 min using a Synergy H1M plate reader (BioTek). For each antifolate, triplicate assays were performed at 20 different drug concentrations. To determine the initial slope for each drug concentration, the average absorbance value of the reference well was subtracted to account for the effect of the antifolate addition on the raw absorbance. Corrected absorbance values were converted to changes in absorbance over time by subtracting the absorbance at the first measurement from all subsequent measurements. Finally, correlation coefficients were compared over different time spans to identify a range with consistent, linear increases in absorbance for all three curves. Initial velocities obtained from these slopes were graphed against the antifolate concentrations, and a hyperbola fit [$y = -b \times x / (K_i + x) + b$, where b is the y -intercept] was used to determine a K_i for each antifolate (KaleidaGraph version 4.1).

Crystallization of Human GARFTase

GARFTase-His was buffer exchanged into 25 mM Tris-HCl (pH 8), 200 mM NaCl, 10 mM β -ME, and a 3-fold molar excess of α,β -GAR, and antifolate (stock solution dissolved in DMSO) was added for a final protein concentration of 10 mg/mL. The protein/ligand solution was incubated at 4 °C for 2 h before crystallization trials were set up. Using 0.1 M Tris-HCl (pH 7.5), 0.333 M NaCl, 18% polyethylene glycol (PEG) 4000, and a 2% PEG 400 precipitant solution, sitting drop plates were set up with 1 μ L of protein, 1 μ L of precipitant, and 0.2 μ L of either 32 mM N -nonyl β -D-thiomaltoside or 9 mM N -decyl β -D-thiomaltoside (Hampton Research, Aliso Viejo, CA). Plates were incubated at 4 °C, and multifaceted cube- and obelisk-shaped crystals were obtained within a few days. Crystals were frozen in liquid nitrogen after being transferred through a cryoprotectant gradient that increased the PEG 400 concentration of the mother liquor to 14% in 2% increments. Cryoprotectant solutions contained α,β -GAR and antifolate in the same concentrations as the crystallization solution.

X-ray Data Collection and Structure Determinations

Data collection was performed at Lawrence Berkeley National Laboratory Advanced Light Source beamline 4.2.2 using the Taurus CMOS detector. All data sets were processed to a P3₂ space group (HKL2000⁴⁰). Molecular replacement was performed using Protein Data Bank (PDB) entry 1MEJ with waters removed (AG71 and AG94 complexes) or 4PN5 with ligands and waters removed (AG23, AG50, AG117, AG118, AG145, and AG147 complexes) as a search model (PHENIX⁴¹). Subsequent model building and refinement were performed using Coot⁴² and PHENIX, respectively.

RESULTS

The compounds in this study are structurally distinct from the clinically used antifolate PMX in that AGF23, AGF71, AGF94, AGF117, AGF118, AGF145, and AGF147 are all 6-substituted pyrrolo[2,3-*d*]pyrimidine analogues, whereas AGF50 contains a thieno[2,3-*d*]pyrimidine moiety.^{22–27} All contain a terminal L-glutamate separated from the bicyclic ring system by a bridge region composed of three (AGF94) or four (AGF23, AGF50, AGF71, AGF117, and AGF118) carbons, followed by benzoyl (AGF23 and AGF50) or thienoyl (AGF71, AGF94, AGF117, and AGF118) ring moieties (Figure 1). AGF117 and AGF118 are 3',5'- and 2',4'-thiophene regioisomers of AGF71, respectively. AGF145 and AGF147 include six- and seven-carbon side chains, respectively, without an aromatic ring system (Figure 1). In contrast, PMX is a 5-substituted pyrrolo[2,3-*d*]pyrimidine benzoyl antifolate with a two-carbon bridge.

The structurally diverse 6-substituted pyrrolo- and thieno-[2,3-*d*]pyrimidine antifolates all elicit low nanomolar to subnanomolar IC₅₀ cytotoxic values in cell proliferation assays with KB human tumor cells *in vitro*^{22–27} (Table 1). For AGF71 and AGF94, *in vivo* antitumor efficacies were established toward FR-expressing KB and IGROV1 tumors.^{24,25} All are excellent substrates for cellular uptake via FRs and/or PCFT and cause cell death by inhibiting the *de novo* purine nucleotide biosynthesis.^{22–27,43} While the principal intracellular target is GARFTase, as established by an *in situ* metabolic assay that measures incorporation of [¹⁴C]glycine into formyl-GAR in intact cells,^{22–27} given the contributions of membrane transport and polyglutamylation to overall drug efficacy, it is impossible to quantitatively assess either their relative GARFTase inhibitions or the relationships between enzyme inhibition and antifolate binding in a cellular context.

Accordingly, we performed kinetic assays with the isolated formyltransferase domain of GARFTase to determine K_i values and determined crystal structures of the human GARFTase formyltransferase domain in complexes with each of the novel antifolates as a monoglutamate. We hypothesized that the most effective GARFTase inhibitors will have conserved structural motifs and that identification of these motifs will provide scaffolds around which hypothesis-based inhibitors can be designed.

GARFTase Inhibition

An N-terminal His₆-tagged formyltransferase domain of the human GARFTase construct was expressed in *Escherichia coli* and purified via affinity Ni-NTA and size exclusion chromatography. The kinetic assay used purified GARFTase with α,β -GAR and 10-CHODDF as substrates and measured absorbance at 295 nm in the presence of varying concentrations of antifolates.³⁹ From hyperbolic fitting of initial rates against inhibitor concentrations, we calculated K_i values ranging from 7 nM to ~1.1 μ M (Figure 2 and Figure S1). The most potent inhibitors were the AGF117 and AGF118 thienoyl regioisomers of AGF71 with K_i values of <10 nM, whereas AGF23 and AGF71 were slightly less effective, exhibiting 2-fold increased K_i values. Three molecules, AGF94 and its straight chain, nonaroyl analogues AGF145 and AGF147, showed similar, moderate potencies with K_i values ranging from ~70 to 100 nM. In contrast to these 6-substituted pyrrolo[2,3-*d*]pyrimidine

analogues, the thieno[2,3-*d*]-pyrimidine AGF50 exhibited micromolar inhibition, roughly 10- and 150-fold weaker than for AGF147 and AGF117, respectively (Figure 2).

Crystal Structures

To determine how variations in molecular structures of the novel 6-substituted pyrrolo- and thieno[2,3-*d*]pyrimidine antifolates in this study allow for the observed inhibitor potencies, we determined structures for this series with human GARFTase and β -GAR substrate. We hypothesized that the most effective inhibitors would bind GARFTase in conformations distinct from the less potent analogues, possibly through a set of key molecular contacts. To test this, the formyltransferase domain of human GARFTase-His was crystallized in the presence of β -GAR and each of the eight novel antifolates. Diffraction data were collected, and phases were determined via molecular replacement prior to model building and refinement. High-resolution crystallographic models, with minimum Bragg spacings ranging from 1.60 to 2.05 Å, were obtained for human GARFTase-His, β -GAR, and the antifolate ternary complexes (Table S1 and Figures S2–S9).

In contrast to that in published GARFTase structures,^{44,45} the density for β -GAR is clearly evident in all crystallographic structures presented herein (Figure S10). The orientation of the pyrrolo[2,3-*d*]pyrimidine motif, shared by seven of the antifolates in this study, is conserved with six conserved hydrogen bonds with GARFTase (shown for AGF117 in Figure 3; also see Figures S2–S9 for detailed contacts). However, the thieno[2,3-*d*]pyrimidine motif of AGF50 lacks a hydrogen bond with the R897 carboxyl, as N7 is replaced by a sulfur atom (Figures S3 and S11A). In all structures, the atoms in the linker between the bicyclic ring system and the glutamyl tail form hydrophobic contacts with GARFTase; i.e., the sulfur atoms in the thiophene moieties of AGF71, AGF94, AGF117, and AGF118 do not have the potential for polar contacts, and all follow a general path through the binding pocket (Figure S11B). Thus, the impact of differences in inhibitory potencies toward GARFTase based on variations involving the linker regions in the antifolates in this study is modest.

The most significant factor that affects the inhibition potency appears to be the manner in which the inherent length and flexibility of the linkers determine positioning of the molecule, and therefore diversity of interactions, involving the L-glutamate moiety. Indeed, the GARFTase ternary complexes demonstrate diverse binding modes for the individual antifolates through differences in the positioning of the glutamyl tails (Figure 4 shows L-glutamate positioning, and Figures S2–S9 provide detailed interaction maps). This variability is perhaps best observed by comparing how the α - and γ -carboxylates are poised in the binding pocket. Because AGF117 is the most potent GARFTase inhibitor of this series *in vitro*, we compared all conformations of the antifolates in this study to that of AGF117 (Figure 4). Thus, AGF23, AGF71, AGF117, and AGF118 all share the same general binding mode for the glutamyl carboxylates, with polar contacts to R871, R897, and K844 (Figure 4A–C,E). While the contacts with GARFTase are maintained for AGF23 and AGF71, there is a distinct shift such that their glutamyl tails protrude toward residues R897 and K844, while moving away from R871 relative to AGF117 (Figure 4C,E).

In contrast, an $\sim 180^\circ$ rotation for the entire L-glutamate in AGF94 swaps the α - and γ -carboxylate positions relative to AGF117 (Figure 4F). Also, the α -carboxylates of AGF23 and AGF71 are rotated roughly 90° relative to that for AGF117, disrupting a bidentate polar interaction with R871 (Figures S2, S4, and S6). The L-glutamyl moieties of AGF50 and AGF147 are rotated $\sim 90^\circ$ compared to AGF117, resulting in the α -carboxylate and γ -carboxylate, respectively, being removed from the pocket occupied by the other six molecules (Figure 4D,H).

In agreement with the notion that ideal positioning of the glutamyl tail is primarily responsible for the observed differences in inhibitor potency measured *in vitro*, the number of interactions between the antifolates and GARFTase correlates with inhibitor potency. In addition to the conserved interactions with the pyrrolo[2,3-*d*]pyrimidine ring, AGF23, AGF71, AGF94, AGF117, AGF118, and AGF145 all form seven to nine polar contacts with GARFTase through the glutamyl tail, whereas AGF147 and AGF50, the least potent of the series, form five and three possible contacts, respectively (Figures S2–S9). For AGF50, the contacts are all long-distance charge–charge interactions, whereas the more potent analogues form multiple polar contacts (H-bonding or ionic interactions) via their glutamyl tails (Figures S2–S9). For three of the four most potent analogues, AGF23, AGF71, and AGF117, the contacts involve five glutamyl tail atoms, whereas AGF118 forms contacts through four atoms (Figures S2, S4, S6, and S7). Three of the four least potent inhibitors, AGF50, AGF94, and AGF147, form contacts through only two glutamyl tail atoms, while AGF145 makes contacts via four glutamyl tail atoms (Figures S3, S5, S8, and S9). Additional evidence implying a correlation between L-glutamate orientation and antifolate potency toward GARFTase can be found upon examination of the key contacts involving the guanidinyll group of R871. In the four most potent complex structures, a single conformation of R871 is seen in contact with the inhibitor, implying a well-ordered, stable contact (Figures S2, S4, S6, and S7). In the remaining complexes of GARFTase with less potent inhibitors, R871 is modeled in two conformations, each with unique contacts to the inhibitor, implying loss of a single, specific binding mode (Figures S3, S5, S8, and S9). Thus, distinct conformations of the novel pyrrolo- and thieno[2,3-*d*]pyrimidine antifolates in this study seen in our GARFTase crystallographic models closely correlate with GARFTase inhibitor potency, such that the glutamyl tail atoms of the most potent monoglutamyl antifolate inhibitors *in vitro* are optimally positioned to maximize contacts with GARFTase.

DISCUSSION

Prior *in vitro* cell-based studies demonstrate that the eight novel pyrrolo- and thieno[2,3-*d*]pyrimidine antifolates examined in this study are potent cytotoxic molecules that inhibit *de novo* purine nucleotide biosynthesis primarily at GARFTase, the first folate-dependent step.^{22–27} In this report, we used the isolated formyltransferase domain of human GARFTase to compare *in vitro* inhibitor potencies with crystallographic structures of antifolates to improve our understanding of the molecular basis for variations in inhibitor potencies at GARFTase.

Our results demonstrate that the L-glutamate moiety of the most effective antifolate inhibitors is well-positioned in the binding pocket to maximize contact with GARFTase. The

side chains of the most effective inhibitors, AGF117, AGF118, AGF23, and AGF71, all contain a pyrrolo[2,3-*d*]pyrimidine ring system with a four-carbon bridge to a planar aromatic group (benzyl or thiophene) (Figure 1). This allows for similar positioning of the L-glutamate tails to maximize the number of atoms forming contacts with the GARFTase binding pocket. It is interesting that AGF71 and AGF23 differ in their planar aromatic groups, although there is effectively no potency difference, and at most slight differences in positioning of their glutamyl carboxylate tails (Figures 1, 2, and 4). However, the 3',5'- and 2',4'-thiophene regioisomers of AGF71 (AGF117 and AGF118, respectively) are >2-fold more potent than AGF71, reflecting rotation of the α -carboxylate tail to form a bidentate interaction with R871 (Figures 1, 2, and 4 and Figures S6 and S7). The linker of AGF94 is one carbon shorter than that of AGF71, and this difference results in a 3-fold loss of potency associated with an inversion of the α - and γ -carboxylates, which eliminates contacts made through the γ -carboxylate (Figures 1, 2, and 4).

The side chains of linear antifolates AGF145 and AGF147 include seven and eight carbons, respectively, which promote two distinct binding conformations (Figures 1 and 4G,H). The glutamyl tail of AGF145, which inhibits GARFTase with a potency similar to that of AGF94, binds most like AGF118 but lacks the hydrogen bond between the amide nitrogen and the M896 backbone carboxyl (Figure 2 and Figure S8). The glutamyl tail of AGF147, which inhibits GARFTase roughly 1.5-fold weaker than AGF94 and AGF145, is oriented so that only the α -carboxylate is capable of interacting with the enzyme, while the γ -carboxylate is rotated out of the binding pocket (Figure 2 and Figure S9). As the side chain groups form no hydrogen bond or charge-charge interactions with GARFTase, inhibition potency best correlates with glutamyl tail orientation, with the length and flexibility of the linker group determining the glutamate conformation required for efficient binding.

Interestingly, AGF23 and AGF50 are pyrrolo- and thieno-[2,3-*d*]pyrimidine antifolates with identical side chains but exhibit a 63-fold difference in GARFTase inhibition (Figures 1 and 2). This could reflect the nearly 90° rotation of the glutamyl tail of AGF50 relative to AGF23 such that only two AGF50 glutamyl tail atoms can form long-distance charge-charge interactions with R871 and R897 (Figure 4C,D and Figures S2 and S3). For comparison, we previously characterized the GARFTase inhibition by PMX,²⁸ as its monoglutamate, and measured an inhibitory potency similar to that for AGF50. Previous studies reported that PMX can inhibit multiple folate-dependent enzymes in addition to thymidylate synthase, its primary target, including GARFTase, albeit with substantially reduced potencies²⁹ and, more recently, 5-aminoimidazole-4-carboxamide ribonucleotide formyl transferase (AICARFTase).⁴⁶ In KB cells, an IC₅₀ of 4.9 nM for inhibition of cell proliferation by AGF50 was reported (Table 1) and the *in situ* GARFTase IC₅₀ is 13.3 nM,²³ indicating that cellular uptake by FR α and polyglutamylation by FPGS must play an important role in the potent antitumor activity of AGF50. For AGF50, a secondary target in addition to GARFTase, most likely AICARFTase, was also implicated in drug effects.²³

Structures of the human GARFTase in complex with a 10-(trifluoroacetyl)-5,10-dideazaacyclic-5,6,7,8-tetrahydrofolic acid (PDB entry 1NJS) and a truncated substrate, 10-CHODDF (PDB entry 1ZLY),^{44,45} have been previously determined. The 10-trifluoroacetyl antifolate has a reported *K_i* of 15 nM in *in vitro* assays with GARFTase, which is

comparable with the K_i of ~8 nM of AGF117 and AGF118. Both its planar aromatic groups and the entire glutamyl tail overlay well with those of AGF117 and AGF118 in our crystal structures (Figure 5), further supporting the notion that the glutamyl tail orientation for monoglutamates is critical to binding to GARFTase.⁴⁴ For the truncated version of the substrate 10-CHODDF, which was modeled lacking the formyl group and the α -carboxylate of the glutamyl tail, the pteridine ring aligns well with the pyrrolo[2,3-*d*]pyrimidine ring systems of our antifolate–GARFTase complexes.⁴⁵ However, additional comparisons between the truncated 10-CHODDF complex and our structures are not realistic as it is unclear whether the 10-CHODDF is disordered or degraded in the prior work.

While our focus is on understanding the molecular determinants for *in vitro* inhibition of GARFTase in a cell-free context to identify improved inhibition profiles, it is important to note that all the antifolates described herein exhibit low nanomolar to subnanomolar IC₅₀ profiles in inhibiting proliferation of KB human tumor cells *in vitro*, with AGF71, AGF94, AGF117, and AGF118 showing the greatest potencies (Table 1).^{22–27} Notably, the analogues with the greatest antitumor potencies are also among the most effective *in vitro* inhibitors of isolated GARFTase (Figure 2). Of course, in intact cells, the efficacy of classical antifolate analogues reflects their mediated uptake by RFC, PCFT, or FRs and subsequent polyglutamylation, a modification that improves cellular retention and generally inhibition of their cellular targets.^{9,35,47} For the antifolates in this study, selective transport by FRs and PCFT over RFC has been demonstrated, resulting in inhibition of cellular GARFTase,^{22–27} and for a subset of compounds (AGF71 and AGF94), polyglutamylation has been established.⁴⁸ Although the study presented here cannot directly address how positioning of the glutamyl tails in these antifolates bound to GARFTase is influenced by polyglutamylation, the optimal conformation seen in our most potent monoglutamate inhibitors *in vitro* has the γ -carboxylate oriented in a manner that allows for proper positioning of the polyglutamyl modification, based on comparison to a structure of GARFTase with polyglutamylated 10-(trifluoroacetyl)-5,10-dideazaacyclic-5,6,7,8-tetrahydrofolic acid (Figure 5). The correlation between inhibition profiles and optimal structural conformations of novel antifolates in GARFTase complexes indicates that AGF23, AGF117, and AGF118 bind in an optimal mode as monoglutamates and thus are attractive compounds for further design of analogues.

In conclusion, our results show that seven structurally diverse pyrrolo[2,3-*d*]pyrimidine antifolates as their monoglutamates inhibit GARFTase with K_i values ranging from 7 to 100 nM, whereas a structurally analogous thieno[2,3-*d*]pyrimidine monoglutamate analogue is substantially less inhibitory toward GARFTase. For the former, subtle variations in the side chain groups within a hydrophobic pocket likely influence critical contacts involving the glutamyl carboxyl tail, thus altering inhibitor potency. A four-carbon bridge followed by the 3',5'-or 2',4'-substituted thiophene as in AGF117 or AGF118, respectively, places the L-glutamate carboxyl groups in an optimal conformation for binding, correlating with a K_i of ~8 nM. These analogues are also among the most potent inhibitors of proliferation of FR α -expressing tumor cells.²⁶ We anticipate our studies to lead to the development of novel targeted antifolates for the treatment of tumors and inflammatory conditions, whereby an optimal combination of specific transport via FRs and/or PCFT over RFC and potent inhibition of *de novo* purine synthesis by GARFTase could be exploited.

Supplementary Material

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ABBREVIATIONS

AICARFTase	5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase
β-ME	β -mercaptoethanol
DMSO	dimethyl sulfoxide
FPGS	folylpoly- γ -glutamate synthase
FR	folate receptor
GAR	glycinamide ribonucleotide
GARFTase	glycinamide ribonucleotide formyltransferase
K_i	inhibition constant
Ni-NTA	nickel-nitrilotriacetic acid
PCFT	proton-coupled folate transporter
PEG	polyethylene glycol
PMX	pemetrexed
RFC	reduced folate carrier
10-CHODDF	10-formyl-dideazafolate
10-fTHF	10-formyl-tetrahydrofolate

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Figure 1.

Molecular structures of 10-formyl-tetrahydrofolate, the clinically available antifolate PMX, and the antifolates highlighted in this study. The numbering and naming conventions of the antifolate compounds described in this work and present in the deposited structure coordinates are indicated.

Figure 2. GARFTase inhibition constants (K_i) for the series of the antifolates are shown with error bars representing standard deviations from at least three experimental measurements. The K_i value for AGF94 was previously published.²⁸

Figure 3.

Conserved binding mode of the pyrrolo[2,3-*d*]pyrimidine moiety in the 10-fTHF binding pocket of GARFTase. AGF117, shown as orange sticks, serves as a model for the binding mode for pyrrolo[2,3-*d*]pyrimidine-containing compounds. AGF50, a thieno-[2,3-*d*]pyrimidine, binds similarly but lacks a putative H-bond contact with the carbonyl of R897 (Figure S11). Predicted hydrogen bond contacts are shown as dashed lines.

Figure 4.

Structural analyses of antifolate inhibitor conformations in the GARFTase-β-GAR-antifolate ternary complexes. (A) Crystal structure of the most potent inhibitor, AGF117 (orange), bound in the 10-CHO-THF binding pocket of GARFTase with interacting residues from GARFTase shown as sticks. Crystal structures of (B) AGF118, (C) AGF23, (D) AGF50, (E) AGF71, (F) AGF94, (G) AGF145, and (H) AGF147 are shown in a fashion similar to that of panel A, with AGF117 shown as thin black sticks in all panels to highlight variations in the glutamyl tail position among the antifolates. Data processing and

refinement statistics (Table S1), as well as detailed interaction maps for all eight complexes (Figures S2–S9), are included in the Supporting Information. The structure for AGF94 was previously reported.²⁸

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Figure 5. Overlay of the crystal structures of AGF117 (green) and polyglutamylated 10-(trifluoroacetyl)-5,10-dideazaacyclic-5,6,7,8-tetra-hydrofolic acid (orange) bound to GARFTase (PDB entry 1RBZ, unpublished) that indicates that our most potent AGF molecules share a binding mode compatible with binding as polyglutamates. Three additional terminal glutamyl groups observed in one chain of PDB entry 1RBZ that extend across the surface of GARFTase have been removed for the sake of clarity. The AGF117 GARFTase complex structure is shown as ribbons with interacting side chains shown as sticks. Hydrogen bonds and charge–charge interactions between the glutamyl tail of the polyglutamylated molecule and GARFTase side chains are shown as dashed lines.

Table 1

IC₅₀ Values for Inhibition of Cell Proliferation by Eight Novel Antifolates Studied in This Work toward KB Human Carcinoma Cells, Which Express FR α and PCFT, Along with RFC^a

	IC ₅₀ (nM)
AGF23	1.9 $\pm$ 0.7
AGF50	4.9 $\pm$ 1.3
AGF71	0.55 $\pm$ 0.10
AGF94	0.26 $\pm$ 0.03
AGF117	0.17 $\pm$ 0.02
AGF118	0.27 $\pm$ 0.07
AGF145	7.9 $\pm$ 0.72
AGF147	1.10 $\pm$ 0.88

^aThese data were previously published.^{22–27}