Novel Pyrrolo[3,2-d]pyrimidine Compounds Target Mitochondrial and Cytosolic One-carbon Metabolism with Broad-spectrum Antitumor Efficacy


Abstract

Folate-dependent one-carbon (C1) metabolism is compartmentalized into the mitochondria and cytosol and supports cell growth through nucleotide and amino acid biosynthesis. Mitochondrial C1 metabolism, including serine hydroxymethyltransferase (SHMT) 2, provides glycine, NAD(P)H, ATP, and C1 units for cytosolic biosynthetic reactions, and is implicated in the oncogenic phenotype across a wide range of cancers. Whereas multitargeted inhibitors of cytosolic C1 metabolism, such as pemetrexed, are used clinically, there are currently no anticancer drugs that specifically target mitochondrial C1 metabolism. We used molecular modeling to design novel small-molecule pyrrolo[3,2-d]pyrimidine inhibitors targeting mitochondrial C1 metabolism at SHMT2. In vitro antitumor efficacy was established with the lead compounds (AGF291, AGF320, AGF347) toward lung, colon, and pancreatic cancer cells. Intracellular targets were identified by metabolic rescue with glycine and nucleosides, and by targeted metabolomics using a stable isotope tracer, with confirmation by in vitro assays with purified enzymes. In addition to targeting SHMT2, inhibition of the cytosolic purine biosynthetic enzymes, β-glycinamide ribonucleotide formyltransferase and/or 5-aminomimidazole-4-carboxamide ribonucleotide formyltransferase, and SHMT1 was also established. AGF347 generated significant in vivo antitumor efficacy with potential for complete responses against both early-stage and upstage MIA PaCa-2 pancreatic tumor xenografts, providing compelling proof-of-concept for therapeutic targeting of SHMT2 and cytosolic C1 enzymes by this series. Our results establish structure–activity relationships and identify exciting new drug prototypes for further development as multitargeted antitumor agents.

Introduction

Metabolic reprogramming to support tumor progression has emerged as a hallmark of cancer (1). Of the many altered metabolic pathways associated with the malignant phenotype, one-carbon (C1) metabolism is particularly notable (2–4). C1 metabolism depends on an adequate supply of tetrahydrofolate (THF) metabolites and generates critical purine, thymidylate, and glycine metabolites essential for cell proliferation and tumor progression (3–5). Purine nucleotides are synthesized de novo in the cytosol, whereas thymidylate is synthesized in both the cytosol and nucleus (3–6). C1 enzymes such as thymidylate synthase (TS) and the purine biosynthetic enzymes β-glycinamide ribonucleotide (GAR) formyltransferase (GARFTase) and 5-aminomimidazole-4-carboxamide (AICA) ribonucleotide (AICAR) formyltransferase (AICARFTase) (the third and ninth steps in de novo purine biosynthesis, respectively) are important therapeutic targets for cancer (7–9). Serine biosynthesis from glycine in the cytosol involves serine hydroxymethyltransferase (SHMT) 1 and uses C1 units from 5,10-methylene-THF (5,10-me-THF) (2–4). SHMT1, like TS, is localized to both the cytosol and nucleus (5,6); however, nuclear SHMT1 does not appear to be an important source of C1 units for TS (6,10).
Cytosolic and mitochondrial C1 metabolic pathways are interconnected by exchange of serine, glycine, and formate (Fig. 1). Extracellular folates are transported into cells by the reduced folate carrier (RFC), proton-coupled folate transporter (PCFT), and folate receptors (FR, refs. 11, 12). Whereas cytosolic folates are transported into mitochondria via the mitochondrial folate transporter (SLC25A32; refs. 13, 14), mitochondrial folates do not exchange with those in the cytosol (15). In cancer cells, the 3-carbon of serine is the major source of C1 units, and in mitochondria, serine catabolic enzymes including SHMT2, 5,10-me-THF dehydrogenase 2 (MTHFD2), and 10-formyl-THF synthetase (reverse; MTHFD1L) generate glycine and C1 units (i.e., formate) to sustain C1-dependent nucleotide and amino acid biosynthesis in the cytosol (Fig. 1). 10-Formyl-THF is synthesized from formate in the cytosol by the bifunctional enzyme MTHFD1. 10-Formyl-THF is utilized for purine nucleotide biosynthesis and can be further converted by MTHFD1 to 5,10-me-THF for TS and SHMT1.

Several studies have implicated mitochondrial C1 metabolism as critical to the malignant phenotype (16–19). A study (18) of messenger RNA profiles for 1,451 metabolic enzymes spanning 1,981 tumors across nineteen different cancer types and 951 matched normal tissues identified SHMT2 and MTHFD2 among the top five most differentially expressed genes, highlighting an oncogenic role of mitochondrial C1 metabolism. Metabolomics analyses of 219 extracellular metabolites from the NCI-60 cancer cell lines showed that glycine consumption and the glycine biosynthetic pathway correlated with cell proliferation (16). These findings, combined with evidence of functional shortages of amino acids (e.g., glycine) in tumors (20), suggested a therapeutic opportunity for SHMT2 targeting in cancer.

SHMT2 is induced by hypoxic stress in Myc-transformed cells (21) and is critical to tumor cell survival in the hypoxic, nutrient-poor tumor microenvironment (17, 21). SHMT2 (or MTHFD2) knockout (KO) cells are viable and tumorigenic (albeit with decreased growth rates) in nutrient-rich conditions, as reversal of cytosolic SHMT1 (serine-to-glycine) provides sufficient C1 units to sustain some level of de novo nucleotide biosynthesis (22). However, SHMT1 only restores a small fraction of the C1 pools in wild-type (WT) cells (22). Furthermore, SHMT1 does not generate sufficient glycine for protein, nucleotide, and glutathione biosynthesis, rendering both SHMT2 and MTHFD2 KO cells as glycine auxotrophs (4). This was the impetus for studies of pyrazolopyran compounds (e.g., SHIN1) with dual SHMT1/SHMT2 inhibition (23). While structurally unrelated to folates, these compounds bound to the folate-binding site in SHMT2 and showed in vitro antitumor efficacy (particularly with B-cell cancers).

In this report, we describe a novel series of 5-substituted pyrrolo[3,2-d]pyrimidine compounds that inhibit SHMT2, and also SHMT1, GARFTase, and/or AICARFTase. Our lead compounds (AGF291, AGF320, and AGF347) show broad-spectrum in vitro antitumor efficacies against H460 non-small cell lung cancer (NSCLC), HCT116 colon cancer, and MIA PaCa-2 pancreatic cancer cells. For AGF347, in vitro findings were extended in vivo to MIA PaCa-2 tumor xenografts, providing compelling proof-of-concept of the therapeutic potential of our multitargeted SHMT2 therapeutics for cancer.

Figure 1.

C1 metabolism is compartmentalized in the cytosol and mitochondria. Folates enter the cell through the plasma membrane-facilitated folate transporters, PCFT and RFC, and enter the mitochondria via SLC25A32. Serine catabolism in the mitochondria beginning with SHMT2 generates glycine and formate, the latter of which is required for downstream cytosolic de novo purine biosynthesis (by GARFTase and AICARFTase) as 10-formyl-THF, and thymidylate biosynthesis, following conversion to 5,10-me-THF by MTHFD1. SHMT1 catalyzes conversion of glycine to serine in the cytosol. AIC1 is metabolized to AICAR (ZMP), the AICARFTase substrate which circumvents the GARFTase step. The arrows denote the net flux of C1 metabolism in proliferating cells, although most reactions are reversible. Both SHMT1 and TS are also localized to the nucleus; however, nuclear SHMT1 is not an important source of C1 units for TS (6, 10).

Materials and Methods

**Chemicals**

[2,3,3-3H]L-Serine (98%) was purchased from Cambridge Isotope Laboratories, Inc. Leucovorin ([6R,S]-5-formyl-THF) was provided by the National Cancer Institute (Bethesda, MD). Pemetrexed (Alimta; PMX) was purchased from LC Laboratories. Gemcitabine (Gemzar) was purchased from Fresenius Kabi USA, LLC. Serine-, glycine- and folate-free RPMI1640 was custom-
ordered culture from Thermo Fisher Scientific and supplemented with tissue-culture grade glycine (Thermo Fisher Scientific) or serine (Sigma-Aldrich), as needed. Other chemicals were obtained from commercial sources in the highest available purities. Chemical synthesis of the pyrrolopyrimidine compounds is described in the Supplementary Methods.

**Molecular modeling and computational studies**

Molecular modeling was performed for all analogues with the human SHMT2 crystal structure (PDB: 5V7I; ref. 23) using the induced fit docking protocol of Maestro (refs. 2, 3; see Supplementary Methods). The compounds were also docked into rabbit SHMT1 (PDB: 1LS3; ref. 24) binding sites. The docking scores of the analogues are reported in Supplementary Table S1.

**Cell culture and proliferation/protection assays**

The HCT116 cell lines including the SHMT1, SHMT2, and MTHFD2 KO cells were described previously (22, 23). The wild-type HCT116 and H460 human tumor cell lines were obtained from the ATCC, whereas MIA PaCa-2 cells were provided by Dr. Wayne Flintoff (University of Western Ontario, Ontario, Canada; ref. 25). From this parental R2 cell line, human RFC and PCFT were individually transfected to generate the isogenic CHO cell lines designated PC43-10 (RFC) and PC43-10 (PCFT-#89, FRα-#65; Roche Diagnostics). Transcript levels were normalized to β-actin and GAPDH. Primer sequences are available upon request.

**Generation of H460 SHMT2 knockdown cell line**

H460 SHMT2 knockdown (KD) cells were generated by transduction with MISSION lentivirus particles (Sigma-Aldrich) containing shRNA targeting SHMT2 (TRCN0000034805) or nontargeted control (NTC) lentivirus as described previously (29). Single clones were isolated and cultured expanded. The extent of SHMT2 KD was confirmed by Western blotting (below).

**Real-time PCR**

Cells were harvested from 60-mm dishes or T25 flasks at approximately 80% confluence and RNAs extracted using TRIzol reagent (Invitrogen). CDNAs were synthesized with random hexamers, MuLV reverse transcriptase, and RNase inhibitor (Applied Biosystems), and purified with a QIAquick PCR Purification Kit (Qiagen). Quantitative real-time RT-PCR was performed using a Roche LightCycler 480 (Roche Diagnostics) with gene-specific primers to the major folate transporters and Universal Probe Library probes (SHMT2-#83, RFC-#32, PCFT-#89, FRα-#65; Roche Diagnostics). Transcript levels were normalized to β-actin and GAPDH. Primer sequences are available upon request.

**Gel electrophoresis and Western blots**

H460 WT, H460 NTC, H460 SHMT2 KD, HCT116 WT, and HCT116 SHMT2 KO cell lines were plated (1 × 10^5 cells/dish) in 60-mm dishes and harvested when the cells were approximately 80% confluent. Cells were disrupted by sonication and cell debris removed by centrifugation (1,800 rpm, 5 minutes, 4°C). The soluble cell fraction was assayed for protein (30). Equal amounts of protein (37 μg) from each sample were electrophoresed on 10% polyacrylamide gels with SDS (31) and transferred to polyvinylidene difluoride membranes (Thermo Fisher Scientific; ref. 32). To detect SHMT2, membranes were incubated for 72 hours with rabbit anti-SHMT2 primary antibody (1:127,622; 22). Cell Signaling Technology). The blots were developed by incubating in IRDye800CW-conjugated goat anti-rabbit IgG secondary antibody (LICOR Biosciences) for 50 minutes and scanning with an Odyssey infrared imaging system (LICOR Biosciences). Protein loading was normalized to β-actin using anti-β-actin mouse antibody (Sigma-Aldrich).

**In vitro-targeted metabolomics by liquid chromatography-mass spectrometry**

Cells were seeded in triplicate 60-mm dishes in folate-free RPMI1640 (contains glycine and unlabeled serine) supplemented with 10% dialyzed FBS, 1% penicillin/streptomycin, 2 mmol/L L-glutamine, and 25 mmol/L leucovorin in a humidified atmosphere at 37° C in the presence of 5% CO_2_ and 95% air. The CHO cell lines were cultured in α-MEM supplemented with 10% bovine calf serum, 1% penicillin/streptomycin solution, and 2 mmol/L L-glutamine. The transfected CHO cell lines (i.e., PCFT4 and PC43-10) were cultured in the presence of 1 ng/mL of G418.

**Real-time PCR analysis by Genetica DNA Laboratories (H460, November 2015; MIA PaCa-2, April 2019).**
protein from the post-extraction pellet (solvulized with 0.5 N NaOH) by the Folin–phenol method (30). Samples were run on a ThermoElectron Corporation Exactive mass spectrometer operating in negative ion mode (33). Separation used reverse-phase ion-pairing chromatography on a 100 mm/2.5 μm Synergy Hydro-RP C18 column (Phenomenex). Individual metabolites were identified from their exact masses and comparison of retention times with standard compounds using the MAVEN software suite (34). Values below the limit of detection were assigned a value of 100 for normalization.

Enzyme expression and purification

N-terminal His-tagged GARFTase (formyltransferase domain; residues 100-302) was expressed and purified (35). Full-length human AICARFTase/IMP cyclohydrolase (ATIC), SHMT1 (residues 7-478, Uniprot ID P34896), SHMT2 (residues 42-504, Uniprot ID 34897), and MTHFD2 (residues 36-333, Uniprot ID P13995) with N-terminal, cleavable hexahistidine tags were further purified by essentially the same protocol. Samples were further purified on AKTA FPLC (GE Healthcare) on a Superdex 200 16/60 (GE Healthcare) column. Further details are in the Supplementary Methods.

In vitro enzymatic assays and Kᵢ determinations

AICARFTase and GARFTase activities were measured by monitoring formation of THF spectrophotometrically from 10-formyl-THF in the presence of various concentrations of inhibitor (36, 37). SHMT1/2 activities were assayed by a coupled reaction with His-MTHFD2 in 200-fold molar excess with NADH production monitored by fluorescence. Kᵢ values were calculated from the IC₅₀ values [Kᵢ = IC₅₀/[S]/Kₘ+1)], using the Kₘ and substrate concentrations for 10-formyl-THF. The calculated Kᵢ values for 10-formyl-THF with His-ATIC and His-GARFTase were 100 μmol/L and 84.8 μmol/L, respectively. The calculated Kᵢ values for THF with His-SHMT1 and His-SHMT2 were 62.8 μmol/L and 108 μmol/L, respectively. To confirm that MTHFD2 was not inhibited by our inhibitors, MTHFD2 activity was evaluated with an NAD(P)H-Glo Detection System Kit (Promega, Ref G9061). Further details are in the Supplementary Methods.

In vivo efficacy trial with MIA PaCa-2 pancreatic cancer xenografts

Methods for in vivo maintenance of MIA PaCa-2 tumor xenografts and drug efficacy evaluations are analogous to those described previously (36, 38–41). The mouse study in this report was approved by the Wayne State University Institutional Animal Care and Use Committee (IACUC). Female NCr severe compromised immunodeficient (SCID) mice were purchased from Charles River Labs. For the early-stage trial, chemotherapy was initiated one day posttumor implantation with AGF347; for the upstage trial, chemotherapy was initiated 7 days posttumor implantation (when tumors had grown to 100–150 mg) with AGF347. For both designs, dosing for AGF347 was 15 mg/kg/injection every 2 days × 8 (total dose of 120 mg/kg); for gemcitabine, dosing was 120 mg/kg/injection every 4 days × 4 (total dose of 480 mg/kg). Both drugs were administered intravenously (0.2 mL/injection). The tumor masses from both flanks of each mouse were added together, and the total mass per mouse was used for calculations of antitumor activity. Quantitative endpoints include: (i) tumor growth delay [T-C, where T is the median time in days for the control 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]; and (ii) gross log₂ cell kill (LCK), determined by the formula LCK = (T-C, tumor growth delay in days)/3.32 × 10d (tumor doubling time in days determined by growth plot). Qualitative analysis included determination of T/C values (in percent) on all days of tumor measurement using the median total tumor burden for treatment (T) and control (C) groups. The endpoint %T/C value for this study corresponds to the first measurement taken post last treatment (day 16 for early stage or day 21 for upstage), when control tumors were still in exponential growth phase (i.e., 500–1,250 mg). Further details are described in the Supplementary Methods.

From the upstage in vivo study, a separate AGF347 treatment arm (3 mice) was designated for metabolomics analysis in comparison with the untreated control (3 mice). Six hours after the sixth dose of AGF347, mice were sacrificed, and tumors removed and immediately frozen in liquid nitrogen. Frozen isolated tumors were weighed; approximately 50 mg pieces were disrupted with a cryomill (Retsch) in 1-mL ice-cold acetonitrile:methanol:water (40:40:20). Solids were collected by centrifugation, then reextracted with 1 mL lysis solution (above). Combined supernatants were dried down and then resuspended in water to a concentration of 50 mg/mL (original tissue mass) for LC-MS analysis, as described above.

Statistical analysis

All data shown reflect at least three biological replicates unless noted otherwise. Statistical analyses were performed by the Karmanos Cancer Institute Biostatistics Core. The expression levels were assessed for the normality assumption. The log₂ transformation was used when all values were positive and the square root transformation was used when values included zero. Statistical tests were carried out using an unpaired t test. P values were not adjusted for multiple comparisons.

Results

Rationale for design of 5-substituted pyrrolo[3,2-d]pyrimidine compounds targeting mitochondrial C1 metabolism at SHMT2

Given the association of mitochondrial C1 metabolism with malignancy (16–19), including reports of SHMT2 as a potential "onco-driver" (17, 42), it was of interest to develop inhibitors of SHMT2 as antitumor agents. We initially looked for potential SHMT2 inhibitors among prototypes of different classes of GARFTase and AICARFTase inhibitors from our published studies (26, 27, 36, 41, 43, 44). We tested these compounds (Supplementary Fig. S1) for inhibition of proliferation of CHO cells engineered from the transporter-null R2 CHO subline (25) to express human PCFT (R2/PCFT4; ref. 27), as we reasoned that tumor-selective uptake of a putative SHMT2 inhibitor by PCFT (45) would be desirable. Because inhibition of mitochondrial C1 metabolism at SHMT2 should induce glycine auxotrophy (22), and mitochondrial C1 metabolism generates glycine and C1 units (i.e., formate) critical for nucleotide biosynthesis, these experiments were performed in glycine- and nucleoside-free media, and the protective effects of added glycine (130 μmol/L) and/or adenosine (60 μmol/L) were determined. With the exception of AGF147, these compounds inhibited cell proliferation and
purine biosynthesis in R2/PCFT4 cells (reflected in adenosine rescue); however, glycine was not protective with or without adenosine addition, indicating a lack of mitochondrial C1 targeting. Thus, it became imperative to design new analogues.

To engineer SHMT2 activity into our compound series, we merged structural features of our previous 5-substituted pyrrolo[2,3-d]pyrimidine benzoyl and thienoyl compounds (43, 44) with those of 5,10-me-THF (SHMT2 substrate) and 5-formyl-THF (SHMT2 inhibitor; ref. 24; Fig. 2). The resulting 5-substituted pyrrolo[3,2-d]pyrimidine analogues included 3 or 4 bridge carbons linked to benzoyl (i.e., AGF291, AGF300, and AGF299) or thienoyl (i.e., AGF331, AGF318, and AGF320) moieties. On the basis of the reported impact of 2' fluorine substitutions in increasing inhibitory potencies of pyrrolo[2,3-d]pyrimidine compounds (39), we designed 2'fluorinated analogues of AGF300 and AGF299, as well (AGF347 and AGF355, respectively; Fig. 2). Compounds were docked into human SHMT2 (Supplementary Fig. S2) with docking scores (Supplementary Table S1) better than for the pyrazolopyran inhibitor SHIN1 (23) (~5.58 kcal/mol) upon redocking.

**Discovery of 5-substituted pyrrolo[3,2,d]pyrimidine compounds that target mitochondrial C1 metabolism**

We synthesized the novel analogues and screened these for inhibition of cell proliferation. To encompass the major modes of facilitative transport, we initially assessed inhibition by these compounds (from 0 to 1,000 nmol/L) toward PCFT-expressing R2/PCFT4 CHO cells and an isogenic CHO subline engineered to express human RFC (PC43-10; ref. 26). Results were compared to those for folate transporter-null R2 CHO cells (25). IC50 values for “active” compounds are shown in Table 1 for the analogues, along with those for AGF94 (a “pure” GARFTase inhibitor; ref. 41) and PMX. Active compounds toward R2/PCFT4 and PC43-10 cells included AGF291, AGF320, AGF331, and AGF347. These analogues were further tested with human tumor cell lines, including H460 NSCLC, HCT116 colon cancer, and MIA PaCa-2 pancreatic...
NOTE: Proliferation inhibition assays were performed using the engineered CHO cell lines R2 (folate transporter-null), PC43-10 (expresses RFC only), and R2/PCFT4 (expresses PCFT only), and human tumor cell lines, including HCT116 (colon cancer), H460 (lung cancer), and MIA PaCa-2 (pancreatic cancer). For HCT116, both wild-type (WT) and CRISPR/Cas9 SHMT2 KO cells (22, 23) were tested. For SHMT2 KO cells, glycine was added to the media to circumvent the impact of loss of SHMT2, permitting cell growth. Results are shown as mean IC50 values (standard deviations), corresponding to the concentrations that inhibit growth by 50% relative to controls included NTC and SHMT2 shRNA KD cells. For H460 cells, adenosine (60 μmol/L) was completely protective up to 10 μmol/L AGF94, whereas glycine (130 μmol/L) had no effect. We tested the protective effects of AICA (320 μmol/L), which is metabolized to AICAR (ZMP, AICARFTase substrate), thus circumventing GARFTase in de novo purine biosynthesis (ref. 41). As AICA was completely protective (Supplementary Fig. S4), GARFTase must be the intracellular target for AGF94 (41). For AGF291, AGF347, and AGF320, combined adenosine and glycine was substantially protective (Fig. 3). These results strongly suggest that these compounds target both mitochondrial C1 metabolism and cytosolic de novo purine biosynthesis. Thymidine provided no protection from any of the compounds and did not increase the extent of rescue by glycine and adenosine. For some analogues, notably AGF320, growth inhibition was modestly (and incompletely) reversed by AICA (with glycine), suggesting a secondary target (below). Analogous results were obtained with HCT116 and MIA PaCa-2 tumor cells (Supplementary Fig. S4). These results establish that AGF291, AGF320, and AGF347 likely target both mitochondrial and cytosolic C1 metabolism.

Identification of the mitochondrial enzyme target for AGF291, AGF320, and AGF347 by targeted metabolomics

To confirm the intracellular enzyme targets of the lead compounds AGF291, AGF320, and AGF347, we performed targeted metabolomics with LC/MS using a [2,3,3-2H]serine tracer in HCT116, H460 and MIA PaCa-2 tumor cells. The cells were processed for LC/MS analysis of total serine, and of isotopically labeled M+3, M+2, M+1, and M+0 serine (M+n represents species with n deuterium atoms). Results for inhibitor-treated cells were compared with those for untreated cells. For WT HCT116 cells, the results were compared with those for SHMT1 KO, MTHFD2 KO, and SHMT2 KO cells (22). For H460 cells, controls included NTC and SHMT2 shRNA KD cells.

KO of cytosolic SHMT1 (SHMT1 KO) in HCT116 cells had no impact on total serine pools. Serine pools were elevated approximately 10-fold in the HCT116 sublines with mitochondrial C1 KO s (SHMT2 KO and MTHFD2 KO) relative to WT controls (Fig. 4B). This further supports the notion that SHMT2 rather than SHMT1 is the primary catabolic enzyme for serine in HCT116 cells (22). In AGF291-, AGF320- and AGF347-treated HCT116 cells, serine also increased approximately 10-fold, indicating a profound loss of serine catabolism. Analogous results were seen with the H460 and MIA PaCa-2 tumor cell lines, including the H460 SHMT2 KD cells (Supplementary Fig. S5A and S5B).

The flux of [3H]metabolites originating from [2,3,3-2H]serine tracer is depicted in Fig. 4A. In proliferating tumor cells, C1 metabolism flows in a clockwise manner, with serine catabolized in mitochondria (starting with SHMT2) and regenerated in the cytosol (via SHMT1; ref. 22). The reactions are reversible (e.g., cancer cells, characterized by expression of PCFT and RFC but not FRTs (refs. 46, 47; Supplementary Fig. S2). IC50 values for growth inhibition are in Table 1. Although there were differences in inhibitor sensitivities among the assorted tumor models, AGF291, AGF320, and AGF347 were consistently the most active of the series.

We again used glycine and nucleoside rescue studies (above) in H460, HCT116, and MIA PaCa-2 cells treated with AGF291, AGF320, or AGF347 to identify the targeted pathways. Results were compared with those for AGF94 and are shown in Fig. 3 for H460 cells. Adenosine (60 μmol/L) was completely protective up to 10 μmol/L AGF94, whereas glycine (130 μmol/L) had no effect. We tested the protective effects of AICA (320 μmol/L), which is metabolized to AICAR (ZMP, AICARFTase substrate), thus circumventing GARFTase in de novo purine biosynthesis (ref. 41). As AICA was completely protective (Supplementary Fig. S4), GARFTase must be the intracellular target for AGF94 (41).

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serine is resynthesized from formate in the mitochondria; ref. 22). The compartmental metabolism of serine can be revealed by analyzing the isotope tracing (“scrambling”) patterns from [2,3,3-2H]serine in HCT116 cells (22, 23). These patterns include both unmetabolized (M+3) and metabolized (M+2 and M+1) forms of serine. The latter species are derived from the recombination of unlabeled glycine with doubly labeled (M+2) 5,10-me-THF and the synthesis of serine from an unlabeled glycine and a singly labeled 5,10-me-THF derived from a formate/10-formyl-THF precursor (M+1), respectively (Fig. 4A).

In WT HCT116 cells fed [2,3,3-2H]serine, most of the serine was catabolized (only 10% M+3 serine remained; Fig. 4C). A large fraction of serine was incompletely labeled (scrambled; i.e., M+1 or M+2), reflecting resynthesized serine from M+1 or M+2 precursors. SHMT1 KO led to a drop (~60%) in the M+1 fraction without a change in the M+2 serine fraction, consistent with

![Figure 3](targeting_mito_cytosol.png)

**Figure 3.**
In vitro antitumor efficacy and identification of targeted pathways and enzymes by novel pyrrolo[3,2-d]pyrimidine analogues in H460 tumor cells by proliferation assays. Dose–response growth inhibition curves are shown for AGF94, an established GARFTase inhibitor (41), and for AGF291, AGF320, and AGF347 without additions, or in the presence of adenosine (60 μmol/L) and/or glycine (130 μmol/L). The results are mean values ± standard deviations for three biological replicates.

![Figure 4](targeting_mito_cytosol_diagram.png)

**Figure 4.**
Targeted metabolomics analysis to identify intracellular enzyme targets of AGF291, AGF320, and AGF347. A, A schematic of serine isotope scrambling and dTTP isotope analysis is shown. Heavy (2H) atoms in serine (red circles) enter the C1 flux in the cytosol through reversal of the SHMT1 reaction and TS leading to dTMP and dTTP (as M+2). In the mitochondria, 2H atoms in serine (blue circles) are metabolized via SHMT2, MTHFD2, and MTHFD1 to formate which is converted to 10-formyl-THF and 5,10-me-THF, leading to dTMP and dTTP (as M+1). Most steps are reversible as noted. Adapted from ref. 22. Total serine pools (B) and the corresponding serine isotope distributions (C) for WT and KO HCT116 sublines, and for inhibitor-treated WT cells, are shown. Total GAR (D) and AICAR (E) pools are shown for WT and SHMT2 KO H460 sublines, including inhibitor-treated WT cells, with and without 1 mmol/L formate. F and G, Relative adenine nucleotide pools (AMP, ADP, and ATP) and dTTP pools with isotope distributions are shown for untreated and inhibitor-treated MIA PaCa-2 cells. For D and E, results for inhibitor-treated and SHMT2 KD cells were normalized to vehicle-treated WT ± formate or NTC ± formate samples, as appropriate. Data are mean values ± SDs for three technical replicates. *, **, *** or **** are used in place of * to specify a significant increase or decrease, respectively. Statistical comparisons were with vehicle-treated WT ± formate or NTC ± formate samples, as appropriate (ns, not significant).
SHMT1 in the cytosol being responsible for bulk synthesis of serine from M+1 5,10-me-THF, and unlabeled (media) glycine (22). In SHMT2 KO cells, the M+3 fraction was >60% of the total serine (Fig. 4C), consistent with a profound loss of serine catabolism. Reflecting this and the depletion of formate downstream of SHMT2, there was approximately 90% decrease in M+1 serine (22). M+2 serine also decreased (~25%) compared with WT cells. Although similar results were seen for the M+1 and M+3 serine fractions with MTHFD2 KO cells (decreased ~75% and increased 3.5-fold, respectively, from WT levels), M+2 serine was increased (~1.7-fold) rather than decreased (Fig. 4C). This reflects accumulation of M+2, 10-me-THF when MTHFD2 is lost, which drives synthesis of M+2 serine from the largely unlabeled glycine pool. Thus, changes in serine isotope labeling from [2,3,3-2H]serine (particularly in the M+2 fraction) are diagnostic for specific perturbations in folate metabolism, and also inform upon the particular enzymatic step that is inhibited.

Treatment of WT HCT116 cells with AGF291, AGF320, or AGF347 closely recapitulated the effects of the SHMT2 KO, including a substantial increase in M+3 serine fraction (~55%–60% of total serine) and decreased M+2 serine (~2–3-fold) compared with WT cells, accompanied by nearly complete loss of M+1 serine (Fig. 4C). Similar results were obtained with SHMT2 KD H460 cells and inhibitor-treated WT H460 and MIA PaCa-2 cells (Supplementary Fig. S5C and SSD, respectively). These results identify SHMT2 as the likely mitochondrial target for AGF291, AGF320, and AGF347.

Identification of the de novo purine biosynthetic pathway as a direct target for AGF291, AGF320, and AGF347

Both GARFTase and AICARFTase require 10-formyl-THF derived from formate, most of which is generated via mitochondrial C1 metabolism from serine (ref. 22; Figs. 1 and 4A). Consistent with this, loss of SHMT2 in H460 SHMT2 KD cells induced significant increases in purine intermediates which are dependent on C1 pools (i.e., 10-formyl-THF), including GAR (GARFTase substrate; 21-fold; Fig. 4D) and AICAR (AICARFTase substrate; 65-fold; Fig. 4E). Likewise, treatment of H460 cells with AGF291, AGF320, and AGF347 (10 μmol/L) increased GAR (10–2,300-fold) and AICAR (40–1,500-fold) relative to untreated controls (Fig. 4D and E). Similar increases in GAR and AICAR pools resulted in inhibitor-treated HCT116 and MIA PaCa-2 cells (Supplementary Fig. S5E–S5H). For the HCT116 sublines, the increases in GAR and AICAR upon inhibitor treatments generally exceeded those resulting from the SHMT2 KO.

To assess the possibility that AGF291, AGF320, and AGF347 directly inhibit cytosolic enzyme targets in de novo purine biosynthesis (i.e., GARFTase and/or AICARFTase), we treated the H460 cells with 1 mmol/L formate, to replenish the cytosolic C1 pool while circumventing the mitochondrial C1 pathway. We reasoned that formate treatment of SHMT2 KD cells should restore levels of GAR and AICAR to those seen in NTC (WT) cells. However, if the cytosolic enzymes were directly inhibited, formate should not effectively reverse GAR and/or AICAR accumulation. In H460 SHMT2 KD cells, treatment with formate completely reversed elevated GAR (Fig. 4D) and AICAR (Fig. 4E) to NTC levels. However, for inhibitor-treated H460 cells, reversal by formate was incomplete, albeit to different extents for different compounds. These results implicate direct targeting of GARFTase and/or AICARFTase by the novel inhibitors, in addition to SHMT2.

We measured the effects of the inhibitors on de novo purine biosynthesis, as reflected in total cellular pools of AMP, ADP, and ATP. In MIA PaCa-2 cells, all the adenine nucleotides were comparably suppressed (~50%) with inhibitor treatments (Fig. 4F). In HCT116 cells (Supplementary Fig. S5J), AMP and ADP pools were impacted more (~50%–70%) than ATP (30% suppression), although SHMT2 KO had no effect. In H460 cells (Supplementary Fig. S5J), SHMT2 KD decreased adenine nucleotide pools (~70%–80%), yet in response to the inhibitors, AMP increased (~50%), ADP decreased (up to ~60%), and ATP was essentially unaffected. Thus, in spite of the requirement for adenosine (with glycine) for complete rescue of all three cell lines from the inhibitory effect of the pyrrolopyrimidine compounds (Fig. 3; Supplementary Fig. S4), the extent of adenine nucleotide depletion varied.

Identification of SHMT1 as a target for pyrrolo[3,2-d]pyrimidine analogues

As SHMT1 (UniProtKB: P34897) maintains 66% sequence identity (48) to SHMT1 (UniProtKB: 34896), we considered the possibility that AGF291, AGF320, and AGF347 could also target cytosolic SHMT1. By molecular modeling (Supplementary Fig. S2), these analogues bound to rabbit SHMT1 (UniProtKB: 07511, 93% homology with human SHMT1; ref. 49) with docking scores from ~8.9 to ~11.14 kcal/mol (Supplementary Table S1), raising the possibility that they may directly inhibit the human enzyme, as well.

To gauge potential cytosolic SHMT1 inhibition by our compounds, we traced [2,3,3-2H]serine into dTTP (dTMP) via C1 transfer from 5,10-me-THF to dTMP by TS (ref. 22; Fig. 4A). In all three cell lines, WT cells incubated with [2,3,3-2H]serine generated M+1 dTTP with little-to-no M+2 dTTP (Fig. 4G; Supplementary Fig. S5K and S5L). This confirms preferential [2,3,3-2H]serine metabolism through the mitochondrial C1 pathway to [3H]formate and into dTTP (M+1) (22). KD of SHMT2 in H460 cells or KO of SHMT2 in HCT116 cells induced a robust M+2 dTTP signal, reflecting serine-to-glycine flux through SHMT1 in the cytosol (22). Consistent with SHMT2 targeting, treatment with AGF291, AGF320, or AGF347 (10 μmol/L) resulted in decreased M+1 and total dTTP in the cell lines (Fig. 4G; Supplementary Fig. S5K and S5L). However, in MIA PaCa-2 cells were these changes accompanied by increased M+2 dTTP (reflecting a SHMT1 compensation response; ref. 22); the other two cell lines showed no measurable M+2 dTTP. These results likely reflect (i) sufficient residual flux through SHMT2 to preserve the primary M+1 labeling of dTTP, and/or (ii) direct targeting of SHMT1, along with cell line–specific differences in SHMT1 activity and inhibition by our compounds. In spite of decreased dTTP pools, thymidine did not rescue cells from effects of any of our inhibitors (Supplementary Fig. S4), a marked difference from TS-targeted inhibitors such as PMX (7). Thus, decreased dTTP likely reflects a secondary impact of targeting SHMT2/SHMT1 rather than directly targeting TS.

Further evidence for direct SHMT1 targeting by our inhibitors involves the substantially increased antiproliferative effects of AGF291, AGF320, and AGF347 toward HCT116 SHMT2 KO cells compared with WT cells (21-fold for AGF291, 6-fold for AGF320, and 10-fold for AGF347; Table 1). Conversely, sensitivities to AGF94 and PMX were similar between WT and SHMT2 KO cells. These results mirror the increased potency toward SHMT2 KO...
cells relative to WT HCT116 cells seen with SHIN1 (23) and further suggest that our pyrrolo[3,2-d]pyrimidine compounds inhibit SHMT1 in addition to SHMT2.

**In vitro confirmation of enzyme targets**

To confirm the enzyme targets (SHMT2, GARFTase, AICARFTase, and SHMT1) identified from our metabolomics experiments, we performed in vitro assays using purified recombinant enzymes. Our results demonstrate direct inhibition of SHMT2 by AGF320, AGF330, and AGF347 (Kₐ of 0.63, 0.056 and 2.19 μM/L, respectively; Table 1). While the compounds inhibited AICARFTase within a 4–5-fold range, AGF330 was the most potent GARFTase inhibitor, corroborating the metabolomics results (Fig. 4D and E). For SHMT1, inhibitions paralleled those of potent GARFTase inhibitor, corroborating the metabolomics

In vitro inhibition of SHMT1 in addition to SHMT2.

**In vivo efficacy study of AGF347 with MIA PaCa-2 tumor xenografts**

We performed an in vivo efficacy trial with AGF347 in a head-to-head comparison with gemcitabine toward MIA PaCa-2, an aggressive tumor model that reaches an euthanasia endpoint within 2 weeks of implant. We initially used early-stage disease as a primary test of drug efficacy. Fourteen days before SC tumor implant, the mice were fed a folate-depleted diet to ensure that serum folate levels approximated those of humans (36, 38, 40, 41, 51). The mice were unselectively distributed to each group's treatment and control arms (5 mice per group), and dosed with AGF347 (15 mg/kg) every 4 days × 8; total dose of 120 mg/kg) or gemcitabine (120 mg/kg/kg) injection every 4 days × 4; total dose of 480 mg/kg) just below their respective maximum tolerated doses (MTDs). Gemcitabine was efficacious, with a median tumor burden on day 16 of 420 mg (range 284–552 mg) compared with 1,189 mg (range 601–1,711 mg) for the control cohort; AGF347 was distinctly superior, yielding a median tumor burden of 0 mg (range 0–276 mg). Median T/C values (see Materials and Methods for definition of quantitative endpoints) on day 16 were 35% for gemcitabine and 0% for AGF347 (Fig. 5A). Tumor growth delays (median T/C to reach 1,000 mg) were 6 days for gemcitabine, compared with 39 days for 4 of 5 mice treated with AGF347 and one tumor-free survivor (TFS) on day 136 (121 days post last dose). To confirm whether immune factors contributed to this "curative" response, this mouse was "rechallenged" with MIA PaCa-2 tumor (bilateral SC implant). The tumor reached 1,000 mg 16 days post-implant, with comparable growth and doubling time to the control group (median, 15.5 days; range, 13–20 days; Fig. 5A).

Both drugs were well tolerated, with modest weight loss as the only adverse symptom [for AGF347, 1% (−0.2 g) median nadir on day 17; for gemcitabine, 6.9% (−1.4 g) median nadir on day 3]. After cessation of therapy, host recovery time was rapid for AGF347 (1 day) compared with gemcitabine (14 days). Thus, at equitoxic dose levels, AGF347 showed superior antitumor efficacy over gemcitabine (>25 logs of cell kill compared with 1.1 logs for gemcitabine), with a 4-fold decreased dose requirement, no acute or long-term toxicities other than reversible weight loss, rapid host recovery time, and a tumor growth delay of >38 days, with 1 of 5 tumors (Table S2).

Studies were extended to upstage MIA PaCa-2 xenografts. For these experiments, after implantation, MIA PaCa-2 tumors were allowed to grow for 7 days (100–150 mg), unselectively distributed (5 mice/group), then treated as for the early-stage trial (Fig. 5B). AGF347 and gemcitabine were both efficacious, with median tumor burdens on day 21 of 733 mg (range 373–1,835 mg) and 524 mg (range 247–1,045 mg), respectively, compared with 1,736 mg (range 1,117–2,046 mg) for the control cohort. AGF347 effected two partial remissions (PRs), including one complete response (CR); no PRs or CRs were seen with gemcitabine. The PRs involved substantial decreases in tumor burden (688 to 220 mg, and 400 to 0 mg) and occurred on day 31 (10 days post last dose). Overall tumor growth delays (median T/C to reach 1,000 mg) of 11 days (gemcitabine) and 7 days (AGF347) were recorded. For the PRs, AGF347 induced tumor growth delays of 26 and 52 days, as compared to 16 and 25 days for the best gemcitabine-responding mice. Both drugs were well tolerated, with modest weight loss during treatment [AGF347, 1% (−0.2 g) median nadir on day 21 with full recovery on day 24; gemcitabine, 5.1% (−1.0 g) median nadir on day 20 with full recovery on day 24].

For matched cohorts of mice maintained on a standard diet (results in approximately 10-fold increased serum folate), antitumor activity of AGF347 was ablated (Supplementary Fig. S7). Thus, excessive levels of circulating folates largely abolish the antitumor activity of AGF347.

To confirm that the enzyme targets of AGF347 identified in vitro (GARFTase, AICARFTase, and SHMT1/2) were inhibited in vivo, tumor samples from a separate cohort of AGF347-treated and control mice (n = 3 each) were harvested 6 hours after the sixth injection of AGF347 (tumors > 1,000 mg). The tumors were homogenized and metabolites analyzed by targeted metabolomics (Fig. S5 and D). In vivo administration of AGF347 induced substantially increased CAR (200-fold) and AICAR (500-fold), consistent with direct inhibition of GARFTase and AICARFTase as seen in vitro (Fig. 4D and E); total serine trended up but did not differ significantly from the control, likely due to exchange with circulating serine pools. We measured changes in purine nucleotide pools in vivo, including 5-fold increased AMP, approximately 2-fold decreased ADP and approximately 10-fold decreased ATP. This severe perturbation of energy charge resulted in a compensatory upregulation of cytochrome c oxidase activity (ref. 51; Supplementary Fig. S8), consistent with cytotoxic drug effects over the chronic course of in vivo treatment.

**Discussion**

The clinical utility of standard chemotherapy drugs is often limited by toxicities toward normal tissues (reflecting a lack of tumor selectivity) and/or drug resistance. Discovery of new and potent inhibitors of tumor-selective pathways remains a formidable challenge. In this report, we identified novel inhibitors of C1 metabolism in mitochondria and cytosol with in vivo antitumor activity. Serine
catabolism by mitochondrial C1 metabolism is the principal source of C1 units for cytosolic de novo purine and thymidylate biosynthesis, and a source of reducing equivalents and ATP (2–5, 21). We targeted SHMT2, the first enzyme in the mitochondrial C1 pathway, with 5-substituted pyrrolo[3,2-d]pyrimidine analogues, rationally designed by molecular modeling from the SHMT2 crystal structure (23) and based on structural similarities to our previously reported 5-substituted pyrrolo[2,3-d]pyrimidine compounds (43, 44) and N-substituted THF metabolites. We identified lead compounds AGF291, AGF320, and AGF347 that inhibited in vitro proliferation of a broad spectrum of tumor subtypes including lung (H460), colon (HCT116), and pancreatic (MIA PaCa-2) cancers.

We assessed critical enzyme targets for our compounds through glycine and nucleoside rescue, and by targeted metabolomics with a [2,3,3-2H]serine tracer, and identified SHMT2 in mitochondria as an intracellular target, along with SHMT1, GARFTase, and AICARFTase in the cytosol. Inhibition of all these targets was confirmed by in vitro assays with purified recombinant enzymes. While the in vitro assays used “mono-glutamyl” inhibitors, greater inhibition should result following metabolism to polyglutamates (7, 9). Inhibition of SHMT1 by our analogues prevents metabolic “compensation” by reversal of SHMT1 catalysis and synthesis of glycine in response to loss of SHMT2 activity (22, 23).

A number of pharmacodynamic factors could contribute to the in vitro antitumor effects of the novel analogues described herein. These include facilitated transport across the plasma membrane by PCFT and/or RFC and into mitochondria (potentially by SLC25A32; refs. 13, 14), and metabolism to drug polyglutamates (7, 9). Variations in these parameters likely account for differences in relative antiproliferative activities toward the assorted tumor models. As AGF347 showed the most potent in vitro activity of the series, this compound was tested in vivo with both early-stage and upstage MIA PaCa-2 xenograft models in SCID mice. AGF347 exhibited potent in vivo efficacy superior to that of gemcitabine, even at a 4-fold decreased total dose, resulting in sustained tumor growth delay and a 20% complete response rate. For the upstage trial, for the PRs, substantial decreases in tumor burden occurred 10 days after the last dose, likely reflecting a long-term impact of AGF347 polyglutamates, associated with target inhibition.

Primary inhibition of SHMT2 in mitochondria results in decreased glycine and C1 units for cytosolic biosynthesis.
(4, 23), and complete ablation through genetic means leads to defective mitochondrial respiration due to impaired synthesis of respiratory chain proteins (52, 53). Other effects include decreased NADPH and glutathione levels with impacts on mitochondrial redox balance and reactive oxygen species (21), and decreased ATP synthesis due to impaired oxidative phosphorylation (52, 53) and C1-flux through MTHFD1L (54). These are exacerbated by direct inhibitions of the de novo purine biosynthetic enzymes GARFTase and AICARFTase by our analogues. Although adenosine (with glycine) was essential for in vitro rescue of the tumor cell lines from our lead compounds, their effects on purine nucleotide pools were variable in both magnitude and direction. This suggests that depletion of critical subcellular purine nucleotide pools may not be reflected in measurements of total cellular purine nucleotides. The sustained treatment for AGF347 in vivo resulted in tumor killing with net energy depletion resulting in an increased AMP/ATP ratio. This was accompanied by activation of cytochrome c oxidase, possibly reflecting post-translational modification (dephosphorylation; ref. 52).

Our results with active 5-substituted pyrrolo[3,4-d]pyrimidine compounds expand upon earlier findings with nonfolate pyrazolopyran inhibitors of human SHMT2 (23) and, to our knowledge, this series represents the first bona fide inhibitors of this intracellular target with demonstrated in vivo antitumor efficacy. Thus, inhibition of SHMT2, coupled with direct inhibition of multiple C1-dependent targets including de novo purine biosynthesis and SHMT1, affords a valuable and exciting new platform for future drug development.

Disclosure of Potential Conflicts of Interest

J.D. Rabinowitz reports receiving other commercial research support from Raze, has ownership interest (including patents) in Raze and Princeton Patent, and has consultant/advisory board relationship with Princeton University. This series represents the first bona fide inhibitors of this intracellular target with demonstrated in vivo antitumor efficacy. Thus, inhibition of SHMT2, coupled with direct inhibition of multiple C1-dependent targets including de novo purine biosynthesis and SHMT1, affords a valuable and exciting new platform for future drug development.

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