Introduction

Metabolic reprogramming is a hallmark of cancer (1). Of the altered metabolisms in cancer, one-carbon (1C) metabolism is especially noteworthy. While 1C metabolism in the cytosol has been an important therapeutic target for cancer since the inception of modern chemotherapy (typified by aminopterin, methotrexate, and 5-fluorouracil; refs. 2, 3), increasing attention has been focused on mitochondrial 1C metabolism and its importance to the malignant phenotype as a critical source of 1C units, glycine, reducing equivalents, and ATP, which have spurred the discovery of novel compounds that target these reactions, with particular focus on 5,10-methylene tetrahydrofolate dehydrogenase 2 and serine hydroxymethyltransferase 2 (15). In this review, we discuss key aspects of 1C metabolism, with emphasis on the importance of mitochondrial 1C metabolism to metabolic homeostasis, its relationship with the oncogenic phenotype, and its therapeutic potential for cancer.

Folate Homeostasis and Compartmentation of Cellular 1C Metabolism

Folates encompass a group of water soluble compounds within the vitamin B9 family composed of pteridine, p-aminobenzoic acid, and L-glutamate moieties (15). While many species from bacteria to plants synthesize folates de novo, mammals cannot (4, 16). Accordingly, folate cofactors must be acquired through the diet (e.g., leafy green vegetables) as reduced forms or as folic acid in fortified foods.

Reflecting their hydrophilic nature, circulating folates have limited capacities to diffuse across plasma membranes. Accordingly, mammalian cells have evolved sophisticated uptake systems (1) to facilitate folate transport across plasma membranes, most notably the reduced folate carrier (RFC; SLC19A1; refs. 17–19) and the proton-coupled folate transporter (PCFT; SLC46A1; refs. 18, 19). The ubiquitously expressed RFC is the major uptake mechanism for folates into tissues and tumors from the systemic circulation (17–19). PCFT is a folate-anion antiporter and exchanges reduced folates for organic anions such as organic phosphates (17–19). PCFT is a proton–folate symporter that facilitates absorption of dietary folates at the acidic pH (~ pH 6) of the upper gastrointestinal (GI) tract (19). While PCFT is also detected in the kidney, liver, placenta, and spleen (20, 21), it is not a major folate transporter in most normal tissues as its activity is very low in tissues outside the upper GI tract secondary to bicarbonate inhibition (at neutral pH; ref. 22). PCFT is optimally active at acidic pH (pH 5–5.5), with detectable activity up to pH 6.5–7 (23). PCFT is expressed in tumors including non–small cell lung cancer (24), malignant pleural mesothelioma (25), epithelial ovarian cancer (26), and pancreatic cancer (27), where it functions in the cellular uptake of folates and related compounds at the acidic pH characterizing the microenvironments of many tumors (20, 23).

Following internalization, folates are compartmentalized in the cytosol and the mitochondria (28), with a smaller pool in the nucleus (Fig. 1; ref. 29). In the cytosol, folate cofactors participate in 1C-dependent metabolism, leading to the synthesis of thymidylate, purine nucleotides, serine, and methionine (15). Cytosolic and mitochondrial 1C pathways are interconnected by an interchange between serine, glycine, and formate (Fig. 1; refs. 4, 5, 28), with uptake of folates from the cytosol into mitochondria via a mitochondrial folate transporter (MFT; SLC25A32; refs. 30, 31). MFT is the only known transporter of folates from the cytosol into the mitochondrial matrix (31) and is a member of the mitochondrial carrier family, which includes the ATP/ADP exchange carrier and the phosphate carrier (32).

In the cytosol and mitochondria, folates are substrates for alternate isofoms of poly(γ-glutamate synthase (FPGS), representing splice variants encoded by a single gene (33). FPGS catalyzes the conjugation of up to eight additional glutamate residues to the
γ-carboxyl of the terminal glutamate of folate substrates (34). Polyglutamyl folates are the preferred substrates for C1 transfer reactions (34). Furthermore, cytosolic folate polyglutamates are retained in cells (34), and mitochondrial folate polyglutamates do not exchange with cytosolic forms (33).

In mitochondria, folates are required for 1C metabolism originating from serine (Fig. 1). Serine catabolism involves three primary steps, catalyzed by SHMT2, MTHFD2 (or methylene tetrahydrofolate dehydrogenase 2-like, MTHFD2L), and methylene tetrahydrofolate dehydrogenase 1-like (MTHFD1L; ref. 4). The net result is generation of glycine and 1C units, with MTHFD1L catalysis resulting in conversion of 10-formyl tetrahydrofolate (10-CHO-THF) to formate, which passes to the cytosol. Serine catabolism in mitochondria serves as the principal source of 1C units and glycine for cellular biosynthesis, including de novo synthesis of purine nucleotides and thymidylate in the cytosol (4–7). Cells deficient in mitochondrial 1C metabolism or MFT are glycine auxotrophs and can require exogenous formate for survival (31, 35, 36). Mitochondrial 1C metabolism is also an important source of NAD(P)H and glycine for glutathione (GSH) synthesis and ATP (refs. 4, 6, 7, 37; see below).

In the cytosol, 10-CHO-THF is resynthesized from formate and tetrahydrofolate by MTHFD1 (Fig. 1), a trifunctional enzyme that includes dehydrogenase, cyclohydrolase, and 10-formyl tetrahydrofolate synthetase activities (38). This provides 10-CHO-THF substrate for de novo purine biosynthesis (by glycaminamide ribonucleotide formyl transferase [GARFTase] and 5-aminomimidazole-4-carboxamide ribonucleotide [AICARFTase]) and 5,10-methylene tetrahydrofolate (5,10-CH2-THF) for thymidylate synthase (TS) and other reactions including SHMT1 (Fig. 1). De novo purine biosynthesis includes 10 sequential reactions from the cytosol.
phosphoribosyl pyrophosphate (PRPP) to IMP (Fig. 1; ref. 39). To facilitate an efficient flux of pathway intermediates, these enzymes assemble into a structure termed the "purinosome" (39), which sequesters pathway intermediates and reduced folates, and colocalizes with mitochondria to consume five moles of ATP per mole of IMP synthesized. SHMT1 converts glycine to serine with 1C units from 5,10-CH₂-THF (Fig. 1). Thymidylate is synthesized from dUMP and 5,10-CH₂-THF by TS, generating dihydrofolate (DHF) which, in turn, is reduced to the active tetrahydrofolate form by dihydrofolate reductase (DHFRED. ref. 15). Interestingly, TS and DHFR (as DHFR-like 1 or DHFRL1) are also expressed in mitochondria to protect the integrity of mitochondrial DNA (40). While the impact of noncytosolic thymi-
dylate biosynthesis on therapeutic targeting with antifolate drugs (below) is not entirely clear, evidence suggests a role in response to TS inhibitors (41).

5,10-CH₂-THF is metabolized in the cytosol to 5-methyl tetra-
hydrofolate by 5,10-methylene tetrahydrofolate reductase (MTHFR; Fig. 1; ref. 42). 5-Methyl tetrahydrofolate is a methyl donor in the conversion of homocysteine to methionine by the vitamin B12–dependent enzyme, methionine synthase (MTR; ref. 43). Furthermore, methionine is converted by methionine adenosyltransferase (5-adenosyl methionine synthetase, SAM5) into S-adenosyl methionine (SAM), which is required for methylation of DNA, phospholipids, and proteins (44). Thus, serine biosynthesis in the mitochondria supports cellular methylation reactions via 5-methyl tetrahydrofolate–dependent methylation of homocysteine and de novo synthesis of ATP.

The 1C reactions depicted in Fig. 1 (beginning with serine in mitochondria) can be viewed as proceeding in a clockwise direction. This reflects the high NAD(P)⁺/NAD(P)H ratios in mitochondria that favor serine oxidation to formate, and low NAD(P)⁺/NADPH ratios in the cytosol that favor conversion of formate to 10-CHO-THF by MTHFD1 and eventually to serine (via SHMT1; ref. 28). When the mitochondrial 1C pathway is lost (e.g., deletion of SHMT2 or MTHFD2), rapid depletion of cytosolic 10-CHO-THF reverses the thermodynamic favorability of the MTHFD1 reaction, resulting in a compensatory reversal of the cytosolic 1C flux (serine → formate) to meet cellular 1C demand (45). However, this compensation is incomplete as these cells exhibit signs of 1C stress (reflected in increased cellular ACAIR and glycine auxotrophy (4, 35, 45). Interestingly, whereas SHMT1 and MTHFD1 reversal compensates for the loss of the mitochondrial 1C pathway, SHMT2 regulates transamination of SHMT2 via direct binding to SHMT2 mRNAs when cellular glycine and folate levels are high (46).

In addition to SHMT1 and SHMT2, an alternatively transcribed SHMT2 isoform (SHMT2α), lacking a mitochondrial targeting sequence (47), is transcribed from a distinct gene (48) and is at least partly localized to the nucleus along with TS, DHFR, SHMT1, and MTHFD1 (29). These proteins undergo posttranslational modifications involving the small ubiquitin-like modifier (SUMO) and are translocated from the cytosol to the nucleus at the onset of S phase where they associate with the DNA repair/replication machinery ("replisome;" ref. 49) to generate nuclear thymidylate, which limits uracil misincorporation into DNA (29) during repair and replication. MTHFD2 was reported (50) to promote tumor cell proliferation by localizing to the nucleus, suggesting a noncanonical role in tumor progression. MTHFD2 has also been reported to interact with RNA processing proteins to regulate DNA repair and regulation (51) and to play a role in controlling global N6-methyl-
ladenosine methylation, including hypoxia-inducible factor (HIF) 2α mRNA (52).

### Serine Biosynthesis, Mitochondrial 1C Metabolism, and Cancer

Synthesis of serine is upregulated in cancer (53, 54). Serine is synthesized from 3-phosphoglycerate (3-PG) with 3-phosphoglycerate dehydrogenase (PGDH) as the first committed step (Fig. 1; ref. 54). PGDH is overexpressed in breast cancers and melanomas, in part, due to gene amplification (53, 55). The 3-phosphopyruvate product is transaminated by phosphoserine aminotransferase 1 (PSAT1) into 3-phosphoserine (3PS), which is converted into serine by phosphoserine phosphatase (PSPH; ref. 54). Serine is an allosteric inhibitor of PGDH (56) and regulates its own synthesis. Furthermore, serine is a ligand and allosteric activator of pyruvate kinase M2 (PKM2), which is expressed in proliferating cancer cells. Thus, under conditions of serine deprivation, PKM2 activity is reduced and more glucose-derived carbon is channeled into serine biosynthesis to support cell proliferation (57). Serine is actively transported from the cytosol into mitochondria by siderophlexin 1/3 (SFXN1/SFXN3; Fig. 1; ref. 58).

Serine catabolism is often activated in cancer, with the genes encoding SHMT2 and MTHFD2 among the most overexpressed metabolic genes in all human cancers as compared with normal tissues (59). Metabolomics analyses of 219 extracellular metabolites from the NCI-60 cancer cell lines showed that glycine consumption and serine catabolism including SHMT2, MTHFD2, and MTHFD1L are closely correlated with cancer cell proliferation (60). High levels of expression of these enzymes in cancer cells may, in part, reflect their regulation by MYC as MYC binds to the promoters for SHMT2 and MTHFD2, as well as for MTHFD1L (61, 62). MTHFD2 is a tumor-selective target which is not significantly expressed in differentiated adult cells (63). Thus, targeting mitochondrial 1C metabolism at a number of levels would likely afford selective tumor inhibition, sparing normal tissues.

In MDA-MB-231 breast cancer cells and tissue-tropic metastatic subclones, serine catabolic enzymes in mitochondria are further upregulated, suggesting their critical roles as drivers of proliferation of a subset of metastatic breast cancers (64). Overexpression of SHMT2 and/or MTHFD2 has been associated with poor prognosis for a number of cancers including breast cancer (12, 59, 64, 65), non-small cell lung cancer (66), pancreatic cancer (11), gliomas (67), cholangiocarcinoma (10), and GI cancers (including esophageal, gastric, and colon cancers; ref. 9). SHMT2 expression is also increased in invasive breast cancer, adrenocortical carcinoma, chromophobe renal cell carcinoma, and papillary renal cell carcinoma, including late-stage tumors (64). This suggests that targeting SHMT2 and/or MTHFD2 could be promising for treating late-stage tumors.

Serine catabolism in mitochondria serves as the principal source of 1C units for cellular biosynthesis, including de novo synthesis of purine nucleotides and thymidylate in the cytosol, and SHMT2 provides >85% of glycine for proteins, purines, and GSH in tumor cells (4–8). Mitochondrial 1C metabolism is a major source of NAD(P)H for synthesis of macromolecules and protection against oxidative stress (6, 37, 68). In mitochondria, glycine is required for heme biosynthesis (69).

SHMT2 catalyzes the conversion of serine to glycine with the generation of 5,10-CH₂-THF (Fig. 1). SHMT2 is essential for cell survival under hypoxic (6) or ischemic (14) conditions and is upregulated by HIF1α in a MYC-dependent manner (6). As MYC-transformed cells rely on SHMT2 to sustain 1C and glycine pools, and on NAD(P)H for cell survival under hypoxic conditions (6), therapeutic targeting of SHMT2 (below) should be selective against hypoxic MYC-transformed tumors that are resistant to other
Modalities such as radiation. Knockout of SHMT2 (in HCT116 and Jurkat cells) was accompanied by increased glycolytic flux, suggesting defects in oxidative phosphorylation (70, 71). SHMT2 knockout impairs formulation of the initiating methionyl tRNA (formyl- Met-tRNA), resulting in decreased translation of mitochondria-encoded complex I and IV subunits, leading to reduced basal and maximal respiratory capacities (71). 5,10-CH₂-THF is also important for synthesis of the taurinomethylidene base of other tRNAs (e.g., lysine and leucine) in mitochondria (70).

SHMT2 is a substrate of histone deacetylase (HDAC) enzymes, which modulates enzyme activity. Desuccinylation of SHMT2 by SIRT5, a class III HDAC and member of the sirtuin family (72), promotes carcinogenesis through activation of SHMT2 catalytic activity (73). While the enzymatically active SHMT2 tetramer is stabilized by pyridoxal phosphate (74), the inactive dimer binds the deubiquitinating BRCC36 isopeptidase complex (BRISC), preventing degra
duction of plasma membrane type 1 IFN receptors and promoting inflammatory signaling (75). HDAC11 deacylates SHMT2 and prevents its association with BRISC; this permits ubiquitination and sequestration of type I IFN receptors (76). As HDAC11 is overexpressed in multiple cancers, HDAC11-mediated SHMT2 deacylation could enable broad spectrum suppression of immune response by cancer cells (77).

MTHFD2 is a bifunctional enzyme with dehydrogenase and cyclo
hydrolase activities that converts 5,10-CH₂-THF to 10-CHO-THF with synthesis of NADH from NAD⁺ (Fig. 1; ref. 78). MTHFD2 can also use NADP⁺ as a cofactor to generate mitochondrial NADPH (63). MTHFD2 was originally reported to be expressed in transformed, embryonic, and undifferentiated adult cells, whereas MTHFD2L is expressed in differentiated adult cells and at all stages of embryogenesis (79). While recent studies found that normal and cancer cells express both enzymes, MTHFD2L is unlikely to have an important role in cancer (80). For MTHFD2, regulation by MYC and mTOR (81, 82) is consistent with its essential role in supporting the increased biosynthetic demands of rapidly proliferating cells (78). In lung cancer, MTHFD2 was implicated in gefitinib resistance and cancer stem–like properties by depleting cellular AICAR (83).

Downstream from MTHFD2 is the enzyme MTHFD1L (Fig. 1; ref. 78), which catalyzes the reverse 10-formyl tetrahydrofolate synthetase reaction by which 10-CHO-THF is converted to tetrahydrofolate, formate, and ATP. Generation of ATP in this step is significant such that when combined with that from NADH and oxidative phosphorylation, each formate generated from serine yields 3.5 ATPs (84). In proliferating cancer cells, synthesis of 1C units exceeds the 1C demand for purine biosynthesis (84). Indeed, formate “overflow” is a characteristic of oxidative cancers and is associated with tumor invasion (85). Formate generated in the mitochondria and exported to the cytosol serves an important “protective” role in limiting loss of unsubstituted tetrahydrofolate by oxidative stress (86).

Glycine is the product of SHMT2 catalysis and itself can be a source of 1C units via the mitochondrial glycine cleavage system (GCS; ref. 87). The GCS including glycine decarboxylase (GDC) converts glycine to CO₂, NH₃, and 5,10-CH₂-THF (Fig. 1). While the importance of the GCS as a source of reducing potential is uncertain, overexpression of GDC was reported to drive tumor formation in lung adenocarcinomas (88). Furthermore, in gliomas with high levels of SHMT2, the GCS is important for clearing glycine because loss of activity results in accumulation of the toxic glycine metabolites, aminocacetone and methylglyoxal (14). Increased glycine can also impair cell growth and decrease NAD(P)H and 5,10-CH₂-THF, possibly due to a reversal of SHMT2 catalysis (89).

Mitochondrial NADPH can also be generated by the catalysis of 10-CHO-THF to CO₂ and tetrahydrofolate by aldehyde dehydroge
gnase 1 family member L2 (ALDH1L2; Fig. 1; ref. 90). ALDH1L2 is approximately 72% homologous to its cytosolic counterpart, aldehyde dehydrogenase 1 family member L1 (ALDH1L1). Although ALDH1L1 is involved in regulating cell proliferation through its control of tetrahydrofolate pools, ALDH1L2 is a major source of NADPH in mitochondria (68, 90).

Serine is converted to glycine via SHMT2 and is a precursor of cysteine (via the transsulfuration pathway), both of which are important for synthesis of GSH (7). MTHFD2 with ALDH1L2 (Fig. 1) provide reducing equivalents (such as NADH and NADPH), which are essential for redox homeostasis and resistance to oxidative stress (63, 68). Knockdown of SHMT2, MTHFD2, or ALDH1L2 increased reactive oxygen species, with concomitant decrease in cellular ratios of NAD(P)H to NAD(P)⁺ and reduced to oxidized GSH (68). This increased redox stress results in increased cell death that could be rescued by N-acetylcysteine (6). ALDH1L2 levels are elevated in many tumor types (90) and ALDH1L2 was implicated in metastasis in a mouse melanoma model, associated with its role in counteracting oxidative stress (91). Thus, mitochondrial 1C metabolism is important for redox homeostasis and oxidative stress. Furthermore, NFR2, a key regulator of antioxidant response (37, 92–94), regulates expression of genes involved in serine synthesis (PDGH and PST1) and catabolism (SHMT2) via ATF4 (66, 95). This, in turn, regulates cellular responses to amino acid starvation.

p53 is associated with the capacity of cancer cells to adapt to serine starvation and oxidative stress, and, indeed, p53 is activated upon serine deficiency, resulting in p21 cell-cycle arrest (96). This diverts metabolism toward GSH synthesis to combat oxidative stress. The protective effects of p53 under serine-depleted condi
tions suggest that p53-null tumors could be particularly vulnerable to serine depletion (96).

The unique demands of tumor cells for 1C units and glycine, and for redox balance under hypoxia (4–6, 8), combined with the high levels of expression of serine biosynthetic and catabolic pathways in cancers versus normal tissues (53, 54, 59, 60), suggest that SHMT2 and MTHFD2 could be important therapeutic targets for cancer. Thus, targeting mitochondrial 1C metabolism would likely afford selective tumor inhibition, sparing normal tissues. In the following sections, we describe progress toward developing therapeutics for direct targeting of SHMT2 and MTHFD2 in cancer.

**Discovery of MTHFD2 Inhibitors for Cancer**

In acute myeloid leukemia (AML), overregulation of MTHFD2 is commonly associated with several treatments that cause AML-related death and differentiation (i.e., 1,25-dihydroxy vitamin D3, PMA, all trans retinoic acid, JQ1, and EPZ004777; ref. 81). This is particularly pronounced in FLT3-ITD AMLs with poor prognoses to standard therapies (61). In breast cancer, MTHFD2 overexpression correlated with metastasis and invasion (97), and with poor prognosis (13). Knockdown of MTHFD2 in breast cancer cells (98) increased the dependence on extracellular glycine that could not be rescued by formate supplementation. Metabolomics analyses in these MTHFD2-knockdown cells revealed decreased mitochondrial 1C metabolism, coupled with increased glycolytic and glutaminolytic fluxes (98).

MTHFD2 has 40% sequence identity with MTHFD1, the cytosolic isozyme (78). Although early crystallization of MTHFD1 in complex with inhibitors (99) identified critical catalytic residues,
such as Lys56, Ser49, and Cys147, the development of small-molecule inhibitors of MTHFD2 has remained challenging. Initial efforts to identify MTHFD2 inhibitors focused on the antibacterial benefits from inhibiting the bacterial MTHFD2 ortholog, FolD (100). A high-throughput screening assay to identify inhibitors of FolD in *Pseudomonas aeruginosa* identified several compound leads; however, these showed modest enzyme inhibition and could not provide scaffolds for further development as MTHFD2 inhibitors. The macrolide keto-carboxylic acid, carolacton (Fig. 2), produced by the myxobacterium *Sorangium cellulosum* inhibited FolD, as well as MTHFD2, in the low nanomolar range (101). However, carolacton was a poor inhibitor of tumor cell proliferation, as the EC$_{50}$ values against human cancer cell lines (i.e., HCT116, KB, and U937) *in vitro* were in excess of 10 $\mu$mol/L, with drug export cited as the main hurdle (101).

Studies with LY345899 (Eli Lilly; Fig. 2) yielded the first crystal structure of an inhibitor complexed with MTHFD2, NAD$^+$, and inorganic phosphate (102). While LY345899 inhibited human MTHFD2 (IC$_{50}$ = 663 nmol/L), it was a more potent inhibitor of MTHFD1 (IC$_{50}$ = 96 nmol/L; ref. 102); however, there was no loss of viability for tumor cells (U2OS) and Hs-S87T) treated with LY345899 *in vitro* (102). Although LY345899 was reported to suppress growth of a SW620 colorectal cancer xenograft and a colorectal cancer patient-derived xenograft (PDx) *in vivo* (103), it is unclear whether this response was due to inhibition of MTHFD1 or MTHFD2.

A novel isozyme-selective MTHFD2 inhibitor, DS44960156 (Fig. 2), with a tricyclic coumarin scaffold, was initially discovered via high-throughput screening, and then modified by structure-based drug design (104). DS44960156 was >18-fold more selective toward human MTHFD2 (IC$_{50}$ = 1.6 $\mu$mol/L) than MTHFD1 (IC$_{50}$ > 30 $\mu$mol/L; ref. 104). Further optimization of this scaffold yielded DS18561882 (Fig. 2), with potent inhibition of MTHFD2 (IC$_{50}$ = 6.3 nmol/L) and 90-fold selectivity for MTHFD2 over MTHFD1 (105). When administered orally, DS18561882 demonstrated remarkable *in vivo* efficacy against triple-negative breast cancer MDA-MB-231 xenografts in nude mice with minimal treatment-related toxicity (105). DS18561882 would seem to be a promising candidate for future clinical evaluation.

**Discovery of SHMT2-targeted Therapeutics for Cancer**

SHMT2 is 60% homologous to SHMT1 (35), suggesting that inhibitors of SHMT2 would likely target both enzymes. Knockdown of SHMT1 in HCT116 colon cancer xenografts in immunocompromised mice had no impact on tumor proliferation, whereas SHMT2 knockdown slightly suppressed cell proliferation (36). Knockdown of both SHMT1 and SHMT2 exerted profound inhibition of tumor progression (36), suggesting that dual inhibition of both SHMT1 and SHMT2 is essential (45).

Classic antifolates, including lometrexol, pemetrexed, and methotrexate, were tested as inhibitors of SHMT1 *in vitro* (106, 107), all with modest activity. Lometrexol showed a $K_i$ of 20 $\mu$mol/L (106); the $K_i$ for pemetrexed was 19.1 $\mu$mol/L (107), approximately 500-fold less potent than that for TS (109 nmol/L; ref. 108). Toward human SHMT2, an IC$_{50}$ value of approximately 100 $\mu$mol/L was reported for lometrexol (109). Analogous results were reported for pemetrexed with SHMT2 (35). Although inhibitors may be greater for polyglutamyl drug forms, these results, nonetheless, suggest that any biological effects of classic antifolates resulting from direct targeting of SHMT1 and SHMT2 are likely to be minor.

Early efforts to generate targeted small inhibitors of SHMT proteins initially focused on herbicidal pyrazolopyran compounds originally described as inhibitors of plant SHMT (Fig. 3A; ref. 110). Optimization of these compounds (Fig. 3B) yielded molecules with antimalarial activity (111). Although these were inhibitors of *Plasmodium falciparum* and *P. vivax* SHMTs *in vitro* (submicromolar IC$_{50}$), they were poorly active against rat L6 myoblasts or HepG2 human hepatoma cells (111). Additional pyrazolopyran compounds were tested against...
A549 and H1299 human lung cancer cells (112) and a lead compound (compound 2.12) was reported to preferentially inhibit SHMT1 over SHMT2 and effect apoptosis with an LD\textsubscript{50} of 34 nmol/L.

The pyrazolopyran series was further optimized by Rabinowitz and colleagues for inhibition against human SHMT1 and SHMT2. The first study using these optimized compounds (113) showed potent (nanomolar) inhibition of isolated human SHMT1 and SHMT2. The IC\textsubscript{50} value for the lead compound RZ-2994 (later renamed SHIN1 for “SHMT inhibitor 1”; Fig. 3C) toward human T effector cells was in the low micromolar range, suggesting its therapeutic potential. Although not an analog of folic acid, a crystal structure of SHIN1 in complex with SHMT2 and effect apoptosis with an LD\textsubscript{50} of 34 nmol/L, with complete pharmacologic rescue by formate and glycine, suggesting on-target inhibition of SHMT2 (36). The IC\textsubscript{50} value for SHIN1 in SHMT2-knockout HCT116 cells decreased nearly two orders of magnitude to approximately 10 nmol/L, reflecting potent inhibition of SHMT1 in addition to SHMT2, whereas the IC\textsubscript{50} value in SHMT1-knockout cells was indistinguishable from that in wild-type cells (36). This confirmed that efficacy toward wild-type HCT116 cells was primarily due to its inhibition of SHMT2 rather than SHMT1. However, inhibition of both enzymes is essential, as this prevents metabolic compensation by reversal of SHMT1 catalysis (serine → glycine) and synthesis of glycine in response to loss of SHMT2 (45).

Interestingly, enhanced potency of SHIN1 toward 8988T pancreatic cancer cells and diffuse large B-cell lymphoma cells revealed distinct metabolic vulnerabilities of these cancer types that could be exploited by SHMT1 and SHMT2 inhibition (36). 8988T cells exhibit defects in mitochondrial 1C metabolism with an overreliance on SHMT1, whereas B-cell lymphomas have intrinsic defects in glycine uptake that render these cells overly reliant on glycine synthesis from serine by SHMT2 (36). Addition of formate did not rescue B-cell lymphomas from the effects of SHIN1 as with HCT116 cells, but rather paradoxically potentiated SHIN1 effects. Cytotoxicity was not due to 1C depletion, but instead was due to glycine deficiency being exacerbated by formate excess, which drives SHMT catalysis in the glycine-consuming (glycine → serine) direction. Despite these promising in vitro results, SHIN1 showed a disappointing lack of in vivo anti-tumor efficacy (36), likely due to its poor pharmacokinetics and/or metabolic instability.

To improve on these shortcomings of SHIN1, Rabinowitz and colleagues synthesized a next-generation pyrazolopyran compound, SHIN2 (Fig. 3D; ref. 114). Like SHIN1, SHIN2 induced metabolic derangements consistent with inhibition of SHMT1 and SHMT2. SHIN2 showed potent in vivo inhibition of NOTCH1-induced T-cell acute lymphoblastic leukemia (T-ALL) xenografts in a mouse model at comparatively high doses (200 mg/kg twice a day, 11 days), with efficacy comparable with that of the standard-of-care treatment methotrexate. Moreover, SHIN2 demonstrated efficacy in a methotrexate-resistant PDX T-ALL model. Combination treatment with SHIN2 and methotrexate in the PDX T-ALL model showed a synergistic response, possibly because of methotrexate-induced depletion of cellular tetrahydrofolic acids, resulting in decreased competition for SHIN2 binding and greater inhibition of SHMT1 and SHMT2.

Discovery of Multi-targeted Inhibitors of SHMT2 and Cytosolic 1C Metabolism at SHMT1 and de novo Purine Biosynthesis

As mitochondrial 1C metabolism from SHMT2 is the major source of 1C units for de novo purine biosynthesis in the cytosol, molecules targeting SHMT2, along with de novo purine biosynthesis at GARFTase and/or AICARFTase, should afford especially potent antitumor agents (35). Primary inhibition of mitochondrial 1C metabolism at SHMT2 would deplete cytosolic formate (e.g., 10-CHO-THF) pools required for nucleotide biosynthesis, potentiating drug efficacy by reducing competition for inhibitor binding at these cytosolic enzyme targets. Moreover, concurrent inhibition of SHMT1 would augment inhibition at SHMT2 by preventing metabolic compensation involving reversal (serine → glycine) of the SHMT1 reaction, analogous to SHIN1 and SHIN2 (36, 114).

To achieve this, Matherly, Gangjee, Dann, and colleagues combined structural features from 5-substituted pyrrolo[2,3-d]pyrimidine inhibitors of de novo purine biosynthesis (115) with those from 5-formyl tetrahydrofurfurale (a SHMT inhibitor; ref. 116) and 5,10-CHI-THF (SHMT2 product), to generate novel 5-substituted pyrrolo[3,2-d]pyrimidine benzoyl and thiophenyl analogs (35). The lead compounds of this series, AGF291, AGF320, and AGF347 (Fig. 4), demonstrated broad range in vitro efficacy toward human tumor cell lines expressing PCFT, including non-small cell lung cancer (H460), colon cancer (HCT116), and pancreatic adenocarcinoma (MIA PaCa2, AsPC1, BxPC3, CFPAC, and HPAC; refs. 27, 35) cells. SHMT2 inhibition was confirmed by targeted metabolomics and flux analysis with [2,3,3-\textsuperscript{3}H]serine, accompanied by direct inhibition of GARFTase and AICARFTase in de novo purine biosynthesis and reduced purine nucleotide pools (35). In vivo antitumor efficacy with curative potential was confirmed with AGF347 in early- and late-stage Mia PaCa2...
pancreatic adenocarcinoma xenografts in SCID mice at modest dosing (120 mg/kg total, every 2 days × 8; ref. 35). Toxicity was modest and consisted of limited weight loss that was completely reversible upon completion of therapy.

A follow-up study demonstrated that AGF347 accumulated in both the cytosol and mitochondria nearly exclusively (>98%) as polyglutamate conjugates (27). In mitochondria, AGF347 accumulation was mediated, at least in part, by MFT, albeit seemingly less than for folates (27). Treatment of HCT116 cells with AGF347 under hypoxic (0.5% O2) conditions resulted in elevated reactive oxygen species (ROS), accompanied by decreased reduced and total GSH, analogous to HCT116 SHMT2-knockout cells (27). The extent of ROS induction with AGF347 varied with different tumor cell lines (27) However, unlike knockout of SHMT2, treatment of HCT116 cells with AGF347 did not suppress mitochondrial respiration (27), likely due to incomplete inhibition of this mitochondrial target (71).

Conclusions and Future Outlook

In spite of recent advances in targeted therapies for cancer, the classic antifolates, typified by methotrexate and pemetrexed, remain vital components of the therapeutic armamentarium (2). These agents have found important clinical applications for cancer in the United States and abroad. Most recently, PCFT-targeted pyrrolo[2,3-d]pyrimidine antifolate inhibitors were described, with tumor targeting based on their selective membrane transport by PCFT under acidic conditions of the tumor microenvironment (20, 23). Notably, all these agents inhibit 1C metabolism at cytosolic enzymes involved in nucleotide biosynthesis (2, 20, 23).

It is now recognized that 1C metabolism involves compartmentalization of folate cofactors and 1C pathways between the cytosol and mitochondria (4, 7, 8, 28), which extends to the nucleus (29). Even within a particular cellular compartment, regulatory networks and protein associations (e.g., purinosome; ref. 39) can occur, designed to ensure an efficient flow of 1C units for biosynthesis of nucleotides and key amino acids, and/or for methylation of critical genes and/or proteins. It is interesting that enzymes previously considered to localize exclusively in the cytosol are now recognized to be expressed as distinct, but functionally homologous, forms in the mitochondria and nucleus, including SHMT1, SHMT2, and SHMT2α, and MTHFD1, MTHFD1L, and MTHFD2 (4). In some cases, these enzymes appear to perform noncanonical functions in the nucleus or mitochondria (117), although this is controversial. Even within mitochondria, functional redundancies exist between normal tissues (MTHFD2L) and tumors (MTHFD2; refs. 78, 79).

It is of further interest that the mitochondrial 1C enzymes, MTHFD2 and SHMT2, are among the most differentially expressed metabolic enzymes between tumors and normal tissues (59). Serine catabolism in mitochondria ensures that the unique metabolic requirements of hypoxic tumors are met for 1C units, glycine, reducing equivalents, and ATP (4–6, 8), while also ensuring that proteins required for mitochondrial respiration are efficiently translated (70, 71). Although the evolutionary rationale for compartmentalization of 1C metabolism continues to emerge, studies suggest its importance for preserving labile tetrahydrofolates from oxidation (86). Furthermore, compartmentalization of 1C metabolism between cytosol and mitochondrial ensures a directional flux of 1C units, in relation to the high NAD(P)⁺/NAD(P)H ratios in mitochondria that favor serine oxidation to formate. In the cytosol, low NADP⁺/NADPH ratios favor synthesis of 10-CHO-THF from formate and tetrahydrofolate (via MTHFD1), and synthesis of serine from glycine and 5,10-CH₂-THF (via SHMT1; refs. 28, 86).

Thus, MTHFD2 and SHMT2 are important biomarkers for cancer, the levels of which correlate with tumor aggressiveness and disease progression (9–12, 59, 64–67). Although these enzymes limit 1C units and glycine for cellular biosynthesis in the cytosol, for HCT116 colon cancer cells, SHMT2 knockout results in GSH depletion as the most significant metabolic change, because supplementation with GSH or N-acetylcysteine completely rescues cells from cytotoxicity (45). This suggests that antioxidant synthesis, rather than synthesis of proteins or purine nucleotides, is the major driver of demand for 1C metabolism in mitochondria, at least in HCT116 cells. Indeed, as serum glycine can be abundant, therapeutic efforts to target SHMT2 (or MTHFD2) seem ideally suited for intrinsically glycine-deficient tumors (e.g., diffuse large B-cell lymphoma; ref. 36).
Promising lead inhibitors have been described for SHMT2 (SHIN1, SHIN2, and AGF347; refs. 35, 36, 114) or MTHFD2 (DS18461882; ref. 105). As compensatory metabolic changes in cytosolic fluxes (e.g., SHMT1 reversal) accompany loss of SHMT2 or MTHFD2 and result in viable and tumorigenic cells (35, 45), it will be important to combine targeting mitochondrial 1C metabolism by small molecules with approaches for targeting MYC (bromodomain and extra-terminal motif inhibitors; ref. 124), as MYC regulates both SHMT2 and MTHFD2 (6, 62, 81).

In conclusion, the pervasive importance of mitochondrial 1C metabolism to the malignant phenotype demonstrates the immense value of the therapeutic targeting of this critical pathway at SHMT2 and/or MTHFD2, along with other enzymes. By any measure, these new agents afford a valuable and exciting platform for future anti-cancer drug development.

Disclosure of Potential Conflicts of Interest

L.H. Matherly and A. Gangjee report collaboration with Flag Therapeutics, Inc., an early-stage oncology company focused on the development of novel therapies. No potential conflicts of interest were disclosed by the other authors.

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