ANTIFOLATE MODULATORS OF AMP-ACTIVATED PROTEIN KINASE SIGNALING AS CANCER THERAPEUTICS

Scott Rothbart
Virginia Commonwealth University

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ANTIFOLATE MODULATORS OF AMP-ACTIVATED PROTEIN KINASE SIGNALING AS CANCER THERAPEUTICS

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

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September 2010
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“Research is to see what everybody else has seen, and to think what nobody else has thought.”

- Albert Szent-Györgyi
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Chemicals and reagents

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<table>
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<th>Description</th>
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<tbody>
<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>α</td>
<td>alpha</td>
</tr>
<tr>
<td>ACC</td>
<td>acetyl CoA carboxylase</td>
</tr>
<tr>
<td>AdoMet</td>
<td>S-adenosyl-methionine</td>
</tr>
<tr>
<td>AICA</td>
<td>5-aminoimidazole-4-carboxamide</td>
</tr>
<tr>
<td>AICAR</td>
<td>5-aminoimidazole-4-carboxamide ribonucleoside</td>
</tr>
<tr>
<td>AICART/AICARFT</td>
<td>5-amino-4-imidazolecarboxamide ribonucleotide formyltransferase</td>
</tr>
<tr>
<td>AK</td>
<td>adenosine kinase</td>
</tr>
<tr>
<td>AKT</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>ALL</td>
<td>acute lymphocytic leukemia</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine-5’-monophosphate</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>APRT</td>
<td>adenine phosphoribosyltransferase</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine-5’-triphosphate</td>
</tr>
<tr>
<td>AU</td>
<td>absorbance units</td>
</tr>
<tr>
<td>β</td>
<td>beta</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>=CH⁺</td>
<td>methenyl</td>
</tr>
<tr>
<td>=CH₂</td>
<td>methylene</td>
</tr>
<tr>
<td>CH₃</td>
<td>methyl</td>
</tr>
<tr>
<td>CHO</td>
<td>formyl</td>
</tr>
<tr>
<td>cm</td>
<td>centimeter</td>
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DDATHF  5,10-dideaza-\( \text{H}_4 \text{PteGlu} \), lometrexol
Deptor  DEP-domain-containing mTOR-interacting protein
dFBS  dialyzed fetal bovine serum
DHFR  dihydrofolate reductase
DNA  deoxyribonucleic acid
dUMP  \( 2' \text{-deoxy-uridylate, uridylylate} \)
dUTP  \( 2' \text{-deoxyuridine-5-triphosphate} \)
eEF2  eukaryotic elongation factor 2
FBS  fetal bovine serum
FGAR  N-formylglycinamide ribonucleotide
fmol  femtomole
FPGS  folylpoly-\( \gamma \)-glutamate synthetase
FPLC  fast protein liquid chromatography
fZMP  \( 5 \text{-formylaminoimidazole-4-carboxamide ribonucleotide} \)
g  grams
GAR  Glycinamide ribonucleotide formyltransferase
GART/GARFT  glycaminamide ribonucleotide formyltransferase
\( \text{H}_2 \text{PteGlu} \)  dihydrofolate
\( \text{H}_4 \text{PteGlu} \)  \( 5,6,7,8 \)-tetrahydrofolate
HGPRT  hypoxanthine-guanine phosphoribosyltransferase
HPLC  high-performance liquid chromatography
Hx  hypoxanthine
IMP  inosine monophosphate
IMPCH  inosine monophosphate cyclohydrolase
IRS   insulin receptor substrate
kB    kilobase
KD    knockdown
kDa   kilodalton
LCA   Lilly compound AICART inhibitor
M     molar
m     meters
MEF   mouse embryonic fibroblast
MFT   mitochondrial folate transporter
mg    milligram
ml    milliliter
mLST8 mammalian lethal with Sec12 protein 8
mM    millimolar
mmol  millimole
MPM   malignant pleural mesothelioma
MTHFR 5,10-methylene-H₄PteGlu₉ reductase
mTOR  mammalian target of rapamycin
MTX   methotrexate, 2-amino-10-methyl-folic acid
nM    nanomolar
nm    nanometers
nmol  nanomole
NSCLC non-small cell lung cancer
PABA  para-aminobenzoic acid
PBS  phosphate-buffered saline
PCFT  proton-coupled folate transporter
PI3K  phosphatidylinositol-3-OH kinase
PIP2  phosphatidylinositol-4,5-bisphosphate
PIP3  phosphatidylinositol-3,4,5-triphosphate
pmol  picomole
PPAT  phosphoribosylpyrophosphate amidotransferase
PRAS40  proline-rich AKT substrate 40 kDa
Protor  protein observed with Rictor
PRPP  5-phosphoribosyl-1-pyrophosphate
PTEN  phosphatase and tensin homolog
PTX  pemetrexed, LY231514, Alimta®
PVDF  polyvinylidene fluoride
Raptor  regulatory associated protein of mTOR
RFC  reduced folate carrier
rh  recombinant human
Rictor  rapamycin-insensitive companion of mTOR
RNA  ribonucleic acid
RTK  receptor tyrosine kinase
RTX  raltitrexed, Tomudex®, ZD1694
SAR  structure-activity relationship
SHMT  serine hydroxymethyltransferase
<table>
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<tr>
<th><strong>SIN1</strong></th>
<th>stress-activated protein kinase interacting protein</th>
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<tr>
<td><strong>siRNA</strong></td>
<td>small interfering RNA</td>
</tr>
<tr>
<td><strong>TCA</strong></td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td><strong>TdR</strong></td>
<td>thymidine</td>
</tr>
<tr>
<td><strong>TMP</strong></td>
<td>thymidylate, thymidine-5-monophosphate</td>
</tr>
<tr>
<td><strong>TS</strong></td>
<td>thymidylate synthase</td>
</tr>
<tr>
<td><strong>TSC</strong></td>
<td>tuberous sclerosis complex</td>
</tr>
<tr>
<td><strong>TSC1</strong></td>
<td>hamartin</td>
</tr>
<tr>
<td><strong>TSC2</strong></td>
<td>tuberin</td>
</tr>
<tr>
<td><strong>TTP</strong></td>
<td>thymidine-5-triphosphate</td>
</tr>
<tr>
<td><strong>µg</strong></td>
<td>microgram</td>
</tr>
<tr>
<td><strong>µl</strong></td>
<td>microliter</td>
</tr>
<tr>
<td><strong>U.S. FDA</strong></td>
<td>United States Food and Drug Administration</td>
</tr>
<tr>
<td><strong>γ</strong></td>
<td>gamma</td>
</tr>
<tr>
<td><strong>ZMP</strong></td>
<td>5-aminoimidazole-4-carboxamide ribonucleotide</td>
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Abstract

ANTIFOLATE MODULATORS OF AMP-ACTIVATED PROTEIN KINASE SIGNALING AS CANCER THERAPEUTICS

By Scott Barri Rothbart, B.S.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2010

Mentor: Richard G. Moran, Ph.D.
Professor, Department of Pharmacology & Toxicology

Since its discovery, it was appreciated that the antifolate pemetrexed had multiple targets within folate metabolism. This laboratory was instrumental in showing that pemetrexed elicited its primary action as a thymidylate synthase inhibitor. Unusual for an antifolate, pemetrexed showed significant clinical activity against malignant pleural mesothelioma and non-small cell lung cancer. Accordingly, the FDA recently issued first-line approvals for pemetrexed in these diseases, leading us to question whether the effects of pemetrexed on other folate-dependent targets could explain this atypical clinical activity of the drug. Studies in this dissertation showed that in addition to thymidylate synthase inhibition, pemetrexed was also an inhibitor of aminomidazolecarboxamide ribonucleotide formyltransferase (AICART), the second folate-dependent enzyme of de novo purine synthesis. Consequent of AICART inhibition, pemetrexed caused robust activation of a key energy-sensing regulatory enzyme of the PI3K-AKT signal transduction pathway, AMP-activated protein kinase (AMPK). AMPK activation resulted from
accumulation of the AMP-mimetic, ZMP, behind the AICART block. Constituents of the PI3K-AKT cascade are frequently deregulated in human carcinomas, uncoupling nutrient supply from proliferative capacity. Therefore, interventions that reinstate control over aberrant signaling along this axis, such as AMPK activation, are of significant cancer therapeutic interest. The cellular consequences of AMPK activation in response to pemetrexed were assessed. In particular, effects on the downstream target of PI3K-AKT signaling, the mammalian target of rapamycin complex 1 (mTORC1), were studied. Unlike targeted mTORC1 inhibitors, such as rapamycin and its analogs, pemetrexed-mediated activation of AMPK also signaled to mTOR-independent controlling elements of protein and lipid synthesis, highlighting additional benefits of AMPK activating agents that extend beyond effects on mTOR signaling. We therefore propose that the unusual activity of pemetrexed in mesothelioma and non-small cell lung cancer is due in part to effects on signaling processes downstream of AMPK activation. These findings present a novel approach to AMPK activation secondary to an AICART block, define pemetrexed as a molecularly targeted agent, and ultimately extend the utility of antifolates beyond their traditional function.
CHAPTER 1

BACKGROUND AND SIGNIFICANCE

Structural, absorptive, and transport characteristics of naturally occurring folates

Antifolates have been a mainstay in cancer therapeutic regimens for over 50 years. Much of our understanding of the cellular metabolism and utilization of dietary folates can be credited to basic and clinical research on their synthetic antagonists. Folates were first discovered in the early 1940’s as the factors in yeast and liver extracts responsible for reversing macrocytic anemia in pregnant women (1). The generalized chemical structure of the folate molecule was deduced from the isolation and crystallization of folic acid from spinach (2). Folic acid, the most oxidized form of folate, consists of three distinct moieties. A 2-amino-4-hydroxy-pteridine ring is conjugated by a methylene group to para-aminobenzoic acid (PABA), which forms a peptide linkage to glutamic acid (Fig 1-1). While folic acid is the most stable form of the folate molecule, modified forms are utilized for cellular metabolism. These folic acid metabolites (herein referred to collectively as “folates”) are reduced to di or tetrahydro forms in the 5,6 and 7,8 positions of the pteridine ring, respectively. The N₅ or N₁₀ nitrogen atoms of 5,6,7,8-tetrahydrofolate (H₄PteGlu) are routinely conjugated to methyl (CH₃), formyl (CHO), methenyl (=CH⁺), or methylene (=CH₂) groups. Additional glutamate residues are processively added to the γ-carboxyl tail by the enzyme folylpoly-γ-glutamate synthetase (FPGS), forming H₄PteGluₙ(2-9) (Fig 1-1) (3, 4).

Although humans can synthesize all components of the folate molecule individually, they do not produce the dihydropteroate synthase enzyme activity essential to conjugate the pteridine and PABA ring (pteroid acid), or the dihydrofolate synthetase enzyme activity usually associated
Figure 1-1. Chemical structures of folic acid and tetrahydrofolate.
with bacterial FPGS. Thus, humans obtain folates solely from dietary and supplemental sources. These hydrophilic B_{9} vitamins are abundant in raw green vegetables, peanuts, legumes, citrus fruits, and liver. However, most folates are destroyed in the cooking process. Cereals, grains, and flour have been fortified with folic acid (1.4 µg folate/gram of product) in the United States since 1998 (5). Folate deficiency in pregnant women is causal of fetal neural tube defects. Deficiency is also associated with cardiovascular disease and blood disorders such as megaloblastic anemia, and can manifest as malnourishment or malabsorption from acute and chronic alcoholism, or secondary to pathologies that affect nutrient absorption by the small intestine (6).

The most abundant natural forms of folate found in the diet are polyglutamated derivatives of 5-CH_{3}-H_{4}PteGlu (Figure 1-1). Polyglutamation limits the transport of folates across membranes. Therefore, γ-glutamyl carboxypeptidases, located in the brush-border of the proximal jejunum, hydrolyze 5-CH_{3}-H_{4}PteGlu polyglutamates to monoglutamates, optimal for transport (7). Folate monoglutamates (including folic acid) are transported across the apical surface of the proximal jejunum in a facilitated manner primarily by the proton-coupled folate transporter (PCFT). The acidic microenvironment of the small intestine is favorable for PCFT-mediated folate transport, which symports folates optimally at pH 5.5 against its concentration gradient with protons along their concentration gradient into the enterocytes (8, 9). It is during intestinal uptake that folic acid from fortified foods is reduced to 5-CH_{3}-H_{4}PteGlu. The efflux mechanism of 5-CH_{3}-H_{4}PteGlu across the basolateral membrane into the vasculature is not known, but most likely involves facilitated transport by multidrug resistance-associated protein 3 (10).
Circulating 5-CH$_3$-H$_4$PteGlu is first delivered to the liver, where it can be passed through to the systemic circulation, secreted into the bile for reabsorption, or can be polyglutamated for storage. Systemic 5-CH$_3$-H$_4$PteGlu transport into cells is facilitated primarily by the reduced folate carrier (RFC) in neutral pH microenvironments (11, 12). The driving force for folate uptake by the RFC is likely the anion gradient generated by retained intracellular organic phosphates (13). 5-CH$_3$-H$_4$PteGlu can also be transported into cells by folate receptor-mediated endocytosis, a process less efficient than RFC-mediated transport.

Once inside peripheral cells, folates are rapidly polyglutamated by FPGS (14). Polyglutamation is essential to trap folates within the cell, and it also increases the affinity of folates for their target enzymes. Folate polyglutamates are distributed relatively equally between the cytosol and mitochondria (15). This compartmentalization of cellular folate polyglutamates is facilitated by cytosolic and mitochondrial FPGS, isoforms synthesized from the same gene (16, 17). Folates are transported into the mitochondria by the mitochondrial folate transporter (MFT), a family member of the inner mitochondrial membrane transport carriers that was cloned and characterized by our lab (18-20). It is likely that folate monoglutamates are the substrates for mitochondrial transport, suggesting substrate competition between cytosolic FPGS and the MFT (16, 20).

**Folates and one-carbon metabolism**

Folate polyglutamates donate and accept single carbon units in transfer reactions for essential cellular biosynthetic processes. Folate metabolism is compartmentalized between the cytosol and mitochondria, each of which are supplied with their own pool of folate polyglutamates as described above (21). In the cytosol, folates are essential for the vitamin B$_{12}$-
mediated conversion of homocysteine to methionine. Methionine is synthesized from the transfer of a CH₃ group of polyglutamated 5-CH₃-H₄PteGlu to homocysteine by methionine synthase in a vitamin B₁₂-dependent reaction. Methionine is conjugated to adenosine by methionine adenosyltransferase to produce S-adenosyl-methionine (AdoMet), one of the most frequently used cellular co-substrates. AdoMet serves as the CH₃ donor for DNA, RNA, neurotransmitter, histone, and other protein methyltransferase reactions (22). AdoMet-dependent methylation of DNA and histone intimately links folates to processes such as DNA replication, transcription, and the DNA-damage response through the regulation of chromatin architecture and organization. AdoMet deficiency, resulting from folate malnourishment or defects in folate metabolism, has been linked to certain types of cancers, heart disease, Alzheimer’s, Downs Syndrome, alcoholic liver disease, and developmental disorders such as neural tube defects (23, 24).

Cytosolic folate polyglutamates also serve as cofactors for thymidylate and purines needed for the biosynthesis of DNA and RNA (Figure 1-2). The methionine synthase reaction releases H₄PteGluₙ, which is reversibly converted to 5,10-methylene-H₄PteGluₙ by serine hydroxymethyltransferase (SHMT), interconverting serine and glycine in the process. 5,10-methylene-H₄PteGluₙ is formylated to 10-CHO-H₄PteGluₙ. This reaction is likely dependent on the proliferative needs of the cell, as 2 moles of 10-CHO-H₄PteGluₙ are utilized in de novo purine synthesis by β-glycinamide ribonucleotide formyltransferase (GART) and 5-amino-4-imidazolecarboxamide ribonucleotide formyltransferase (AICART) as carbon donors for assembly of the purine skeleton. These enzymes are discussed in detail in chapter 2. 10-CHO-H₄PteGluₙ can also be hydrolyzed to 5,10-methenyl-H₄PteGluₙ, which is then reduced to 5,10-methylene-H₄PteGluₙ. 5,10-methylene-H₄PteGluₙ reductase (MTHFR) irreversibly converts
Figure 1-2. Key therapeutic targets of folate metabolism. Drug discovery efforts targeting folate metabolism over the last 20 years emphasized pharmacophores away from the traditional target of folate metabolism, DHFR, and towards enzymes requisite for thymidylate and purine biosynthesis. Adapted from Muhsin M et al., 2004. Nat. Rev. Drug Disc. 3(10):825-826 (25)
5,10-methylene-\(\text{H}_4\)PteGlu\(n\) back to 5-CH\(3\)-\(\text{H}_4\)PteGlu\(n\). Thymidylate synthase (TS) also uses 5,10-methylene-\(\text{H}_4\)PteGlu\(n\) as a carbon donor for the reductive methylation of 2’-deoxy-uridylate (dUMP) to thymidylate (TMP). Dihydrofolate reductase (DHFR) then rapidly converts the reduced \(\text{H}_2\)PteGlu\(n\) back to \(\text{H}_4\)PteGlu\(n\). The central role of \(\text{H}_4\)PteGlu\(n\) in the maintenance of cytosolic one-carbon metabolism can be appreciated from this description (Figure 1-2).

In the mitochondria, folate polyglutamates are essential for glycine and formate synthesis. Like in the cytosol, \(\text{H}_4\)PteGlu\(n\) is the essential cofactor for these reactions, and is converted to 5,10-methylene-\(\text{H}_4\)PteGlu\(n\) by a second pool of SHMT, interconverting serine and glycine in the process. Interestingly, cells deficient in mitochondrial folate pools (due to a loss of MFT function) are dependent on glycine supplementation for survival. Why cytosolic SHMT cannot supply the glycine necessary for survival is unknown.

**Discovery and development of antifolates as cancer therapeutics**

Following the discovery that dietary folates could rescue megaloblastic anemia, it was postulated that acute leukemias might be a disease of folate deficiency due to the morphological similarities between these megaloblasts and acute leukemic bone marrow blasts (26). However, this hypothesis was disproven by studies in children showing that folate supplementation intensified acute leukemias, and that a folate-deficient diet caused remission (27). It was therefore proposed by Sidney Farber that these leukemias were dependent on folates for proliferation and their proliferation rate was limited by the supply of folates. Farber and colleagues at Children’s Hospital in Boston obtained folate antagonists synthesized by Lederle Laboratories, and were for the first time able to induce remission of childhood acute lymphocytic leukemia (ALL) with one of these folate analogs, aminopterin (4-amino-folic acid) (Figure 1-3)
Aminopterin was initially used clinically to treat childhood leukemias, but was ultimately replaced by a close structural analog with a superior therapeutic index, methotrexate (2-amino-10-methyl-folic acid) (29). Almost 50 years later, methotrexate is still used in multidrug treatment regimens for childhood ALL, and has also proven effective in combating autoimmune diseases like psoriasis and rheumatoid arthritis (30, 31).

Shortly after the discovery of methotrexate and its analogs, the primary folate-dependent therapeutic target of this drug was identified to be DHFR (32). Methotrexate is a tight-binding inhibitor of DHFR ($K_i \sim 0.004$ nM) (33). DHFR inhibition by methotrexate prevents DNA and RNA synthesis by preventing the reduction of $H_2PteGlu_n$ to $H_4PteGlu_n$, the vital precursor for thymidylate and purine biosynthesis cofactors (Figure 1-2). Additional $H_4PteGlu_n$-dependent reactions, such as serine-glycine interconversions and methionine synthesis, are also hindered.

It is now appreciated that, like natural folates, methotrexate is polyglutamated in cells. This is important, as polyglutamation of methotrexate enhances intracellular retention of the drug and permits methotrexate to inhibit DHFR for longer periods of time in the face of an expanding $H_2PteGlu_n$ pool (35). Polyglutamation also increases the spectrum of target inhibition. While methotrexate polyglutamation does not enhance DHFR inhibition, it does increase the potency of methotrexate for TS ($K_i(glu1) \sim 13,000$ nM, $K_i(glu5) \sim 47$ nM) and AICART ($K_i(glu1) \sim 143,000$ nM, $K_i(glu5) \sim 56$ nM) (36, 37). However, this enhanced affinity for other folate-dependent targets is likely irrelevant to the therapeutics of such a tight-binding DHFR inhibitor, since the cofactors for these reactions (5,10-methylene-$H_4PteGlu_n$ and 10-CHO-$H_4PteGlu_n$, respectively) are already depleted in the absence of $H_4PteGlu_n$. Significant to cancer therapy, polyglutamation adds a layer of selectivity to methotrexate, as metabolites accumulate in tumor cells to a much greater extent than in bone marrow and intestinal tissues (38).
Figure 1-3. Classes of clinically evaluated antifolates and representative compounds from each class

DHFR Inhibitors

Aminopterin

Methotrexate

TS Inhibitors

CB3717

Raltitrexed

GART Inhibitors

DDATHF

Multitargeted Antifolates

Pemetrexed
Thousands of methotrexate analogs were synthesized and tested as cancer therapeutics, with the goal of maintaining the potency for DHFR while enhancing the substrate specificity of transport and polyglutamation, but limiting the uptake in normal tissues. Most attempts failed, as methotrexate was indeed a superior drug in this sense. However, recent studies emerged showing the analog pralatrexate (10-propargyl-10-deazaaminopterin, Folotyn®) met these criteria and showed remarkable responses in T-cell lymphomas. This study ultimately led to the U.S. Food and Drug Administration (FDA) approval of pralatrexate for relapsed peripheral T-cell lymphoma, a rare form of non-Hodgkins lymphoma, in 2009 (39).

**Second-generation antifolates move away from DHFR inhibition**

Antifolate drug discovery efforts over the past few decades shifted focus away from the 2,4-diamino-pteridine pharmacophore (targeting DHFR) and towards pharmacophores targeting the folate-dependent enzymes of thymidylate and purine biosynthesis. The first potent antifolate thymidylate synthase inhibitor (Ki ~ 3 nM) to come from this effort was CB3717 (40). CB3717 had antitumor activity in breast, ovarian, and liver cancers, but was ultimately withdrawn from the clinic due to life-threatening renal toxicity from poor solubility at low pH (41, 42).

Keeping this same 5,8-dideazafolate pharmacophore, analogs of CB3717 were synthesized in an attempt to increase solubility. Raltitrexed (Tomudex®, ZD1694) was identified from this effort. Raltitrexed is a 2-desamino-2-methyl-N\(^{10}\)-substituted-5,8-dideazafolate analog with a thiophene substitution for the PABA ring (Figure 1-3) (43). Compared to CB3717, these characteristics not only increased solubility, they made raltitrexed a superior substrate for both RFC-mediated transport and polyglutamation by FPGS (43). In addition to intracellular trapping of raltitrexed metabolites, polyglutamation increased the potency of raltitrexed as a TS inhibitor.
by more than 100-fold. Raltitrexed showed significant clinical response rates in colorectal and breast cancer patients. It is currently in widespread use outside the United States for the treatment of colorectal cancer, but never gained U.S. FDA approval, as it was not determined superior to the current standard of care for colorectal cancer, 5-fluorouracil (5-FU, also a TS inhibitor) with leucovorin (44, 45).

The discovery of the first antifolate inhibitor of *de novo* purine biosynthesis, 5,10-dideaza-H$_4$PteGlu (DDATHF, lometrexol) was somewhat serendipitous (Figure 1-3). The structure was originally proposed by G. Peter Beardsley as a potential TS inhibitor, but cell culture end-product reversal experiments performed in our lab showed it was targeting purine synthesis (46). Subsequently, enzyme kinetic studies demonstrated that DDATHF was a potent inhibitor of GART, the first folate-dependent enzyme of *de novo* purine synthesis (47-49). DDATHF is transported into cells via the RFC as well as the PCFT, and is a good substrate for FPGS (46). Polyglutamates showed potent antitumor activity against a broad spectrum of carcinomas, but the development of DDATHF was halted in Phase I clinical testing due to induction of severe thrombocytopenia (50). Importantly, the unfavorable toxicity of DDATHF was ablated with oral folic acid supplementation (51, 52). These findings set a precedent for future clinical regimens to include folic acid and vitamin B$_{12}$, although the protective mechanism is not fully understood.

**Pemetrexed: a multi-targeted antifolate**

DDATHF not only faced troubles in the clinic, it was also a medicinal chemist’s nightmare. Synthesis involved 23 steps, producing a mixture of diastereomers about the 6-position, and separation of these isomers resulted in extremely low yields (47). Pemetrexed
(LY231514, Alimta®) (Figure 1-3) was discovered from synthetic approaches aimed at eliminating this chirality of carbon 6 of DDATHF, in which a pyrrolopyrimidine ring replaced the 5-deazapteridine (53). Surprisingly, this modification also changed the target profile. Pemetrexed polyglutamates were potent inhibitors of thymidylate synthase both in vitro ($K_i \approx 1.3$ nM) and in cell culture. However, it was apparent from end-product cell culture reversal experiments that higher doses of pemetrexed had a significant secondary target, reversible with the addition of preformed purine (53, 54). This suggested that, like its predecessor DDATHF, pemetrexed was also targeting *de novo* purine synthesis. *In vitro* kinetic analysis of recombinant human TS, DDATHF, AICART, and mouse GART indicated that most potent purine synthesis target was GART (Table 1-1) (54). Data presented in Chapter 2 challenges the interpretation of the results of this study, showing that the *de novo* purine synthesis cellular target of pemetrexed is AICART, the second folate-dependent enzyme of this pathway. At the time, the effects of pemetrexed on purine synthesis by Shih *et al* were duly noted, but the therapeutic effect of the drug was attributed primarily to TS inhibition since the drug was 60-times less potent as a GART inhibitor. Of significance, DHFR inhibition is irrelevant to the therapeutics of a potent TS inhibitor like pemetrexed, due to depletion of $H_2$PteGlu, the cofactor of the DHFR reaction (55). Indeed, gene amplification of DHFR did not correlate with resistance to pemetrexed. However, TS amplification and FPGS mutations are common resistance mechanisms to pemetrexed (56).

Pemetrexed has several favorable properties that contribute to its therapeutic efficacy. Early studies identified pemetrexed as one of the most efficient substrates for FPGS ever tested, permitting much greater cellular accumulation and extended target inhibition at lower doses than other antifolates (53, 56, 57). Pemetrexed uses both the RFC and the PCFT for transport. Importantly, pemetrexed is transported efficiently by the PCFT in both low and neutral
Table 1-1. Inhibitory activity of pemetrexed and its polyglutamates against folate-dependent enzymes.

<table>
<thead>
<tr>
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<th>Ki (mean ± s.e. nM)</th>
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<tr>
<td></td>
<td>rhTS</td>
</tr>
<tr>
<td>PTX</td>
<td>109 ± 9.0</td>
</tr>
<tr>
<td>PTX-glu3</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>PTX-glu5</td>
<td>1.3 ± 0.3</td>
</tr>
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</table>

pH microenvironments, and in tumors with compromised RFC-mediated transport, an inherent resistance mechanism to antifolates (58-61). It has been suggested that the PCFT is in fact the primary transporter of pemetrexed in solid tumors, whose extracellular microenvironment is acidified due to profuse lactic acid secretion, a byproduct of aerobic glycolysis, the so-called “Warburg Effect” (62).

*The unusual clinical utility of pemetrexed suggests alternative targets*

Cell-based and clinical studies with pemetrexed have demonstrated a broad spectrum of activity in carcinomas of the bladder, breast, cervix, colon, gastrointestinal tract, and pancreas (63, 64). Additionally, pemetrexed is active in malignant pleural mesothelioma (MPM) and non-small cell lung cancer (NSCLC), which is atypical for an antifolate and particularly for a thymidylate synthase inhibitor. MPM is a rare, highly aggressive malignancy of the pleural cavities lining the lungs and chest wall whose genesis is highly associated with exposure to asbestos fibers (65). While the U.S. Environmental Protection Agency banned the material use of asbestos in the early 1970’s, symptoms of MPM typically present up to 50 years following initial exposure (66).

The median survival following diagnosis of MPM is only 11 months, and the disease is resistant to most chemotherapeutic regimens, including methotrexate or 5-FU with leucovorin (67-69). The clinical utility of pemetrexed in MPM was recognized from two studies in regions of Europe where asbestos exposure was abnormally high, and hence, was enriched for MPM patients (70, 71). Pemetrexed was combined with cisplatin based on observed synergism in an MPM cell line, and this combination was the first therapeutic regimen to show a survival benefit in this disease (64, 72). The U.S. FDA ultimately approved pemetrexed plus cisplatin for first-
line treatment of MPM patients who are not candidates for surgery based on an increased survival (16 months overall following diagnosis) compared to cisplatin alone (67). Pemetrexed (500 mg/m\(^2\)) is given in a 10-minute infusion once every 21 days. This is likely an effective treatment modality due to the retention of polyglutamated metabolites in target tissues following plasma clearance (73). Like its predecessor DDATHF, folic acid and B\(_{12}\) supplementation is indicated to limit overt toxicity (67).

The recent approvals of pemetrexed for the treatment of NSCLC come at the crossroads of a global lung cancer epidemic. Over 1 million people are diagnosed with lung cancer each year, 90% of which are of non-small cell origin (74). Diagnosis is matched by the annual mortality rate, placing lung cancer as one of the top ten cause of death in the world (75). Cigarette smoking is the primary risk factor. The World Health Organization classifies lung cancers by histological subtypes, with 99% of lung carcinomas meeting the criteria of small cell and non-small cell. NSCLC is further subdivided among squamous and nonsquamous adenocarcinoma and large cell histologies (76).

NSCLC is highly progressive, with a median survival of 6 months following diagnosis if left untreated (77). The clinical success of pemetrexed against MPM suggested it might be beneficial in treatment regimens for NSCLC. This hypothesis was tested in a large clinical trial comparing pemetrexed to docetaxel (Taxotere\(^\circledR\)), which at the time was standard of care for second-line treatment of NSCLC (78). While the median survival time of patients treated with pemetrexed and docetaxel were comparable (8.3 vs. 7.9 months median survival, respectively), pemetrexed was significantly less toxic. This led to 2004 FDA approval of pemetrexed for second-line treatment of NSCLC.
On the heels of this study was a head to head trial of pemetrexed in combination with cisplatin compared to the first-line treatment option, gemcitabine (Gemzar®) with cisplatin (79). Overall, the survival rates on each arm were identical (10.3 months), but when the statistics were separated based on histologies, it was clear that pemetrexed/cisplatin was advantageous in the nonsquamous population, with median survival rates approaching 16 months. Recent studies have equated elevated TS expression to pemetrexed resistance among squamous histologies, although our studies would suggest TS-independent genetic factors might be involved (80). Accordingly, the FDA approved pemetrexed/cisplatin for first-line treatment of nonsquamous NSCLC in 2008, representing the first antifolate approved as a first-line cancer agent in the U.S. in over 50 years.

In 2009, pemetrexed became the first drug approved by the FDA for maintenance therapy of NSCLC (81). This treatment strategy entails administering pemetrexed prior to disease progression following a platinum-based treatment cycle. Although this limits the treatment-free period following therapy, the low overt toxicity of pemetrexed combined with best supportive care has shown significance in survival benefit.

**Scope of the dissertation**

In an era of cancer biology dominated by the development and trial of molecularly targeted therapeutics, the clinical future of conventional cytotoxic agents like antifolates remains uncertain. However, with the recent first-line approvals of the multi-targeted antifolate pemetrexed for the treatment of MPM and NSCLC, the 21st century ushered in a new wave of interest in antifolate research. The response of MPM and NSCLC to pemetrexed was a bit surprising, as other TS inhibitors (raltitrexed and 5-FU/leucovorin) showed minimal response
rates in clinical studies (82, 83). This suggested that the TS-independent effects of pemetrexed might be of therapeutic importance. Studies in this dissertation contributed to the elucidation of a novel mechanism by which pemetrexed and its analogs elicit their antitumor effects. In Chapter 2, a series of whole-cell biochemical experiments are presented that identified AICART as the relevant secondary cellular target of pemetrexed. AICART inhibition caused a striking accumulation of the purine synthesis intermediate 5-aminomidazole-4-carboxamide ribonucleotide (ZMP), behind the metabolic block. ZMP signaled to AMPK, mimicking the activating effects of AMP on this vital cellular energy sensor. Additionally, effects of accumulated ZMP on AICART activity were studied in vitro. AMPK activation by pemetrexed and ensuing downstream consequences are studied in Chapter 3, with particular focus on signaling through the mTOR pathway. For the first time, we showed that the consequences of folate-dependent target inhibition extend beyond folate metabolism to gain control of cellular signaling pathways commonly unrestrained in tumors. In Chapter 4, the therapeutic outcomes of TS and AICART inhibition by pemetrexed are studied individually. We conclude that while inhibition of folate-dependent processes remains an essential component of the therapeutics of pemetrexed, our studies suggest that the full spectrum of antitumor activity elicited by the drug is in part attributed to effects outside folate metabolism.
CHAPTER 2
DISCOVERY OF AICART AS THE SECOND FOLATE-DEPENDENT TARGET OF PEMETREXED

E.C. Taylor and colleagues at Princeton University first synthesized pemetrexed as part of a series of analogs of the GART inhibitor DDATHF that eliminated the C-6 chiral center (Figure 1-3) (53). Pemetrexed was selected for further evaluation because studies showed it potently inhibited (IC50 ~ 30 nM) the growth of both mouse L1210 and human CCRF-CEM lymphoblastic leukemic cells. Cell culture end-product reversal experiments have proven enormously useful in defining the folate-dependent targets of antifolates. For instance, addition of thymidine (TdR) to cell culture media, converted intracellularly to thymidylate (TMP; the end-product of the thymidylate synthase reaction) by thymidine kinase, negates the effect of thymidylate synthase inhibition by providing an exogenous source of the reaction end-product. Figure 2-1 illustrates this effect, showing that high doses of the pure antifolate thymidylate synthase inhibitor, raltitrexed, do not inhibit clonogenic survival in the presence of TdR. Surprisingly, when end-product reversals were applied to pemetrexed, it was clear that pemetrexed was not behaving like its predecessor DDATHF, but more like the TS inhibitor raltitrexed. However, TdR rescued the growth inhibitory effect of pemetrexed only at low drug concentrations (53). Higher concentrations of drug required addition of TdR and a preformed purine to rescue growth inhibition. This result suggested that pemetrexed (or its polyglutamates) were primarily targeting thymidylate biosynthesis, but had inhibitory activity against de novo purine biosynthesis at higher concentrations.
Figure 2-1. The cytotoxicity of the thymidylate synthase inhibitor raltitrexed is reversed by addition of thymidine to cell culture media. Clonogenic survival assays were done as described in the Methods section on H460 cells exposed to the indicated concentrations of raltitrexed for 48 hours. In rescue conditions, 5.6 µM thymidine was used, and remained present in culture media throughout the duration of the experiment.
Thymidylate biosynthesis

Thymidylate synthase (TS) catalyzes the reductive methylation of 2’-deoxyuridine-5-monophosphate (dUMP, uridylate) to thymidine-5-monophosphate (TMP, thymidylate) using the cofactor 5,10-methylene-H₄PteGlu₈ as both the one-carbon source and the reducing agent. TMP serves as the only de novo source of thymidine-5-triphosphate (TTP) for DNA synthesis, making TS one of the most evolutionarily conserved enzymes in nature (84). Therefore, TS was identified early as a target for anticancer therapeutics, and the enzyme kinetics as well as the consequences of inhibition have been extensively characterized. Steady-state kinetic analysis indicated that TS follows an ordered-sequential mechanism with the substrate dUMP binding first, followed by 5,10-methylene-H₄PteGlu. H₂PteGlu then dissociates, followed by the product TMP (85, 86). Folate polyglutamates, including antifolates, have been shown to bind TS in the absence of substrate under phosphate-buffered conditions, suggesting a mixed reaction order (87). However, this observation is likely influenced by phosphates competing with dUMP for binding, combined with polyglutamation greatly enhancing the affinity of cofactors for TS (88).

X-ray crystal structures of TS from multiple species in various states of catalysis have shown dramatic conformational changes associated with cofactor binding that bring the substrate and cofactor together in a deep pocket protected from solvent where the reaction takes place (89-91). A brief description of the reaction mechanism is important to understand how the enzyme is inhibited pharmacologically. Details of this mechanism are described by C.W. Carreras and D.V. Santi (92). Catalysis begins by nucleophilic attack of an essential active-site cysteine (Cys195 of human TS) to C6 of dUMP (93). This converts C5 to a nucleophilic enolate intermediate that accepts C11 of 5,10-methylene-H₄PteGlu, activated by formation of an iminium ion at N5. With substrate, cofactor, and Cys195 now covalently linked, the proton at
C5 of the pyrimidine is abstracted, and H₄PteGlu is released by β-elimination. The 6-hydrogen of 5,10-methylene-H₄PteGlu is transferred to the methylene intermediate as a hydride (H⁻¹) ion, and Cys195 is released by β-elimination to give the products TMP and H₂PteGlu. The kinetic constants derived from human TS are listed in Table 2-1 (94).

Inhibition of thymidylate synthase results in “thymineless death,” a term coined by S.S. Cohen in the early 1950’s from observations made by culturing a thymidine auxotrophic strain of *E. coli* in the absence of thymidine (95). In aerated cultures lacking thymidine, cell viability was diminished but turbidity increased, suggesting unbalanced growth, that is, a condition in which DNA synthesis was inhibited while RNA and protein synthesis continued. This led to the conclusion that irreversible DNA lesions were causal of thymineless death (96, 97). It is now known that the cytotoxic effect of TS inhibition results from both a diminution of TTP as well as accumulation of 2’-deoxyurididine-5-triphosphate (dUTP) (98). The rise in dUTP is credited to both accumulation of dUMP behind the TS block combined with saturation of dUTPases that catabolize dUTP to dUMP. Since DNA polymerases do not show substrate preference for TTP over dUTP, uracil is misincorporated into newly synthesized DNA at a high rate (99). Uracil glycosylases recognize and excise uracil, but dUTP is likely misincorporated again during the repair process. These ineffective cycles of excision and repair ultimately lead to DNA strand breaks that trigger apoptosis. As one would expect, the cytotoxic effects of TS inhibition are seen only in S-phase of the cell cycle. It would therefore seem likely that inhibition of S-phase entry would limit the cytotoxicity of a TS inhibitor. This concept will be revisited in Chapter 4.
Kinetic constants for TS were derived from Santi DV et al. J. Biol. Chem. 1989. 264(16):9145-9148. Kinetic constants for GART were derived from Sanghani SP et al. Biochemistry. 1997. 36(34):10506-10516. Kinetic constants for AICART were derived from research presented in the results section of this chapter.

Table 2-1. Kinetics constants for several recombinant human enzymes of folate metabolism

<table>
<thead>
<tr>
<th></th>
<th>TS</th>
<th>GART</th>
<th>AICART</th>
</tr>
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<tbody>
<tr>
<td>$k_{cat}$ (s$^{-1}$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dUMP</td>
<td>2.5</td>
<td>7.5</td>
<td>2.2</td>
</tr>
<tr>
<td>(6R,6S)-5,10-CH$_2$-H$_4$PteGlu</td>
<td>8 ± 0.3</td>
<td>0.9 ± 0.2</td>
<td>32.5 ± 6.5</td>
</tr>
<tr>
<td>$K_m$ (uM)</td>
<td>3.4 ± 0.4</td>
<td>1.1 ± 0.2</td>
<td>3.3 ± 0.5</td>
</tr>
</tbody>
</table>
The first clinically used thymidylate synthase inhibitor was the pyrimidine analog 5-fluorouracil (5-FU). Synthesized by C. Heidelberger and colleagues, it was rationalized that 5-FU would produce a preferential toxicity to tumors, because they utilized uracil for nucleic acid biosynthesis to a greater extent than normal tissues (100). 5-FU is a prodrug, metabolized intracellularly to FdUMP as well as to FUTP and FdUTP. The latter allows for misincorporation directly into RNA and DNA, although incorporation of 5-FU into DNA is a rare event. The incorporation of 5-FU into RNA is a major effect of the drug and has been implicated in toxicity to the GI tract and hematopoiesis (101). FdUMP competes with dUMP for binding to TS. When catalysis is initiated by Cys195, an essentially irreversible covalent ternary complex forms between FdUMP, 5,10-methylene-H₄PteGlu, and the enzyme (Figure 2-2) (85). This results in accumulation of dUMP and the ensuing events described above. The formation of this ternary complex is likely dependent on binding of 5,10-methylene-H₄PteGlu to the enzyme, causing a conformational shift to bring the substrates into close proximity. Indeed, it has been shown that 5,10-methylene-H₄PteGlu is essential to stabilize the ternary complex and prevent FdUMP dissociation (85, 102). In agreement, the sensitivity of tumors to 5-FU was improved by cotreatment with 5-CHO-H₄PteGlu (leucovorin) by enhancing TS inhibition through ternary complex stabilization (42). Based on these studies, 5-FU was combined with leucovorin clinically, and has been a mainstay in the treatment of colorectal cancer in the U.S. for the past 20 years.

Folate-based inhibitors of TS were pursued partly due to the clinical successes of 5-FU and methotrexate. It was rationalized that a more specific TS effect would be obtained from folate antagonism than from pyrimidine antagonism, as the off-target and overt toxic effects of 5-FU from non-selective DNA and RNA incorporation were well documented (103).
Figure 2-2. Crystal structures of thymidylate synthase with fluoropyrimidine (left) and antifolate (right) inhibitors bound illustrate differences in modes of inhibition. *E. coli* TS (PDB Code: 1TSN) is depicted on the left in a ternary complex with FdUMP (blue) and 5,10-CH$_2$-H$_4$PteGlu (orange). Cys195 is in red. Human TS (PBD Code 1HVY) is depicted on the right in a closed conformation with raltitrexed (purple) and dUMP (yellow) bound to Cys195 (red). Images generated with PyMOL (www.pymol.org).
Additionally, on the basis of the ordered-sequential mechanism of TS, accumulation of dUMP behind a TS block was thought to limit FdUMP ternary complex formation through direct competition (104). On the contrary, dUMP accumulation was expected to enhance ternary complex formation with a folate-based inhibitor (103). Furthermore, competition with 5,10-methylene-H$_4$PteGlu would likely be irrelevant, as this cofactor is maintained at low intracellular levels (14).

In an attempt to direct antifolates away from DHFR and towards TS, a series of 10-substituted 5,8-dideazafolic acid analogs were synthesized. Carbon substitutions at N5 and N8 of a quinazoline ring and substitutions at the N10 position significantly enhanced TS binding (40). From this series came raltitrexed, whose cytotoxic effect was abolished by addition of thymidine to culture medium, indicating a primary effect on TS (105, 106). A crystal structure of raltitrexed bound to human TS with dUMP suggests antifolate binding locks TS in a closed conformation (Figure 2-2) (107). Raltitrexed is an efficient substrate for the RFC ($K_m \sim 2.5 \mu M$) and FPGS ($K_m \sim 1.3 \mu M$ as a pentaglutamate) (106). Polyglutamates of raltitrexed potently inhibit TS ($K_i \sim 1 \text{nM}$) (105, 106). While most cell lines were significantly more sensitive to raltitrexed than 5-FU/Leucovorin, clinical studies evaluated by the U.S. FDA determined raltitrexed was not superior to 5-FU/Leucovorin in the treatment of colon cancer (44, 45, 105).

**De novo purine biosynthesis**

Humans rely on salvage and *de novo* pathways for the synthesis of purines for DNA and RNA synthesis (Figure 2-3). The precursor of the adenosine and guanosine nucleotides is inosine monophosphate (IMP). The salvage pathway regenerates IMP from degraded nucleic acids. The free bases hypoxanthine and guanine are converted to IMP in a single step by
hypoxanthine-guanine phosphoribosyltransferase (HGPRT) using 5-phosphoribosyl-1-pyrophosphate (PRPP) as a cofactor. The ribose form of hypoxanthine, inosine, is converted to IMP by inosine kinase. Additionally, adenine can be converted directly to AMP by adenine phosphoribosyltransferase (APRT) using PRPP. Nucleotide salvage is indeed essential to humans, as deficiency in HGPRT causes accumulation of uric acid, resulting in Lesch-Nyhan syndrome (108).

Proliferating cells have a high demand for purine nucleotides due to active replication and transcription. Therefore, they rely heavily on the synthesis of purines de novo, an energy-consuming process consisting of a series of 10 enzymatic reactions in which the inosine skeleton is assembled stepwise on the backbone of PRPP (Figure 2-3). Salvage of preformed purines like hypoxanthine or inosine negates the effect of de novo purine synthesis inhibition (Figure 2-3). In addition to providing the end-products of the pathway, de novo purine synthesis is also subjected to several mechanisms of feedback inhibition by salvage nucleotide synthesis. First, the de novo and salvage pathways compete for PRPP, the substrate for the first and committed step of de novo purine synthesis catalyzed by phosphoribosylpyrophosphate amidotransferase (PPAT). Therefore, consumption of PRPP by the more energy-efficient process of salvage synthesis diminishes de novo synthesis (109). Additionally, the activity of PPAT is inhibited by purine nucleotides IMP, AMP, GMP, and ATP (110). For these reasons, we supplement cell culture media with serum in which nucleic acids have been removed by dialysis. This limits the salvage synthesis of purines, allowing us to study the biochemistry and pharmacology of de novo synthesis that is unrestricted by feedback regulation. Two reactions of de novo purine biosynthesis are dependent on the folate cofactor 10-CHO-H₄PteGlu (111). Glycinamide
Figure 2-3. The folate-dependent steps of *de novo* purine biosynthesis and mechanisms of salvage. *De novo* purine biosynthesis consists of ten sequential enzymatic reactions in which 5-phosphoribosyl-1-pyrophosphate (PRPP) is converted to inosine monophosphate (IMP). IMP is converted to AMP and GMP thorough additional enzymatic steps (not shown). The two folate-dependent formyl transfer reactions of this pathway are catalyzed by GART and AICART, using the substrates glycaminamide ribonucleotide (GAR) and ZMP respectively to produce formylglycinamide ribonucleotide (FGAR) and N-formylaminoimidazole-4-carboxamide ribonucleotide. ZMP can be produced by salvage of its cell-permeable precursors, AICA and AICAR. Adenine phosphoribosyltransferase (APRT) uses PRPP to convert the free base AICA to ZMP, and the nucleobase AICAR is phosphorylated to ZMP by adenosine kinase. Additionally, IMP can be produced by salvage of the preformed purine, hypoxanthine, by hypoxanthine-guanine phosphoribosyltransferase (HGPRT) and the cofactor PRPP.
ribonucleotide formyltransferase (GART) catalyzes the third step of the *de novo* pathway, the conversion of glycinamide ribonucleotide (GAR) to N-formylglycinamide ribonucleotide (FGAR) (111, 112). Aminoimidazolecarboxamide ribonucleotide formyltransferase (AICART) catalyzes the penultimate step of *de novo* purine synthesis, the transfer of a CHO group from 10-CHO-H₄PteGlu to the exocyclic NH₂ group of 5-aminoimidazole-4-carboxamide ribonucleotide (ZMP) to form 5-formylaminoimidazole-4-carboxamide ribonucleotide (fZMP). Addition of a *de novo* pathway intermediate between these steps allows for delineation between inhibition of these two reactions. 5-aminoimidazole-4-carboxamide (AICA) is converted intracellularly to the purine synthesis intermediate ZMP by adenine phosphoribosyltransferase (APRT). ZMP is conveniently positioned downstream of GART, and upstream of AICART. Therefore, addition of AICA to culture media rescues the effect of GART inhibition, but not AICART inhibition. This strategy helped define GART as the folate-dependent target of the antifolate DDATHF and, surprisingly, suggested AICART as the purine synthesis target of pemetrexed. The data from these pivotal experiments are discussed in detail at the beginning of the results section of this chapter.

*Glycinamide ribonucleotide formyltransferase*

Human GART occupies the C-terminal domain of a monomeric trifunctional enzyme that also catalyzes steps 2 (glycinamide ribonucleotide synthetase, GARS) and 5 (aminoimidazole ribonucleotide synthetase, AIRS) of *de novo* purine synthesis (Figure 2-3) (113). Dead-end inhibitor studies with recombinant human enzyme suggested that GART followed an ordered-sequential mechanism with 10-CHO-H₄PteGlu binding first (114). However, previous studies in our lab with recombinant mouse GART provided evidence for a random-sequential binding
order, since the folate-antagonist DDATHF and the endogenous substrate GAR bound with equal affinity in the presence or absence of one another (115). This binding mechanism was also confirmed by stopped-flow kinetics with \textit{E. coli} GART and is now commonly accepted as the mode of binding of substrates to bacterial and mammalian GART (116). The kinetic constants derived from mouse GART are listed in Table 2-1 (115).

Our lab with collaborators at Princeton and Yale Universities discovered that DDATHF was the first potent antifolate inhibitor of GART (46, 47). DDATHF is an analog of $\text{H}_4\text{PteGlu}$ with carbon substitutions at the N5 and N10 positions (Figure 1-3). The growth of tumors in culture and \textit{in vivo} is strongly inhibited by DDATHF, validating GART as a cancer therapeutic target (46, 47). DDATHF monoglutamates are tight binding inhibitors of recombinant mouse GART ($\text{Ki} \sim 5.6 \text{ nM}$), and polyglutamation enhances the potency by more than more than 10-fold ($\text{Ki} \sim 0.11 \text{ nM}$) (115). Analysis of nucleotide pools in CCRF-CEM cells following a short DDATHF exposure (4 hours) indicated depletion of both ATP and GTP pools, an effect sustained for greater than 24 hours (117). Hypoxanthine and AICA both prevented purine depletion, further indicating GART as the primary target. Thymidylate pools were not depleted with DDATHF, and growth inhibition of cells in culture was not rescued by addition of thymidine, collectively indicating that DDATHF was not inhibiting thymidylate synthase (117).

A series of DDATHF analogs were synthesized for structure-activity relationship (SAR) studies to determine the features of the molecule essential for interaction with GART (47, 48, 115, 118). From these studies, it was observed that the co-product of the reaction, (6S)-$\text{H}_4\text{PteGlu}$ was a weak GART inhibitor, and inhibition was competitive with the commonly-used \textit{in vitro} substrate 10-CHO-5,8-dideazafolate. Additionally, the 2-amino group on the pteridine ring was determined to be essential for inhibition, suggesting a role of hydrogen bonding for this
functional group. The observation that the carbon-substituted N5 derivative of H₄PteGlu (5-deaza-H₄PteGlu) was a potent GART inhibitor suggested this modification alone was enough to direct the molecule to GART. A subsequent structural study with recombinant *E. coli* GART bound to 5-deaza-H₄PteGlu and GAR provided a basis for the observations made with SAR (Figure 2-4) (119). Hydrogen bonding stabilized the modified pteridine ring at positions 1, 2, 4, and 8. Additionally, the potency increase by carbon-substituting the N5 position was confirmed. Since there was no ligand-enzyme interaction at this position, it was proposed that desolvating a NH group would be more costly than for a methylene group. The crystal structure also suggested a higher degree of stabilization of the pteridine ring compared to the rest of the molecule and showed the glutamate moiety protruding towards the surface of the enzyme, not contributing to binding in the active site.

**Aminomidazolecarboxamide ribonucleotide formyltransferase**

Human AICART occupies the C-terminal domain (residues 199-592) of a bifunctional polypeptide encoded by the ATIC (*purH*) gene (120). The N-terminal domain (residues 1-198) encodes inosine monophosphate cyclohydrolase (IMPCH), which catalyzes the final step of *de novo* purine synthesis, the cyclization of the inosine ring. While the multi-subunit nature of *de novo* purine synthesis enzymes is conserved in eukaryotes, ATIC is the only multi-protein complex of *de novo* purine synthesis in bacteria. This evolutionary conservation suggested a crosstalk between the two enzymes, possibly in the form of substrate channeling. However, when expressed as individual domains, each reaction proceeded at a rate equivalent to the catalysis as a fusion protein (120). Moreover, single-turnover kinetics were consistent with a lack of substrate channeling between the enzymes (121). A more likely explanation for this
Figure 2-4. Crystal structure of *E. coli* GART with 5-deaza-H₄PteGlu and GAR supports SAR studies with recombinant mouse GART. The carbons of 5-deaza-H₄PteGlu are depicted in green. Relevant hydrogen bonds with *E. coli* GART (PDB code 1CDE) are depicted as dashed lines. GAR is depicted in red. Image generated with PyMOL (www.pymol.org).
evolutionary conservation was suggested by kinetic analysis of the reaction, in which the formyl transfer was reversible, highly favoring the reverse reaction producing $10\text{-CHO-}H_4\text{PteGlu}$ and $\text{ZMP}$ ($k_{\text{cat}} \sim 6.7 \text{ s}^{-1}$) over the forward reaction producing $H_4\text{PteGlu}$ and formyl-$\text{ZMP}$ ($k_{\text{cat}} \sim 2.9 \text{ s}^{-1}$) (121). Therefore, having IMPCH in close proximity to cyclize the newly synthesized formyl-$\text{ZMP}$ would drive the AICART reaction towards product formation. The crystal structure of the avian bifunctional enzyme placed the AICART and IMPCH active sites within 50 Å, and provided no evidence for substrate channeling (122).

Although GART and AICART both utilize the same folate cofactor and carry out similar reactions, there are very little mechanistic and structural similarities between these two enzymes. In contrast to the random-sequential order of the GART reaction, the AICART reaction obeys an ordered-sequential mechanism, in which $10\text{-CHO-}H_4\text{PteGlu}$ binds first, followed by ZMP (121, 123). The kinetic constants derived from recombinant human AICART expressed and purified in this dissertation are listed in Table 2-1. An alignment study with several enzymes that use $10\text{-CHO-}H_4\text{PteGlu}$ revealed a supposedly conserved cofactor binding site (124). However, mutagenesis studies of multiple residues in the human GART and AICART domains corresponding to this conserved site indicated a loss of activity for GART, but no effect on AICART activity (120). These findings clearly illustrated that there were fundamental differences in the active site structures of these two enzymes, and support the argument that a competitive inhibitor of one formyltransferase would not necessarily be an inhibitor of another. Indeed, studies in this dissertation and by others have shown that the potent antifolate GART inhibitor (6R)-DDATHF is not an AICART inhibitor (46, 125, 126). Structural studies of avian and human AICART/IMPCH illustrated major differences in AICART organization and active site structure compared to TS, DHFR, and GART. The bifunctional AICART/IMPCH protein
crystallizes as a homodimer, and a small region rich in β-sheets bridges the AICART and IMPCH domains (122). While active sites of IMPCH reside exclusively in each of the monomers, the active site of AICART was uniquely identified at an interface between homodimers of the enzyme (Figure 2-5). Prior cross-linking and sedimentation equilibrium dialysis experiments suggested that dimerization was indeed required for AICART activity, and the structural data supports this conclusion (127, 128).

A recent report by M. Vincent and colleagues at Universitaires Saint-Luc (Brussels) identified a young patient with biallelic mutations in the AICART domains of the ATIC gene, producing inactive enzyme from both alleles (129). This important study set a precedent for our observations consequent to AICART inhibition by pemetrexed, detailed in this chapter. The patient was born healthy, but her condition rapidly deteriorated. By 6 months, the patient had psychomotor delay, partial occipital seizures, congenital blindness, and noticeable dysmorphic features of the face. A follow-up visit at age 4 identified massive accumulation of the cell-permeable catabolite of ZMP, 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), in the patient’s urine. Low levels of urinary AICAR have been detected in patients with folate and vitamin B₁₂ deficiencies, ALL, and HGPRT deficiencies (Lesch-Nyan syndrome) (130, 131). However, AICAR measured in this patient was 300-fold over levels in control urine, in which AICAR was undetectable (129). Relevant to our studies, high concentrations of ZMP (as well as ZDP and ZTP) were measured in erythrocytes from this patient compared to control patients. It was subsequently determined that AICART activity was lost in cultured fibroblasts from this patient. Genetic analysis determined that one ATIC allele showed a frameshift in exon 2, producing an unstable mRNA. This frameshift was also present on one allele from the healthy mother. The patient’s other ATIC allele had a K426R mutation in exon 13 of the AICART
Figure 2-5. Crystal structure of human AICART/IMPCH bound to ZMP and a sulfonyl-containing antifolate illustrates that the AICART active site is formed by homodimerization. The sulfonyl-containing antifolate BW1540U88UD (white ball and stick) and ZMP (orange ball and stick) are depicted in the active site of human AICART (PDB Code 1P4R). The crystal structure for AICART is a homodimer, and the active site is at the interface of monomer 1 (brown) and monomer 2 (green). Image generated with PyMOL (www.pymol.org).
domain, also present in the healthy father. Recombinant AICART with the K426R mutation was inactive and structural studies with avian AICART suggested this residue was essential for stability of the tertiary structure (122, 129). Interestingly, the activity of IMPCH in this pediatric case was 40% of control, consistent with a frameshift in one allele of the ATIC gene, and that these two fused reactions were indeed independent of one another, as indicated by IMPCH enzyme activity in the allele with the K426R mutation within the AICART domain (129).

Defining the folate-dependent targets of pemetrexed

Pemetrexed was discovered to be a TS inhibitor serendipitously, as it was synthesized from a drug discovery program targeting GART (53). An in vitro enzymology study conducted by scientists at Eli Lilly and Company (Indianapolis, IN) identified the secondary target of pemetrexed as glycinamide ribonucleotide formyltransferase (GART), the first folate-dependent enzyme of de novo purine synthesis (54). A summary of their kinetic measurements is presented in Table 1-1. The reported $K_i$ of GART for pentaglutamates was 65 nM, which was quite weak compared to DDATHF ($K_i \sim 0.3$ nM) (49). Interestingly, AICART was concluded from this study to be a minor target of pemetrexed, with a reported $K_i$ of 260 nM for the pentaglutamate. The results of this study were taken as definitive, and for the next decade, GART inhibition was credited as the second mechanism of pemetrexed action. Studies presented in this section begin by describing a pivotal CCRF-CEM end-product reversal experiment performed by Dr. Moran that suggested the second target of pemetrexed was not GART, but AICART. The results that follow indeed define the elusive secondary target of pemetrexed as the second folate-dependent enzyme of de novo purine synthesis, AICART.
MATERIALS AND METHODS

Chemicals and reagents

Pemetrexed (LY231514), (6R)-DDATHF, and LCA (Lilly compound AICART inhibitor) were obtained from Eli Lilly and Company (Indianapolis, IN). In some experiments, pemetrexed was purchased from LC Laboratories (#P-7177 Woburn, MA). ^3^H-Pemetrexed was obtained from Eli Lilly and Company at a specific activity of 10.6 Ci/mmol and was purified by HPLC prior to use as described below. ^1^C-glycine was from Moravek Biochemicals (#MC-408 Brea, CA) and was supplied at a specific activity of 53 mCi/mmol. (6S)-5-Formyl-5,6,7,8-tetrahydrofolic acid, calcium salt (#16.221) and (6R,S)-5,10-methenyl-5,6,7,8-tetrahydrofolic acid chloride (#16.230) were from Schircks Laboratories (Jona, Switzerland). AG1X-8 100-200 mesh anion exchange resin, chloride form (#140-1443) was from Bio-Rad Laboratories (Hercules, CA). Safety Solve scintillation cocktail (#111177) was from Research Products International (Mount Pleasant, IL). The pET-28A bacterial expression vector (#698643) originally from Novagen/EMD (Darmstadt, Germany), was a generous gift from Dr. David Williams (VCU Department of Pathology). The BL21(DE3) Escherichia coli strain (#C6000-03), TRIzol Reagent (#15596026), SuperScript III First-strand Synthesis System (#18080), high-fidelity PCR supermix (#12532016), 1 kB plus molecular mass ladder (#10787018), and T4-DNA ligase (#15224-041), TOP-10 chemically competent E. coli (#C4040), and primers were from Invitrogen (Carlsbad, CA). Restriction enzymes were from New England Biolabs (Ipswich, MA). The DNA Gel Extraction Kit (#28704) was from Qiagen (Valencia, VA). The PureYield Plasmid Midiprep System (#A2492) was from Promega (Madison, WI). 2YT Broth (#22712020) was from Invitrogen. B-PER Bacterial Protein Extraction Reagent (#78248) was from Thermo Scientific (Rockford, IL). Bradford Reagent (#5000006) was from Bio-Rad.
Laboratories. All other reagents were from Fisher Scientific (Waltham, MA) or Sigma Aldrich (St. Louis, MO) and were of the highest available purity.

Some routinely used stock solutions for rescue experiments were made as follows. A hypoxanthine stock solution (5 mM) was made by dissolving 0.034 g of hypoxanthine in 47 ml of 75 mM HCl. The concentration was determined by measuring absorbance at 250 nm from a 1:100 dilution of the stock solution in 1x PBS using an extinction coefficient of 10.6 mM$^{-1}$ cm$^{-1}$. The volume was adjusted accordingly with 75 mM HCl for a final concentration of 5 mM. This solution was filter-sterilized and was stable at 4°C for at least 1 year. Hypoxanthine typically was used at a final concentration of 32 µM in rescue experiments unless otherwise noted. Stock solutions of thymidine (560 µM) were routinely made by dissolving 0.00678 g of thymidine powder in 48 ml of 1x PBS. The concentration was determined by measuring absorbance at 267 nm from a 1:10 dilution of the stock solution in 1x PBS using an extinction coefficient of 9.7 mM$^{-1}$ cm$^{-1}$. The volume was adjusted accordingly with 1x PBS for a final concentration of 560 µM. This solution was filter-sterilized and was stored in single-use aliquots at -20°C. Thymidine was typically used at a final concentration of 5.6 µM in rescue experiments unless otherwise noted. HEPES/MOPS buffer (20 mM HEPES, 10 mM MOPS, pH 7.4) was added to all cell culture media containing hypoxanthine to buffer the HCl. A 40x stock solution of HEPES/MOPS was made and stored at 4°C for at least 1 year. 19.06 g HEPES and 8.37 g MOPS were dissolved in 70 ml 1 N NaOH. The pH was adjusted to 7.4 with approximately 3 ml of 6 N HCl. The volume was adjusted to 100 ml before being filter-sterilized and stored at 4°C.
Cell culture

All cell lines were from the American Type Culture Collection (ATCC) (Manassas, VA) unless otherwise noted. The CCRF-CEM human lymphoblastic leukemia cell line was maintained at a density between \(10^5\) and \(10^6\) cells/ml in RPMI-1640 medium (#11875 Gibco/Invitrogen Carlsbad, CA) supplemented with 10% dialyzed fetal bovine serum (dFBS) (#100108 Gemini Bio-Products West Sacramento, CA). CEM cells were grown at 37°C with 5% CO\(_2\) and given fresh media every 2-3 days. AICART (-/-) human skin fibroblasts were a generous gift from Marie-Francois Vincent (Universite Catholique de Louvain, Brussels, Belgium) and were maintained in DMEM (#11995 Gibco/Invitrogen Carlsbad, CA) supplemented with 15% FBS (#100106 Gemini Bio-Products West Sacramento, CA); experiments were performed in dFBS. Carcinoma cell lines were also maintained in RPMI-1640 medium supplemented with 10% dFBS and were grown at 37°C with 5% CO\(_2\). Carcinoma cell lines were passaged by seeding in T-75 flasks at a density of \(10^6\) cells/dish every 2-3 days. Passaging of adherent cell lines included washing with 1x phosphate buffered saline (#10010 Gibco/Invitrogen) and trypsinizing for 5 minutes at 37°C with 1x trypsin-EDTA (#15400 Gibco/Invitrogen).

Cellular end product reversal experiments

Adherent cells were seeded at a density of \(10^4\) cells/well of a 12-well plate and allowed to adhere overnight. Conditions were usually plated in triplicate. Fresh media containing drugs and rescue agents was added the next day. Rescue experiments typically lasted 72 hours, and fresh media containing drugs and rescue agents was added if cells were incubated for longer periods of time. Following the incubation period, cells were washed 1x with PBS, trypsinized in
2ml 1x trypsin-EDTA, and 1 ml of a single-cell suspension was counted electronically using a Z1 Coulter Particle Counter (Beckman Coulter Brea, CA). Data is presented as percent cell growth of experimental samples relative to rescue agent controls in the absence of drug.

**N-Formyl glycinamide ribonucleotide synthesis from \(^{14}\)C-glycine**

The isolation of \(^{14}\)C-N-Formyl glycinamide ribonucleotide (FGAR) by ion-exchange chromatography was adapted from a procedure previously published by our laboratory (46). AG1X-8 100-200 mesh anion exchange resin, chloride form (5 g) was suspended in 25 ml of 4 M formic acid. The slurry was stirred at room temperature for 10 minutes and filtered through Whatman #1 using a Buchner funnel. The filtered resin then was washed with 60 ml of 4 M formic acid and 2 L ddH\(_2\)O to neutralize the resin. The equilibrated resin was then resuspended in 25 ml of 50 mM formic acid and stored in a glass bottle at room temperature.

Following drug treatments, 7 x 10\(^6\) CCRF-CEM cells were pelleted by centrifugation at 1,000 rpm for 5 minutes in a 50 ml conical tube, washed 1x with PBS, and resuspended in 2 ml 37°C RPMI 1640 without glutamine or serum. A final concentration of 10 \(\mu\)M azaserine or PBS was added and the cell suspension was incubated for 30 min at 37°C mixing occasionally. Glutamine at 2 mM and 0.25 \(\mu\)Ci/mL of \(^{14}\)C-glycine were added, and the incubation continued at 37°C for 1 hour. Cells were centrifuged at 3,000 rpm for 10 minutes, washed 1x with cold 1x PBS, spun at 1,000 rpm for 10 minutes, and resuspended in 2 ml cold 5% (w/v) trichloroacetic acid. Samples were vortexed vigorously and held on ice for 5 minutes before being centrifuged at 7,000 rpm for 10 minutes at 4°C to remove debris (macromolecules). The supernatant, which contained free nucleotides, was transferred to a new tube. The supernatant was extracted twice with cold diethyl ether by adding an equal volume of ether to each tube, spinning at 1,000 rpm
for 5 minutes, and discarding the top ether layer as radioactive liquid waste. An aliquot (1.5 ml) of the aqueous layer was loaded onto plastic Pasteur pipettes columns whose tops were removed, plugged with glass wool, and packed with a 6-7 cm bed volume of equilibrated AG1X-8 anion exchange resin. The column was eluted first with 10 ml 0.5 M formic acid, then with 10 ml 4 M formic acid. Scintillation cocktail (5 ml) was added and $^{14}$C was counted for 2 minutes per vial on a Multi-Purpose Scintillation Counter (#LS 6500 Beckman Coulter). A typical chromatogram of azaserine-blocked CCRF-CEM cells is depicted Figure 2-6, with the area under fractions 12-14 representing labeled FGAR.

**High performance liquid chromatography analysis of ZMP**

ZMP was quantified from whole-cell acid-soluble extracts using a procedure modified from Corton *et al* (132). Typically, $10^6$ cells were plated on 100 mm dishes and allowed to adhere overnight prior to drug treatment. For suspension cultures, $10^6$ cells were resuspended directly in media containing drugs. Following drug exposure, cultures were washed 1x with PBS, scraped, and densities were determined electronically using a Z1 Coulter Particle Counter (Beckman Coulter). Cells were pelleted by centrifugation at 1,500 rpm for 5 minutes and resuspended in 5% (w/v) trichloroacetic acid (TCA) at a density of 5,000 cells/$\mu$l TCA. Suspensions were vortexed vigorously and held on ice for 5 minutes before being spun at 7,500 rpm for 10 minutes to remove debris. Supernatants were transferred to new tubes and extracted twice with an equal volume of cold diethyl ether under a fume hood to remove the acid. The extraction procedure consisted of centrifugation at 1,500 rpm for 5 minutes and removal of the upper ether-containing layer. Extracts were passed through a 0.45 $\mu$m syringe filter before being stored at -80°C until ready for HPLC analysis.
Figure 2-6. FGAR accumulates in CEM cells treated with azaserine. 14C-FGAR was isolated by anion-exchange chromatography in 1 ml fractions as described in the Methods section.
ZMP was analyzed by high-performance liquid chromatography (HPLC) using a Spectra-Physics P4000 pump, UV2000 absorbance reader set to monitor a wavelength of 280 nm, and a Datajet integrator. A strong anion exchange (SAX) 250 x 2 mm HPLC column (#00G4149B0 Phenomenex Torrance, CA) fitted with a guard column (# KJ04282, #AJ04310 Phenomenex Torrance, CA) was equilibrated with Buffer A (5 mM NH₄H₂PO₄, pH 2.8) at a flow rate of 0.2 ml/min. Typically, 5 µl acid-soluble extract was injected onto the column in a volume of 300 µl Buffer A. After passing Buffer A over the column for an additional 2 minutes, ZMP was eluted from the column using a linear gradient of 100% Buffer A to 100% Buffer B (750 mM NH₄H₂PO₄, pH 3.9) over 25 minutes at a flow rate of 0.2 ml/min. ZMP had a retention time of approximately 16 minutes under these conditions (Figure 2-7A). Buffer B was flowed over the column for an additional 8 minutes following each run to clean the column, and the mobile phase was returned to Buffer A over the next 10 minutes. Total time from injection to injection was approximately 45 minutes. To determine ZMP concentration from extracts, results were fitted to a standard curve generated from synthetic ZMP (Figure 2-7B). The detection limit using this protocol was approximately 25 pmol ZMP.

**Expression and Purification of recombinant human ATIC**

The general strategy for expression and purification of recombinant human ATIC was similar to that of Beardsley et al (120, 123), and entailed cloning the human ATIC cDNA into the pET-28A bacterial expression vector with BamHI and Nhel restriction sites. The resulting construct, pET28A_ATIC, now in frame with a N-terminal 6-His affinity purification tag, was transformed into BL21(DE3) cells for expression. His-tagged ATIC was purified over a nickel
Figure 2-7. Identification and quantification of ZMP by HPLC. ZMP was analyzed by HPLC from whole-cell acid soluble extracts as described in the Methods section. (A) A representative HPLC chromatogram measuring ZMP from CEM cells treated with 500 µM AICAR. (B) A standard curve was generated with synthetic ZMP.
affinity column and was polished on a gel filtration column before being stored at -20°C. Detailed procedures are outlined below.

Total RNA Isolation: Total RNA was extracted from HCT116 cells grown to 75% confluency on a 100 mm tissue-culture dish with TRIzol Reagent according to the manufacturers protocol. All materials and reagents used were sterile and RNAse-free. Diethylpyrocarbonate (DEPC) H$_2$O (0.01% v/v) was prepared by incubating at room temperature overnight and autoclaving before use. Pipettes were cleaned with RNAse ZAP (Invitrogen), and crosslinked with a UV Stratalinker 2400 (#400075 Stratgene La Jolla, CA). Cells were placed on ice, washed 1x with cold PBS, and lysed directly in 2 ml cold TRIzol Reagent. Cells were scraped, the slurry was transferred to a 14 ml round bottom Falcon tube, and the sample was incubated at room temperature for 5 minutes. An aliquot (400 µl) of chloroform was added to the sample in the fume hood. The sample was shaken vigorously for 15 seconds and incubated at room temperature for 3 minutes. The sample was spun at 7,000 rpm for 15 minutes at 4°C. The mixture separated into 3 phases. RNA remained exclusively in the aqueous upper phase. This aqueous phase was transferred to a fresh 14 ml tube and 1 ml isopropanol was added to precipitate the RNA. This sample was incubated at room temperature for 10 minutes before being centrifuged at 7,000 rpm for 10 minutes at 4°C. The supernatant was removed, and the RNA pellet was washed 1x with 75% ethanol in DEPC H$_2$O. The sample was mixed and spun at 7,000 rpm for 5 minutes at 4°C. The ethanol was removed and spun again to remove excess ethanol. The pellet was resuspended in 200 µl DEPC H$_2$O. For storage purposes, 75 µl dissolved RNA was suspended in 225 µl 100% ethanol and stored at -80°C. This sample could then be re-precipitated and solubilized if necessary. RNA concentration and purity was determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies
Wilmington, DE). Pure RNA has an OD$_{260/280}$ of 2.0. The OD$_{260/230}$ ratio was also used as a secondary indicator of RNA purity, and values below the range of 1.8-2.2 indicated the presence of copurified contaminants. RNA integrity was also determined by resolving RNA on a 1% TAE agarose gel stained with ethidium bromide at 100 volts for 30 minutes. A distinct banding pattern and intensity difference between 28s and 18s rRNA indicated that the RNA was intact.

**cDNA Synthesis:** cDNA was reverse-transcribed from 5 µg of total RNA using the SuperScript III First-strand Synthesis System from Invitrogen. SuperScript III Reverse Transcriptase is similar to the Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT), but has been engineered to diminish RNase H activity and enhance thermal stability. RNA was mixed with a final concentration of 5 µM oligo(dT)$_{20}$ primer and 1 mM dNTP mix in a volume of 10 µl adjusted with DEPC-H$_2$O. The mixture was incubated at 65°C for 5 minutes, then placed on ice for at least 1 minute. The cDNA synthesis mix was prepared in a separate tube by combining 2 µl of 10x RT buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 4 µl 25 of mM MgCl$_2$, 2 µl of 0.1 M DTT, 1 µl of RNaseOUT (40 U), and 1 µl of SuperScript III Reverse Transcriptase (200 U) per reaction. 10 µl of the cDNA synthesis mix was added to the RNA/primer mixture, gently mixed, and cDNA was reverse-transcribed by incubating at 50°C for 50 minutes. The reaction was terminated by incubating at 85°C for 5 minutes before being held on ice. 1 µl of RNase H (2 U) was added to each tube and the tube was incubated for 20 minutes at 37°C. cDNA was stored at -20°C until use.

**Primer design:** Primers to amplify the 1.77 kilobase human ATIC cDNA were designed based on the published cDNA sequence (GenBank accession #U37436.1). The sense primer (1) was designed to incorporate a *NheI* restriction site (GCTAGC) directly upstream of the first in-
frame ATG (start codon). The antisense primer (2) was designed to incorporate a BamHI restriction site (GGATCC) directly downstream of the first in-frame TAG (stop codon).

(1) 5’ GAGTTAAGCTAGCATGGCTCCCGGCCAGC 3’
(2) 5’ CCCATGGATCCTCGTGAAAGAGCCGAAG 3’

Primers were analyzed using the OligoAnalyzer from Integrated DNA Technologies (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer). Primers were designed with a 50-60% GC content, melting temperature between 55-75°C, and were 18-30 nucleotides in length. Six random nucleotides were added upstream of the restriction site on the 5’ to allow for recognition and digestion by restriction enzymes. Strings of 3 or more G’s, C’s, or T’s were avoided in the 3’ end, but 1 G or C was placed at the 3’ end to help tack down the primer during annealing. Lyophilized primers were dissolved in HPLC-grade H2O to stock concentrations of 100 µM and were further diluted in HPLC-grade H2O to a working stock concentration of 7.5 µM before being added to Polymerase Chain Reactions (PCR). Primers were routinely stored as 100 µM stocks at -20°C as well as 7.5 µM working stocks in single-use aliquots.

**Cloning the human ATIC cDNA:** The human ATIC open reading frame was amplified by PCR from HCT116 cDNA using Platinum PCR SuperMix High Fidelity from Invitrogen. This SuperMix contains anti-Taq DNA polymerase antibody bound to recombinant Taq DNA polymerase, Mg²⁺, dNTPs, and *Pyrococcus* species GB-D thermostable polymerase. The polymerase activity of antibody-bound Taq is inhibited, allowing reactions to be set up at room temperature. Taq polymerase activity is restored by heating the reaction to 94°C (“hot start”) (133, 134). *Pyrococcus* species GB-D thermostable polymerase has proofreading ability due to its 3’ to 5’ exonuclease activity. A typical 25 µl PCR reaction contained 300 nM sense and antisense primers, 22 µl high-fidelity PCR SuperMix and 1 µl template cDNA. Negative control
reactions were always ran in the absence of cDNA. Amplifications were carried out on a DNA Engine Peltier Thermal Cycler (Bio-Rad Laboratories) using the following program: 1) 1 cycle of 94°C for 4 minutes, 2) 35 cycles of 94°C for 30 seconds, followed by a 57.1-62.8°C annealing gradient for 30 seconds, and then 72°C for 2 minutes (1 min/kB), 3) 1 cycle of 72°C for 15 minutes, and 4) a 4°C hold. Reactions were ran next to a 1 kB plus molecular mass ladder on a 1% TAE agarose gel stained with ethidium bromide at 100 volts for 30 minutes, and the 1.77 kB fragment was excised from the gel and purified using a Qiagen Gel Extraction Kit as described below. This kit contains silica-membrane columns that fit into microcentrifuge tubes, allowing purification by centrifugation at 17,900 x g in a tabletop centrifuge at room temperature. The excised agarose slice was dissolved in a high-salt buffer (1 mg/3 μl) at 50°C for 10 minutes, gently mixing several times. The solubilized DNA was bound to the silica column, washed twice with an ethanol-containing buffer, and eluted in 30 μl of 10 mM Tris-HCl pH 8.5. DNA concentration and purity was determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). DNA was used only if the OD260/280 was greater than 1.8.

The amplified human ATIC cDNA and pET28A bacterial expression vector were both sequentially digested with *NheI* and *BamH1* according to the manufacturers double-digest recommendation (http://www.neb.com/nebecomm/doubledigestcalculator.asp). These restriction sites were chosen because they are both present in the vector only once (both in the multiple cloning site), and they are not present in the endogenous human ATIC cDNA sequence. The doubly digested vector and insert were then gel purified with a Qiagen gel extraction kit as described above and ligated with T4-DNA Ligase. Ligation reactions were carried out at room temperature for 2 hours and contained 4 μl of 5x Ligase Reaction Buffer (250 mM Tris-HCl pH 7.6, 50 mM MgCl2, 5 mM ATP, 5 mM DTT, and 25% (w/v) polyethylene-glycol-8000), 30 fmol
vector, 90 fmol insert, and T4 DNA Ligase (1 U) in a total volume of 20 µl (q.s. with sterile ddH₂O).

The ligated construct was transformed into Invitrogen TOP-10 chemically competent *E. coli* as described. One vial of TOP-10 chemically competent *E. coli* was thawed on ice for 10 minutes. An aliquot (2 µl) of the ligated construct was added to the thawed *E. coli*, the tube was mixed gently, and placed on ice for 5 minutes before being heat-shocked for 30 seconds at 42°C without shaking. The tube was returned to ice, and 250 µl of room temperature S.O.C. media was added. The transformation tube was shaken horizontally (200 rpm) at 37°C for 1 hour. An aliquot of the transformation (20-100 µl) was plated on kanamycin-resistance plates (50 µg/ml kanamycin in LB agar) and incubated at 37°C overnight. Colonies were picked, inoculated in 25 ml LB cultures (with 50 µg/ml kanamycin), and shaken at 225 rpm overnight at 37°C. Plasmids were isolated as described below from the transformed bacteria using the Promega PureYield Plasmid Midiprep System. This kit uses a silica-membrane column attached to a vacuum manifold, and is designed to purify 100-200 µg of plasmid DNA from 25-100 ml overnight bacterial cultures. Overnight cultures were pelleted at 5,000 x g for 10 minutes and resuspended in a buffer containing 50 mM Tris-HCl pH 7.5, 10 mM EDTA pH 8.0, and 100 µg/ml RNase A. Cells were lysed in a solution containing 0.2 M NaOH and 1% SDS, mixed 5 times by inversion, and incubated at room temperature for 3 minutes. A neutralization solution containing 4.09 M guanidine HCl pH 4.8, 759 mM potassium acetate, and 2.12 M glacial acetic acid was added, and the sample was mixed by inversion 5 times. The lysate was centrifuged at 15,000 x g for 15 minutes to remove cellular debris. The cleared lysate was decanted onto a silica-membrane column, and vacuum was applied to bind DNA to the column. The column was washed with 20 ml of a buffer containing 60% ethanol, 60 mM potassium acetate, 8.3 mM Tris-HCl pH 7.5, and
0.04 mM EDTA pH 8.0. DNA was eluted in 500 µl nuclease-free water, and quantity and integrity of DNA was measured using the NanoDrop as described above. Clones were screened for the presence of the insert by digestion with NheI and BamHI, and verified plasmids were sent for sequencing at the VCU Nucleic Acids Research Core Facility using gene-specific primers designed to cover every 200 base pairs of the human ATIC cDNA to ensure high-quality sequencing runs.

**rhATIC Expression:** pET28A_ATIC clone 2 was chosen for transformation into Invitrogen BL21(DE3) E. coli. The transformation procedure was performed as described above for TOP-10 E. coli. Transformants were plated on kanamycin-resistance LB-agar plates and grown overnight at 37°C. A streak of colonies was inoculated into a 5 ml 2YT Broth (with 50 µg/ml kanamycin) and grown overnight at 37°C with shaking at 225 rpm. A 1 ml fraction of this inoculate was mixed with a final concentration of 20% sterile (autoclaved) glycerol, and stored at -80°C (expression starter cultures could readily be inoculated from ice chips taken off this plasmid glycerol stock). A 1:50 dilution of an overnight culture was diluted in fresh 2YT Broth (with 50 µg/ml kanamycin) and grown at 37°C with shaking at 225 rpm until the OD$_{600}$ measured 0.6 (approximately 1.5 hours). The incubation temperature was dropped to 30°C and cultures were induced with 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 3 hours. Cultures were pelleted by centrifugation at 4,000 rpm for 10 minutes, washed 1x with cold 0.85% NaCl, and frozen at -80°C until purification.

**rhATIC Purification:** Thawed cell pellets were lysed in B-PER Bacterial Protein Extraction Reagent (4 ml/gram of cell pellet) supplemented with 150 mM NaCl. BPER is a proprietary mild nonionic detergent in 20 mM Tris HCl pH 7.5. The lysate was incubated at room temperature for 10 minutes and was spun at 10,000 rpm for 10 minutes to separate soluble
and insoluble protein. The majority of ATIC protein expressed was present in the insoluble fraction (Figure 2-8A). Inducing expression at 16°C overnight or expressing in a strain of bacteria sensitive to IPTG concentration (TunerDE3) did not increase the soluble protein yield. Scaling my expression cultures up to 1 L did produce a sufficient amount of soluble protein for my needs, so I luckily did not have to attempt purification from inclusion bodies. Soluble human ATIC was purified with the help of Dr. David Williams using an AKTA Purifier fast protein liquid chromatography (FPLC) system (GE Lifesciences Piscataway, NJ) fitted with a P-900 pump, a UV-900 absorbance detector, and a Frac-920 fraction collector. Soluble protein was loaded onto a nickel-charged purification column. This column was 16 mm in diameter and contained a 5 ml bed volume of chelating sepharose (#17057501 GE Lifesciences) charged with NiCl₂. The loading capacity of the resin was 20 mg/ml bed volume. The column was equilibrated with 5 bed volumes of Buffer A (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.2 mM 2-mercaptoethanol) at a flow rate of 4 ml/min. Protein absorbance was monitored at 280 nm as well as 330 nm to detect aggregates. Nonspecific proteins were washed off first with 5 column volumes of 4% Buffer B (total imidazole = 20 mM) (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.2 mM 2-mercaptoethanol, 500 mM imidazole), then with 5 column volumes of 12% Buffer B (total imidazole = 60 mM), and ATIC was eluted over 5 column volumes with a stepwise gradient from 20-50% Buffer B (total imidazole = 100-250 mM). Fractions of equivalent volume from each step of the nickel purification procedure were ran on a 7.5% SDS-PAGE gel and visualized with a coomassie staining solution (#1610786 Bio-Rad) (Figure 2-8A). Eluted fractions containing protein (as detected by absorbance at 280 nm) were pooled and loaded onto a HiLoad 26 mm/60 inch Superdex 75 gel filtration column (#17107001 GE Lifesciences) equilibrated with Buffer C (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 50 mM KCl, 5 mM EDTA,
5 mM DTT) at a flow rate of 1.8 ml/min. The loading capacity of this column was 12 ml. Absorbance was monitored at 280 nm, and the chromatogram showed one prominent peak eluting at the correct size (62 kDa) based on a calibration of the column proteins of known size. The fractions containing a protein of ≈ 62 kDa were pooled and concentrated with an Amicon Ultra Centrifugal Filter Device with a 3 kDa molecular weight cutoff (#UFC900324 Millipore Billerica, MA) by centrifuging at 4,000 rpm for 30 minutes. The protein concentration was determined using the Bradford Reagent according to the manufacturers protocol with BSA as a standard. An equal volume of glycerol was added to the concentrated protein, which was stored at -20°C in Buffer C. The yield from a 1 L culture was approximately 5.5 mg of protein. Purity was greater than 99% as determined by SDS-PAGE and coomassie staining, in which no trace proteins were detected when 50 µg of purified protein was electrophoresed (Figure 2-8B).

Production of 10-CHO-H₄PteGlu from 5-CHO-H₄PteGlu

The formyl donor 10-CHO-H₄PteGlu was synthesized by acid-catalyzed dehydration of (6S)-5-CHO-H₄PteGlu to form (6R)-5,10-CH⁺-H₄PteGlu. (6R)-5,10-CH⁺-H₄PteGlu was then hydrolyzed to (6R)-10-CHO-H₄PteGlu by a basic pH shift. Because of the instability of 10-CHO-H₄PteGlu, it was produced on demand from 5-CHO-H₄PteGlu for use in enzyme assays. The synthesis procedure that follows is based on the method of Stover and Schirch (6).

Typically, 500 nmol (6S)-5-CHO-H₄PteGlu was dissolved in 0.1 N HCl (pH 1.5) to a final concentration of 500 µM. The solution was protected from light and rotated at room temperature. The formation of (6R)-5,10-CH⁺-H₄PteGlu was detected by monitoring an increased absorbance spectrophotometrically at 352 nm (ε = 25 mM-1 cm-1 at pH 1.5) after a 1:50 dilution in 0.1 N HCl. Complete conversion was attained after a 2.5-hour incubation. The
Figure 2-8. Recombinant human AICART expression and purification. Recombinant human AICART was expressed and purified as described in the Methods section. (A) I: insoluble, S: soluble, FT: Ni-NTA column flow through, W1: 20 mM imidazole wash, W2: 60 mM imidazole wash, E1: 100 mM imidazole elution, E2: 175 mM imidazole, E3: 250 mM imidazole. (B) S: soluble, FT: Ni-NTA column flow through, Ni-NTA: pooled nickel column elutions (100 and 175 mM imidazole), GF: pooled gel filtration elutions.
solution also had a characteristic yellow tinge. (6R)-5,10-CH\textsuperscript{+}-H\textsubscript{4}PteGlu is stable in solution for several months as long as the pH is maintained below 2.0 and the temperature does not exceed 40\degree C (6). (6R)-5,10-CH\textsuperscript{+}-H\textsubscript{4}PteGlu was stoichiometrically converted to (6R)-10-CHO-H\textsubscript{4}PteGlu by adding a final concentration of 50 mM 2-mercaptoethanol to the solution and adjusting the pH to 8.5 with 5N KOH. The pH was adjusted carefully, as the solution was not buffered. The reaction was again shielded from light and rotated at room temperature. The reaction was monitored by a decrease in absorption at 352 nm, and was complete by 1 hour. The solution also no longer had a yellow tinge. (6R)-10-CHO-H\textsubscript{4}PteGlu was placed on ice, shielded from light, and was used in assays the same day. In some instances, (6R,6S)-5-CHO-H\textsubscript{4}PteGlu or (6R,6S)-5,10-CH\textsuperscript{+}-H\textsubscript{4}PteGlu powder was used as starting material.

**AICART activity assay**

The AICART activity assay was based on previously reported methods in which the formation of H\textsubscript{4}PteGlu was monitored spectrophotometrically at 298 nm (135, 136). Assays were performed on a Hewlett Packard UV-VIS 8453 Spectrophotometer with ChemStation data acquisition software (Agilent Technologies Santa Clara, CA). Reactions were 1 ml in volume and were done in 1-cm pathlength quartz cuvettes (#14385916A Fisher) at room temperature. Unless otherwise noted, reactions contained 1x assay buffer (33 mM Tris-HCl pH 7.4, 25 mM KCl, 5 mM 2-mercaptoethanol), 100 \mu\text{M} (6R)-10-CHO-H\textsubscript{4}PteGlu, 50 \mu\text{M} ZMP, and 3 \mu\text{g} enzyme. Blank measurements were taken, and the reactions were started by the addition of enzyme followed by a quick plunge with a cuvette mixer (# Z370835). Linear absorbance readings at 298 nm were taken every 2 seconds for 20 seconds, and an initial velocity was measured as absorbance units (AU)/second. The concentration of H\textsubscript{4}PteGlu formed in a given
reaction was calculated using $\epsilon = 19.7 \text{ mM}^{-1} \text{ cm}^{-1}$ at pH 7.4, the difference in extinction coefficients for 10-CHO-H$_4$PteGlu plus ZMP and H$_4$PteGlu plus CHO-ZMP (136). Stock solutions of pemetrexed were dissolved in 10x assay buffer. Data was analyzed using the Kinetics module of Sigma Plot v10.0.
RESULTS

End-product reversal studies define the folate-dependent cellular targets of antifolates

Cell growth reversal experiments performed in our lab using L1210 mouse leukemic cells (46, 47) and more recently by R.G. Moran in CEM human leukemic cells (Figure 2-9A) defined (6R)-DDATHF as a GART inhibitor (125). Complete reversal of the growth inhibitory effect of (6R)-DDATHF in CEM cells indicated that the antifolate was targeting de novo purine biosynthesis. To determine whether the first (GART) or second (AICART) folate-dependent enzyme of de novo purine synthesis was inhibited by (6R)-DDATHF, the purine synthesis intermediate AICA was included in culture medium. The nucleobase AICA is metabolized to ZMP by APRT and PRPP, introducing a metabolic intermediate between GART and AICART (Figure 2-3). Therefore, if the secondary target of pemetrexed were GART, inclusion of thymidine and AICA in the medium would completely reverse growth inhibition (46, 47), whereas, if AICART were inhibited, either no effect or a mild exacerbation of growth inhibition would be expected. The growth inhibitory effect of (6R)-DDATHF was indeed rescued by AICA, demonstrating that (6R)-DDATHF is primarily a GART inhibitor (Figure 2-9A).

Using this same strategy, R.G. Moran performed cell growth reversal experiments to define the folate-dependent targets of pemetrexed (Figure 2-9B) (125). The growth of CEM human leukemic cells was inhibited by pemetrexed at low nM concentrations. Inclusion of thymidine in the culture medium partially rescued growth inhibition by pemetrexed, shifting the IC50 approximately 10-fold. Growth inhibition by pemetrexed was not rescued by inclusion of hypoxanthine, but inclusion of both thymidine and hypoxanthine reversed the growth inhibitory effects of pemetrexed even at high concentrations. This reversal pattern is in agreement with previous literature (53, 54) and has been interpreted to mean that pemetrexed is primarily a
thymidylate synthase inhibitor and also has a second site of action along de novo purine biosynthesis, only affected at higher drug concentrations. Others have shown that AICA by itself at 300 µM did not change the potency of pemetrexed against CEM cells (54). This data was uninformative, as hypoxanthine by itself had no rescue effect on pemetrexed, confirming the effect on purine synthesis as secondary to thymidylate synthase inhibition. R.G. Moran repeated this experiment, adding AICA (320 µM) in the presence of thymidine (to alleviate effects of pemetrexed on thymidylate synthase), which mildly enhanced the growth inhibition by pemetrexed (Figure 2-9B) (125). The data presented in Figure 2-9B was our first indication that the purine synthesis target of pemetrexed was most likely AICART, not GART.

CEM cells were chosen for this initial reversal experiment, because it is fairly simple and reproducible to electronically count cells in suspension. Indeed, most of the initial studies defining pemetrexed as an AICART inhibitor were done in the CEM background (125). However, since pemetrexed is clinically indicated for MPM and NSCLC, it was essential to extend our studies in leukemic cells to carcinomas. The H460 human NSCLC cell line was subjected to end-product reversal experiments in the presence of pemetrexed (Figure 2-10A). The low nM growth inhibitory effect of pemetrexed was similar in H460 to that in CEM. Likewise, inclusion of thymidine shifted the IC50 approximately 10-fold, and inclusion of thymidine and hypoxanthine completely rescued the growth inhibitory effects of pemetrexed even at high concentrations (Figure 2-10A). Thus, it was concluded that the growth inhibitory
Figure 2-9. Reversal of CEM cell growth inhibition by AICA suggests that the second target of pemetrexed is AICART, not GART. Exponentially growing CEM human leukemic cells were treated with the indicated concentrations of (A) (6R)-DDATHF or (B) pemetrexed alone (no add), or in the presence of thymidine (TdR, 5.6 µM), hypoxanthine (Hx, 32 uM), AICA (320 µM), or a combination of TdR with either Hx or AICA. Drug and modifying agents were added simultaneously and drug-containing media was changed at 48 hours. Cell growth was determined after 96 hours by Coulter counting and cell number is expressed relative to controls without drug. These experiments were performed by RG Moran.
Figure 2-10. Thymidine and hypoxanthine reversal profiles of growth inhibition induced by pemetrexed in H460 and HCT116 cells. Exponentially growing (A) H460 human colon carcinoma cells and (B) HCT116 human non-small cell lung carcinoma cells were treated with the indicated concentrations of pemetrexed alone (closed circles), or in the presence of 5.6 µM thymidine (open circles), 32 µM hypoxanthine (closed triangles), or a combination of TdR with Hx (open triangles). Drug and modifying agents were added simultaneously and cell growth was determined after 72 hours electronically by Coulter counting. Cell number is expressed relative to controls without drug. Cultures were set up in triplicate, and error bars are representative of standard deviation from three biological and two technical replicates.
effects of pemetrexed in H460 cells, like CEM, were due to a primary inhibition of thymidylate synthase and secondary inhibition of a purine synthesis intermediate, likely AICART. While AICA was not included in these reversal experiments, a more extensive analysis of AICART inhibition by pemetrexed in H460 cells is presented in Chapter 3.

Cell culture reversal studies were not always a completely clear indicator of the growth inhibitory effects of pemetrexed. A case in point is seen in an experiment on the HCT116 human colon tumor cell line (Figure 2-10B). The growth of HCT116 cells was potently inhibited by pemetrexed at low nM concentrations, with an IC50 shifted a half log left of both CEM and H460. Exposure to drug in the presence of thymidine only shifted the growth inhibitory effect of pemetrexed approximately 3-fold, suggesting that inhibition of thymidylate synthase contributed less to the growth inhibitory effect than in CEM and H460. Although the most sensitive folate-dependent step in intact HCT116 cells was thymidylate synthase, the secondary target in purine synthesis was affected even at minimal inhibitory concentrations of pemetrexed. Unlike CEM and H460 cells, inclusion of thymidine and hypoxanthine did not completely rescue growth inhibition at higher doses of pemetrexed (Figure 2-10B). This surprising result suggested that pemetrexed might have targets outside the folate pathway in HCT116 cells. After confirming this puzzling result with several biological repeats, I questioned whether the lack of complete reversal was due to insufficient concentration of rescue agents. The concentrations of rescue agents commonly used in our reversal studies, 5.6 µM thymidine and 32 µM hypoxanthine, were chosen from optimization studies done in L1210 cells by R.G. Moran (137). The effect of increasing concentrations of thymidine in HCT116 cultures was tested in the presence of 1 µM pemetrexed with and without hypoxanthine (Figure 2-11A). Increasing the concentration of thymidine in the presence of pemetrexed but absence of hypoxanthine had no added rescue effect
Figure 2-11. HCT116 growth inhibition in the presence of pemetrexed and thymidine is not rescued any further by increasing the thymidine (A) or hypoxanthine (B) concentration. Exponentially growing HCT116 human colon carcinoma cells were treated with the indicated concentrations of pemetrexed (PTX), thymidine (TdR), and/or hypoxanthine (Hx). Drug and modifying agents were added simultaneously, and cell growth was determined after 72 hours electronically by Coulter counting. Cell number is expressed relative to controls without drug. Cultures were set up in triplicate, and error bars are representative of standard deviation from three biological and two technical replicates.
in HCT116. In the presence of hypoxanthine and pemetrexed, thymidine concentrations above 5.6 µM were growth inhibitory. Therefore, insufficient thymidine could not explain the lack of complete HCT116 growth rescue in the presence of pemetrexed and hypoxanthine. A similar experiment was performed with increasing hypoxanthine concentrations, but no additional rescue effect was observed (Figure 2-11B). Several hypotheses regarding this conundrum in HCT116 cells that integrate data from Chapters 2 and 3 are presented in the Chapter 3 discussion section of this dissertation.

**Effects of pemetrexed on GART in intact cells**

In the lab’s original determination of the site of action of (6R)-DDATHF along the *de novo* purine biosynthesis pathway, $^{14}$C-FGAR accumulation was measured in azaserine-blocked mouse leukemic cells following drug exposure (46, 138, 139). This method takes advantage of several key metabolic aspects of the pathway: 1) The enzyme immediately downstream of GART, FGAR amidotransferase, is sensitive to the glutamine analog azaserine. 2) Glycine is incorporated into the purine skeleton by the enzyme immediately upstream of GART, GAR synthetase (Figure 2-3). When cells were incubated with azaserine and subsequently pulse-labeled with $^{14}$C-glycine, labeled FGAR accumulated, and could be isolated by ion-exchange chromatography as described in the Methods section. If GART was inhibited, the incorporation of radiolabel into FGAR would be prevented. This was demonstrated in intact CEM cells treated with growth-inhibitory concentrations of (6R)-DDATHF, indicating GART as a primary target (Figure 2-12). On the contrary, growth inhibitory concentrations of pemetrexed only gradually inhibited $^{14}$C-FGAR accumulation to an extent that did not reach 50% even at high concentrations (Figure 2-12). Thus, any effect of pemetrexed on GART appears to be limited
Figure 2-12. **14C-FGAR accumulation in CEM cells suggests that pemetrexed is a weak inhibitor of GART.** Cells were exposed to the indicated concentrations of either (6R)-DDATHF or pemetrexed with 5.6 μM thymidine for 24 hours. Nucleotides were then extracted from azaserine-blocked cells pulsed with 14C-glycine, and radiolabeled FGAR was separated by anion-exchange chromatography as described in the Methods section. Results are representative of pooled averages from two biological replicates.
and does not correlate with growth inhibition observed in the presence of thymidine (Figure 2-9B).

**ZMP accumulates to high levels in intact cells following AICART inhibition**

Inhibition of AICART results in accumulation of the reaction substrate, ZMP, behind the metabolic block. This was illustrated by measuring ZMP content in human skin fibroblasts from a patient identified to have AICART inactivating mutations in both alleles of the ATIC gene, the product of which produces the bifunctional enzyme, AICART and IMP-CH (Table 2-2). These cells were generously provided to us by Dr. Marie Vincent at Universitaires Saint-Luc (Brussels). To determine whether ZMP accumulated in these cells, HPLC was used to separate and quantify ZMP from whole-cell acid-soluble extracts (Figure 2-7). ZMP concentrations were calculated from a standard curve established with synthetic ZMP. To convert moles of ZMP to molarity, it was estimated that $10^6$ cells contained 1 µl cell water. ZMP levels in AICART -/- human skin fibroblasts were measured to 1.16 mM, greater than 1000-fold above those seen in the normal human lung fibroblast, WI-38 (Table 2-2). Interestingly, ZMP was only detected in these cells when grown in media containing dialyzed FBS. ZMP was undetectable when grown in normal FBS, which often contains free nucleotides as a result of breakage of red blood cells during the serum isolation process. Therefore, *de novo* purine synthesis was likely feedback inhibited by salvage of purine nucleotides in the presence of undialyzed FBS.

Figure 2-13 depicts a seminal experiment of this dissertation, showing that the ZMP pool in CEM cells expanded at growth inhibitory concentrations of pemetrexed. This indicated that the *de novo* purine synthesis target of pemetrexed or its metabolites was AICART, not GART. If GART inhibition played a role in the secondary effect of pemetrexed, ZMP accumulation would
Table 2-2. ZMP accumulates in human skin fibroblasts devoid of AICART activity.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>ZMP (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AICART -/- HSF</td>
<td>1.16 ± 0.34</td>
</tr>
<tr>
<td>WI-38</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Human skin fibroblasts (HSF) from a patient with inactivating mutations in both alleles of the AICART gene or WI38 human lung fibroblasts were cultured in medium containing dialyzed fetal bovine serum and harvested for ZMP measurements in mid-log growth as described in the Methods section. Results are presented as averages ± standard deviations from two biological and two technical replicates.
Figure 2-13. ZMP accumulation in CEM cells indicates that pemetrexed is an inhibitor of AICART. Cells were exposed to the indicated concentrations of either (6R)-DDATHF or pemetrexed with 5.6 μM thymidine for 48 hours. Nucleotides were then extracted, and ZMP was separated by HPLC as described in the Methods section. The area units under HPLC peaks corresponding to ZMP were converted to moles using the equation derived from a standard curve generated with synthetic ZMP. The cytosolic volume of 10⁶ cells was roughly equivalent to 1 μl cell water. Error bars are representative of standard deviation from two biological and two technical replicates.
not have been measurable, as observed with growth inhibitory concentrations of the GART inhibitor (6R)-DDATHF (Figure 2-13). Based on *in vitro* kinetic studies, it had also been suggested that pemetrexed was a DHFR inhibitor (54). However, if pemetrexed substantially inhibited DHFR, 10-CHO-H4PteGlu would be diminished, the GART reaction would not proceed, and thus ZMP would not accumulate because it would not be synthesized. To test this hypothesis, we measured the effect of the DHFR inhibitor methotrexate on ZMP accumulation in HCT116 cells (Figure 2-14). Although it had been previously reported that methotrexate inhibited AICART as well (36, 37), ZMP was undetectable in methotrexate-treated HCT116 cells (Figure 2-14). While methotrexate likely inhibited AICART in these cells, this experiment proved that an AICART block would be irrelevant when DHFR was also inhibited and would not result in ZMP accumulation.

When CEM cells were treated with AICA in the presence of pemetrexed, the accumulation of ZMP was exacerbated (Figure 2-15). This implied that blockage of *de novo* purine synthesis at the AICART step was restricting the flow of intermediates through this pathway. It is important to note that the concentrations of AICA used in this challenge did not cause measurable ZMP accumulation in the absence of pemetrexed (Figure 2-15). This suggested that AICART was either not rate-limiting to the flow of *de novo* purine synthesis, as suggested previously by Wilson *et al* (140), or that the rate of conversion of AICA to ZMP by APRT at this concentration was slower than the rate or conversion of ZMP to FAICAR by AICART (see Figure 2-3).

The massive level of ZMP measured in pemetrexed-treated CEM cells was quite surprising and impressive, reaching 1-2 mM following 48-hour exposure to pemetrexed and thymidine. This was over a 1000-fold increase in the steady-state concentration of ZMP in
Figure 2-14. ZMP does not accumulate in response to methotrexate treatment in HCT116 cells. HCT116 cells were exposed to 1 µM pemetrexed (PTX) or 1 µM methotrexate (MTX) for 24 hours in the presence of 5.6 µM thymidine (TdR). Nucleotides were then extracted, and ZMP was separated by HPLC as described in the Methods section. Synthetic ZMP (50 pmol) was added to the samples labeled + ZMP as an internal standard.
Figure 2-15. Expansion of the ZMP pool by AICA in pemetrexed-treated CEM cells indicates an AICART block. Exposure to AICA for 48 hours increased the intracellular ZMP levels in a dose-dependent manner in pemetrexed-treated, but not untreated CEM cells. Nucleotides were then extracted, and ZMP was separated by HPLC as described in the Methods section. Error bars are representative of standard deviation from two biological and two technical replicates.
untreated CEM cells, which was undetectable by our HPLC method, with an estimated sensitivity of 20 pmol ZMP. This result was extended to carcinoma cell lines, in which ZMP measurements were made at a single dose of pemetrexed (1 µM) and a single exposure time (24 hours) (Table 2-3). ZMP accumulated greater than 1000-fold in all cell lines tested, illustrating the conserved biochemical nature of this effect. The variability in ZMP measurements across cell lines was unexpected, especially in the TE85 osteosarcoma cell line, which accumulated 12.4 mM ZMP (Table 2-3). Of note, researchers at Eli Lilly Research Laboratories also observed a human cell line that accumulated greater than 15 mM ZMP following pemetrexed treatment (personal communication).

**ZMP inhibits cell growth**

The question arose as to whether the levels of ZMP measured in pemetrexed-treated tumor cells was sufficient to contribute to the growth inhibitory effects of the drug. This seemed likely, as concentrations of pemetrexed that caused dose-dependent accumulation of ZMP correlated with the thymidine-insensitive growth inhibitory concentrations of pemetrexed in HCT116 cells (Figure 2-16). To approach this question, we compared the levels of ZMP that accumulated in pemetrexed-treated cells with the levels of ZMP in cells treated with growth-inhibitory concentrations of AICAR, whose growth-suppressive effects are thought to be exclusively dependent on conversion to ZMP (141, 142). In CEM cells, exposure to growth-inhibitory concentrations of AICAR resulted in the accumulation of ZMP that was measured to be 0.4-2 mM (Figure 2-17A). A re-plot of this data showed a strong correlation ($r^2 = 0.9563$) between intracellular ZMP concentration and growth inhibition (Figure 2-17B). We therefore
Table 2-3. ZMP accumulates in human carcinoma cell lines following exposure to pemetrexed and thymidine.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Origin</th>
<th>ZMP (nmol / 10^6 cells) = mM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TdR</td>
</tr>
<tr>
<td>HCT116</td>
<td>Colon</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>H460</td>
<td>NSCLC</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>MCF7</td>
<td>Breast</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>WIDR</td>
<td>Colon</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>TE85</td>
<td>Osteosarcoma</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>A549</td>
<td>Lung</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>OVCAR-8</td>
<td>Ovarian</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>HELA</td>
<td>Cervical</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>HT1080</td>
<td>Fibrosarcoma</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>H526</td>
<td>SCLC</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Exponentially growing cells were treated with 1 µM pemetrexed and 5.6 µM thymidine for 24 hours. ZMP was measured by HPLC from trichloroacetic acid extracts as described in the Methods section. 1 mM ZMP is equivalent to 1 nmol ZMP in 1 µl cell water. ZMP in all cell lines tested in the presence of thymidine alone was less than 1 µM. Results are presented as averages ± standard deviations from three biological replicates.
Figure 2-16. The secondary growth-inhibitory mechanism of pemetrexed correlates with ZMP accumulation. HCT116 cells were treated with pemetrexed for 72 hours in the absence (circles) or presence (open triangles) of thymidine. ZMP (closed triangles) was measured by HPLC as described in the Methods section following 15 hour exposure to pemetrexed and thymidine. Measured values of ZMP in nmol/10^6 cells were converted to mM concentrations in cell water. Cultures were set up in triplicate, and error bars for cell growth are representative of standard deviation from three biological and two technical replicates. ZMP measurements are representative of standard deviation from two biological and two technical replicates.
Figure 2-17. ZMP inhibits growth in CEM cells. (A) CEM cells were exposed to the indicated concentrations of AICAR for 24 hours and ZMP (open circles) was analyzed by HPLC. Cell number (closed circles) was measured by Coulter counting. (B) A strong correlation exists between cell growth after 24 hours and the intracellular ZMP pool from panel A. Cultures were set up in triplicate, and error bars for cell growth are representative of standard deviation from three biological and two technical replicates. ZMP measurements are representative of standard deviation from two biological and two technical replicates.
concluded that ZMP levels measured in pemetrexed-treated cells were sufficient to be causal of the growth inhibition observed in the presence of thymidine.

If ZMP was the growth-inhibitory trigger for pemetrexed in the absence of thymidylate synthase inhibition, how was it that a preformed purine such as hypoxanthine could then completely rescue growth inhibition? Rescue of depleted purine nucleotides could not explain this question, as pemetrexed does not deplete GTP or ATP pools (Figure 3-7) (143). We performed a dose response of hypoxanthine rescue in CEM cells treated with pemetrexed and thymidine and made ZMP measurements at each of these doses. Surprisingly, we determined that hypoxanthine dose-dependently inhibited the accumulation of ZMP in CEM cells, which correlated with the growth rescue effect (Figure 2-18). We hypothesized that this was due to the negative feedback effect of intracellular nucleotides made from hypoxanthine on PPAT, the first and committed step of de novo purine synthesis, switching the cells from de novo synthesis to purine salvage.

The dynamics of ZMP accumulation

ZMP levels were measured as a function of time in HCT116 cells exposed to 1 µM pemetrexed (Figure 2-19). A time course of ZMP accumulation following pemetrexed exposure showed that, after an initial delay of a few hours, ZMP accumulated linearly a rate of 0.2 mM/hour up to 15 hours, and remained elevated at high levels out to 48 hours (Figure 2-19). Longer time points were not measured, but if the trend of this graph continued, ZMP levels would remain elevated above 1 mM for greater than 96 hours with continuous exposure to drug. We hypothesized that the plateau of ZMP accumulation between 15 and 24 hours was a reflection of an equilibrium being reached whereby the rate of accumulation (corresponding with
Figure 2-18. Hypoxanthine dose-dependently prevents the accumulation of ZMP and growth inhibition by pemetrexed in CEM cells. CEM cells were exposed to 1 µM pemetrexed and 5.6 µM thymidine for 48 hours with the indicated concentrations of hypoxanthine in the medium. Cell growth (closed circles) was determined by Coulter counting and ZMP (open circles) was measured by HPLC. Cultures were set up in triplicate, and error bars for cell growth are representative of standard deviation from three biological and two technical replicates. ZMP measurements are representative of standard deviation from two biological and two technical replicates.
Figure 2-19. ZMP accumulates as a function of time in HCT116 cells exposed to pemetrexed. Cells were exposed to 1 μM pemetrexed and 5.6 μM thymidine for 0, 4, 7, 15, 24, and 48 hours. Acid-soluble extracts were analyzed by HPLC as described in the Methods section. The area units under HPLC peaks corresponding to ZMP were converted to moles using the equation derived from a standard curve generated with synthetic ZMP. The cytosolic volume of $10^6$ cells was roughly equivalent to 1 μl cell water. Error bars are representative of standard deviation from two biological and two technical replicates.
the flow of purine synthesis) was being matched or slightly exceeded by the rate of ZMP metabolism to either higher order phosphates or to AICAR and AICA, which could efflux from the cell.

Interesting to us was the observation that after 15 hours of drug exposure, ZMP levels slowly diminished over time even in the continued presence of drug. The concentrations of pemetrexed that cause accumulation of ZMP in vitro in HCT116 cells (0.01-1 µM) (Figure 2-16) are maintained in serum of patients treated with this drug for more than 48 hours (144). However, serum levels of pemetrexed are probably not an accurate reflection of intracellular concentration of pemetrexed metabolites, as polyglutamation shifts the kinetics of the bidirectional transport equilibrium. Therefore, it is likely that intracellular pemetrexed concentrations are maintained at several orders of magnitude greater than concentrations measured in serum. We therefore wanted to determine the dynamics of ZMP accumulation after drug removal, which would likely be a more accurate reflection of the behavior of this effect in patients treated with pemetrexed. The schematic for this experiment is depicted in Figure 2-20. HCT116 cells were treated with 1 µM pemetrexed and thymidine for 15 hours to allow for maximal accumulation of ZMP. Cells were then either maintained in pemetrexed and thymidine supplemented media or bathed in media that did not contain drug. Acid-soluble extracts were taken at several time points following the pemetrexed pre-incubation, and ZMP measurements were made. We expected that ZMP levels would remain elevated in the absence of pemetrexed, because polyglutamation of pemetrexed would allow for intracellular retention of potent metabolites of the drug. We were surprised to determine that ZMP levels steadily decreased in the absence of pemetrexed (Figure 2-20).
Figure 2-20. Dynamics of ZMP accumulation in HCT116 cells in response to pemetrexed withdrawal. HCT116 cells were exposed to 1 µM pemetrexed and 5.6 µM thymidine for 15 hours (time 0) and drug either remained in the media (open circles) or was removed (closed circles) at this time. Whole-cell acid-soluble extracts were made from cells at the indicated time points following the initial 17 hour drug exposure, and were analyzed by HPLC for ZMP content as described in the Methods section. Error bars are representative of standard deviation from two biological replicates.
The pemetrexed analog, LCA, is an AICART inhibitor

When pemetrexed was discovered to be a potent anti-cancer agent, medicinal chemists at Eli Lilly synthesized many analogs of pemetrexed in an attempt to enhance the drug-like properties of the compound. Our lab received several hundred analogs on the pemetrexed pharmacophore. When we discovered that pemetrexed had AICART as a second target, we sought to discover pemetrexed analogs from this library that had AICART as a primary target. Previous studies suggested that compounds with an extra methylene group between C9 and C10 (homofolates) were inhibitory to the folate-dependent enzymes of *de novo* purine synthesis and had minimal effects on TS (145-147). Several members of this pemetrexed analog library were indeed homofolates, one of which was LCA (Figure 1-3). We therefore sought to determine whether LCA was an AICART inhibitor.

R.G. Moran performed an end-product reversal experiment in CEM cells that first suggested LCA was an AICART inhibitor (Figure 2-21). LCA inhibited the growth of CEM cells at low nM concentrations. Unlike pemetrexed, thymidine only slightly rescued the growth inhibitory effects of LCA. Hypoxanthine, however, shifted the growth inhibition curve roughly 5-fold, but did not fully rescue the antiproliferative effect of the drug. Conversely, AICA did not have any rescue effect on LCA-treated cells either when added by itself or when added with thymidine, suggesting an AICART block. Inclusion of both thymidine and hypoxanthine completely rescued the growth inhibitory effects of LCA (Figure 2-21). This result suggested that LCA had the opposite selectivity of pemetrexed. Thus, it likely inhibited AICART primarily, and had a secondary effect on thymidylate synthase. This was
Figure 2-21. The reversal pattern of CEM cell growth inhibition by LCA suggests that the primary target is AICART, with a secondary inhibition of thymidylate synthase. Exponentially growing CEM human leukemic cells were treated with the indicated concentrations of LCA alone (no add), or in the presence of thymidine (TdR, 5.6 µM), hypoxanthine (Hx, 32 µM), AICA (320 µM), or a combination of TdR with either Hx or AICA. Drug and modifying agents were added simultaneously and drug-containing media was changed at 48 hours. Cell growth was determined after 96 hours by Coulter counting and cell number is expressed relative to controls without drug. These experiments were performed by RG Moran.
quite exciting to us that the selectivity of the compound could be altered just by a one-carbon addition to the pemetrexed skeleton.

To definitively identify AICART as the primary site of action for LCA along de novo purine synthesis, ZMP accumulation in response to LCA was determined. Indeed, ZMP accumulated dose-dependently in response to LCA treatment in CEM cells across growth-inhibitory concentrations of this compound (Figure 2-22). This data in combination with Figure 2-21 indicated that the primary target of LCA was AICART. For the first time, we had an antifolate in hand that was primarily an AICART inhibitor. In fact, LCA was likely a more potent inhibitor of AICART in vivo than pemetrexed, as measured by the extent of ZMP accumulation at equivalent doses. In a screen of three different cancer cell lines (CEM, HCT116, and H460), ZMP accumulated to a greater extent following LCA treatment than pemetrexed treatment (Figure 2-23). The therapeutic effects of LCA will be more extensively discussed in Chapter 4 of this dissertation.

**Recombinant human AICART is sensitive to substrate inhibition in vitro**

Based on in vitro $K_i$ measurements for pemetrexed with recombinant mouse GART and human AICART, it was determined that GART was the primary de novo purine synthesis target of pemetrexed (54) (Table 1-1). However, our whole-cell biochemical data suggested otherwise. It was not completely clear what was causing the discrepancy between the in vitro and in situ kinetics. We therefore expressed and purified recombinant human AICART (Figure 2-8) in an attempt to address this contradiction.

We chose a continuous spectrophotometric assay to measure AICART activity by monitoring the formation of the co-product of the AICART reaction, $\text{H}_4\text{PteGlu}$ at 298 nm. Our
**Figure 2-22.** ZMP accumulates dose-dependently in CEM cells exposed to LCA. Cells were exposed to the indicated concentrations of LCA for 24 hours. Acid-soluble extracts were analyzed by HPLC as described in the Methods section. The area units under HPLC peaks corresponding to ZMP were converted to moles using the equation derived from a standard curve generated with synthetic ZMP. The cytosolic volume of $10^6$ cells was roughly equivalent to 1 µl cell water. Error bars are representative of standard deviation from two biological and two technical replicates.
Figure 2-23. Comparing ZMP accumulation across cell lines in response to pemetrexed and LCA. The indicated cell lines were exposed to 1 µM pemetrexed and 5.6 µM thymidine or 1 µM LCA for 48 hours. Acid-soluble extracts were subjected to HPLC analysis of ZMP as described in the Methods section.
measured kinetic parameters for AICART are listed in Table 2-1, and are mostly consistent with parameters measured by others (121). The $K_m$ for 10-CHO-H$_4$PteGlu was determined for both the 6R and 6R,6S isomers (Figure 2-24 and Table 2-1), both routinely synthesized from 6R or (6R,6S)-5-CHO-THF (see Methods section). In most experiments, 100 $\mu$M 10-CHO-THF was used, because higher concentrations were inhibitory to AICART (Figure 2-25). While interesting, this observation is likely not physiologically relevant, as the concentration of 10-CHO-THF in the cell rarely rises above 10 $\mu$M (14).

The $K_m$ for ZMP was also determined by a continuous spectrophotometric kinetic analysis of product formation (Figure 2-26). Indeed, the previous $K_i$ determinations for pemetrexed were performed with 50 $\mu$M ZMP and 100 $\mu$M 10-CHO-THF (54) (Table 1-1). However, the levels of ZMP we measured from whole-cell acid-soluble extracts were greater than 1 mM in all cell lines tested (see Table 2-3). This is greater than 100 times the measured $K_m$ for ZMP and greater than 20 times the substrate concentration used to determine the $K_i$ of pemetrexed. We therefore sought to determine whether AICART activity was affected by elevated substrate concentrations. We extended the concentration used for $K_m$ measurements out to 1.5 mM ZMP, and were surprised to see a drastic diminution in enzyme activity beyond 500 $\mu$M ZMP (Figure 2-27). In fact, at 1.5 mM ZMP, AICART activity was diminished by greater than 90% from its maximal velocity. This finding is of importance, because it suggests AICART inhibition is enhanced by accumulation of substrate as a result of continued flow of the de novo purine synthesis pathway. Therefore, even weak inhibition of AICART may dramatically affect enzyme activity as substrate accumulates. Additionally, this result suggests that a dead-end complex may be forming between AICART and ZMP. It is therefore likely that substrate
inhibition of AICART by ZMP is contributing to the inhibitory effect of pemetrexed in cells, a finding that was likely not accounted for in the initial *in vitro* kinetic workup.
Figure 2-24. Km determination for 10-CHO-THF on recombinant human AICART. Initial velocity measurements were taken in the presence of the indicated concentrations of (A) 6R-10-CHO-THF or (B) 6R,6S-10-CHO-THF as described in the Methods section. All reactions contained 3 µg AICART and 50 µM ZMP. Error bars are representative of standard deviation of triplicate measurements.
Figure 2-25. 10-CHO-THF inhibits AICART activity at high concentrations. Initial velocity measurements were taken in the presence of the indicated concentrations of 6R-10-CHO-THF as described in the Methods section. All reactions contained 3 µg AICART and 50 µM ZMP. Error bars are representative of standard deviation of triplicate measurements.
Figure 2-26. Km determination for ZMP on recombinant human AICART. Initial velocity measurements were taken in the presence of the indicated concentrations of ZMP as described in the Methods section. All reactions contained 3 µg AICART and 100 µM 6R-10-CHO-THF. Error bars are representative of standard deviation of triplicate measurements.
Figure 2-27. Recombinant human AICART is sensitive to substrate inhibition by ZMP. Initial velocity measurements were taken in the presence of the indicated concentrations of ZMP as described in the Methods section. All reactions contained 3 µg AICART and 100 µM 6R-10-CHO-H₄PteGlu. Error bars are representative of standard deviation of triplicate measurements.
DISCUSSION

Chemical synthesis aimed at eliminating the chiral center of carbon 6 of DDATHF resulted in the discovery of pemetrexed, a potent inhibitor of thymidylate synthase (53). However, it was quickly appreciated that pemetrexed had other folate-dependent targets contributing to its therapeutics (53, 54). The successful clinical utility of pemetrexed in the treatment of MPM and NSCLC, diseases typically unresponsive to thymidylate synthase inhibitors (82, 83), led us to consider the biological relevance of thymidylate synthase-independent targets of pemetrexed. Although in vitro enzymology studies suggested the secondary target of pemetrexed was GART (54), data presented in this chapter defined the secondary target of pemetrexed as the second folate-dependent enzyme of de novo purine synthesis, AICART (Figure 2-3). Central to the elucidation of AICART as the second target of pemetrexed were two key observations. First, the discovery that AICA did not have any growth-rescue effect on CEM cells treated with pemetrexed in the presence of thymidine, and slightly potentiated the growth-inhibitory effect of the drug (Figure 2-9), suggested that passage through de novo purine synthesis was blocked at AICART (Figure 2-3). Second, the dose-dependent accumulation of the AICART reaction substrate, ZMP, following pemetrexed treatment, an effect not seen in response to the GART inhibitor (6R)-DDATHF, illustrated that the flow of de novo purine synthesis was inhibited at the AICART step by pemetrexed (Figure 2-13).

Why has AICART inhibition by pemetrexed or its metabolites not been previously recognized?

In vitro kinetic analysis of folate-dependent targets of pemetrexed and its polyglutamated metabolites discounted AICART inhibition as a biologically relevant effect of the drug (54) (Table 1-1). Inhibition assays with a fixed concentration of ZMP (50 µM) defined the $K_i$ of
monoglutamated and pentaglutamated pemetrexed as 3.58 µM and 0.265 µM, respectively. The $K_i$ measured against GART as a pentaglutamate was 0.065 µM, which was more favorable, but still weak. Regardless, from this study, the de novo purine synthesis target of pemetrexed was labeled as GART, and remained as such for the next decade. Our studies, however, defined pemetrexed or its metabolites as a potent AICART inhibitor in situ, with minimal inhibitory activity against GART. The massive accumulation of ZMP subsequent to an AICART block by pemetrexed suggested the drug potently inhibited the enzyme (Table 2-3). Additionally, siRNA knockdown of AICART by greater than 95% resulted in significantly less ZMP accumulation than pemetrexed treatment as shown in Chapter 3 of this dissertation.

These observations, coupled with the previous enzymology of in vitro AICART inhibition, suggested the behavior of the pemetrexed-AICART interaction in situ differed from the behavior in the controlled in vitro environment. We propose that an in situ metabolic trapping mechanism explains this discrepancy (Figure 2-28A). The AICART reaction obeys an ordered-sequential binding mechanism, in which 10-CHO-H$_4$PteGlu$_n$ binds first, followed by ZMP (121, 123). As ZMP accumulates behind an AICART block by pemetrexed, the binary AICART-pemetrexed complex will reform a ternary inhibited complex whenever ZMP dissociates. This would prevent the dissociation of pemetrexed from the binary complex, thus metabolically trapping the enzyme in inhibited complexes. This trapping mechanism is similar to that described for FdUMP to thymidylate synthase in the presence of an expanded pool of 5,10-methylene-H$_4$PteGlu$_n$ induced by co-treatment of 5-FU with leucovorin (42, 85).

We therefore propose that the $K_i$ of pemetrexed and its polyglutamates for AICART would decrease as the ZMP concentration increases. This effect would not have been measured with the fixed concentration of ZMP (50 µM) used in the prior in vitro studies (54). We
Figure 2-28. Proposed kinetic mechanisms for inhibition of AICART by ZMP. (A) ZMP metabolically traps AICART in a ternary complex with pemetrexed. (B) ZMP traps AICART in a dead-end complex.
expressed and purified recombinant human AICART (Figure 2-8) and attempted to prove our hypothesis by repeating the *in vitro* $K_i$ studies in the presence of pemetrexed and increasing concentrations of ZMP. However, we ran into a dilemma. We discovered that AICART was extraordinarily sensitive to substrate inhibition by ZMP at concentrations greater than 500 µM (Figure 2-27). This finding was interesting in and of itself, as it suggests that ZMP forms a dead-end complex with AICART at these physiologically relevant concentrations of ZMP measured in pemetrexed-treated cells (Figure 2-28B). However, this effect also limits our ability to use activity measurements to test the metabolic trapping hypothesis. Confounded by effects of substrate inhibition, we were only able to use this kinetic approach to test the metabolic trapping hypothesis at concentrations of ZMP that did not exceed 500 µM. Comparing the concentration-dependent inhibitory effect of pemetrexed on AICART activity at 50 µM and 500 µM ZMP, we did not measure enhanced enzyme inhibition at the higher ZMP concentration (Figure 2-29). We therefore propose to determine the extent of trapping by direct binding measurements. One could theoretically determine the extent of metabolic trapping by incubating recombinant human AICART with $^3$H-pemetrexed and increasing concentrations of ZMP, and passing the ternary complex through a Sephadex G-50 column. These experiments are underway, and the assay workup will be similar to that of the microassay for FPGS activity, previously developed by our lab (148).

*Is there biological relevance to the variability of ZMP accumulation across cell lines?*

Our initial observation that ZMP accumulated following pemetrexed treatment in CEM human leukemic cells (Figure 2-13) prompted us to question the generality of this effect in cells of varying histologies (Table 2-3). While all cell lines in our screen measured ZMP
Figure 2-29. ZMP (500 µM) does not increase the potency of pemetrexed as an AICART inhibitor. Initial velocity measurements were taken in the presence 50 µM ZMP (closed circles) or 500 µM ZMP (open circles) with the indicated concentrations of pemetrexed as described in the Methods section. All reactions contained 3 µg AICART and 100 µM 6R-10-CHO-THF. The ordinate is expressed as such to normalize the data for substrate inhibition by ZMP at 500 µM. Error bars are representative of standard deviation of triplicate measurements.
accumulation greater than 1000-fold above control, there was significant variability in static measurements across and even among histologies. What could explain this variability, and more importantly, was there additional therapeutic gain when the ZMP pool was expanded beyond a certain point? This latter question will be addressed first.

Comparing ZMP accumulation with growth inhibition in response to pemetrexed (Figure 3-12) illustrated no correlation between these measurements. For instance, while the TE85 osteosarcoma cell line measured ZMP accumulation above 12 mM, by far the highest measurement in our screen, TE85 was the fifth most sensitive cell line to pemetrexed. Indeed, the H460 NSCLC cell line was as sensitive to pemetrexed as TE85, but accumulated roughly 6-fold less ZMP (Table 2-3). This suggests that other factors, either biochemical or genetic, contribute to the variability among these cell lines to growth inhibition, independent of excessive ZMP accumulation. I hypothesize that there is a threshold of ZMP accumulation that must be exceeded to trigger AMPK activation and subsequent signaling events downstream of AMPK (detailed in Chapter 3), and the $V_{\text{max}}$ of AMPK likely occurs at ZMP concentrations well below levels we are measuring in response to pemetrexed. In support of this hypothesis, studies by Corton et al showed that the half maximal effect of ZMP on activation of purified rat liver AMPK was 164 µM ± 44 (132). Additionally, an unpublished experiment by A.C. Racanelli in our lab showed that AMPKα was maximally hyperphosphorylated at T172 (see Chapter 3 for details) in CEM cells following a 0.1 µM pemetrexed treatment for 48 hours (A.C. Racanelli, Ph.D. dissertation, 2009). Figure 2-13 shows that ZMP levels at this concentration and time point are just below 1 µM.

Several hypotheses were considered to identify the cause of variability in ZMP concentrations measured across cell lines. First, it was suggested that variability could arise
from differences in the flow of intermediates through the de novo purine synthesis pathway. However, the doubling time for each cell line tested was roughly 20-24 hours, suggesting that the rate of DNA synthesis and subsequent necessity for purines across cell lines would be roughly equivalent. Therefore, drastic differences in the flow of de novo purine synthesis would be an unlikely explanation of the variability. To test this, however, one could measure the rate of de novo purine synthesis in these cell lines by measuring the extent of $^{14}$C-glycine or $^{14}$C-formate incorporation into ATP as a function of time.

Alternatively, but not necessarily mutually exclusive from the above hypothesis, the rate of metabolism of ZMP may be different across these cell lines. For instance, ZMP that accumulates behind the AICART block may be catabolized to the cell-permeable precursors AICAR and/or AICA. Additionally, ZMP is converted to the –di and-triphosphate nucleotide forms, and measurable amounts of ZTP are detected by HPLC in acid-soluble extracts within 15 minutes of AICAR treatment in rat hepatocytes (132). Our HPLC protocol could be adapted to optimally measure ZDP and ZTP to determine whether this was occurring. Furthermore, an HPLC protocol was previously described to measure AICAR and/or AICA with a C18 reverse phase column (129, 149), but interpretation of these measurements may be complicated, as these metabolites can efflux from the cell.

Most likely, the variability in ZMP accumulation across cell lines is due to differences in transport and metabolism of pemetrexed. For instance, variability in FPGS activity would influence the intracellular concentration of polyglutamated metabolites of pemetrexed, known to be more potent inhibitors of AICART than the parent compound (54). Differences in pemetrexed uptake across cell lines may also contribute to variability, and this hypothesis could be tested experimentally with $^3$H-labeled pemetrexed uptake studies. It would be interesting to
compare expression of the RFC and PCFT across these cell lines to determine whether transporter expression correlates with the ZMP measurements. Regardless of differences in ZMP concentrations, I suspect that the levels of ZMP measured in all cell lines tested is well above the threshold to trigger activation of AMPK and provide the signal necessary to promote signaling events described in Chapter 3.

**Defining the pharmacophore of an AICART inhibitor**

The discovery that pemetrexed targets AICART is of interest both from a therapeutic standpoint (see Chapters 3 and 4) as well as from a drug development standpoint. It is not understood why the substitution of a pyrrolopyrimidine ring for the 5-deazapteridine of DDATHF (Figure 1-3) directs pemetrexed away from GART and towards TS and AICART. The observation that addition of an extra methylene group between the C9 and C10 position of the folate backbone (homofolate) directed compounds towards the folate dependent enzymes of de novo purine synthesis (145-147) led us to the discovery that LCA had AICART as its primary target (Figures 2-21 and 2-22). Therefore, our studies are beginning to define the pharmacophore of an AICART inhibitor, and the discovery of potent and selective inhibitors of AICART is warranted based on the consequences of AICART inhibition described in this dissertation.

To date, very few potent inhibitors of AICART have been discovered. Structure-based drug design efforts by the Burroughs Wellcome group resulted in the synthesis of two sulfamido-bridged 5,8-dideazafolate analogs (BW1540 and BW2315) that potently inhibited (Kᵢ ≈ 7 nM) AICART (150). Although these compounds were potent AICART inhibitors *in vitro*, they were weakly cytotoxic in human colon cancer cell lines, with IC₅₀ values of approximately 4 µM
(150). This suggests that these compounds are poor substrates for transport and polyglutamation. Nevertheless, crystal structures of BW1540 and BW2315 bound in the active site of human AICART (PDB codes 1P4R and 1PLO and Figure 2-5) revealed key structural features of the AICART folate-binding site that may be exploited in future drug discovery efforts to direct inhibitors away from GART and TS and towards AICART. One such unique feature was the presence of an oxyanion hole in the AICART active site (residues 450-468), previously shown to stabilize the transition-state intermediates of several enzymes of the α/β hydrolase family (151, 152). Hydrogen bonding interactions between the sulfonyl oxygens of these inhibitors and nitrogen atoms of several residues in this oxyanion hole were suggested to be a primary determinant of the positioning of these inhibitors in the AICART active site, and likely stabilize the transition state during the formyl-transfer reaction (153). Neither GART nor TS possess this structural feature (153).

The Moran lab has acquired a library of pemetrexed and DDATHF analogs synthesized primarily by chemists at Eli Lilly. We propose a cell-based screening assay to determine the extent of AICART inhibition by these compounds. Based on our findings with LCA, which came from this same library set, we believe the likelihood of a hit is good. Using ZMP accumulation as an indicator of AICART inhibition, we will build a layer of kinetics into this assay by performing the screen in situ, an important factor that was disregarded in the structure-based drug design scheme described above. In addition to kinetics, an additional advantage of an in situ screen as opposed to an in vitro screen (which we are now equipped to do) is the ability to rule out potential interference by other folate-dependent targets. This is important, as there is clear hierarchy that exists in folate-dependent target inhibition. For instance, a compound that is an AICART inhibitor, but also potently inhibits GART, would make AICART inhibition
irrelevant, as no ZMP would be synthesized subsequent to GART inhibition. Likewise, an AICART inhibitor that is also a potent DHFR inhibitor (like methotrexate; see Figure 2-14) would not accumulate ZMP, since 10-CHO-H₄PteGlu would be depleted, and therefore *de novo* purine synthesis intermediates would not be synthesized.

Several studies have led to the discovery of non-folate-based AICART inhibitors. Crystallographic comparisons of both the apo and substrate-bound forms of AICART indicated that the active site was fairly rigid, and no major conformational changes took place upon ligand binding (122, 150). Therefore, virtual ligand docking has been used to screen for novel AICART inhibitors (153). In a virtual screen of 2,000 compounds selected from the NCI diversity set, 44 compounds were identified as potential AICART inhibitors. *In vitro* kinetic analysis identified 8 of these compounds as μM inhibitors, and one compound, NSC30171, had a $K_i$ of 154 nM (153). The discovery that the AICART active site is constructed by homodimerization (Figure 2-5) suggests compounds that hinder dimerization would be selective as AICART inhibitors over other folate-dependent enzymes that function as monomers. This concept was explored by screening a 40,000-member peptidomimetic library for dissociative AICART inhibitors (154). One inhibitor from this screen, Cappsin 1, was identified to be a low μM noncompetitive, dissociative inhibitor of AICART. Additionally, Cappsin 1 did not inhibit GART or DHFR. It would be of importance to determine whether these novel AICART inhibitors described in this section display similar biological effects observed with pemetrexed (see Chapter 3 and 4).

**Diminution of ZMP following drug washout**

To determine whether ZMP levels remained elevated following pemetrexed removal, we performed an experiment in HCT116 (and H460) cells in which a 17 hour treatment with
pemetrexed was followed by drug washout. Time points were taken intermittently following washout and ZMP measurements were made (Figure 2-20). We were surprised and a bit puzzled by the result. Our data clearly showed that in the absence of pemetrexed, ZMP levels were diminishing with time at a rate of 0.08 nmol/10^6 cells hr^-1. Our expected outcome for this experiment was that ZMP would continue to accumulate following washout, as polyglutamation would trap pemetrexed metabolites intracellularly, and target inhibition would continue in the absence of an exogenous source of drug.

Several hypotheses were generated that might explain this data. One possibility is that pemetrexed monoglutamates are the inhibitory forms of the drug towards AICART. Since pemetrexed is such an efficient substrate for polyglutamation by FPGS (53, 56, 57), drug washout would remove the “active” form of the drug. Any remaining intracellular monoglutamates would be polyglutamated and would no longer target AICART. Since pemetrexed is such a good FPGS substrate, in order for the cell to maintain a sufficient pool of pemetrexed monoglutamates, FPGS would need to be saturated with substrate. If the metabolic trapping mechanism described above is occurring, the potency of pemetrexed monoglutamates as AICART inhibitors may be sufficient to promote ZMP accumulation. Interestingly, in vitro, pemetrexed monoglutamates are more potent inhibitors of AICART than GART (Table 1-1). Studies addressing the transport and polyglutamation profile of ^3H-pemetrexed are warranted to address this hypothesis.

Another possibility is that efflux of pemetrexed is rapid, and the presence of an exogenous source of drug maintains an equilibrium necessary for target inhibition. Again, transport studies with ^3H-pemetrexed would allow us to address this hypothesis. Upon removal of drug from the media, it is presumed from this data that pemetrexed is no longer inhibiting
AICART. Therefore, the rate of diminution in the ZMP pool measured would be a reflection of ZMP metabolism to either higher order phosphates or to AICAR and AICA. To determine whether an AICART block is still present in the absence of exogenous drug, cells could be treated with 320 µM AICA. If AICART were still inhibited, ZMP would accumulate (Figure 2-15).
CHAPTER 3
PEMETREXED TRIGGERS AN ENERGY-SENSITIVE METABOLIC CHECKPOINT SECONDARY TO AICART INHIBITION

The metabolic block induced consequential to AICART inhibition by pemetrexed resulted in massive intracellular accumulation of ZMP, presumably due to continued flow of intermediates through \textit{de novo} purine synthesis in the presence of a downstream blockade. The unusual therapeutic profile of pemetrexed coupled to the observation that ZMP was not accumulating in response to DDATHF or methotrexate led us to question how this effect that distinguished pemetrexed from other antifolates was contributing to the cellular response to the drug. Our studies were guided in the direction of signaling through AMP-activated protein kinase (AMPK), because of parallel literature using the riboside of ZMP, 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), as a chemical tool to activate AMPK. AICAR was shown \textit{in vitro}, \textit{in situ}, and \textit{in vivo} to activate AMPK following its metabolism to ZMP (132, 155, 156). We therefore suspected that pemetrexed was indirectly modulating signaling processes through AMPK secondary to ZMP accumulation behind the AICART block. This hypothesis was examined in the experiments described in this chapter.

\textit{AMP-activated protein kinase – a cellular energy sensor}

Signaling mediated by AMP-activated protein kinase (AMPK) is the primary cellular regulatory mechanism for maintaining energy homeostasis. Its activation in response to diminished cellular ATP levels promotes catabolic processes to generate energy while inhibiting anabolic processes that consume it (157). Cancer cells routinely possess genetic abnormalities in PI3K-AKT and MAPK signaling that hyperactivate the mammalian target of rapamycin complex
in the absence of extracellular stimuli, leading to autonomous growth and proliferation (Figure 3-1) (158, 159). AMPK constitutes an endogenous controlling mechanism that limits mTORC1 activity. Therefore, modulation of AMPK signaling presents as a probable point of therapeutic intervention for molecularly targeted cancer agents (160).

The Energy Charge Hypothesis: AMPK was originally identified as a kinase allosterically modulated by adenosine-5’-monophosphate (AMP) (161). We now know that AMPK responds to changes in the AMP:ATP ratio, the most sensitive indicator of cellular energy status (162, 163). The first observation that nucleotides themselves controlled cellular signaling processes was by D.E. Atkinson, who showed that AMP and ATP reciprocally regulated enzymes of the glycolytic pathway (164). At the time, it was thought that nucleotides acted directly on these enzymes, which led Atkinson to postulate this sensitivity to AMP and ATP as the energy charge hypothesis. It is now recognized that AMPK is the nucleotide sensor in this cascade, not the target enzymes themselves.

That AMPK is sensitive to the AMP:ATP and not the ADP:ATP ratio is attributed to the reaction equilibrium maintained by adenylate kinase (Reaction 1). Healthy cells keep the ATP:ADP ratio at approximately 10:1 by ATP synthase (Reaction 2) to ensure adequate ATP for cellular processes. The driving force for the ATP synthase reaction comes from the downhill flow of protons across the inner mitochondrial membrane, a gradient maintained by oxidative phosphorylation pumping protons against this gradient (165).

\[
\begin{align*}
(1) & \quad 2\text{ADP} \leftrightarrow \text{ATP} + \text{AMP} \\
(2) & \quad \text{ADP} + \text{Pi} \Rightarrow \text{ATP}
\end{align*}
\]

ATP synthase disrupts the equilibrium imposed by adenylate kinase. Therefore, to maintain equilibrium, the adenylate kinase reaction is driven from right to left. The leftward adenylate
Figure 3-1. Cellular signaling upstream and downstream of mTORC1 and mTORC2. The mTOR signaling pathway senses and responds to changes in cellular nutrient, energy, and redox status. The best characterized of the two mTOR complexes, mTORC1, controls proliferative capacity in part by regulating processes such as CAP-dependent translation, ribosome biogenesis, and autophagy. mTORC2 contributes to the negative-feedback regulation of mTORC1 signaling, and has also been implicated in regulating ion transport, cell growth, and cytoskeletal organization.
kinase reaction coupled with the ATP synthase reaction maintains the ATP:AMP ratio at approximately 100:1 under conditions of ATP homeostasis. Under conditions of cellular stress, the rate of ATP consumption increases, driving the adenylate kinase reaction from left to right to maintain equilibrium, generating AMP in the process. Dividing both sides of the equilibrium reaction of adenylate kinase (\([ATP][AMP] = [ADP]^2 K\)) by \([ATP]^2\), it can be determined that the AMP:ATP ratio varies by the square of the ADP:ATP ratio. For example, if the ADP:ATP ratio rises by 10-fold, the AMP:ATP ratio would rise by 100-fold. Therefore, it can be seen how the AMP:ATP ratio is a much more sensitive indicator of compromised energy status than the ADP:ATP ratio (163).

**Subunit Structure:** AMPK is a heterotrimeric protein consisting of a catalytic \(\alpha\) subunit and regulatory \(\beta\) and \(\gamma\) subunits. Humans have seven genes that encode isoforms of the three subunits, creating the potential for great diversity in complex formation. Isoform expression seems to be tissue-specific, but much is still to be learned about the regulation and characteristics of the different isoforms both as monomers and as part of the trimeric complex. Two genes encode \(\alpha\) subunits. \(\alpha1\) is ubiquitously expressed while \(\alpha2\) is most abundant in skeletal and cardiac muscle as well as in the liver (166). Both \(\alpha\) subunits share 90% sequence identity in their N-terminal catalytic domains, which contain a conserved threonine residue (T172) in an activation loop, a feature shared by other serine/threonine protein kinases. Phosphorylation of this residue was thought to be essential for kinase activity of the trimer (167, 168). However, data presented in this chapter as well as from others suggests this essentiality of T172 phosphorylation may be substrate-specific (169). The \(\alpha\) subunit also contains an autoinhibitory domain, postulated from structural studies to bind the catalytic domain and constrain its mobility (170). The C-terminal domains of the \(\alpha\) subunit isoforms share 60% sequence identity and
contain the binding site for the β subunit (171, 172). Two genes encode isoforms of the β subunit. β1 is ubiquitously expressed and β2 is most abundant in skeletal muscle (173). The C-terminal domain of the β subunit acts as a scaffold, binding both the α and γ subunits (171, 174). The β subunit also contains a glycogen-binding domain, which is thought to allow AMPK to sense stored cellular energy in addition to free energy (175, 176). Three genes encode isoforms of the γ subunit, which contain binding sites for AMP and ATP (177). The N-termini of the three isoforms have a conserved region that binds to the β subunit (178). Structural studies show the γ subunit contains four tandem cystathione-β-synthase (CBS) motifs that generate two Bateman domains, common in proteins that bind adenosine-containing ligands (171, 177). AMP binds cooperatively to two of the CBS motifs, and their binding is competitive with ATP. A third AMP was tightly bound in the crystal structure, and it was hypothesized that this AMP did not exchange with ATP. The fourth site did not contain either AMP or ATP (171). Binding assays showed enhanced association of AMP to the γ subunit as concentrations rose through the µM range, even in the presence of physiologic ATP concentrations (171).

**Mechanism of Activation:** The binding of AMP to the γ subunit allosterically activates the kinase by several known mechanisms. Structural studies suggest that AMP binding to the γ subunit causes a conformational shift in the α subunit, relieving the constrained conformation imposed by the autoinhibitory domain (170). Additionally, AMP allosterically modulates the conformation of residues surrounding T172 in the α subunit, protecting this key residue from phosphatases. Therefore, it is suggested that AMP protects T172 from dephosphorylation rather than promoting its phosphorylation (179, 180). It is not clear whether the above-mentioned consequences of AMP binding are mutually exclusive, but both seem to be necessary for full
activation of the kinase. Importantly, ZMP mimics both effects of AMP, the allosteric activation of AMPK as well as inhibition of T172 dephosphorylation (132).

**Upstream Kinases:** AMPKα T172 is the substrate for several known upstream kinases. The predominant T172 kinase in most cells is thought to be LKB1 (181-183). LKB1 was originally identified as a tumor suppressor gene product mutated in Peutz-Jeghers syndrome, a premalignant familial disorder characterized by benign hamartomas of the gastrointestinal tract (184). Of significance, LKB1 is also frequently mutated in lung adenocarcinomas (Figure 3-2), and it has been suggested that the tumor suppressor function of LKB1 is mediated through effects downstream of AMPK activation (185-187). LKB1 expression is ubiquitous and it functions as part of a complex with the accessory proteins STRAD and MO25 (182). LKB1 is thought to constitutively phosphorylate AMPKα T172, a mark easily removed by phosphatases in the absence of protection by allosteric binding of AMP (see above) (181). In the presence of calcium or a calcium ionophore, AMPKα T172 is also phosphorylated by calmodulin-dependent protein kinase kinases (CAMKKs), especially CAMKKβ (188-190). Phosphorylation of AMPKα T172 by CAMKKβ has been shown in the absence of elevated AMP, suggesting CAMKKβ itself or downstream effectors elicit control of T172 phosphatases. While calcium alone stimulates activity of AMPK, AMP does synergize with calcium to further stimulate AMPK activity, supporting the conclusion that full activity requires both T172 phosphorylation and allosteric modulation (191). Most recently, a third upstream kinase of AMPKα T172 has been identified, transforming growth factor-β-activated protein kinase-1 (TAK1), which may play a role in the autophagy response induced by AMPK (see Chapter 4) (192-194).
Figure 3-2. The most commonly mutated genes upstream of mTORC1 in human non-small cell lung cancer cell lines. Mutation data from the Wellcome Trust Catalog of Somatic Mutations in Cancer (COSMIC) database was compiled from 67 human non-small cell lung cancer cell lines.

Compiled from: http://www.sanger.ac.uk/perl/genetics/CGP/core_line_viewer?action=cell_lines
**Downstream targets of AMPK**

The signals transmitted by AMPK to downstream targets temporarily stimulate energy-producing processes and limit nonessential energy-consuming processes to restore cellular ATP homeostasis. To accomplish this, AMPK inhibits both protein synthesis and lipid metabolism, and promotes the mobilization and breakdown of glucose. A list of several well-characterized direct targets of AMPK is presented in Table 3-1 (195-209). The downstream targets of AMPK predominantly studied in this dissertation converge on mTORC1, which is discussed in detail as follows.

**PI3K-AKT signaling:** The PI3K-AKT signal transduction pathway responds to both extracellular and intracellular signals to coordinate cell growth and proliferation by promoting the anabolic processes of protein and lipid biosynthesis, while limiting catabolic processes such as autophagy (210). Effectors of the pathway are activated by environmental stimuli such as growth factors, oxygen, amino acids, and glucose (Figure 3-1). Deregulation of components of this signaling network are hallmarks of most human cancers and a number of benign hamartoma syndromes, conditions in which cell growth and survival are uncoupled from environmental triggers. Therefore, therapeutic interventions that restrict uncontrolled signaling through the PI3K-AKT pathway are being heavily pursued for the management of these diseases (158, 211).

**Signals converging on mTOR:** Class I phosphatidylinositol-3-OH kinases (PI3K) are heterodimeric proteins consisting of a regulatory p85 subunit and a catalytic p110 subunit (212). The binding of growth factors to receptor tyrosine kinases (RTK) promotes the autophosphorylation of tyrosine residues on the cytoplasmic tails of the receptor. This recruits PI3K and adaptor proteins such as insulin receptor substrates (IRS1 and IRS2) to the RTK, where PI3K is activated by phosphorylation. The GTPase RAS can also recruit, bind, and
Table 3-1: Direct Targets of AMP-Activated Protein Kinase

<table>
<thead>
<tr>
<th>Target</th>
<th>Phosphorylation Site</th>
<th>Target Effect</th>
<th>Pathway Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Protein Synthesis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raptor</td>
<td>S792</td>
<td>↓ Interaction</td>
<td>↓ mTORC1 signaling</td>
<td>195</td>
</tr>
<tr>
<td>TSC2</td>
<td>S1387, T1271</td>
<td>↑ Activity</td>
<td>↓ mTORC1 signaling</td>
<td>196</td>
</tr>
<tr>
<td>eEF2 Kinase</td>
<td>S398</td>
<td>↑ Activity</td>
<td>↓ translation elongation</td>
<td>197</td>
</tr>
<tr>
<td><strong>Lipid Metabolism</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACC</td>
<td>S79</td>
<td>↓ Activity</td>
<td>↓ fatty acid synthesis</td>
<td>198</td>
</tr>
<tr>
<td>HMG-CoA-Reductase</td>
<td>S872</td>
<td>↓ Activity</td>
<td>↓ cholesterol synthesis</td>
<td>199</td>
</tr>
<tr>
<td>Hormone-sensitive lipase</td>
<td>S554</td>
<td>↓ Activity</td>
<td>↓ lipolysis</td>
<td>200</td>
</tr>
<tr>
<td><strong>Carbohydrate Metabolism</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GLUT4</td>
<td>N/A</td>
<td>↑ Membrane localization</td>
<td>↑ glucose uptake</td>
<td>201</td>
</tr>
<tr>
<td>Glycogen synthase</td>
<td>S8</td>
<td>↓ Activity</td>
<td>↓ glycogen synthesis</td>
<td>202</td>
</tr>
<tr>
<td>Phosphofructokinase 2</td>
<td>S466, S461</td>
<td>↑ Activity</td>
<td>↑ glycolysis</td>
<td>203</td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mTORC2</td>
<td>S171</td>
<td>↑ Cytosolic localization</td>
<td>↓ transcription</td>
<td>204</td>
</tr>
<tr>
<td>Endothelial NO synthase</td>
<td>S1177</td>
<td>↑ Activity</td>
<td>↑ NO</td>
<td>205</td>
</tr>
<tr>
<td>p300</td>
<td>S89</td>
<td>↓ Interaction</td>
<td>↓ transcription</td>
<td>206</td>
</tr>
<tr>
<td>HNF4-a</td>
<td>S313</td>
<td>↓ DNA binding</td>
<td>↓ transcription</td>
<td>207</td>
</tr>
<tr>
<td>ChREBP</td>
<td>S568</td>
<td>↓ DNA binding</td>
<td>↓ transcription</td>
<td>208</td>
</tr>
<tr>
<td>p53</td>
<td>S15</td>
<td>↑ Activity</td>
<td>↑ transcription, ?</td>
<td>209</td>
</tr>
</tbody>
</table>
activate PI3K through its association with the p110 subunit at the plasma membrane (213). The catalytic p110 subunit of PI3K converts the membrane lipid phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3). This catalysis is antagonized by the tumor suppressor gene product phosphatase and tensin homolog (PTEN), second only to p53 in mutation frequency among human tumor suppressor genes (158, 212). The p110 subunit of PI3K is also commonly hyperactivated by mutation in breast, colon, brain, and gastrointestinal tumors (214, 215).

PIP3 functions as a second-messenger in the PI3K signaling cascade, recruiting lipid-binding pleckstrin-homology (PH) domain-containing proteins, such as protein kinase B (AKT), to the plasma membrane. Activation of the oncogene product AKT requires PIP3-mediated recruitment to the plasma membrane through its N-terminal PH domain, where it is phosphorylated in its activation loop at T308 by another PH domain-containing protein, PDK1 (216, 217). While the T308-phosphorylated form of AKT is weakly active, maximal activity is attained by an additional phosphorylation in a hydrophobic region of the C-terminal regulatory domain at S473, catalyzed by the mammalian target of rapamycin complex 2 (mTORC2) (216-219). Structural studies initially showed AKT phosphorylated solely at T308 crystallized in an inactive conformation, and subsequent allosteric phosphorylation at S473 induced a conformational shift in the catalytic domain to a more active conformation (220). More recently, it was shown that membrane recruitment of AKT through its PH domain interacting with PIP3 was able to induce a conformational change allowing PDK1 access to T308, a structural change likely missed in the preceding in vitro crystallography (221). Membrane localization is essential for S473 phosphorylation by mTORC2, supporting the role of PI3K in this modification. Importantly, S473 phosphorylation was not inhibited in PDK1-deficient cells or following
treatment with PDK1 inhibitors, but conversely, T308 phosphorylation was undetectable in several nonphosphorylatable S473 AKT mutants. This suggests that S473 phosphorylation by mTORC2 may proceed and facilitate phosphorylation of T308 by PDK1, rendering the kinase fully active (222, 223).

Structural components of mTOR complexes: The downstream targets of AKT are plentiful, and generally promote cell survival through inhibition of apoptosis, as well as growth and proliferation through direct and indirect stimulation of mTORC1 (224). This uniquely places AKT downstream of mTORC2 and upstream of mTORC1. Each of the mTOR complexes is an assembly of distinct components, commonly nucleated by the mTOR catalytic serine/threonine kinase subunit. mTOR was aptly named following its identification as the target of the potent macrolide ester rapamycin, which forms a complex with its intracellular receptor FKBP12 and binds in the N-terminus of the catalytic subunit (225-227). FKBP12-rapamycin binds to mTORC1 complexes but not mTORC2 complexes (228, 229). However, FKBP12-rapamycin is able to bind free mTOR (227, 230, 231). While acute exposure to rapamycin (less than 1 hr) potently inhibits mTORC1 signaling, prolonged exposure to rapamycin (24 hr) inhibits mTORC1 and mTORC2 by binding to newly-synthesized mTOR catalytic subunits, perturbing new complex formation (232). The mechanism by which rapamycin acutely inhibits complexed mTORC1 is not fully understood, but structural studies suggest rapamycin disrupts the binding of the Raptor (regulatory associated protein of mTOR) subunit unique to the mTORC1 complex (233, 234).

In addition to Raptor, the mTORC1 complex consists of mLST8 (mammalian lethal with Sec12 protein 8), PRAS40 (proline-rich AKT substrate 40 kDa), and Deptor (DEP-domain-containing mTOR-interacting protein). Raptor is proposed to regulate the assembly of the
mTORC1 complex and to define the substrate specificity of mTORC1 by recruiting downstream targets that contain TOR signaling motifs to the catalytic mTOR subunit of the complex (235-237). mLST8 binds to the mTOR kinase domain, and is also a component of the mTORC2 complex assembly. Its function is poorly understood, and knockout studies in mice show mLST8 is dispensable for mTORC1 signaling, but is essential to the stability and activity of mTORC2 (238). PRAS40 and Deptor were recently identified as endogenous inhibitors of mTORC1, and activation of the kinase promotes the direct phosphorylation and subsequent dissociation of these negative regulators (239, 240). The observation that PRAS40 has a TOR signaling motif suggests the inhibitor may function by competing with mTORC1 for substrate binding (241). The mechanism by which Deptor inhibits mTORC1 is unknown, but it is also an endogenous inhibitor of mTORC2 (239).

The mTORC2 complex consists of Rictor (rapamycin-insensitive companion of mTOR), SIN1 (stress-activated protein kinase interacting protein), Protor (protein observed with Rictor), mLST8, and Deptor. Rictor has been implicated in stabilizing the mTORC2 complex through its interaction with SIN1 and Protor. Compared to mTORC1, little is known about the biological functions of mTORC2. This is partly because our understanding of mTORC1 biology was facilitated by studies utilizing its potent inhibitor, rapamycin. However, important functions of mTORC2 are emerging, such as the discovery that mTORC2 is the kinase upstream of AKT S473 (242). Knockdown of Rictor limits cell motility as well as actin polymerization, but the molecular mechanisms of these effects are poorly understood (228, 229). Additionally, mTORC2 phosphorylates SGK1 (serum-and-glucocorticoid-induced protein kinase 1) which, similarly to AKT, phosphorylates and prevents the nuclear translocation of the FoxO1 (forkhead box protein O1) and FoxO3a apoptosis-inducing transcription factors (243, 244).
Opposing influences of AKT and AMPK on mTORC1: AKT activates mTORC1 through direct and indirect mechanisms. Directly, AKT phosphorylates the endogenous mTORC1 inhibitor PRAS40 at T246, which promotes its dissociation and sequestration to the cytosolic anchor protein 14-3-3 (240, 245). Indirectly, AKT stimulates mTORC1 kinase activity through inhibitory phosphorylation of S939 and T1462 on the Tuberin (TSC2) subunit of the Tuberous Sclerosis Complex (TSC) (246, 247). The TSC complex is a heterodimer consisting of Hamartin (TSC1) and TSC2. TSC2 is a GTPase activating protein, that, when bound to TSC1, triggers the GTPase activity of the G-protein Rheb, promoting its conversion to a GDP-bound inactive form (248). When bound to GTP, Rheb stimulates the activity of mTORC1 through a mechanism that is not completely understood. Therefore, the TSC complex inhibits mTORC1 signaling through its GTPase activating effect on Rheb. Interestingly, Rheb has been shown to associate with the mTOR catalytic subunit as well as mLST8, and both are components of mTORC1 and mTORC2 complexes (249). However, studies thus far have not found evidence to support GTP-Rheb as a modulator of mTORC2 activity (250).

The discovery of AMPK as a negative regulator of signaling through the PI3K-AKT-mTOR axis arose partly from the identification of LKB1 as a major upstream kinase of AMPK (181-183). As described above, mutations in LKB1 are causal of Peutz-Jeghers syndrome, characterized by benign hamartomas of the gastrointestinal tract (184). Several clinical features of Peutz-Jeghers syndrome are shared by other hamartoma syndromes such as Cowden’s disease, caused by inactivating mutations in the PTEN, as well as TSC syndrome, resulting from mutations in the TSC1 and TSC2 genes (211, 251). Common to these signaling molecules is their convergence on mTORC1. Indeed, it has been shown that AMPK opposes both the direct and indirect influences of AKT on mTORC1 activity. Specifically, AMPK phosphorylates
Raptor at S792, promoting its dissociation from the mTORC1 complex and subsequent sequestration to 14-3-3, preventing the recruitment of mTORC1 substrates (195). Additionally, AMPK phosphorylates TSC2 at S1387 and T1271, marks that activate the TSC complex, promoting mTORC1 inhibition through inactivation of Rheb-GTPase activity (196). Therefore, AMPK activation promotes inhibition of signaling processes mediated by mTORC1 by opposing the activation of two studied nodes of signal transduction by AKT, namely TSC1/2 and mTORC1.

In addition to counteracting AKT signaling, AMPK also opposes the influence of aberrant MAPK signaling converging on the TSC complex. The primary effector of the MAPK signaling pathway is the GTPase RAS, which frequently harbors mutations that constitutively activate the kinase in colon, thyroid, pancreatic, melanoma, and lung tumors (252). As described above, RAS can directly stimulate PI3K signaling, which would in itself stimulate AKT and subsequently, mTORC1. However, mTORC1 hyperactivity is retained when AKT is inhibited in the presence of hyperactive K-RAS, and vise versa (253). This illustrates that signaling mediated by PI3K and RAS can independently converge on mTORC1. Indeed, RAS signaling through the classical MAPK cascade to extracellular signal-regulated kinase (ERK) indirectly promotes mTORC1 signaling. TSC2 S664 is a direct substrate for ERK, which perturbs the TSC complex formation, and thus drives mTORC1 signaling through deregulation of Rheb-GTP (254, 255). Additionally, it has been reported that phosphorylation of TSC2 at S1798 by RSK, a downstream substrate of ERK, inhibits Rheb-GTPase activity (256).

*Signaling downstream of mTORC1:* The best characterized function of mTORC1 is the regulation of the initiation step of 7-methyl guanosine CAP-dependent translation, in which a multiprotein complex of translational initiation factors, collectively known as the eIF4F
complex, binds the 5’ CAP of mature mRNAs and recruits the 40S ribosomal subunit (Figure 3-3). Subsequently, the ribosome-bound initiation complex scans the mRNA from 5’ to 3’ until it encounters an optimal AUG translational start codon. The 60S ribosomal subunit is then recruited along with elongation factors, and translation elongation begins (257). The eIF4F initiation complex consists of the initiation factors eIF4E, eIF4G, and eIF4A. eIF4E directly binds the CAP structure, and recruits eIF4G and the helicase eIF4A, which functions to linearize hairpins in the 5’ UTR of mRNA as the complex scans for the start codon (Figure 3-3). The processivity of eIF4A is greatly enhanced by the binding of eIF4B (258). mTORC1 signals directly to key controlling elements of the eIF4F initiation complex, 4E-binding protein 1 (4EBP1) and 40S ribosomal protein S6 kinase (S6K1) (259). 4EBP1 is a translation inhibitor that functions by competing with eIF4G for binding to eIF4E (Figure 3-3). Therefore, when 4EBP1 is bound to eIF4E, the translation initiation complex cannot assemble, and CAP-dependent translation is inhibited. mTORC1 phosphorylates 4EBP1 at four known residues (T37, T45, S65, and T70), diminishing its affinity for eIF4E, thus promoting CAP-dependent translational initiation.

S6K1 is also an important translational regulatory target of mTORC1. S6K1 was originally identified as the kinase upstream of the 40S ribosomal protein S6, whose phosphorylation is essential for protein synthesis (259). S6K1 activation requires phosphorylation at two sites, T389 by mTORC1 and T229 by PDK1 (235). S6K1 phosphorylates eIF4B at S422, a modification necessary to promote recruitment of eIF4B to the initiation complex, where it stimulates the helicase activity of eIF4A (260) (Figure 3-3). In addition to its direct regulatory roles on protein synthesis, S6K1 also indirectly influences translation by promoting ribosome biogenesis (261).
Figure 3-3. Regulation of CAP-dependent translation initiation. (A) The binding of 4E-BP1 to the CAP-binding protein eIF4E inhibits translation by preventing the recruitment of initiation factors and the 40S ribosome to capped mRNAs. Phosphorylation of 4E-BP1 by mTORC1 inhibits it from binding eIF4E, which promotes CAP-dependent translation initiation. (B) S6K and RSK promote cap-dependent translation by phosphorylating eIF4B, which then enhances the helicase activity of eIF4A. Helicase activity of the CAP-dependent initiation complex is necessary to unwind hairpinned structures in the 5’ untranslated regions of capped mRNAs.

S6K1 is also an important feedback inhibitor of AKT signaling, controlling a negative feedback loop essential to regulate mTORC1 activity. Evidence of a feedback loop surfaced from studies with TSC-null mouse embryonic fibroblasts (MEFs) that have hyperactive mTORC1 signaling, but are insensitive to insulin and have diminished AKT activity (262, 263). Subsequent studies demonstrated that S6K1 phosphorylates the insulin receptor (an RTK) scaffold protein IRS1 at S302 (264). Phosphorylation disrupts the binding of IRS1 to its receptor, which diminishes signaling mediated by PI3K. Moreover, free IRS1 is sequestered by 14-3-3 and degraded by the 26S proteasome (265). More recently, S6K1 was also shown to diminish mTORC2-mediated phosphorylation of AKT S473 by phosphorylating Rictor at T1135, promoting its sequestration to 14-3-3 (266). This feedback mechanism is likely the reason why hamartomas in TSC patients are less aggressive, as PTEN inhibition in TSC2-null tumors circumvents the feedback loop and drives tumor progression (255, 267). There are important therapeutic implications associated with this feedback loop as well. For instance, in TSC-null cells, chronic rapamycin treatment partially restores insulin sensitivity through inhibition of feedback control (264, 268). However, in tumors presenting with hyperactivating mutations along the PI3K-AKT-mTOR signaling axis, chronic rapamycin treatment potentiates the oncogenic effects of AKT, an unfavorable consequence of mTORC1 inhibition (269). In the research described in this chapter, we discovered that mTORC1 signaling is sensitive to this feedback regulation by AKT hyperactivity in response to pemetrexed. Furthermore, we show that inhibition of mTORC1 can be prolonged for therapeutic gain by dual targeting of AMPK and AKT, suggesting a rational combination therapeutic approach.
Pharmacological and environmental modulation of AMPK signaling

A number of pharmacological agents, endogenous ligands, and environmental cues have been identified as having AMPK-activating effects in cells. The mechanisms by which these stimuli activate AMPK are as diverse as the chemical space they occupy, partly because AMPK is sensitive to stresses that alter ATP homeostasis. For simplicity, activators will be grouped into classes based on known mechanisms of activation of the kinase. Classes of AMPK-activating agents discussed below include AMP-mimetics, inhibitors of oxidative phosphorylation, mitochondrial ATP synthase inhibitors, inhibitors of glycolysis, modulators of cytosolic calcium, and direct activators.

AICAR and now pemetrexed fall into the AMP-mimetic group. AICAR was the first agent identified as an AMPK activator, and much of our understanding of downstream signaling mediated by AMPK is credited to studies with this compound. AICAR is transported into cells by the adenosine transporter and is converted to ZMP by adenosine kinase (AK) ((270). The cellular effects of AICAR are ablated in cells lacking AK or cells co-treated with an AK inhibitor, confirming ZMP as the active metabolite (141, 142). The development of AICAR as a cancer therapeutic agent has been limited, partly because of its limited bioavailability in vivo and its ability to compete for transport at the adenosine receptor (270, 271). Of note, the concentrations of pemetrexed required to activate AMPK are several orders of magnitude less than AICAR. Nevertheless, AICAR has served as an excellent positive control for effects of pemetrexed mediated by accumulated ZMP.

Agents that deplete cellular ATP levels through various mechanisms often have a resultant AMPK-activating effect. The antidiabetic biguanides (metformin, phenformin) and thiazolidinediones (troglitazone, rosiglitazone, and pioglitazone) are representative of AMPK-
activating agents that inhibit oxidative phosphorylation, thus altering the AMP:ATP ratio by diminishing mitochondrial ATP production. Metformin has been a standard of care in the treatment of type-2 diabetes for over 50 years, and it is now appreciated that the effects of metformin on glucose uptake and metabolism are partially mediated by AMPK activation secondary to inhibition of complex I of the electron transport chain (272, 273). Although studies have suggested that metformin activates AMPK through various other mechanisms, metformin was unable to activate AMPK in HEK293 cells that were transfected with an AMPKγ mutant that rendered the kinase insensitive to elevated AMP levels (274). This illustrated that the effects of metformin on AMPK are mediated exclusively by alterations in the AMP:ATP ratio. Interestingly, chronic metformin administration in animal models of spontaneous breast cancer and diet-induced prostate cancer decreased tumorigenesis (275, 276). Indeed, epidemiological studies have noted a negative correlation of cancer incidence among diabetic patients taking metformin (277, 278). Resveratrol and quercetin are plant-derived polyphenols that elicit their AMPK-activating effects through inhibition of mitochondrial ATP synthase (279). The effects of resveratrol on insulin sensitivity, glucose tolerance, and mitochondrial biogenesis were ablated in AMPKα1 or α2 knockout mice, illustrating the centrality of AMPK in these beneficial effects of resveratrol (280). While resveratrol likely mediates its effects on AMPK exclusively through this mechanism, quercetin may affect AMPK in additional ways, since the above-described HEK293 mutant cell line is not completely insensitive to quercetin, as it is with resveratrol (274).

Drug discovery efforts by Abbott Laboratories (Abbott Park, IL) identified A-769662 as the first direct AMPK activator (281). A-769662 has a thienopyridone core structure and was initially shown to activate AMPK in vitro and significantly decrease plasma glucose in a diabetic
mouse model. A-769662 mimicked both effects of AMP, allosteric activation as well as protection of AMPKα T172, but it did not compete with AMP for binding (157). Consistent with this result, γ mutant HEK293 cells insensitive to AMP were sensitive to A-769662 (274). Conversely, AMPKβ mutants completely lacking the glycogen binding domain were insensitive to A-769662 while sensitivity to AMP remained (179). Interestingly, S108 in the glycogen-binding domain was identified as a critical residue for A-769662 action, but the significance of this residue is unknown. While the binding site of A-769662 is elusive, these data suggest that A-769662 either directly interacts with the β subunit or that allosteric binding causes a conformational shift in the β subunit essential for activation of the kinase. Like pemetrexed, activation of AMPK by A-769662 did not require the upstream kinase LKB1.

While AMPK classically responds to variation in the AMP:ATP ratio, AMPK was discovered to be sensitive to changes in intracellular calcium homeostasis (188-190). A plausible explanation of why the kinase has adapted to respond in this way is that calcium triggers many energy-consuming cellular processes, such as membrane trafficking, neurotransmitter secretion, and the operation of ion channels and pumps (191). The predominant effector of AMPK in response to calcium is CAMKKβ, as isoform-specific siRNA and the CAMKK inhibitor STO-609 ablates residual AMPK activity in LKB1-null Hela cells following treatment with the calcium ionophore A23187 (188). As described above, CAMKKβ is a known alternative upstream kinase of AMPKα T172. This residue is hyperphosphorylated in response to A23187, an effect blocked by STO-609 (188-190). Activation of AMPK in response to calcium occurs in the absence of allosteric binding of AMP, as γ mutant HEK293 cells insensitive to AMP were as sensitive to A23187 as wild type cells, and the effect was blocked by STO-609 (274).
In addition to pharmacological activators, AMPK is sensitive to a number of pathological stressors and physiological stimuli such as the endogenous hormones leptin, adiponectin, ghrelin, and endocannabinoids (282). AMPK is activated in response to hypoxia and ischemia, and this activation is thought to be causal of the increased glycolytic flux observed in cardiac tissue under these conditions (283). Activation is likely the result of inhibition of oxidative phosphorylation and subsequent changes to the AMP:ATP ratio. In neuronal tissue, it is unclear whether AMPK activation under ischemic conditions is protective or detrimental (284). Interestingly, glial cell AMPKα2 seems to be more sensitive to hypoxia than α1, and regulation of the kinase might occur at both the mRNA and protein level (285). In skeletal muscle, AMPKα2 is activated in response to exercise (286). This effect is likely a direct result of muscle contraction rather than hormonal release, as the kinase is stimulated experimentally by electrical-induced contraction of rat epitrochlearis muscles, albeit to a lesser extent than by perfusion with AICAR (287). In a recent study, it was shown that treatment of sedentary C57B/6J mice with AICAR (500 mg/kg/day) for 4 weeks actually mimicked effects seen with exercise. Notably, mice had a decreased ratio of epididymal fat mass to body weight ratio with no subsequent weight loss, increased oxygen consumption, and mice ran longer (23%) and farther (44%) in a treadmill endurance test than vehicle-treated mice (288).
MATERIALS AND METHODS

Chemicals and reagents

SAMS peptide (#1344) was from Tocris Bioscience (Ellisville, MO). Protein G sepharose (#17061801) and m⁷GTP-Sepharose (27502501) were from GE Lifesciences (Piscataway, NJ). AMPKα1 antibody (#07350) for immunoprecipitation was from Upstate (Billerica, MA). [γ-32P]-ATP (#NEG002) was from Perkin Elmer (Waltham, MA) and was supplied at a specific activity of 10 Ci/mmol. GSK690693 was from SYN Thesis Med Chem (Melbourne, Australia). OSU-03012 was a gift from Dr. Paul Dent (VCU Department of Neurosurgery). DharmaFECT transfection reagent #2 (#T200201), Dharmacon siGENOME SMARTpool siRNAs targeting human ATIC (#M008292010005), AMPKα1 (#M005027020005), and non-targeting siRNA pool #1 (#D0012061305) were purchased from Thermo Scientific (Rockford, IL). AICAR (#A611700) was from Toronto Research Biochemicals (Toronto, Canada). Trans35S-Label (#51006) was from MP Biochemicals (Santa Ana, CA) and was provided at a specific activity of 1175 Ci/mmol. Complete EDTA-free Protease Inhibitor Cocktail tablets (#11873580001) were from Roche Applied Science (Indianapolis, IN). Thirty % Acrylamide/BIS solution 37.5:1 (#1610158), Laemmli Sample Buffer (#1610737), and Dual Color Precision Plus Protein Standard (#1610374) were from Bio-Rad Laboratories. Immobilon polyvinylidene fluoride (PVDF) membrane (#IPVH00010) was from Millipore (Billerica, MA). StartingBlock Blocking Buffer (#37542), Goat anti-rabbit IgG (#31462), and Goat anti-mouse IgG (#31348) secondary antibodies were from Thermo Scientific. Blotting Grade Blocker Non-fat Dry Milk (#1706404) was from Bio-Rad Laboratories. A list of antibodies and their sources can be found in Table 3-2. All other reagents
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were from Fisher Scientific (Waltham, MA) or Sigma Aldrich (St. Louis, MO) and were of the highest available purity.

**Immunoblotting**

*Total Protein Isolation:* Protein was typically harvested from 2-5 x 10^6 cells grown on 100 mm dishes. Prior to total protein harvest, one protease inhibitor cocktail tablet was dissolved in 50 ml 1x PBS and placed on ice. Tissue culture plates were kept cold throughout the harvesting procedure. Cells were washed once with cold 10 ml PBS (containing protease inhibitor), scraped, and pelleted at 1,000 rpm for 5 minutes at 4°C. Cells were lysed in cold buffer containing 62.5 mM Tris-HCl pH 6.5, 2% SDS, 5% glycerol, 5% 2-mercaptoethanol, 50 mM NaF, and a 1x concentration of Complete EDTA-free Protease Inhibitor Cocktail. Lysates were sheared through 21-gauge needles 30 times before being spun at 14,000 rpm for 2 minutes. This shearing procedure was repeated, and lysates were spun at 14,000 rpm for 5 minutes. The protein concentration was determined using the Bradford Reagent according to the manufacturers protocol with BSA as a standard. Typical protein concentration was 2-5 µg/µl. Protein was placed in single-use aliquots and stored at -80°C.

*SDS-PAGE and Protein Transfer:* Gel electrophoresis and wet membrane transfers were performed using the Mini PROTEAN-3 Cell system (#1653301) from Bio-Rad Laboratories. Total protein was mixed with an equal volume of Laemmli Sample Buffer, boiled for 5 minutes, and 20 µg was loaded onto 1.5 mm SDS-polyacrylamide gels, poured according to the recipe provided with 30% Acrylamide/Bis 37:5:1 (#1610158) from Bio-Rad Laboratories. An aliquot (5-10 µl) of Dual Color Precision Plus Protein Standard was also loaded onto every gel for mass determination. Typically, protein was resolved on 7.5% or 12% gels in running buffer (25 mM
Tris base, 250 mM glycine, 0.1% SDS) at 50 volts for 30 minutes (or until protein migrated out of the stacking gel) followed by 120 volts for 1-1.5 hours to optimally separate the protein of interest.

Polyvinylidene fluoride (PVDF) membrane that had been pre-soaked in methanol was rinsed in water along with the gels, and, PVDF, gels, and sponges for the transfer were equilibrated for at least 20 minutes in cold transfer buffer (25 mM Tris, pH 8.3, 192 mM glycine, 10% methanol). Six pieces of Whatman paper per gel were cut to the approximate size of an electrophoresis plate, and the transfer sandwich was assembled as follows. Layering on the black face of a transfer cassette, 3 pieces of Whatman paper (dipped in transfer buffer) were placed on top of 1 sponge. Air bubbles were removed by rolling with a 5 ml plastic pipette. The gel was centered on the Whatman paper, and a PVDF membrane was placed on top of the gel. Again, it was important to remove all air bubbles from the transfer sandwich by rolling. It also helped to keep the sandwich as wet as possible with transfer buffer during assembly. 3 pieces of Whatman paper (dipped in transfer buffer) were placed on top of the membrane, followed by another sponge. The transfer cassette was closed, placed in its holder in the gel box along with a plastic ice block, and the gel box was then filled with cold transfer buffer. Transfers were either ran on ice or in the cold room (4°C) at 100 volts for 45 minutes (one gel) or 50 minutes (2 gels). Following the transfer, membranes were dipped in methanol and dried on the lab bench for 15 minutes before being immunoblotted.

Antibody Detection: A general antibody detection procedure is described in this section. For detailed conditions for each antibody, refer to Table 3-2. Dried membranes were soaked briefly in methanol and non-specific proteins were blocked for 1 hour in either StartingBlock Buffer or 5% Blotting Grade Blocker Non-fat Dry Milk 0.1% TBS-T (0.5 M Tris-HCl pH 7.5,
0.14 M NaCl, 2.7 mM KCl, 0.1% Tween 20). Membranes were washed three times for 5 minutes in 0.1% TBS-T. Primary antibodies diluted in either StartingBlock Buffer or 5% BSA (#A4503) from Sigma were incubated on the membranes overnight at 4°C with rotation in sealed plastic bags to minimize antibody consumption. Membranes were washed 3 times with 0.1% TBS-T for 5 minutes and incubated for 1 hour in horseradish peroxidase-conjugated secondary antibody. Membranes were then washed 3 times with 0.1% TBS-T for 10 minutes. It was found that washing more stringently during this step greatly diminished non-specific background during exposure. Membranes were incubated with West Pico or West Dura SuperSignal chemiluminescence substrate (Pierce) for 5 minutes. Blots were exposed to autoradiography film and processed on an automated film developer. If the chemiluminescent conditions were not known, West Pico was applied first. If no signal was apparent, the blot was rinsed with 0.1% TBS-T, and West Dura (diluted by 40% with PBS) was applied. Signal was usually observed with one of these conditions, and rarely was a more stringent detection reagent applied. Blots presented in this dissertation are representative of findings from at least two biological replicates.

**AMPK activity assays**

Immunoprecipitated AMPKα1 activity was measured as the amount of radiolabelled phosphate transferred to the SAMS peptide based on the method described by Hardie *et al* (289). HCT116 cells were plated at a density of $10^6$ cells/100 mm dish and were drug-treated the following day. Cells were washed once with PBS and scraped before being broken in cold lysis buffer (50 mM Tris-HCl pH 7.4, 50 mM NaF, 5 mM Na pyrophosphate, 1 mM EDTA, 1 mM EGTA, 250 mM mannitol, 1% (v/v) Triton X-100, 1 mM DTT, 1 mM benzamidine, 0.1 mM
PMSF, and 5 μg/ml soybean trypsin inhibitor). Protein content was determined by Bradford assay using BSA as a standard.

For each sample, a 10 μl bed volume of protein G sepharose was equilibrated with IP buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 50 mM NaF, 5 mM Na pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1% (v/v) Triton X-100, 1 mM DTT, 0.1 mM benzamidine, 0.1 mM PMSF, and 5 μg/ml soybean trypsin inhibitor) by washing three times. Ten μg of AMPKα1 antibody (Upstate) was bound to the equilibrated protein G beads by incubating for 45 minutes at 4°C with rotation. Antibody-bound beads were washed 5 times with cold IP buffer and incubated overnight with 100 μg of protein lysate at 4°C with rotation. The AMPKα1 IP was washed 4 times with cold IP buffer containing 1 M NaCl followed by 2 washes with cold buffer containing 62.5 mM HEPES pH 7.0, 62.5 mM NaCl, 62.5 mM NaF, 6.25 mM Na pyrophosphate, 1.25 mM EDTA, 1.25 mM EGTA, 1 mM DTT, 1 mM benzamidine, 1 mM PMSF, and 5 μg/ml soybean trypsin inhibitor. The IP was resuspended in 60 μl of assay buffer (50 mM HEPES pH 7.4, 1 mM DTT, and 0.02% (v/v) Brij-35).

Typical reactions were performed in 25 μl of assay buffer containing 0.2 mM SAMS peptide, 0.2 mM AMP, 0.2 mM [γ-32P]-ATP (final specific activity ~500 cpm/pmol), 5 mM MgCl2, and 5 μl (approximately 8 μg) of immunoprecipitated AMPKα1. Reactions were incubated in a water-bath shaker at 37° and were quenched by spotting onto Whatman P81 paper. Papers were washed carefully by slow mixing twice for 5 minutes in 500 ml of 1% (v/v) phosphoric acid, once in 500 ml H2O, and once in 100 ml acetone before being counted in scintillation fluid.
**High performance liquid chromatography analysis of ATP**

Whole-cell acid-soluble extracts were made as previously described in Chapter 2. ATP was analyzed by HPLC using a Spectra-Physics P4000 pump, UV2000 absorbance reader set to monitor a wavelength of 254 nm, and a Datajet integrator. A strong anion exchange (SAX) 250 x 2 mm HPLC column (#00G4149B0 Phenomenex Torrance, CA) fitted with a guard column (#KJ04282, #AJ04310 Phenomenex Torrance, CA) was equilibrated with Buffer A (7.5 mM NH₄H₂PO₄, pH 4.5, 2% CH₃CN) at a flow rate of 0.2 ml/min. Typically, 5 µl acid-soluble extract was injected onto the column in a volume of 300 µl Buffer A. After passing Buffer A over the column for an additional 2 minutes, ATP was eluted from the column using a linear gradient of 100% Buffer A to 60% Buffer B (750 mM NH₄H₂PO₄ pH 4.9, 2% CH₃CN) over 25 minutes at a flow rate of 0.2 ml/min. ATP had a retention time of approximately 32 minutes under these conditions (Figure 3-4A). 60% Buffer B was flowed over the column for an additional 8 minutes following each run to clean the column, and the mobile phase was returned to Buffer A over the next 10 minutes. Total time from injection to injection was approximately 45 minutes. To determine ATP concentration from extracts, results were fitted to a standard curve generated from synthetic ATP (Figure 3-4B).

**RNA interference**

Cells were plated at 2 x 10⁵ cells/well of a 6-well plate late in the day. siGENOME SMARTpool siRNAs (Dharmacon) (50 nM) were transfected with 0.1% DharmaFECT reagent according to the manufacturer’s instructions. The transfection media remained on the cells for 24 hours, at which time cells were washed with PBS and fresh media was replaced. Longer incubations resulted in visually apparent toxicity under a microscope. All experiments were
Figure 3-4. Identification and quantification of ATP by HPLC. ATP was analyzed by HPLC from whole-cell acid soluble extracts as described in the Methods section. (A) A representative HPLC chromatogram measuring ATP from CEM cells treated with 1 µM pemetrexed and 5.6 µM thymidine for 24 hours. (B) A standard curve was generated with synthetic ATP.
controlled with the DharmaFECT transfection reagent in the absence of siRNA (mock), as well as a non-targeting siRNA SMARTpool (scrambled). Drug treatments were started 48 hours after transfection, and protein was harvested after 72 hours, unless otherwise noted. AICART siRNA transfected using this method produced greater than 90% and less than 95% knockdown in HCT116 cells that was apparent by 48 hours and persisted 72 hours post-transfection (Figure 3-5). A similar result was seen with AMPKα1 knockdown (Figure 3-11A).

**Clonogenic survival assays**

Cells were plated in triplicate from a single cell suspension at 100/60 mm dish and allowed to attach overnight. Cells were then treated continuously with media supplemented with drugs (and rescue agents) for the indicated times. Plates were washed with PBS, and fresh media was added every 2-3 days until control colonies were easily counted (typically 7-10 days). When rescue agents were included, they remained in the media throughout the duration of the experiment. Plates were then washed twice with PBS, fixed with methanol for 20 minutes, and stained with 5% Wright-Giemsa reagent for 20 minutes. Stain was washed off with H₂O, and plates were dried overnight at room temperature. All visible colonies were counted manually.

**Assaying for global protein synthesis**

To determine global translational effects of pemetrexed in HCT116 cells, protein synthesis rates were determined by ³⁵S-methionine incorporation following drug exposure. Cells were plated at a density of 2 x 10⁶ cells/150 mm dish late in the day and treated with drugs the following morning. Drug-containing culture media was aspirated and the cells were washed once with PBS before being trypsinized and counted. Cells (4 x 10⁵) were transferred to a 1.5 ml
Figure 3-5. AICART knockdown in HCT116 cells. HCT116 cells were transfected with Dhamacon siGENOME SMARTpool siRNAs targeting human AICART as described in the Methods section. Immunoblot analysis confirmed siRNA knockdown (KD) of AICART 48 and 72 hours after transfection (upper panel). Total protein (20 µg) was loaded into each lane in the upper panel. Quantification of AICART KD (lower panel) after 72 hours indicated greater than 95% knockdown in these cells. Mock indicates treatment with transfection reagent alone.
microcentrifuge tube and spun at 1,000 rpm for 5 minutes. Pelleted cells were resuspended in 500 µl RPMI 1640 with 10% dFBS and 0.5 µCi Trans$^{35}$S-Label. A typical batch of Trans$^{35}$S-Label contained greater than 70% $^{35}$S-methionine. Triplicate reactions were incubated in a 37°C water bath with shaking for 0, 10, 20, 40, and 60 minutes, and were quenched by adding 500 µl 20% TCA (10% TCA [final]), vortexing vigorously, and holding on ice. When all incubations were completed, samples were spun at 7,500 rpm for 5 minutes. The supernatant was aspirated, and the pellet was washed twice with 2 ml 5% TCA. Pellets were resuspended in 200 µl 23 M formic acid, and 90% was of the total volume counted in scintillation fluid. One µl of label was counted with each experiment to correct for decomposition. Counts were expressed as nmol $^{35}$S-methionine incorporated/min/10$^6$ cells.

$m^7$GTP-CAP pulldown

To determine whether pemetrexed treatment enhanced residence of 4E-BP1 at the $m^7$-guanosine CAP, cell lysates were incubated with $m^7$GTP-sepharose beads, which have been previously shown to bind both eIF4E and 4E-BP1 (290). Following the indicated drug treatments, cells were lysed on ice for 30 min in IP buffer (25 mM HEPES pH 7.5, 1% NP40, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 1 mM PMSF, 0.1% 2-mercaptoethanol, and 1x Roche Complete Protease Inhibitor Tablet). Cleared lysate (500 µg) was incubated with 40 µl of a 50% slurry of $m^7$GTP-Sepharose (GE Lifesciences) for 2 hr at 4°C with rotation. Cap complexes were washed with IP buffer four times, resuspended in Laemmlli Sample Buffer, and boiled for 5 min before being resolved on a 12.5% SDS-PAGE gel and immunoblotted as described above.
RESULTS

AMPK is activated by accumulated ZMP following AICART inhibition by pemetrexed

As described above, AMPK is sensitive to changes in the cellular AMP:ATP ratio. As such, AMPK signals to key controlling elements of protein, lipid, and carbohydrate metabolism to balance nutrient metabolism with energy supply (Table 3-1) (157). ZMP is a mimetic of AMP, the primary allosteric activator of AMPK (132). The binding of AMP or ZMP to the AMPKγ subunit causes both relief of autoinhibition of the catalytic AMPKα subunit as well as a conformational shift in the AMPKα subunit, likely protecting T172 from phosphatases (179, 180). AMPKα T172 phosphorylation has been proposed to be essential for kinase activity, and is therefore a conventional marker for AMPK activation (167, 168). In HCT116 cells treated with pemetrexed, AMPKα T172 became robustly hyperphosphorylated (Figure 3-6A). A 3-fold increase in kinase activity of AMPKα1 was also measured in HCT116 cells treated with pemetrexed (Figure 3-6B). Kinase activity was assayed in vitro using the AMPK-specific SAMS peptide as substrate and immunoprecipitated AMPKα1 from HCT116 cells pretreated with pemetrexed. The data in Figure 3-6 suggests that the hyperphosphorylation induced by pemetrexed is sufficient to activate the kinase, while the increased phosphorylation measured with thymidine alone was not sufficient to activate the kinase. This increase in T172 phosphorylation in response to thymidine treatment is not completely understood and was not always observed. AMPK activity was not measurable in immunoprecipitates from AMPKα2 in either HCT116 or H460 cells, confirming that α1 is the primary catalytic subunit in these cells.

In addition to increases in AMP (or ZMP), AMPK is also activated in response to decreases in cellular ATP levels. Since pemetrexed inhibits AICART, an intermediate of de novo purine synthesis, it was necessary to determine the effect of pemetrexed on ATP pools.
Figure 3-6. Pemetrexed induces AMPKα phosphorylation and activity in HCT116 cells. HCT116 cells were treated with 1 μM pemetrexed in the presence of 5.6 μM thymidine to offset the effects of TS inhibition. (A) The phosphorylation of T172 on AMPKα was detected by western blot from 20 μg total protein, and (B) the kinase activity of 8 μg immunoprecipitated AMPKα1 was measured using the SAMS peptide as a substrate as described in the Methods section. In (B), error bars are representative of standard deviation from three technical replicates.
Previous studies have shown that pemetrexed does not diminish cellular ATP (291), and indeed, we showed a similar result in CEM cells (Figure 3-7). In fact, ATP levels were slightly elevated in response to pemetrexed, consistent with previous observations (291). Therefore, we suspected that the AMPK response to AICART inhibition was a result of ZMP accumulation, not ATP depletion (291).

To definitively link AICART inhibition to AMPK activation, we determined whether the effects of pemetrexed treatment on AMPK activity could be recapitulated by depletion of AICART. HCT116 cells were transfected with a pool of siRNA’s directed against AICART mRNA (Figure 3-5). Indeed, AMPKα T172 phosphorylation was increased following AICART knockdown, confirming the intermediacy of AICART inhibition in this effect (Figure 3-8A). Surprisingly, the effect of approximately 95% knockdown of AICART (Figure 3-5) on ZMP accumulation (Figure 3-8B) was less pronounced than that seen following treatment with pemetrexed (Figure 2-16). This suggested that pemetrexed treatment in these cells had a stronger inhibitory effect on AICART than we initially suspected. Accumulation of ZMP in AICART knockdown cells exposed to a low concentration of AICAR (100 µM) indicated that the flow of de novo purine synthesis was becoming restricted by knockdown of AICART (Figure 3-8B). However, ZMP accumulation even under conditions of AICART knockdown with an AICAR challenge in HCT116 cells did not match that seen following pemetrexed treatment in these cells, in which the accumulation of up to 4 mM ZMP was commonly seen (Figure 2-16). Therefore, it appears that AICART activity is not limiting the flux of de novo purine synthesis, as previously postulated (140). Importantly, this result indicates that extensive inhibition of AICART is needed to cause the substantial accumulation of ZMP following pemetrexed treatment.
Figure 3-7. ATP is not depleted in CEM cells treated with pemetrexed. CEM cells were treated with the indicated concentrations of pemetrexed and 5.6 μM thymidine for 48 hours. ATP levels from whole-cell acid-soluble extracts were quantified by anion-exchange HPLC as described in the Methods section. The area units under HPLC peaks corresponding to ATP were converted to moles using the equation derived from a standard curve generated with synthetic ATP. The cytosolic volume of 10^6 cells was roughly equivalent to 1 μl cell water. Data points represent averages of two replicates.
Figure 3-8. AICART knockdown recapitulates effects of pemetrexed in HCT116 cells. siRNA knockdown (KD) of AICART mimics the effects of pemetrexed on (A) AMPKα phosphorylation and (B) ZMP accumulation in HCT116 cells. Cells were exposed to siRNA for 24 hours and harvested after 72 hours. Where indicated, cells were treated with 100 µM AICAR for the last 4 hours of incubation. In (A), 20 µg of total protein was loaded into each lane. In (B), error bars represent standard deviations from two biological and two technical replicates. * Represents measurements below the detection limit of the HPLC (<0.001 µM).
"mTORC1 inhibition by pemetrexed is dependent on AMPK activity"

AMPK both directly and indirectly inhibits mTORC1 through phosphorylation of Raptor and TSC2, respectively (Figure 3-1) (195, 196). The effect of pemetrexed on AMPK-dependent Raptor phosphorylation was determined in HCT116 cells. In response to pemetrexed, striking hyperphosphorylation of Raptor S792 was measured (Figure 3-9). Phosphorylation status of the downstream target of mTORC1, S6K1, was used as an indicator of effects on mTORC1 activity. Pemetrexed robustly inhibited S6K1 phosphorylation by mTORC1 at T389 (Figure 3-9). Together, these data suggested that pemetrexed was inhibiting mTORC1 signaling through effects on AMPK. In support of these findings, a competitive inhibitor of the AMPKα ATP-binding site, compound C (272), blocked the hypophosphorylation of S6K1 T389 induced by pemetrexed (Figure 3-10). This implied that mTORC1 inhibition by pemetrexed depended on the catalytic activity of AMPK. While the primary target of compound C is AMPK, off-target effects of compound C likely complicate the specificity of this inhibitor as a chemical probe for signaling events mediated by AMPK. To definitively link pemetrexed-induced mTORC1 inhibition to AMPK activation, the effects of pemetrexed on mTORC1 signaling were determined in HCT116 cells transiently transfected with siRNA pools to AMPKα1 (Figure 3-11). Interestingly, AMPKα1 knockdown had a significant cytotoxic effect in HCT116 cells, suggesting that disruption of the balance of AMPK signaling either by activation or inhibition might be a justified avenue of therapeutic intervention. Knockdown of AMPKα1 prevented the hyperphosphorylation of Raptor at S792 and the hypophosphorylation of S6K1 at T389 induced by pemetrexed (Figure 3-11B). These effects were measured even in the presence of elevated ZMP levels, indicating that pemetrexed was still inhibiting AICART in these cells (Figure 3-
Figure 3-9. Downstream effects of pemetrexed-induced activation of AMPK on mTORC1 signaling. Immunoblots on HCT116 cells treated with pemetrexed for 15 hours were probed for p-S792 of the Raptor subunit of the mTORC1 complex and for p-T389 of S6K1 as described in the Methods section. Total protein (20 µg) was loaded into each lane.
Figure 3-10. Inhibition of mTORC1 signaling by pemetrexed is dependent on AMPK activity. The AMPKα inhibitor, compound C (1 µM), blocks the effects of pemetrexed on mTORC1 signaling following 15 hour exposure to both agents in HCT116 cells. All treatments contained 0.1% DMSO and 5.6 µM thymidine. Total protein (20 µg) was loaded into each lane.
Figure 3-11. Pemetrexed inhibits mTORC1 through activation of AMPKα1 in HCT116 cells. (A) Depletion of AMPKα1 by siRNA knockdown in HCT116 cells 48 hours after transfection (B) blocks the effects of pemetrexed on mTORC1 signaling. siRNA pools were added to HCT116 cells for 72 hour with 1 μM pemetrexed and 5.6 μM thymidine present during the last 15 hours. Total protein (20 μg) was loaded into each lane. The bottom panel indicates that ZMP still accumulated in siRNA-treated cells, as expected, and results are presented as averages ± standard deviations from two biological and two technical replicates.
11B). Importantly, knockdown of AMPKα1 depleted all AMPKα detectable by western blot with an antibody that recognized both α1 and α2 isoforms of AMPK (Figure 3-11A), further indicating that AMPKα2 was not expressed in HCT116 cells. We therefore concluded that the pemetrexed-induced accumulation of ZMP behind an AICART block activated AMPK, signaling downstream through Raptor phosphorylation to hinder the kinase activity of the mTORC1 complex (Figure 3-1). Additionally, AMPK signaling through TSC likely contributes to the observed mTORC1 inhibition by pemetrexed, and it will be important to determine the extent of inhibition through this axis in comparison to the direct effect on Raptor.

**LKB1 is not required for AMPK activation in response to pemetrexed**

The tumor suppressor gene product, LKB1, is the predominant upstream kinase of AMPKα T172 in most cells (181-183). Phosphorylation of AMPKα T172 has been shown to be requisite for the kinase activity of the trimer (167, 168), and genetic deletion of LKB1 renders cells insensitive to several agents that function through AMPK activation. Since loss-of-function mutations in LKB1 occur with high frequency in primary non-small cell lung carcinomas (Figure 3-2) (185), and pemetrexed is clinically indicated for the treatment of NSCLC, we sought to determine whether the effects of pemetrexed on signaling through AMPK would be affected by loss of LKB1.

In a screen of carcinoma cell lines for the secondary growth-inhibitory effect of pemetrexed, we identified several cell lines that had loss-of-function mutations in LKB1, the human H460 NSCLC, A549 lung carcinoma, and Hela cervical carcinoma (Figure 3-12). Somewhat surprisingly, these cell lines were all sensitive to pemetrexed. The degree of
Figure 3-12. Sensitivity of carcinoma cells to pemetrexed with thymidine and to rapamycin. Cells were exposed to 1 µM pemetrexed and 5.6 µM thymidine or to 25 nM rapamycin for 72 hour. Growth was assessed by Coulter counting. Cell line genotypes were determined from the Sanger Institute Cancer Cell Line Project Consortium website (http://www.sanger.ac.uk/genetics/CGP/CellLines/) and are listed as mutant (M) for LKB1, PI3K, and KRAS. Mutancy for LKB1 indicates loss of function, while PI3K and KRAS mutations confer hyperactivity. Cultures were set up in triplicate, and error bars are representative of standard deviation from triplicate measurements.
sensitivity to pemetrexed, however, could not be predicted by LKB1 mutation status alone, as H460 was the second most sensitive cell line in our screen, A549 fell in the middle, and Hela was among the least sensitive. We chose two cell lines for further investigation, H460 and Hela, and confirmed that these cells lacked LKB1 expression by western blot (Figure 3-13A). H460 has a premature stop at codon 37, the most common loss-of-function mutation in LKB1 (292), and Hela has a biallelic deletion of the gene (293). Cell growth in the presence of several concentrations of pemetrexed with thymidine showed that both cell lines were indeed sensitive to pemetrexed (Figure 3-13B). Compared to HCT116 cells, which express wild type LKB1, pemetrexed was 10-fold less potent at inhibiting growth in H460 and Hela cells (Figure 3-13). Interestingly, the effect of proliferation of Hela cells plateaued at approximately 60% growth inhibition, even at high concentrations of pemetrexed, while H460 had a similar growth inhibitory effect as HCT116 at high concentrations. We do not yet understand this effect.

To determine whether inhibition of mTORC1 signaling was preserved in LKB1-null carcinomas treated with pemetrexed, we used S6K1 T389 phosphorylation as a marker for mTORC1 activity. To our surprise, S6K1 T389 was hypophosphorylated in both H460 and Hela cells, proving that LKB1 was not necessary for the inhibitory effect of pemetrexed on mTORC1 (Figure 3-14). HCT116 cells were carried as a positive control through this experiment, and total protein lysates from all three cell lines were loaded onto the same gels, allowing for quantitative comparisons to be made across samples. Interestingly, S6K1 expression was significantly higher in HCT116 than in H460, and Hela had very weak expression of S6K1. Because of this, the exposure level for these blots was dependent on expression in Hela. Therefore, HCT116 total and phosphorylated S6K1 was overexposed, as was H460 phosphorylated S6K1. While a clear
Figure 3-13. Sensitivity of LKB1-null cell lines to growth inhibition by pemetrexed. (A) LKB1 expression was assessed by immunoblot. Total protein (20 µg) was loaded into each lane. (B) Growth was determined by Coulter counting after 72 hour exposure to 1 µM pemetrexed in the presence of 5.6 µM thymidine. Cultures were set up in triplicate, and error bars are representative of standard deviation from triplicate measurements.
Figure 3-14. Signaling downstream of mTORC1 is inhibited in LKB1-null cells following pemetrexed treatment. Cells were exposed to 1 µM pemetrexed and 5.6 µM thymidine for 15 hours. Immunoblots were done on equivalent amounts of total protein, assayed by the Bradford method. Total protein (20 µg) was loaded into each lane. Blots were exposed to the level at which HELA S6K1 was detectable, resulting in overexposure of H460 and HCT116. Hypophosphorylation is also detectable by the pronounced mobility shift in the total S6K1 blot in pemetrexed-treated lanes.
diminution in S6K1 T389 phosphorylation was measured in all three cell lines, the degree of hypophosphorylation of S6K1 T389 correlated with the degree of sensitivity to pemetrexed (i.e., HCT116 being the most sensitive and Hela the least). Regardless, this result indicated that pemetrexed-mediated mTORC1 inhibition was indeed maintained in the absence of LKB1.

In HCT116 cells, we used chemical (compound C) and genetic (AMPKα1 siRNA knockdown) approaches to determine whether the effects of pemetrexed on mTORC1 activity were mediated through AMPK activation. By both criteria, they were. Likewise, in H460 cells, blocking the catalytic activity of AMPK with compound C ablated the inhibitory effect of pemetrexed on S6K1 T389 phosphorylation (Figure 3-15). This confirmed that pemetrexed-mediated mTORC1 inhibition was mediated by AMPK activation in the absence of LKB1. Unlike what was seen in HCT116, compound C induced S6K1 T389 hypophosphorylation in the absence of pemetrexed in H460 cells, an effect not completely understood at this time.

In LKB1-null cell lines, enhanced phosphorylation of AMPKα T172 did not always correspond with AMPK activity. For example, in H460 cells, hyperphosphorylation of AMPKα T172 in response to pemetrexed treatment was not detectable, because this residue was already hyperphosphorylated in the absence of treatment (Figure 3-16). However, in addition to the above-mentioned result with S6K1 T389 phosphorylation, several observations discussed later in this chapter clearly indicated that AMPK was activated by pemetrexed exposure. 1) The direct target of AMPK, acetyl CoA carboxylase (ACC), was hyperphosphorylated at S79 in H460 and Hela cells (Figure 3-17). ACC catalyzes the committed reaction of de novo fatty acid synthesis, the conversion of acetyl CoA to malonyl CoA. Phosphorylation of ACC at S79 by AMPK inhibits the activity of this enzyme (198). 2) Eukaryotic elongation factor 2 (eEF2), the substrate for the direct AMPK target eEF2 kinase, was also hyperphosphorylated at T56 in H460 and Hela
Figure 3-15. Inhibition of mTORC1 signaling by pemetrexed is dependent on AMPK activity, even in the absence of LKB1. The AMPKα inhibitor, compound C (1 µM), blocks the effects of pemetrexed on mTORC1 signaling in the H460 LKB1-null human non-small cell lung carcinoma cell line following 15 hour exposure to both agents. All treatments contained 0.1% DMSO and 5.6 µM thymidine. Total protein (20 µg) was loaded into each lane.
Figure 3-16. Differential effects of AMPKα T172 phosphorylation in response to pemetrexed across cell lines. Cells were exposed to 1 μM pemetrexed and 5.6 μM thymidine or to 250 μM AICAR for 15 hours. Total protein was isolated, quantified, and subjected to immunoblot analysis as described in the Methods section. Total protein (20 μg) was loaded into each lane. H460 blot on the right was overexposed to allow for detection of HELA.
Figure 3-17. Differential effects on acetyl-CoA carboxylase S-79 phosphorylation in response to pemetrexed across cell lines. Cells were exposed to 1 μM pemetrexed and 5.6 μM thymidine or to 250 μM AICAR for 15 hours. Total protein was isolated, quantified, and subjected to immunoblot analysis as described in the Methods section. Total protein (20 μg) was loaded into each lane. H460 blot on the right was overexposed to allow for detection of HELA.
Figure 3-18. Phosphorylation of eukaryotic elongation factor 2 at T56 is augmented in response to pemetrexed in cells of wild type (HCT116) and –null (H460, HEla) LKB1 status following pemetrexed treatment. Cells were exposed to 1 µM pemetrexed and 5.6 µM thymidine for 15 hours. Total protein was isolated, quantified, and subjected to immunoblot analysis as described in the Methods section. Total protein (20 µg) was loaded into each lane.
cells (Figure 3-18). Phosphorylation of eEF2 kinase by AMPK promotes eEF2 kinase to transmit an inhibitory phosphorylation signal to eEF2 by targeting T56 on this essential translation elongation factor (197). AMPK in untreated H460 cells was inactive by these criteria. The inactivity of AMPK that was extensively phosphorylated at T172 in untreated H460 cells led to the conclusion that T172 phosphorylation was necessary, but not sufficient for AMPK activation in these cells, and our data suggested that AMPK activation only occurred following accumulation of ZMP either after pemetrexed or AICAR treatment (Figures 3-16 through 3-18). The effect of pemetrexed on AMPKα T172 phosphorylation in Hela cells was somewhat different. Hyperphosphorylation of AMPKα T172 was measured in Hela cells treated with pemetrexed, but not with AICAR (Figure 3-16). The inability of AICAR to activate AMPK in Hela cells has indeed been reported by others (182). Surprisingly, we measured no accumulation of ZMP in AICAR-treated Hela cells (data not shown). This suggested that Hela cells might have lost adenosine kinase function, preventing the conversion of AICAR to ZMP. This finding warrants further study.

**Feedback suppression of AKT is relieved following pemetrexed-induced mTORC1 inhibition**

The original purpose of the following experiments was simply to characterize the dynamics of mTORC1 inhibition by pemetrexed. We somewhat naively hypothesized that we would see a pattern of AMPK activation that coincided with downstream inhibition of mTORC1 signaling, measured by phosphorylation status of S6K1 and 4E-BP1. Signaling events along the AMPK-mTORC1 axis were measured as a function of time following pemetrexed and thymidine treatment in HCT116 cells. A 48-hour signaling time course was chosen based on the time course of ZMP accumulation measured in these cells. In response to pemetrexed, AMPKα
became hyperphosphorylated at T172 by 15 hours and this phosphorylation persisted for an interval coincident with expansion of the ZMP pool (Figure 3-19). It therefore appeared that the presence of this expanded ZMP pool was sufficient to trigger AMPKα hyperphosphorylation for an extended period of time.

Initially, a burst in mTORC1 signaling was measured at the 7-hour time point, supported by both hyperphosphorylation of 4E-BP1 and S6K1 (Figure 3-20A and 3-21). This was especially apparent from a mobility shift observed on immunoblots of total 4E-BP1 (Figure 3-20A). Since 4E-BP1 has 4 phosphorylation sites sensitive to mTORC1, a substantial shift in size can be seen when probing for total protein. The hyperphosphorylated form runs at approximately 22 kDa on a 12.5% polyacrylamide gel, while the unphosphorylated form runs at approximately 17 kDa (Figure 3-20A). At time 0 (or in untreated cells) total 4E-BP1 looked like a smear across this size range, indicating a mixture of phosphorylated forms. However, at the 7-hour time point, there was a clear mobility shift to the higher phosphorylated form, and this was confirmed by measuring hyperphosphorylation of 4E-BP1 T70. The mechanism by which mTORC1 signaling ramped up before being shut down is unknown to us, as is the therapeutic relevance of this interesting phenomenon.

Inhibition of 4E-BP1 phosphorylation peaked by 15 hours, but recovered slowly, as indicated by western blots probed with phospho-specific antibody and, particularly, by a pan 4E-BP1 antibody (Figure 3-20A). While only a very low percentage of this protein ran at the position of p-4E-BP1 (pan 4E-BP1 blot, Figure 3-20A), there was a substantial expansion of the cellular content of unphosphorylated 4E-BP1 after pemetrexed treatment in HCT116 cells. This suggests that 4E-BP1 was either being overexpressed or that the protein was not susceptible to turnover in its unphosphorylated form following pemetrexed treatment. Because
unphosphorylated 4E-BP1 is known to tightly bind to eIF4E-capped mRNA complexes, preventing the binding of eIF4G and the initiation of cap-dependent translation (Figure 3-3), we hypothesized that CAP-bound 4E-BP1 was stabilized. We measured the binding of 4E-BP1 to eIF4E-cap complexes in extracts of pemetrexed-treated cells. The binding of 4E-BP1 to eIF4E-bound 7-methyl-GTP-beads was enhanced by pemetrexed and was maintained for at least 24 hours after treatment (Figure 3-20B), indicating a prolonged presence of active inhibitor of cap-dependent translation. In contrast, the binding of 4E-BP1 to cap complexes promoted by rapamycin was less than seen with pemetrexed and diminished at longer times (12 hr) after treatment (Figure 3-20B), as previously shown by others (29).

To our surprise, the time-course of pemetrexed effects on S6K1 phosphorylation (Figure 3-21) indicated that S6K1 phosphorylation was initially inhibited by 15 hours, but that hypophosphorylation of S6K1 was transient and partially recovered by 48 hours (Figure 3-21). As in HCT116 cells, hypophosphorylation of S6K1 was transient in H460, peaking at 15-24 hours and partially recovering by 48 hours, suggesting that the negative feedback controls on mTORC1 signaling are also functional in H460 cells, which lack LKB1 (Figure 3-21). Important to note is that the recovery of S6K1 phosphorylation occurred in spite of continued phosphorylation of AMPKα T172 and expansion of the ZMP pool (Figure 3-19 and 3-21), suggesting that an event dominant over continued AMPK activity was being activated.

We sought to determine whether the partial escape from mTORC1 inhibition following pemetrexed was caused by relief of feedback suppression of PI3K and AKT signaling (Figure 3-1). The phosphorylation and stability of IRS-1 was determined with an antibody directed against total IRS-1 protein. As S6K1 became hypophosphorylated at T389, an increased level of IRS-1 was observed (Figure 3-22), presumably signifying expression and stabilization of this protein
Figure 3-19. ZMP accumulation and AMPK activation as a function of time in HCT116 cells exposed to pemetrexed. For Immunoblotting, cells were exposed to 1 μM pemetrexed and 5.6 μM thymidine for the indicated times. Total protein was isolated, quantified, and subjected to immunoblot analysis as described in the Methods section. Total protein (20 μg) was loaded into each lane. For ZMP measurements, cells were exposed to 1 μM pemetrexed and 5.6 μM thymidine for 0, 4, 7, 15, 24, and 48 hours. Acid-soluble extracts were analyzed by HPLC as described in the Methods section. The area units under HPLC peaks corresponding to ZMP were converted to moles using the equation derived from a standard curve generated with synthetic ZMP. The cytosolic volume of $10^6$ cells was roughly equivalent to 1 μl cell water. Error bars are representative of standard deviation from two biological and two technical replicates.
Figure 3-20. Repression of mTORC1 signaling to 4E-BP1 is maintained over time following pemetrexed treatment in HCT116 cells. (A) Cells were exposed to 1 μM pemetrexed and 5.6 μM thymidine for the indicated times. Total protein was isolated, quantified, and subjected to immunoblot analysis as described in the Methods section. Total protein (20 μg) was loaded into each lane. (B) Cells were exposed to 1 μM pemetrexed and 5.6 μM thymidine or 25 μM rapamycin for the indicated times. Thymidine and DMSO controls were exposed for 24 hours. Cell lysates (500 μg) were incubated with m7GTP-Sepharose beads and subjected to immunoblot analysis as described in the Methods section. Sepharose-precipitated protein (350 μg) was loaded into each lane probed with 4E-BP1 antibody, and 150 μg sepharose-precipitated protein was loaded into each lane probed with eIF4E antibody.
Figure 3-21. Repression of mTORC1 signaling to S6K1 partially recovers over time following pemetrexed treatment in HCT116 and H460 cells. Cells were exposed to 1 µM pemetrexed and 5.6 µM thymidine for the indicated times. Total protein was isolated, quantified, and subjected to immunoblot analysis as described in the Methods section. Total protein (20 µg) was loaded into each lane.
Figure 3-22. Recovery of mTORC1 signaling in response to pemetrexed is concurrent with release of feedback inhibition of PI3K and AKT in HCT116 cells. Cells were exposed to 1 µM pemetrexed and 5.6 µM thymidine for the indicated times. Total protein was isolated, quantified, and subjected to immunoblot analysis as described in the Methods section. Total protein (20 µg) was loaded into each lane.
Enhanced phosphorylation of AKT on S473 was also observed coincident with recovery of pemetrexed-mediated inhibition of S6K1 in HCT116 cells (Figure 3-22). Therefore, it appeared that the partial recovery of S6K1 T389 phosphorylation (Fig 4B) was a result of enhanced AKT activity, at least partially reactivating mTORC1. Importantly, reactivation of mTORC1 is not seen following rapamycin treatment since there is a direct block of mTORC1. This is a somewhat obvious, yet important distinction between direct mTORC1 inhibition and inhibition of mTORC1 indirectly through AMPK activation.

In support of this mechanism of feedback hyperactivity of AKT being the cause of transient S6K1 inhibition following pemetrexed, treatment of HCT116 cells with the pan-AKT competitive inhibitor GSK690693 prolonged the inhibitory effect of pemetrexed on S6K1-T389 phosphorylation (Figure 3-23). Additionally, colony formation experiments with this combination indicated that the cytotoxicity of AICART inhibition by pemetrexed was enhanced by exposure to the AKT inhibitor (Figure 3-24). Moreover, an additive cytotoxic effect in HCT116 and H460 cells was measured by clonogenic survival when pemetrexed was combined with the celecoxib-derived PDK1 inhibitor OSU-03012 (Figure 3-25). Collectively, these drugs not only served as tools to identify AKT as a mediator of this rebound effect on mTORC1 signaling, they also suggest that combining pemetrexed with agents that inhibit PI3K signaling is likely a clinically relevant combination strategy.

The effects of AMPK activation by pemetrexed extend beyond mTORC1 inhibition

Our studies thus far have primarily focused on the effects of pemetrexed as an mTORC1 inhibitor. However, the downstream consequences of AMPK activation are well known to extend beyond inhibition of mTOR signaling (Table 3-1). This is illustrated in a comparison of
Figure 3-23. AKT reactivation mediates recovery of mTORC1 signaling after pemetrexed treatment in HCT116 cells. Cells were exposed to 1 µM pemetrexed and the pan AKT inhibitor GSK690693 (100 nM) in the presence of 5.6 µM thymidine for the indicated times. Total protein was isolated, quantified, and subjected to immunoblot analysis as described in the Methods section. Total protein (20 µg) was loaded into each lane.
Figure 3-24. The cytotoxicity of pemetrexed in HCT116 cells is enhanced in combination with GSK690693. Clonogenic survival assays were performed as described in the Methods section. Cells were exposed to 0-10 μM combinations of pemetrexed and GSK690693 for 72 hours. Survival plates representative of additivity are shown in the top panel and enumerations of these colonies are shown in the bottom panel. Cultures were set up in triplicate, and error bars are representative of standard deviation from triplicate measurements.
Figure 3-25. The cytotoxicity of pemetrexed in (A) HCT116 and (B) H460 is enhanced in combination with OSU-03012. Clonogenic survival assays were performed as described in the Methods section. Cells were exposed to 0-10 \( \mu \)M combinations of pemetrexed and OSU-03012 for 72 hours. Colony number was counted manually, and representative combinations showing additivity are depicted relative to control colony formation in the presence of vehicle (0.1% DMSO). Cultures were set up in triplicate, and error bars are representative of standard deviation from triplicate measurements.
growth inhibitory effects of pemetrexed and thymidine with rapamycin (Figure 3-12). The sensitivity of all ten of these cell lines to pemetrexed and to a concentration of rapamycin that would cause complete and prolonged inhibition of mTORC1 (25 nM) were not identical nor even correlated, with most cell lines demonstrating higher sensitivity to AMPK activation by pemetrexed than to direct inhibition of mTOR (Figure 3-12). We highlight that similar sensitivity (or lack-there-of) to rapamycin and its analogs in several of these cell lines was also reported elsewhere (295). From this experiment, we concluded that the secondary effects of pemetrexed causing growth inhibition were due to the downstream signaling from multiple effects of AMPK activation rather than solely to inhibition of mTORC1.

Striking to us was the drastic difference in sensitivity to pemetrexed and rapamycin in HCT116 cells (Figure 3-12). Rapamycin is thought to elicit its antiproliferative effects exclusively through mTOR inhibition, which perturbs ribosome biogenesis and CAP-dependent translation, processes essential for protein synthesis. We therefore attempted to determine the effects of pemetrexed and rapamycin on global protein synthesis in these cells. We pulse-labeled HCT116 cells with $^{35}$S-methionine following an exposure to pemetrexed or rapamycin that was sufficient to inhibit mTORC1 signaling. The rate of protein synthesis (measured by $^{35}$S-methionine incorporation) in these cells following drug exposure was indeed reflective of the growth inhibitory effects of these drugs (Figure 3-26). While rapamycin had an insignificant effect on the rate of protein synthesis in HCT116 cells compared to its vehicle control, pemetrexed inhibited the rate of protein synthesis by greater than 50% in these cells (Figure 3-26). This degree of protein synthesis inhibition was in agreement with reported inhibition following AICAR treatment (296). This finding therefore confirmed that mTORC1-dependent
Figure 3-26. **Global translational effects of pemetrexed and rapamycin in HCT116 cells.**

HCT116 cells were treated with pemetrexed or rapamycin for 24 hours before being pulse-labeled with $^{35}$S-methionine as described in the Methods section. Error bars are representative of standard deviation from triplicate measurements. * Represents $p < 0.05$ as determined by a student’s T-test.
and independent factors downstream of AMPK activation by pemetrexed were contributing to this diminution in protein synthesis in HCT116 cells.

We then questioned whether the differential effect of pemetrexed and rapamycin on protein synthesis could be explained by other signaling events mediated by AMPK. AMPK has been shown to directly target eEF2 kinase (197). Activation of eEF2 kinase transmits an inhibitory phosphorylation signal to eEF2 by targeting T56 on this essential translation elongation factor. Indeed, eEF2 was hyperphosphorylated at T56 in response to pemetrexed (Figure 3-18). We extended these findings to LKB1-null H460 and HELA cells, which also showed increased phosphorylation in response to pemetrexed. This not only confirmed our findings in HCT116 cells, it also gave further support that AMPK signaling mediated by pemetrexed was preserved in the absence of LKB1 (Figure 3-13 and 3-18). Interestingly, when we compared phosphorylation status of eEF2 at T56 (Figure 3-18) across these three cell lines, we observed a high basal level of T56 phosphorylation in untreated HCT116 cells in the absence of detectable phosphorylation of AMPKα T172 (Figure 3-16). This result suggested that either another kinase was responsible for these phosphorylation events, or that AMPK had a basal level of kinase activity in untreated cells that was sufficient to target eEF2 kinase, but not sufficient to propagate inhibitory signals to mTORC1. It is important to note that the basal activity of AMPK in HCT116 cells measured by our in vitro kinase assay (Figure 3-6) was not 0. Therefore, it is likely that this latter hypothesis may explain the kinase activity observed in the absence of a stimulus.

The first identified substrate of AMPK was acetyl CoA carboxylase (ACC), which catalyzes the committed step of de novo fatty acid synthesis, the conversion of acetyl CoA to malonyl CoA (198). Phosphorylation of ACC at S79 by AMPK inhibits the activity of this
enzyme, thus perturbing de novo lipid synthesis. Indeed, the preferred in vitro substrate for AMPK in kinase assays is the SAMS peptide, which encompasses S79 of ACC and surrounding residues (155). In LKB1-null H460 and Hela cells, ACC was hyperphosphorylated at S79 in response to pemetrexed and AICAR (Figure 3-17). Surprisingly, it was difficult to detect increases in phosphorylation of ACC S79 in HCT116 cells. In a direct comparison with H460 lysates, it was determined that, like eEF2, ACC S79 in HCT116 cells was hyperphosphorylated in untreated cells (Figure 3-17). Hyperphosphorylation of ACC S79 under basal conditions was also reported by others (169), and this result questioned whether another kinase was indeed responsible for phosphorylation of ACC. As mentioned above, our data also suggests an alternative hypothesis. The activity of AMPK measured in the absence of an allosteric stimulus may be sufficient to directly target both ACC and eEF2 kinase, but insufficient to target the mTORC1 signaling axis.
DISCUSSION

Mutant hyperactivity of the PI3K-AKT and RAS-MAPK signaling pathways, both converging on mTORC1, are a common phenotype in human carcinomas, and are thought to be causal of uncontrolled cell growth and proliferation in the absence of environmental stimuli (158). Operating as a negative regulator of aberrant signaling within these pathways, AMPK is activated in response to diminished ATP, limiting the energy-consuming processes of protein and lipid metabolism, and promoting the energy-generating process of carbohydrate metabolism, to maintain cellular energy homeostasis (Figure 3-1) (157). In this chapter, we show that AICART inhibition by antifolates stimulates AMPK activity following accumulation of the de novo purine synthesis intermediate, ZMP. This demonstrates a novel therapeutic intervention to prevent aberrant proliferation in tumors with genetic defects in these pro-survival pathways. Activation of AMPK in human carcinoma cells following AICART inhibition by pemetrexed elicits mTORC1-dependent and -independent effects on key controlling elements of translational initiation and elongation as well as lipid metabolism. Additionally, we identified a potential limitation to mTORC1 inhibition through this mechanism, the release of feedback inhibition of AKT signaling. This suggested a rational therapeutic strategy in which pemetrexed might be effectively combined with inhibitors of upstream signaling components of the PI3K-AKT or RAS-MAPK signaling pathways to limit rebound hyperactivity of mTORC1. However, we note that the effects of AMPK activation by pemetrexed extend beyond mTORC1 signaling, placing AICART inhibitors in a class distinct from rapamycin and its analogs.
**The requirements for AMPK activation may be cell and substrate-specific**

In vitro, ZMP mimics the allosteric effects of AMP on the activity of AMPK following binding to CBS motifs in the γ subunit (132, 297). ZMP or AMP binding to the γ subunit is thought to allosterically stimulate the kinase activity of the α subunit both directly through conformational restraint of an autoinhibitory peptide on the α subunit and by protecting AMPKα T172 from dephosphorylation (170, 179, 180). In HCT116 cells, these dual allosteric effects of ZMP appear to contribute to the enhanced kinase activity of AMPK necessary for mTORC1 inhibition (Figure 3-6 and 3-9). Following pemetrexed-mediated ZMP accumulation in HCT116 cells, T172 is maintained in a phosphorylated state, the proposed autoinhibitory domain of AMPKα is presumably restrained, and the kinase is inhibitory towards mTORC1 signaling. However, AMPK activity was easily measurable in immunoprecipitates of untreated HCT116 cells (Figure 3-6). While this basal AMPK activity did not translate inhibitory signals to mTORC1, the mTORC1-independent targets of AMPK, ACC and eEF2, were hyperphosphorylated in HCT116 cells (Figure 3-17 and 3-18) in the absence of detectable T172 phosphorylation or an expanded ZMP pool (Figure 3-16 and 2-16). This suggests that either AMPK displays substrate-specificity, requiring enhanced levels of activity for certain targets, or that another kinase phosphorylates ACC and eEF2 in untreated HCT116 cells. Indeed, others have observed phosphorylation of ACC in cells with no detectable AMPKα T172 phosphorylation (169). The mechanism of the differential phosphorylation in HCT116 cells of mTORC1-independent targets, ACC and eEF2, and of mTORC1-related AMPK targets remains to be elucidated.

The interpretation of signaling events following pemetrexed treatment in cells lacking LKB1 added to the apparent complexity of AMPK activation. In H460 cells, AMPKα T172 was
hyperphosphorylated in the absence of stimulation (Figure 3-16). However, neither ACC nor eEF2 were hyperphosphorylated under these conditions, and S6K1 T389 was not hypophosphorylated (Figure 3-14, 3-17 and 3-18). Only after pemetrexed or AICAR treatment were these targets affected. This suggested that allosteric binding of ZMP to the AMPKγ subunit in H460 cells is required to release autoinhibition of the α subunit, but not to induce a conformational shift necessary to protect AMPKα T172 from phosphatases. Presumably, this residue is already protected. CAMKKβ or Tak1 are candidate alternative upstream kinases responsible for AMPKα T172 phosphorylation in these cells. It is possible that these upstream kinases are inhibiting phosphatase activity towards this residue or that H460 cells simply lack the T172 phosphatase. We therefore concluded that T172 phosphorylation alone is insufficient to fully activate the kinase activity of AMPKα in H460 cells. In accord, an in vitro measurement of AMPKα1 kinase activity in untreated H460 cells showed no basal kinase activity compared to basal activity in HCT116 (data not shown), a finding consistent with our signaling results.

In Hela cells, AMPK responded to pemetrexed in a manner similar to HCT116. Basal phosphorylation of AMPKα T172 was very low (compared to H460) (Figure 3-16), and became hyperphosphorylated in response to pemetrexed. Presumably, T172 was protected from phosphatase activity by ZMP, amplifying the influence of another kinase such as Tak1 or CAMKKβ. Like HCT116 cells, there did appear to be basal kinase activity of AMPK towards ACC and eEF2, but not towards the mTORC1-dependent target, S6K1, which was only affected in the presence of pemetrexed (Figure 3-14, 3-17 and 3-18).

While AMPKα T172 phosphorylation was observed in Hela cells treated with pemetrexed, phosphorylation was not measured following AICAR treatment (Figure 3-16). The inability of AICAR to activate AMPK in Hela cells has been reported by others (182). In these
studies, not only did AICAR fail to stimulate the kinase, but also the downstream effects of AMPK activation were not observed in Hela cells treated with AICAR. The authors concluded that AMPK required LKB1 for activation by ZMP. However, our data refutes this, as pemetrexed-mediated ZMP accumulation stimulates the kinase. Importantly, we found no accumulation of ZMP in AICAR-treated HeLa cells, suggesting a loss of adenosine kinase activity in these cells. In comparison, ZMP was easily measurable in pemetrexed-treated Hela cells (Table 2-3). The lack of ZMP accumulation in Hela following AICAR treatment is interesting, and further study is warranted to permit proper interpretation of prior studies.

Of interest to us was the observation that ZMP induced by pemetrexed stimulated AMPK activity in cells that lost LKB1 function (Figures 3-13 through 3-18), in spite of the fact that the experimental support for LKB1 as the principal kinase for AMPKα T172 phosphorylation is strong (181-183). In H460 cells, the AMPKα subunit is hyperphosphorylated at T172 under basal conditions by a kinase other than LKB1, suggesting a lack of T172 phosphatase activity in these cells. As described above, AMPK is not catalytically active under these conditions but becomes active following the accumulation of ZMP. Central to the proper interpretation of these findings is the elucidation of the upstream kinase responsible for phosphorylating AMPKα T172 in H460 cells.

Based on previous literature, it could be hypothesized that CAMKKβ is the alternate upstream kinase in cells that lack LKB1 (188-190). We could test this by measuring AMPKα T172 phosphorylation in H460 cells following treatment with CAMKKβ siRNA or the pharmacological inhibitor STO-609. Indeed, the observation that CAMKKβ siRNA and STO-609 treatment blocked AMPKα T172 phosphorylation has been previously made in Hela cells, also lacking LKB1 (189). Importantly, studies have shown that while CAMKKα activity
depends on a rise in intracellular calcium, CAMKKβ is constitutively active, although activity does increase in the presence of calcium (287, 298). Therefore, it is not unreasonable to think that CAMKKβ could substitute for LKB1 as the AMPKα T172 kinase.

**Antifolate activation of AMPK by ZMP-independent mechanisms**

The studies presented in this chapter definitively prove that AMPK is activated by pemetrexed in response to accumulation of ZMP. However, AMPK is also sensitive to changes in the AMP:ATP ratio as well as to increased intracellular calcium (157). This suggests that other antifolates, classically recognized to deplete nucleotide pools, may have AMPK-activating effects. In response to pemetrexed, the concentration of ATP and GTP do not diminish (Figure 3-7) (143). This is important, because a depletion of cellular ATP would stimulate AMPK. This was indeed the case with the GART inhibitor (6R)-DDATHF. A.C. Racanelli in our lab showed that AMPK was activated in response to (6R)-DDATHF, an effect reversed by addition of the salvage nucleotide inosine (Racanelli AC, Ph.D. dissertation 2009, Figure 4-8). In comparison to pemetrexed, (6R)-DDATHF depletes both cellular ATP and GTP (46). Based on *in vitro* studies with rat liver AMPK, AMP is a more efficient allosteric activator of AMPK than ZMP (132). This would suggest that depletion of ATP by (6R)-DDATHF might be a more effective means of AMPK activation than ZMP accumulation following pemetrexed treatment. However, when A.C. Racanelli compared the effects of AICAR, LCA and (6R)-DDATHF on inhibition of mTORC1 signaling, she surprisingly showed a more effective blockade in response to the ZMP-mimetic and the AICART inhibitor than to GART inhibition (Racanelli AC, Ph.D. dissertation 2009, Figure 4-6). This therefore suggests that AMPK activation using an AICART inhibitor would be more advantageous than through GART inhibition.
It is not understood why purine pools are not depleted in response to pemetrexed treatment. Given the fact that ZMP pools are expanded in response to pemetrexed to a much greater extent than siRNA knockdown of AICART by approximately 95%, we expect that pemetrexed induced a strong inhibitory effect on AICART, which would hypothetically diminish *de novo* purine synthesis (Table 2-2 and 2-3). I hypothesize that *de novo* ATP synthesis is indeed diminished, but ATP pools are maintained as a result of AMPK activity towards effectors of carbohydrate metabolism (Table 3-1). As next-generation AICART inhibitors are discovered, it will be important to determine the extent of ATP depletion in response to these treatments, as a combination of ZMP accumulation and ATP depletion in response to significant AICART inhibition may have an additive effect on AMPK activity.

The finding that AMPK activity can be modulated by intracellular calcium in the absence of alterations to the AMP:ATP ratio is extremely interesting (188-190). The effects of methotrexate on calcium homeostasis were recently studied in rat hepatocytes (299). Cytosolic calcium in response to methotrexate was elevated, and it was determined that the antifolate enhanced calcium release from the endoplasmic reticulum in a manner similar to IP$_3$ rather than promoting extracellular calcium uptake. The effects of pemetrexed on intracellular calcium homeostasis are unknown. However, if pemetrexed also enhanced cytosolic calcium, this would present an alternative explanation for the AMPK-activating effect of pemetrexed in LKB1-null cells. It may also add to the explanation of why pemetrexed, but not AICAR, is able to activate AMPK in Hela cells. It would also be interesting to determine whether the therapeutic effect of pemetrexed could be potentiated with a calcium ionophore. Importantly, if this proposed effect on calcium homeostasis was independent of effects on folate metabolism, it may help explain
why the growth-inhibitory effects of pemetrexed could not be completely rescued by thymidine and hypoxanthine in HCT116 cells (Figure 2-10B).

**Diverse potential clinical utility of AICART inhibitors**

The finding that the effects of pemetrexed extend beyond folate metabolism to control signaling pathways disrupted in cancer suggests that AICART inhibitors may have therapeutic efficacy in a variety of clinical conditions. The utility of structural analogs of rapamycin for cancer therapeutics has recently been demonstrated by the activity of temsirolimus and everolimus for renal cell carcinoma (300, 301). Based on our results, we would predict that the clinical effectiveness of AICART inhibitors would show overlap with rapalogs. A direct inhibitor of mTOR such as rapamycin, binds to a site adjacent to the kinase domain of mTORC1 as a complex with FKBP12, and inhibits mTORC1 activity at low nanomolar concentrations (259). Prolonged rapamycin exposure has also been reported to inhibit mTORC2 signaling, but the effects of rapamycin are generally taken as due to inhibition of mTORC1 (232). In contrast, AICART inhibitors activate AMPK and inhibit mTORC1 by enhancing two physiological control systems, the phosphorylation of the Raptor subunit of mTORC1 at S792, which prevents or inhibits the formation of a complex between mTOR and Raptor, and phosphorylation of the TSC2 subunit of the tuberous sclerosis complex, an effect which promotes the GTPase activity of Rheb, preventing activation of mTORC1 (195, 196). Pemetrexed-induced accumulation of ZMP does not appear to inhibit the mTORC2 complex, as judged by the enhanced phosphorylation of AKT seen in Figure 3-22.

The pronounced effect of AICART inhibitors on mTORC1 signaling suggests these agents may have therapeutic efficacy in the treatment of tuberous sclerosis complex disorder.
TSC is caused by mutations in the TSC1 and TSC2 genes, and results in a multi-system disorder plagued by benign hamartomas of the brain, kidney, skin, heart, and lungs (302). TSC patients often present with mental retardation, epilepsy, autism, renal angiomyolipomas, and pulmonary lymphangioleiomyomatosis (LAM). The identification that the TSC GTPase activating protein functioned as an upstream regulator of mTORC1 signaling suggested that rapamycin and its analogs may be an effective therapeutic approach to controlling the symptoms of TSC disorder, for which there were no effective therapeutics. The efficacy of rapamycin and its analogs in clinical trials for TSC patients has been promising, and tumor regression as well as a diminution in neurological effects of the disease has been documented (303). However, symptoms and tumors returned upon removal of the drug. Patients were treated with rapamycin continuously for up to 9 months, however, the long-term effects of continuous rapamycin exposure are unknown.

It is likely that AICART inhibitors would also be beneficial to TSC patients. It will be important to test this hypothesis in mouse models of TSC. It would also be expected that removal of an AICART inhibitor would allow TSC symptoms to return. Therefore, studies of chronic AICART inhibitor administration in comparison to chronic rapalog treatment are warranted to determine whether there is any advantage to long-term administration of one over the other. The kinetics of antifolates may make them more favorable for chronic administration than rapamycin, which has a short half-life and would have to be given quite frequently. On the contrary, pemetrexed polyglutamates are retained intracellularly following plasma clearance, and therefore, the drug is given once every 21 days in a 10-minute infusion (73). Pemetrexed is also well tolerated, and is approved for maintenance therapy of NSCLC (80), suggesting long-term administration may be possible without overt toxicity. However, the progressive nature of
NSCLC would likely not provide the clinical data necessary to deem pemetrexed safe for chronic administration over the time scale needed for TSC therapy.

Although AMPK phosphorylation of TSC2 and Raptor exerts a strong inhibition on mTORC1 and downstream targets critical to protein and lipid synthesis, a number of other targets are controlled by AMPK that are not mediated by mTORC1 (Table 3-1) (157). It would be expected that pemetrexed-induced activation of AMPK would also affect these other direct targets of this kinase, and that the overall effects of AICART inhibition would have components additional to the effects seen with rapamycin analogs. This would afford AICART inhibitors a therapeutic advantage over direct mTORC1 inhibitors, and might extend the utility of AICART inhibitors towards diseases and conditions of metabolic origin. Indeed, we show that targets for AMPK whose phosphorylation is not mediated by mTORC1, such as ACC and eEF2, are affected by pemetrexed (Figures 3-17 and 3-18). The multiple effects of AMPK on lipid synthesis suggest AICART inhibitors should be tested as therapeutics for disorders of lipid metabolism, such as hypercholesterolemia and obesity. As described above, AMPK directly inhibits ACC, which catalyzes the committed reaction of de novo fatty acid synthesis, converting acetyl CoA to malonyl CoA (Figure 3-27). Additionally, AMPK directly inhibits HMG CoA reductase (Table 3-1), a key enzyme of de novo cholesterol biosynthesis that is the target of the statin class of cholesterol-lowering drugs. Most recently, the sterol regulatory-element binding proteins (SREBPs) were identified as downstream targets of mTORC1 (304). These transcription factors are essential for the expression of multiple enzymes of de novo fatty acid and cholesterol biosynthesis (Figure 3-27). Indeed, we have shown that pemetrexed inhibits the nuclear localization of SREBP1 (Figure 3-28), but this effect is likely sensitive to unknown variables, as the result was difficult to repeat.
Figure 3-28. Pemetrexed inhibits nuclear localization of the SREBP-1 transcription factor. HCT116 cells were treated with 1 µM pemetrexed and 5.6 µM thymidine for 24 hours. Total protein was isolated, quantified, and subjected to immunoblot analysis as described in the Methods section. Total protein (20 µg) was loaded into each lane. Nuclear SREBP-1 (65 kDa) is a cleavage product from the full-length species (100 kDa), which is bound to the endoplasmic reticulum membrane. This SREBP-1 antibody detects both cytosolic (membrane-bound) SREBP-1 as well as cleaved (nuclear) SREBP-1.
Diminishing the influence of AKT on AMPK-mediated mTORC1 inhibition

Whereas AMPK inhibits mTORC1 through phosphorylation of TSC2 and the Raptor subunit of mTORC1, AKT opposes each of these effects. AKT indirectly inhibits the GTPase activity of the mTORC1 effector Rheb (196, 306, 307), likely through phosphorylation of multiple residues including S939 and T1462 on the human TSC2 subunit (246, 247), while AMPK phosphorylation of TSC2 S1387 and T1271 are requisite for mTORC1 inhibition in response to energy stress (196). AKT also activates mTORC1 by phosphorylating the PRAS40 subunit of mTORC1 at T246, promoting the dissociation of inhibitory PRAS40 from mTORC1 (245), whereas the phosphorylation of Raptor S792 by AMPK results in inhibition of the kinase activity of mTORC1 (195).

A question of significance is whether the effect of AMPK or that of AKT would dominate at each of these two nodes of signal integration. The events we observed during recovery from pemetrexed-induced inhibition of mTORC1 signaling give some insight into this interaction. We show that, in spite of continued activation of AMPK in response to pemetrexed, the inhibition of S6K1 phosphorylation is partially relieved, apparently by activation of the opposing influences of AKT on TSC2 and mTORC1 (Figure 3-21). However, the recovery of 4E-BP1 was less marked than was the recovery of phosphorylation of S6K1, and residence of 4E-BP1 at a synthetic m7GTP-sepharose substrate remained following rebound hyperactivity (Figure 3-20). This suggests that rebound hyperactivity of AKT may present a differential influence on mTORC1 activity towards its substrates. Of course, it is the unphosphorylated form of 4E-BP1 that is central to inhibition of protein synthesis, and that species remained markedly accumulated after pemetrexed treatment.
Others have reported that the stimulation of AKT activity following rapamycin suppression of S6K1 activity did not prevent suppression of S6K1 phosphorylation nor did it interfere with growth suppression by everolimus (Lane HA 2009 8. Ludwig DL 2006 66). This is expected, as rapamycin is directly blocking mTORC1. We note that the relief of feedback induced stimulation of AKT after pemetrexed treatment also did not prevent complete growth suppression of HCT116 cells after 72 hours (Figure 2-16), although though recovery of S6K1 phosphorylation was evident by 24 hours (Figure 3-21). At the moment, we do not understand why the secondary effects of pemetrexed are not reversed by this hyperactivated AKT. However, our results do suggest therapeutic efficacy in limiting rebound hyperactivity of mTORC1 signaling to S6K1 (Figure 3-23 through 3-25). Indeed, we show that inhibition of clonogenic survival in response to pemetrexed can be enhanced by inhibiting AKT, either directly with the competitive inhibitor GSK690693 (Figure 3-24), or indirectly by inhibiting the AKT T308 kinase, PDK1, with OSU-03012 (Figure 3-25). Interestingly, PDK1 inhibition would affect both AKT and S6K1 directly, providing a dual mechanism of controlling rebound hyperactivity (See Figure 3-1).

The realization that the efficacy of rapamycin and its analogs was potentially limited by rebound hyperactivity of AKT has stimulated drug discovery efforts to circumvent this issue. One promising approach is the development of competitive inhibitors of the mTOR catalytic subunit (308-311). This approach would in effect target both mTORC1 and mTORC2, as they share the same mTOR catalytic subunit. Blocking mTORC1 would inhibit the downstream targets S6K1 and 4EBP1, and simultaneously blocking mTORC2 would inhibit AKT and prevent the mTORC2-stimulating effect of S6K1 inhibition. Although these competitive mTOR inhibitors will perturb AKT signaling, they do not directly target hyperactive PI3K. Therefore,
another approach was the discovery of dual competitive inhibitors of mTOR and PI3K. These compounds have been developed and are already being tested against hematological malignancies (312-314). These compounds are proving to be more effective anti-cancer agents than the rapalogs, and it would be very interesting to determine whether additivity can be seen with these compounds in combination with AICART inhibitors.

**Molecular genetics of tumor sensitivity to AMPK activation**

Genetic abnormalities in elements of the PI3K-AKT, RAS-MAPK, and AMPK signaling networks are frequently observed in biopsies of primary human tumors (158, 315, 316). In lung tumors, KRAS was activated in 35% of biopsies, LKB1 was deficient in 23%, and PI3K was activated in 8%; loss of PTEN was a rare occurrence in NSCLC, although it is common in other tumor types, such as prostate and colon cancers (158). PI3K hyperactivating mutations are also common events in colon, breast, prostate, and ovarian carcinomas (317). Determination of how these genetic signatures of tumors affect the sensitivity to AICART inhibitors is a question of importance, and may aid in the ability to screen patients to predict response to a particular treatment regimen.

To our surprise, LKB1 mutations did not confer resistance to the downstream effects of AMPK activation by pemetrexed in the two LKB1-null cell lines we studied (Figure 3-13). Even in the absence of LKB1, AMPK is clearly activated in response to pemetrexed (Figure 3-14 through 3-18). In addition to LKB1 mutations, KRAS is frequently hyperactivated in primary NSCLC and cell lines derived from these tumors (316). KRAS propagates signaling to ERK and RSK, both of which phosphorylate and inhibits TSC2, similarly to AKT (158). Carcinomas with hyperactive mTOR signaling have been predicted to be more sensitive to rapamycin because of
an increased dependence on mTOR signaling for survival and proliferation (219). It remains to be seen whether mutations in KRAS correlates with hypersensitivity to pemetrexed. Interestingly, HCT116 cells were the least sensitive to growth inhibition by rapamycin but the most sensitive to the secondary effect of pemetrexed. Indeed, cell lines with hyperactive PI3K and KRAS (HCT116 and H460) were the most sensitive in our screen (Figure 3-12). It would be worthwhile to extend this observation to other double mutant cell lines to determine whether these two genetic signatures could serve as biomarkers to predict tumor sensitivity to pemetrexed.
CHAPTER 4
DIFFERENTIAL THERAPEUTIC OUTCOMES OF FOLATE-DEPENDENT TARGET INHIBITION BY PEMETREXED

The discovery that pemetrexed was an AMPK-activating agent that elicited a strong inhibitory effect on mTORC1 signaling prompted us to question how this newly-discovered mechanism of action was contributing to the overall therapeutic effect of the drug. While the cytotoxic therapeutic consequences of thymidylate synthase inhibition via induction of apoptosis have been well documented, the therapeutic effects of AICART inhibition as they pertain to AMPK activation and mTORC1 inhibition remained undefined. The overall cytotoxic effect of pemetrexed has been studied, but the individual contribution of each target of pemetrexed has not been explored. We therefore sought to understand how TS and AICART inhibition contributed both individually and in synchrony to the therapeutics of pemetrexed.

The cytotoxicity associated with TS inhibition is apoptotic and S-phase-specific

As described in Chapter 2, the cytotoxic effect of TS inhibition results from both a diminution of TTP as well as accumulation of dUTP, causing ineffective cycles of excision and repair that ultimately lead to DNA strand breaks and induction of apoptotic cell death. (98). Therefore, the cytotoxicity associated with TS inhibition is likely S-phase specific (318). From a time course of CCRF-CEM cell cycle profiles following exposure to pemetrexed, it was demonstrated that cells accumulated at the G1/S border by 12-24 hours, followed by synchronous entry into S-phase (319). By 36 hours, the cell population was mostly accumulated in S-phase, assessed by both flow cytometry and 5-bromo-2-deoxyuridine incorporation, and a significant population was present at a sub-G1 peak, indicative of apoptosis (320). By 48 hours,
the majority of the cell population was apoptotic, supported by the presence of significant DNA fragmentation (319). Interestingly, using thymidine to reverse the effects of raltitrexed, it was demonstrated by clonogenic survival that the cytotoxicity associated with TS inhibition was completely reversible if thymidine was supplemented in media containing inhibitor within the first 24 hours of drug exposure (321). This indicated that the cellular events contributing to irreversible commitment to apoptosis following TS inhibition did not occur within the first 24 hours of drug treatment, consistent with the time of entry into S-phase following pemetrexed treatment.

**Therapeutic effects of AICART inhibition**

From our studies in Chapter 3 detailing the cellular signaling events that ensued following AICART inhibition, we hypothesized that the therapeutic consequences of AICART inhibition would be similar to effects mediated by an AMPK activating agent such as AICAR or an mTORC1 inhibitor such as rapamycin. One of the most widely recognized consequences of AMPK activation and mTORC1 inhibition is the induction of macroautophagy (herein referred to as autophagy). This coordinated process of cellular self-digestion is paradoxically essential for cellular survival, but deleterious if deregulated (322). Autophagy is an adaptive mechanism to cellular starvation in which cytoplasmic contents including misfolded, aggregated, and long-lived proteins, as well as damaged or unneeded organelles are degraded through the lysosomal machinery and recycled for carbon sources necessary for nutrient metabolism (323). Autophagy operates at a low basal level in most cells and is upregulated in response to nutrient or energy starvation, the withdrawal of growth factors, or when the energetic demands of a cell are elevated.
Autophagy occurs in several distinct phases that are tightly controlled by more than 20 evolutionarily conserved proteins encoded by autophagy genes (ATG’s) (322). A serine/threonine kinase complex consisting of the ATG-gene products ULK1, FIP200, and ATG13 induces the autophagy cascade. The process begins with the nucleation and expansion of a phagophore isolation membrane, which likely buds from the endoplasmic reticulum. A class III PI3K complex consisting of Vsp15, Vsp34, Atg14, and Beclin1 mediates phagophore nucleation, and the ATG12 ubiquitin-like conjugation system promotes phagophore expansion. The phagophore engulfs cytosolic components and upon closure is then referred to as an autophagosome. The ATG12 complex also catalyzes the cleavage and lipid conjugation of LC3-I to form LC3-II, promoting the stable association of LC3-II with the autophagosome. Once LC3-II binds the autophagosome, this double-membraned vesicle fuses with a lysosome to form an autolysosome, where the inner membrane and captured contents are degraded. Membrane permeases then release the degraded contents back into the cytosol (322, 324).

mTORC1 is a key negative regulator of autophagy, inhibiting this catabolic process when nutrients and growth factors are abundant (325, 326). It was recently discovered that mTORC1 regulates autophagy by inhibiting the initiating step in the cascade (327-329). mTORC1 phosphorylates both ULK1 and ATG13, which perturbs the association of this multiprotein complex and inhibits its kinase activity. In support of this finding, rapamycin enhanced the kinase activity of ULK1 and promoted the association of this initiation complex with isolation membranes. In contrast, AMPK activation promotes autophagy (330). While the mechanism of activation is likely mediated partially through mTORC1 inhibition, novel mechanisms by which AMPK can directly stimulate the autophagy cascade by signaling to ULK1 are being elucidated (Shaw RJ, conference communication).
Both AMPK-activating agents as well as mTORC1 inhibitors have been shown to induce cell cycle arrest at the G1/S phase boundary. Among other processes, progress from G1 to S phase of the cell cycle requires phosphorylation of the retinoblastoma protein (Rb) by the CDK4/6-cyclin D complex, which releases transcription factors necessary to enhance synthesis of S-phase specific target genes. The cyclin-dependent kinase inhibitor, p21, whose mRNA expression is induced by p53, promotes G1 arrest by preventing CDK complexes from phosphorylating Rb (331-333). Studies have shown that the AMPK-activating agent metformin diminished the expression of cyclin D1 and causes hypophosphorylation of Rb in several prostate carcinoma cell lines (334). Additionally, studies in the HepG2 hepatocellular carcinoma cell line demonstrated that AICAR induced a G1/S checkpoint arrest that corresponded with pronounced stabilization of p53 concomitant with S15 phosphorylation as well as enhanced expression of p21 (335). Rapamycin has also been shown to induce a G1/S arrest, however, p53 is not stabilized, nor is p21 expression enhanced in response to drug (336-340). This would suggest that the effect of AMPK activation on p53 stabilization is independent of mTORC1 inhibition. Indeed, it has been shown in vitro that AMPK can phosphorylate p53 on S15, a posttranslational modification that contributes to p53 stability by perturbing its interaction with MDM2 (209, 341-343).
MATERIALS AND METHODS

Chemicals and reagents

Propidium iodide staining solution (#P3566), RNAse A (#12091-021), and Acridine Orange vital dye (#A3568) were from Invitrogen (Carlsbad, CA). All other reagents were from Fisher Scientific (Waltham, MA) or Sigma Aldrich (St. Louis, MO) and were of the highest available purity.

DNA content/Cell Cycle Analysis

Tissue culture dishes (10 mm) were seeded with 10^6 cells and allowed to adhere overnight. Following drug treatments, wells were washed once with PBS, trypsinized, and counted electronically using a Z1 Coulter Particle Counter (Beckman Coulter Brea, CA). One million cells were transferred to a new tube, pelleted by centrifugation at 1,000 rpm for 5 minutes, and resuspended in 1 ml propidium iodide staining solution (75 µg/ml propidium iodide, 0.1% Triton X-100, 10 µg/ml RNAse A, and 32 mM sodium citrate). Samples were vortexed and placed at 4°C overnight to maximize staining. Prior to analysis, samples were filtered through 35 µM nylon mesh. DNA content was analyzed with a Beckman CoulterEPICS Elite ESP (Enhanced Sorting Performance) multilaser flow cytometer in the Flow Cytometry and Imaging Shared Resource Facility at VCU.

Acridine Orange Staining

Cells were seeded in 6-well plates at a density of 10^5 cells/well and allowed to attach overnight. Following drug treatments, cells were washed once with PBS before being incubated with Acridine Orange vital dye (1 µg/ml in media) for 15 minutes at 37°C. Cells were washed
again with PBS, and staining was visualized within 1 hour on an inverted fluorescence microscope at a wavelength of 480-520 nm.
RESULTS

**Pemetrexed is both a cytotoxic and antiproliferative therapeutic agent**

The cytotoxicity associated with thymidylate synthase inhibition is well documented (Figure 2-1) (321). Indeed, pemetrexed is a potent cytotoxic agent in tumor cell lines and in vivo (344). However, the relative contribution of thymidylate synthase inhibition and AICART inhibition to the therapeutics of pemetrexed is not known. To address this question, we performed clonogenic survival assays in the presence and absence of the growth rescue agents thymidine and hypoxanthine to isolate the effects of pemetrexed on each target individually. In the absence of rescue agents, pemetrexed had a pronounced cytotoxic effect in both HCT116 and H460 cells (Figure 4-1 and 4-2). The toxicity window of pemetrexed in both cell lines was fairly broad, between 0.01 and 10 µM following a 72 hour drug treatment. When this experiment was performed in media supplemented with thymidine to rescue the effect of thymidylate synthase inhibition, the cytotoxic effect of pemetrexed was completely rescued in HCT116 and H460 cells. Upon closer examination (Figure 4-3 and 4-4), it became apparent that although colony number remained the same, colony size was dramatically smaller. This was our first indication that the secondary effect of pemetrexed (AICART inhibition) was antiproliferative, not cytotoxic. Indeed, when both thymidine and hypoxanthine were supplemented in the media, the cytotoxic and antiproliferative effects of pemetrexed were completely rescued, even at high doses (Figure 4-1 and 4-2).

To confirm that AICART inhibition by pemetrexed was antiproliferative, we performed a similar experiment with LCA, whose effects we suspected were mediated primarily through AICART inhibition and secondarily through inhibition of thymidylate synthase (Figure 2-21). Compared to pemetrexed, LCA was 10-fold less potent at inducing cytotoxicity in
Figure 4-1. The cytotoxic effect of pemetrexed in HCT116 cells is rescued in the presence of thymidine and a preformed purine. Clonogenic survival assays were done as described in the Methods section on HCT116 cells exposed to the indicated concentrations of pemetrexed for 72 hours. In rescue conditions, 5.6 µM thymidine and 32 µM hypoxanthine were used, and remained present in culture media throughout the duration of the experiment. Enumerations of these colonies in the absence of rescue agents are depicted in the graph. Cultures were set up in triplicate, and data points are representative of an average of triplicate measurements.
Figure 4-2. **The cytotoxic effect of pemetrexed in H460 cells is rescued in the presence of thymidine and a preformed purine.** Clonogenic survival assays were done as described in the Methods section on H460 cells exposed to the indicated concentrations of pemetrexed for 72 hours. In rescue conditions, 5.6 μM thymidine and 32 μM hypoxanthine were used, and remained present in culture media throughout the duration of the experiment. Enumerations of these colonies in the absence of rescue agents are depicted in the graph. Cultures were set up in triplicate, and data points are representative of an average of triplicate measurements.
Figure 4-3. The effect of pemetrexed in the presence of thymidine is antiproliferative to HCT116 cells. Clonogenic survival assays (A) were done as described in the Methods section on HCT116 cells exposed to 1 µM pemetrexed and 5.6 µM thymidine for 72 hours. Enumerations of these colonies are depicted in (B). Cultures were set up in triplicate, and enumerations are representative of an average of triplicate measurements.
Figure 4-4. The effect of pemetrexed in the presence of thymidine is antiproliferative to H460 cells. Clonogenic survival assays (A) were done as described in the Methods section on H460 cells exposed to 1 µM pemetrexed and 5.6 µM thymidine for 72 hours. Enumerations of these colonies are depicted in (B). Cultures were set up in triplicate, and enumerations are representative of an average of triplicate measurements.
the absence of rescue agents in both HCT116 and H460 cells (Figure 4-5). Importantly, addition of thymidine to culture medium had no effect on the clonogenic survival of either HCT116 or H460 cells in the presence of LCA (Figure 4-5). This result confirmed that the primary target of LCA was not thymidylate synthase. It was interesting to us that LCA retained its cytotoxic effect in the presence of thymidine. This suggested that either LCA had additional targets beyond AICART and thymidylate synthase, that potent AICART inhibition was indeed cytotoxic in and of itself, or that prolonged growth inhibition was suppressing colony formation.

The antiproliferative effect of pemetrexed in the presence of thymidine was extremely interesting to us and had potential therapeutic importance. We therefore sought to determine how the secondary effect of pemetrexed augmented passage through the cell cycle. Initially, HCT116 cells were treated with pemetrexed in the presence and absence of thymidine for 24 hours, and DNA content of propidium iodide-stained cells was analyzed by flow cytometry. In the presence of pemetrexed, a clear accumulation of HCT116 cells in S-phase of the cell cycle was measured (Figure 4-6). This was likely indicative of thymidylate synthase inhibition (318). However, when the effect of thymidylate synthase inhibition was alleviated with extracellular thymidine, it appeared that pemetrexed was arresting cells at the G1/S boundary (Figure 4-6). Over the course of a 24 hours treatment with pemetrexed and thymidine in HCT116 cells, the approximate % distribution of cells in G1:S:G2 changed from 41:18:36 to 55:15:26. We performed flow cytometry as a function of time in H460 cells treated with pemetrexed and thymidine or LCA. Indeed, we saw pronounced accumulation of cells at the G1/S checkpoint with a clear diminution of cells in G2/M in response to each of these treatments (Figure 4-7). Importantly, G1/S arrest was measured in the absence of thymidine in LCA-treated cells, further confirming that thymidylate synthase was not a primary target for this compound.
The cytotoxicity of pemetrexed requires both an apoptotic and autophagic response

The primary cell death mechanism induced by thymidylate synthase inhibition is apoptosis (98). Indeed, pemetrexed has been shown to strongly induce apoptosis in cultures of human tumor cell lines (319). We therefore questioned whether the effect of AICART inhibition by pemetrexed was contributing to the apoptosis induced by the drug. We hypothesized that AICART inhibition would induce autophagy, as there is strong evidence linking mTORC1 signaling to the suppression of autophagy and AMPK signaling to autophagy induction (325, 326, 330). We first queried whether autophagy was induced subsequent to AICART inhibition. We stained LCA-treated HCT116 and H460 cells with the acridine orange, a vital dye that stains neutral compartments green and acidic compartments orange (Figure 4-8). The rationale for this method was the identification of lysosomal compartments, whose formation is upregulated when the autophagic cascade is induced. Clearly, LCA increased the orange staining pattern in both cell lines, and this punctate staining pattern was indicative of an autophagic response.

A more specific indicator of autophagy comes from measuring the sequestration cleaved LC3 to autophagosomes, which then fuse to the lysosome. We collaborated with Dr. Margaret Park, a post-doctoral fellow in Dr. Paul Dent’s lab in the Massey Cancer Center (VCU) to measure LC3 localization to lysosomes. Dr. Park transfected H460 cells with a GFP-tagged LC3 construct, treated cells with pemetrexed in the presence and absence of thymidine, and measured green foci in these cells, indicative of association of LC3 with lysosomes. We observed
Figure 4-5. The antiproliferative effect of LCA is not rescued in the presence of thymidine. Clonogenic survival assays were done as described in the Methods section on HCT116 and H460 cells exposed to the indicated concentrations of LCA for 72 hours. In rescue conditions, 5.6 µM thymidine was used, and remained present in culture media throughout the duration of the experiment.
Figure 4-6. Cell cycle analysis of HCT116 cells following pemetrexed exposure indicates a shift from S-phase accumulation to G1/S arrest in the presence of thymidine. HCT116 cells were exposed to 1 μM pemetrexed and 5.6 μM thymidine for 24 hours. DNA content was measured by propidium iodide incorporation as described in the Methods section.
Figure 4-7. Cell cycle analysis of H460 cells following pemetrexed and thymidine or LCA exposure indicates a G1/S arrest in these cells. Cells were exposed to 1 µM pemetrexed and 5.6 µM thymidine for the indicated times. DNA content was measured by propidium iodide incorporation as described in the Methods section.
Figure 4-8. Acidic vesicles are more prominent in HCT116 and H460 cells treated with LCA. HCT116 and H460 cells were treated with 1 µM LCA for 24 hours before being stained with the vital dye Acridine Orange and visualized under an inverted fluorescence microscope as described in the Methods section.
significant LC3 localization to lysosomes in these cells (Figure 4-9). This not only indicated to us that pemetrexed was inducing autophagy, it also showed that this effect was independent of thymidylate synthase inhibition, as it was occurring in the presence and absence of thymidine (Figure 4-9). This effect also occurred in HCT116 cells, although to a lesser extent.

Importantly, our clonogenic survival data indicated that in the absence of thymidylate synthase inhibition, no cell death was being induced (Figure 4-3 and 4-4). Therefore, we drew the conclusion that autophagy alone was not sufficient to induce cell death in response to pemetrexed. But was autophagy contributing to the apoptotic effect in response to thymidylate synthase inhibition? To test this, we ablated the autophagic response to pemetrexed and queried what happened to the apoptotic response. To ablate autophagy, we used both chemical and genetic means. Chemically, we treated H460 cells with 3-methyladenine, a class III PI3K inhibitor that prevents autophagosome formation (345). Genetically, we directly knocked down Beclin-1 with siRNA. We determined that the apoptotic effect of pemetrexed was severely blunted in the absence of autophagy (Figure 4-10). It appeared that the cytotoxicity of pemetrexed resulted from a combined effect of apoptosis induced by thymidylate synthase inhibition and autophagy induced by AICART inhibition. Importantly, loss of either one prevented the cytotoxicity of the drug.
Figure 4-9. LC3-GFP staining pattern following pemetrexed treatment indicates a commitment to autophagy that is independent of thymidylate synthase inhibition. H460 human non-small cell lung cancer cells were transfected with an LC3-GFP construct and were treated with the indicated concentrations of pemetrexed and 5.6 μM thymidine 24 hours later for 24 hours. Cells were then visualized under an inverted fluorescence microscope for LC3 foci formation, indicative of association with autophagolysosomes. 

Experiment performed by Dr. Margaret Park (Dent Lab, VCU).
Figure 4-10. The apoptotic effect of pemetrexed is ablated in the absence of autophagy. H460 cells were exposed to (A) 3-MA for 4 hours or (B) Beclin 1 siRNA for 24 hours before being exposed to the indicated concentration of pemetrexed for 24 hours. Apoptosis was measured by Annexin V/propidium iodide staining, analyzed by flow cytometry. Measurements are representative of cells scoring positive for both Annexin V and propidium iodide. Experiment performed by Dr. Margaret Park (Dent Lab, VCU). Error bars are representative of standard deviation from two biological and three technical replicates.
DISCUSSION

In this chapter, we determined that AICART inhibition by pemetrexed was cytostatic, not cytotoxic (Figure 4-3 and 4-4). Furthermore, we showed that this antiproliferative AMPK-mediated effect of the drug arrested cells at the G1/S boundary (Figure 4-6 and 4-7). AICART inhibition induced an autophagic response, likely as a result of AMPK activation and mTORC1 inhibition (Figure 4-8 and 4-9). Although autophagy alone did not induce cell death, its induction was essential for the overall cytotoxic effect of pemetrexed (Figure 4-10). From these studies, we propose that the efficacy of pemetrexed against non-small cell lung cancer and mesothelioma is reflective of its combined activity as a thymidylate synthase inhibitor as well as an AMPK-activating agent.

A complex interplay between autophagy and apoptosis

The cellular consequences of autophagy induction are not straightforward, and are likely context-dependent. In situations of stress or nutrient deprivation, autophagy is suggested to play a pro-survival role, recycling cytoplasmic debris for energy (346). On the other hand, autophagy induction under conditions of nutrient sufficiency are damaging to the cell. In this context, autophagy has been classified as a distinct mode of programmed cell death. The finding that pemetrexed induced autophagy in response to AICART inhibition (Figure 4-8 and 4-9), led us to question whether autophagy was contributing to or protecting cells from the cytotoxic effect of pemetrexed. That AICART inhibition was cytostatic, not cytotoxic (Figure 4-3 and 4-4) suggested that autophagy was not promoting cell death in response to pemetrexed. However, inhibition of autophagy by genetic and pharmacological means protected pemetrexed-treated cells from cell death by apoptosis (Figure 4-10). These findings led to the conclusion that the
overall cytotoxic effect of the drug requires both an autophagic and apoptotic response from
combined inhibition of AICART and thymidylate synthase, respectively, and that ablation of
either programmed cell death mechanism perturbs the cytotoxic effect of pemetrexed (Figure 4-
11).

From a biological standpoint, our data suggests that the processes of apoptosis and
autophagy are inter-related. The coordinate control and crosstalk between these two cell death
mechanisms has been an important topic of recent study, and the conclusions, like autophagy
itself, are not straightforward. In certain instances, autophagy and apoptosis have been shown to
be mutually exclusive modes of cell death that could be activated by similar stimuli. For
instance, inhibition of apoptosis with the pan caspase inhibitor Z-VAD.fmk induced autophagic
cell death in mouse fibrosarcoma and human T-cell lymphoma cells (347). Additionally, the
topoisorerase II poison and common apoptosis-inducing agent, etoposide, induced pronounced
autophagic cell death in MEF’s from bax<sup>-/-</sup>-bak<sup>-/-</sup> double-knockout mice, resistant to apoptotic
stimuli (348). It would be very interesting to determine whether bax<sup>-/-</sup>-bak<sup>-/-</sup> double-knockout
MEF’s responded similarly to pemetrexed. In a reciprocal fashion, inhibition of autophagy has
been shown to potentiate the apoptotic response. Under conditions of nutrient starvation, in
which autophagy has been shown to play a cytoprotective role, apoptosis was induced following
siRNA knockdown targeting mRNA from several autophagy genes including Beclin 1 (349).

Studies have also shown that autophagy and apoptosis work coordinately to induce cell
death. Induction of autophagy by overexpression of ULK1 (the mammalian homolog of ATG1
in <i>Drosophila melanogaster</i> and <i>S. cerevisiae</i>) induced apoptotic cell death in <i>Drosophila</i> (350).
A point of integration between these two pathways may be the interaction between the anti-
apoptotic protein, Bcl-2, and Beclin 1. Under conditions of starvation in yeast and mammalian
Figure 4-11. A proposed model for the involvement of apoptosis and autophagy in the induction of cell death by pemetrexed.
cells, binding of Bcl-2 to Beclin 1 inhibited autophagy (351). Moreover, starvation of cells expressing a mutant form of Beclin 1, unable to bind to Bcl-2, promoted autophagic cell death. Therefore, Beclin 1 may promote apoptosis by sequestering Bcl-2. This finding is of relevance to our studies, as knockdown of Beclin 1 in pemetrexed-treated H460 cells diminished the apoptotic response to the drug (Figure 4-10). I hypothesize that as a consequence of Beclin 1 knockdown, Bcl-2 is released to prevent the release of cytochrome C from the mitochondria, inhibiting apoptosis. Thus, our interpretation of the effects of ablation of autophagy by Beclin 1 knockdown in pemetrexed-treated cells may be complicated by this anti-apoptotic effect. To circumvent this potential complication, it would be advantageous to repeat this experiment targeting ULK1 with siRNA rather than Beclin 1.

**Comparison of pemetrexed and raltitrexed illustrates the significance of a secondary target**

Based on kinetic parameters, it could be rationalized that pemetrexed and raltitrexed would have similar therapeutic effects. Both drugs are potent thymidylate synthase inhibitors as pentaglutamates (Ki ~ 1.0 nM) and have similar kinetic properties in terms of RFC transport (K_m ~ 5 µM) and FPGS polyglutamation (K_m ~ 0.8 µM) (54, 57, 352). However, raltitrexed is several orders of magnitude more cytotoxic to tumors in culture and in humans (353, 354). This finding was also confirmed by clonogenic survival assays presented in this dissertation (Figure 2-1 and 4-2). This effect may be partially due to enhanced transport of pemetrexed by the PCFT (58-61). Additionally, pemetrexed has proven effective in treating tumors resistant to raltitrexed (143), further illustrating the importance of thymidylate synthase-independent effects of the drug.
The cytotoxicity associated with thymidylate synthase inhibition occurs following cellular entry into S-phase, where futile cycles of excision-repair of misincorporated dUTP into DNA lead to strand breaks and induction of apoptosis (98, 318). In this chapter, we discovered that in addition to the cytotoxic effect of thymidylate synthase inhibition, pemetrexed also generates an antiproliferative effect consequent of AICART inhibition, arresting cells at the G1/S cell cycle checkpoint (Figure 4-6 and 4-7). This does not occur following raltitrexed treatment, as the cytotoxic effect is completely reversed by addition of thymidine even at higher concentrations of drug (Figure 2-1). Therefore, it is hypothesized that the AICART effect of pemetrexed limits the cytotoxicity associated with thymidylate synthase inhibition by hindering the cellular traverse into S-phase. From our studies in Chapter 2, we showed that hypoxanthine dose-dependently diminished ZMP accumulation in response to pemetrexed treatment across concentrations of hypoxanthine that incrementally rescued growth inhibition (Figure 2-18). Therefore, hypoxanthine may be diminishing the G1/S arrest induced by AMPK activation in pemetrexed-treated cells, and enhancing S-phase entry, where the cytotoxic effects of thymidylate synthase are seen. Regardless, the enhanced clinical efficacy of pemetrexed compared to raltitrexed suggests benefit to combining the cytotoxic thymidylate synthase effect with the antiproliferative AMPK-activating effects subsequent to AICART inhibition, even though the toxicity associated with thymidylate synthase inhibition is diminished.

**Does p53 influence the therapeutic outcome of AICART inhibition?**

Greater than 50% of human cancers harbor mutations in the tumor suppressor p53, making it the most frequently mutated gene in human malignancies (355). Activation of this transcription factor has been shown to induce apoptosis, cell cycle arrest, and senescence under
certain conditions (356). Importantly, the therapeutic consequences of mTORC1 inhibitors and AMPK-activating agents have shown differential sensitivities to p53 in wild type and mutant tumors. The growth of HCT116 p53\(^{-}\) tumor xenografts in nude mice was significantly more inhibited in response to metformin and AICAR treatment than HCT116 p53\(^{+/+}\) tumor xenografts (357). This differential effect was concomitant with enhanced apoptosis in HCT116 p53\(^{-}\) cells, but autophagy induced by both treatments was ablated in the absence of p53. If p53 is indeed essential to the induction of autophagy in response to AMPK activation, our studies would paradoxically predict that p53\(^{-}\) cells would not die by apoptosis in response to pemetrexed. Clonogenic survival assays in p53 isogenic HCT116 and MEFs would be a straightforward way to begin addressing this hypothesis.

Upon glucose starvation, MEFs have been shown to arrest at the G1/S cell cycle checkpoint, an effect dependent on AMPK activation, but independent of mTORC1 inhibition (209). Growth arrest in response to glucose starvation also required p53, and loss of p53 conferred MEFs sensitive to apoptosis in the absence of glucose. This suggests that the cell cycle arrest induced by p53 in response to AMPK activation is cytoprotective. It will be important to determine the cell cycle response to pemetrexed and thymidine in the absence of p53. This data would suggest that cells would no longer arrest at the G1/S checkpoint, which may enhance the cytotoxicity of thymidylate synthase inhibition by pemetrexed. However, several experiments done in our lab suggest that loss of p53 does not enhance the cytotoxicity of pemetrexed and thymidine treatment. S. Agarwal in our lab compared the growth inhibitory effect of pemetrexed and thymidine in p53 isogenic HCT116 and MEFs, and showed that growth inhibition was not enhanced in the absence of p53 in either of these cell types. Additionally, S. M. Anderson performed clonogenic survival assays in p53 isogenic HCT116 cells exposed to
pemetrexed and thymidine for 24 hours, and did not see any toxicity in the absence of p53. In both genetic backgrounds, the secondary effect was antiproliferative. We do not understand why the cellular effects of AMPK activation in the absence of p53 reported by Jones et al are not recapitulated with pemetrexed and thymidine treatment.

An important (but overlooked) result presented by Jones et al showed that expression of the p53 target gene, p21, was not stimulated in response to AMPK activation (209). Binding of two moles of p21 to G1/S-specific cyclin/cyclin-dependent kinase complexes inhibits their activity, and thus prevents traverse through this cell cycle checkpoint. The observation that p21 expression was not induced following pemetrexed-mediated stabilization of p53 was also made by C.L. Heyer in our lab, suggesting that p53 is not directed to the p21 promoter following AMPK activation. If the G1/S arrest induced by pemetrexed requires p53, it will be important to understand how p53 induces this arrest independent of p21 induction. Other p53 target genes, that interact with cyclin/cyclin-dependent kinase complexes, such as GADD45, have been shown to be upregulated in response to DNA damage-induced p53 stabilization, and would be candidates to begin elucidating this mechanism (358).

AMPK activation was also shown to correlate with stabilization of p53 and phosphorylation of S15, a mark thought to stabilize p53 by disrupting the interaction with Mdm2, and prime p53 for further posttranslational modifications that influence its activity (341, 342). The sequence surrounding S15 of human p53 is similar to the sequence surrounding S79 of human ACC, and AMPKα1 immunoprecipitated from glucose-deprived cells were able to phosphorylate a GST-p53 fusion protein containing the N-terminal sequence (residues 1-98) of p53 (209, 343). Of significance, mTORC1 activates a S15 phosphatase (359), suggesting an AMPK-mTORC1 feedback loop to regulate this important posttranslational modification.
Additionally, the p53 target genes, Sestrin 1 and 2, have been shown to stimulate AMPK activity (360). This may serve as a positive feedback loop to keep p53 stabilized, since AMPK activation by Sestrins would promote p53 S15 phosphorylation both directly as well as through inhibition of mTORC1 signaling. If Sestrin gene expression is induced following pemetrexed treatment, this would amplify the AMPK-activating effect, and would define an important role of p53 in the therapeutics of AICART inhibitors. The complex regulation of p53 in response to AMPK activation thus illustrates the importance of elucidating how p53 influences the therapeutic response to this new class of AMPK-activating agents.
CHAPTER 5
PERSPECTIVES

The clinical response of MPM and NSCLC to pemetrexed, atypical for a thymidylate synthase inhibitor, suggested that secondary effects of the drug were of significant therapeutic importance. In this dissertation, a novel mechanism of pemetrexed action was discovered, stemming from inhibition of AICART, the second folate-dependent enzyme of de novo purine synthesis. The metabolic block imposed by AICART inhibition resulted in the intracellular accumulation of the reaction substrate and AMP-mimetic, ZMP. Consequently, we identified pemetrexed to be a potent activator of AMPK, central to the regulation of cellular energy homeostasis. The molecular and therapeutic consequences of AICART inhibition and AMPK activation by pemetrexed were studied, with particular focus on perturbation of the PI3K-AKT-mTOR signaling cascade. Mutations in key regulators of this pathway promote hyperactive signaling in the absence of growth stimuli, uncoupling nutrient supply from the demands of proliferation and survival. AMPK negatively regulates PI3K-AKT-mTOR signaling through several mechanisms, and thus represents a promising cancer therapeutic target to regain control over hyperactivity along this signaling axis. These studies suggest that the therapeutics of antifolates extend beyond classical effects of folate-dependent target inhibition, and raise several questions of considerable importance whose answers will likely influence the development of next-generation antifolates as molecularly-targeted agents.

*Are the secondary effects of pemetrexed demonstrated in cell culture relevant in the clinical setting?* Our hypothesis suggests that the efficacy of pemetrexed in MPM and NSCLC is a result of the AMPK-activating effects of the drug. The implications of AMPK activation by
pemetrexed in humans are of significant importance for the implementation of rational combination therapeutic strategies, biomarker screening for clinical responsiveness, as well as for the development of next-generation antifolates targeting AICART. Therefore, it is essential to quickly determine whether this effect of pemetrexed is clinically relevant. To determine whether pemetrexed inhibits AICART in vivo, a non-invasive and fairly straightforward approach would be HPLC or liquid chromatography-mass spectrometry (LCMS) analysis of human blood and urine for ZMP and the membrane-permeable metabolites, AICAR and AICA. Protocols for extraction and quantification of these metabolites from bodily fluids are well established, and importantly, basal levels are often undetectable (129, 149). The concentrations of pemetrexed that cause ZMP accumulation in cell culture (0.03-1 µM) (Fig 16, 21) are maintained in the serum of patients for more than 48 hours (144), and the cellular effects of pemetrexed on AMPK signaling arise within the first 15 hours of exposure (Fig 42). This would suggest sampling blood or urine 24 hours following drug dosing as an optimal starting point for analysis. Additionally, exposure as a single agent, either in the maintenance or second-line setting, would be most favorable to avoid potential complications in interpretation from exposure to pemetrexed in combination with platinums.

*Does targeted AICART inhibition/AMPK-activation represent an efficacious cancer therapeutic strategy, and could it fit into rational combination therapeutics regimens targeting PI3K-AKT-mTOR signaling?* With the far-reaching effects of AMPK on processes essential to cellular proliferation and survival, therapeutic activation of AMPK represents a promising cancer therapeutic strategy. However, the observation that AICART inhibition by pemetrexed is cytostatic, not cytotoxic suggests that the answer to this question is not as straightforward as one might think. Will all AICART inhibitors be cytostatic? Would permanently preventing the
outgrowth of a tumor rather than eradicating it be an acceptable and effective outcome of cancer therapy? The discovery and study of novel AICART inhibitors will help answer some of these important questions. Indeed, clonogenic survival assays with LCA, whose primary target is likely AICART, suggest that not all AICART inhibitors are cytostatic (Figure 4-5). While LCA likely has a weak TS effect, in the presence and absence of TdR, the antifolate is an impressive inhibitor of clonogenic survival compared to pemetrexed with TdR (Figure 4-1 and 4-2). Interestingly, while the cytotoxicity of pemetrexed requires both the apoptotic thymidylate synthase effect and the autophagic AICART effect, our clonogenic survival data suggests this is not the case with LCA. This may be due to differential potencies of pemetrexed and LCA as AICART inhibitors, or LCA may have other TS-independent targets. A more detailed analysis of the interplay between these two modes of programmed cell death in response to pemetrexed is warranted, and will ultimately be necessary to fully exploit the AICART effect.

The susceptibility of mTORC1 inhibition by pemetrexed to feedback activation of AKT would suggest combining AICART inhibitors with direct or indirect inhibitors of PI3K, AKT, mTORC2, or PDK1 to enhance the effect of AICART inhibition. Indeed, combination strategies to diffuse negative feedback effects are already being implemented with the development of dual inhibitors of mTORC1/mTORC2, S6K1/AKT, and mTORC1/PI3K (361). That caloric restriction and exercise, both implicated in reducing cancer risk, are physiological activators of AMPK, suggests that low doses of AMPK-activating agents combined with an exercise regimen and a controlled diet may be synergistic in the treatment of cancer. Indeed, dose-dependent caloric restriction in mice has shown AMPK activation and mTORC1 inhibition (362), and mice fed high fat diets had decreased AMPK activity and increased mTORC1 signaling (363).
Can the utility of AICART inhibitors/AMPK-activating agents be extended to other diseases and conditions? That AMPK-activating agents have diverse cellular metabolic effects suggests that AICART inhibitors could be developed as therapeutics for conditions extending beyond cancer. One obvious utility would be for the management of Tuberous Sclerosis Complex. Phase II clinical studies with rapamycin analogs as single agents have shown a remarkable response in the regression of hamartomas in TSC patients (364, 365). However, the short half-life of rapamycin requires daily dosing, and the condition progressed quickly following termination of treatment (364). Antifolates have favorable kinetic profiles in terms of transport and polyglutamation, trapping and enhancing the target potency of these compounds. For this reason, pemetrexed is given once every 21 days, and although the parent drug is cleared from systemic circulation fairly rapidly, metabolites are retained intracellularly for extended periods of time (56). Additionally, our data suggests that pemetrexed may be effective in treating Peutz-Jeghers syndrome, as pemetrexed was able to activate AMPK and elicit inhibitory effects on mTORC1 in the absence of LKB1. This is supported by studies showing that rapamycin was efficacious in treating a mouse model of Peutz-Jeghers syndrome, in which a single allele of LKB1 was knocked out (366). The effects of AMPK on the promotion of carbohydrate metabolism and inhibition of lipid synthesis are well documented as well. This would suggest that AICART inhibitors should be tested for their anti-diabetic and cholesterol/fatty acid-lowering effects. Interestingly, metformin use correlates with a decreased incidence of cancer (277, 278), suggesting that AICART inhibitors may have a similar therapeutic profile.

Do tumor genetics predict sensitivity to AICART inhibitors/AMPK-activating agents? It is often stated in the mTOR signaling field that tumors harboring mutations that drive mTORC1
signaling are hypersensitive to rapamycin, as they rely more heavily on mTORC1 signaling for growth and proliferation (219). This hypothesis is indeed supported by mouse models with PTEN, TSC, and AKT mutations, which all respond strongly to rapamycin. Patient sensitivity to AMPK-activating agents might similarly be predicted based on an individual’s mutation profile along the PI3K-AKT-mTOR signaling axis. In our limited screen of carcinoma cell lines, the most sensitive cell types were those with hyperactive mutations in PI3K and KRAS. A more extensive study of genetic predictors of AMPK-sensitivity is warranted to determine whether specific mutations correlate with tumor sensitivity. Our screen also demonstrated that additional markers of sensitivity to AMPK-activating agents beyond mTORC1 signaling are of interest, as there was no correlation between sensitivity to pemetrexed and rapamycin. This suggests additional benefits of AMPK activation that extend beyond mTORC1 signaling. Analysis of signaling events mediated by AMPK to mTORC1-independent metabolic processes in response to AICART inhibitors is essential to the future development of this class of AMPK-activating agents, and will likely uncover more sensitive biomarkers to predict responsiveness to these agents.
LITERATURE CITED


VITA

Scott Barri Rothbart was born on September 23, 1982 in Livingston, New Jersey, and is a United States citizen. He graduated from Pine Ridge High School (Deltana, Florida) in 2001. He was awarded a full academic scholarship to attend the University of Florida (Gainesville, Florida), where he performed undergraduate research studying zinc transport and metabolism under the mentorship of Dr. Robert J. Cousins. Scott received a Bachelor of Science degree in Food Science and Human Nutrition from the University of Florida in 2005. In the fall of 2005, he was accepted into a pre-doctoral training program through the department of Pharmacology and Toxicology in the School of Medicine at Virginia Commonwealth University (Richmond, Virginia), and joined the laboratory of Dr. Richard G. Moran in the spring of 2006. Scott presented his research at the meeting of the Virginia Cancer Researcher’s Society in 2007, and was an invited speaker at the American Association for Cancer Research (AACR) Special Conference in Metabolism and Cancer in 2009, where he received a Scholar-in-Training award. He presented posters on his dissertation research at the Daniel T. Watts Research Symposium in 2008, and at the AACR 101st Annual Meeting in 2010. As a section chairperson for the Virginia Academy of Sciences in 2009, Scott led the organization and implementation of the Medical Sciences section of the 87th annual meeting. In the fall of 2010, Scott received a National Research Service Award to be a trainee in the University of North Carolina (UNC) Lineberger Comprehensive Cancer Center Postdoctoral Training Program. He will be conducting postdoctoral research at UNC Chapel Hill (Chapel Hill, North Carolina) under the mentorship of Dr. Brian D. Strahl in the department of Biochemistry and Biophysics.

Manuscripts resulting from the present dissertation research
