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The Therapeutic Targeting Of Folate Receptor Alpha Positive Tumors Via Folate Receptor Selective Novel 5- And 6- Substituted Pyrrolo [2,3-D]pyrimidine Antifolates"

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THE THERAPEUTIC TARGETING OF FOLATE RECPTOR ALPHA POSITIVE TUMORS VIA FOLATE RECEPTOR-SELECTIVE NOVEL 5-AND 6- SUBSTITUTED PYRROLO [2,3-*D*] PYRIMIDINE ANTIFOLATES

by

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DEDICATION

This dissertation is dedicated to *Seamus Isaiah Ryan*, my sun (son), my world and my very reason to press forward and claim every reward life has to offer.

My sojourn through this process would not have been possible without the love, support and encouragement of my husband, *Paul Seamus Ryan.* Thank you for your steadfast devotion, patience and understanding. You have been my guiding light, offering important insight in some of my darkest moments. You are the quintessential professional and the lessons that you have imparted upon me about maintaining my integrity and professionalism in every situation are some of greatest lessons learned.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Abbreviations

Ade: adenosine

AICA: 5-aminoimidizole-4-carboxamide

AICAR: 5-aminoimidazole-4-carboxamide ribonucleotide

AICARFTase: 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase

AIRS: 5'-Aminoimidazole ribonucleotide synthase

AK: adenosine kinase

AMP: adenosine monophosphate

AMPK: activated monophosphate kinase

AMT: aminopterin

APRT: adenine phosphoribosyl transferase

ASL: adenylosuccinate lyase

ATP: adenosine triphosphate

ATTC: AICARFT/IMP cyclohydrolase

CAIRS: carboxyaminoimidozole ribonucleotide synthase

CFD: cerebral folate deficiency

CHO: Chinese hamster ovary cells

DHFR: dihydrofolate reductase

DPBS: delbecco's phosphate buffered saline

EOC: epithelial ovarian cancer

FA: folic acid added

FAICAR: formyl 5-aminoimidazole-4-carboxamide ribonucleotide

FBP1: folate binding protein 1

FGAM: formyl glycinamide ribonucleotide synthase

FGAR: formyl glycinamide ribonucleotide

FPGS: folylpoly gamma glutamate synthetase

FR: folate receptor

GAR: glycinamide ribonucleotide

GARFTase: glycinamide ribonucleotide formyltransferase

GMP: Guanosine monophosphate

GPI: glycosylphosphatidylinositol

GPAT: glutamine phosphoribosyl pyrophosphate amidotransferase

HBOB: hereditary breast and ovarian cancer

HGSC: high grade serous carcinoma

HNPCC: hereditary nonpolyposis colorectal cancer

IMP: inosine monophosphate

MFT: mitochondrial folate transporter

MFS: major facilitator superfamiliy

MS: methionine synthase

MTA: methylthioadenosine

MTAP: methylthioadenosine phosphorylase

MTHFC: methylene tetrahydrofolate cyclohadrase

MTHFR: methylene tetrahydrofolate reductase

mTOR: mammalian target of rapamycin

MTX: methotrexate

NA: no additions

NTC: non-targeted control

NTD: neural tube defects

OEI: ovarian epithelial inclusion cyst

ORF: open reading frame

OSE: ovarian surface epithelium

PAICS: phosphoribosyl aminoimidazole carboxylase synthase

PCFT: proton-coupled folate transporter

PLP: pyridoxal-5- phosphate

PMX: pemetrexed

PRPP: phosphoribosyl pyrophosphate

PteGlu: pteroyl monoglutamate

RA: rheumatoid arthritis

RFC: reduced folate carrier

SAICARS: phosphoribosylaminoimidazolesuccinocarboxamide synthase

SAM: S-adenosyl methionine

SAR: structure activity relationship

SHMT: serine hydroxymethyltransferase

STIC: serous tubal intraepithelial carcinoma

TAM: tumor associated macrophage

Thyd: thymidine

THF: tetrahydrofolate

TS: thymidylate synthase

UTR: untranslated region

ZMP: AICAR monophosphate (see AICAR)

CHAPTER 1: INTRODUCTION: FOLATES IN HUMAN HEALTH

1.1 The identification and isolation of folate

The functional importance of many essential vitamins and minerals, including Vitamin B9, folicin or folic acid, was unearthed by rigorous studies that examined associated pathologies that resulted from nutrient deficiencies. The history of folates in human health originates in hematology. At this time, investigating the impact of diet on human disease was emerging, with much emphasis being placed on what we now know as nutritional anemias.[1,2] Macrocytic anemia, a subclass of megaloblastic anemia, was prevalent among pregnant women in impoverished communities whose access to proteins, vegetables and fruits was limited.[1] This specific type of anemia is characterized by enlarged erythrocytes which is thought to be a product of impaired DNA synthesis in red blood cells; a cell type with a high rate of turnover.[3,4] In observing the effects of diet in this condition on albino rats and monkeys, Dr. Lucy Wills and colleagues discovered that the administration of yeast, yeast extract (Marmite™) or crude liver extract to individuals subjected to a diet deficient in B vitamins, ameliorated the anemic condition.[1,2,5] This extrinsic factor contained in yeast was later called "Wills' factors" by Drs. Janet Watson and William B. Castle. Through the examination of multiple case studies of macrocytic anemia treated with different fractions of liver extract and beef muscle, Watson and Castle concluded that there was an additional component/ factor present that was responsible for assuagement of marocytic anemia that was different from that which cured Addisonian anemia (pernicious anemia).[5,6] Unbeknownst to the scientists of the time, yeast, plants and other microorganism are cable of *de novo* folate biosynthesis and the liver is the primary site of the body's folate storage.[7,8] Based on the empirical clinical data, there was an eruption of critical studies that examined the treatment potential of liver extract (given orally or parentally) in anemia, which in turn gave rise to multiple

names for folate, based solely on the animal used in the experiment. These included M factor (monkeys), vitamin Bc (chickens), *Lactobacillus* casei factor, and anti-anemia factor, to name a few. The nomenclature used to describe folic acid or folates has undergone many iterations with it being named pteroylglutamic acid due to the presence of a pteridine. The current name, folic acid or folates, was not used until the 1940's, upon isolation and identification of the compound in green leafy vegetation.[1,5,9]

Folate was first isolated from spinach leaves in 1941 by Herschel Mitchell and colleagues and was eponymously named folic acid after the Latin word folium that translates to leaf.[1,10,11] In 1943, E. L. Robert Stokstad and colleagues isolated this very same "factor" from the liver that promoted the growth of *Lactobacillus* casei.[1,12] Stokstad synthesized the first pure crystal form of the unconjugated compound, and from that, he was able to reveal important structural information about the molecule. During his tenure at Berkley's Nutritional Science department, Stokstad began seminal research on folic acid metabolism. Stokstad's research serves as the foundation for antifolate drug development as he identified many of the enzymes involved in the folate metabolic cycle nearly 34 years ago (1979).[5] This early work was credited for leading to the development of the first antifolate, aminopterin (4-aminopteroic acid) (AMT).

Today, folates are well recognized for the role they play in human health, growth and development.[13] One of many well documented examples of the impact of folates on human health is the indisputable role that the vitamin plays in the full development and closure of the neural tube during days 21-28 post-conception or embryogenesis.[14] Adequate folate supplementation in pregnant women has been thought to lead to the prevention of congenital malformations of the central nervous system and consequently a decrease in infant mortality and morbidity as neural tube defects (NTDs) are only second to cardiac defects as the most common congenital

malformations.[15] The abundance of landmark studies that supported this notion lead to a mandatory implementation of folic acid fortification in grain products in the United States.[15] The resulting rapid decline in infants born with spina bifida or anencephaly, as a consequence of neural tubes defects, demonstrated the importance of folate in early development.[14,15] Moreover, deficiencies in this vitamin and mutations in genes associated with folate absorption and/or metabolism have been linked to other disease states and disorders such as vascular disease, certain subtypes of cancer and Alzheimer's disease.[4] This provided concrete evidence that folates were vital to the preservation of normal cellular physiology and were a critical component to human health.

1.2 Folate biology and chemistry

Many anabolic pathways require the addition or modification of carbon groups. Folates are coenzymes that participate in the metabolism of amino acids and nucleotides by donating or transferring the required carbon units, which is referred to as one-carbon metabolism.[9,13] Because folate metabolism plays an integral part in the movement of carbon, it has been described by many as the cornerstone of one-carbon metabolism.[13,16,17] One-carbon metabolism is thought to be comprised of three distinct but interwoven metabolic cycles, the folate metabolic cycle, the methionine cycle and the trans-sulfuration pathway.[18-20] The unifying feature of these three pathways is the transfer of active one carbon units in a number of enzymatic reactions.

Folate is a generic term that describes any compound that participates in similar cellular activities as the vitamin that results in the production of amino acids, purine nucleotides and thymidylate.[21,22] This includes naturally occurring folates in foodstuffs, enzymatically converted species and the synthetic derivative folic acid.[21,22] The chemical name of the non-conjugated vitamin (containing a single γ glutamate residue) is pteroylmonoglutamate (PteGlu).[17] This

molecule is comprised of three groups, including (1) a pterin moiety, which has a methylene group linage at the 6th position to a (2) para amino benzoyl group and (3) L-glutamatic acid.[17] The pteridine ring is of great functional importance in the metabolism of this vitamin. The two nitrogen atoms placed on the ring favors reduction product that leads to di and/or tetra hydrogenation of the ring (**Figure 1.1**).

Folates destined for intracellular accumulation undergo extensive metabolism to assume their role as cofactors in physiological reactions.[8,17,19] This metabolism is thought to principally involve the reduction of the pteridine ring (**Figure 1.1**) (which introduces an asymmetric center at the C6 position), polyglutamylation (the successive addition of glutamate moieties to the γ-glutamyl of the parent molecule) and the transition to oxidative or reductive states.[9,19,23,24] These reactions give rise to a number of active tetrahydrofolate (THF) forms that are distinguishable by the substituents they carry at the N5 or N10 positions.[8] They can include methenyl (CH^+), methylene (CH_2), methyl (CH_3), formyl groups (CHO) and formimino (CH=NH) (**Figure 1.2**).[8] The active tetrahydro forms are the only species that can readily donate or accept single carbons units.[8,19] Naturally occurring folates exist as reduced folylpolyglutamates when within the cell, in which there are multiple glutamate moieties attached to the γ-carboxyl end of the folate molecule.[19,23] The most prevalent form of folate identified in mammals is reduced 5-methyl tetrahydrofolate.[25,26] In contrast, the synthetic vitamin used in food fortification, folic acid, is fully oxidized and tends to be less labile than naturally occurring folates, which become less stable with oxidation.[9] Because of this, naturally occurring folates and folic acid have a different point of entry in the metabolic cycle.

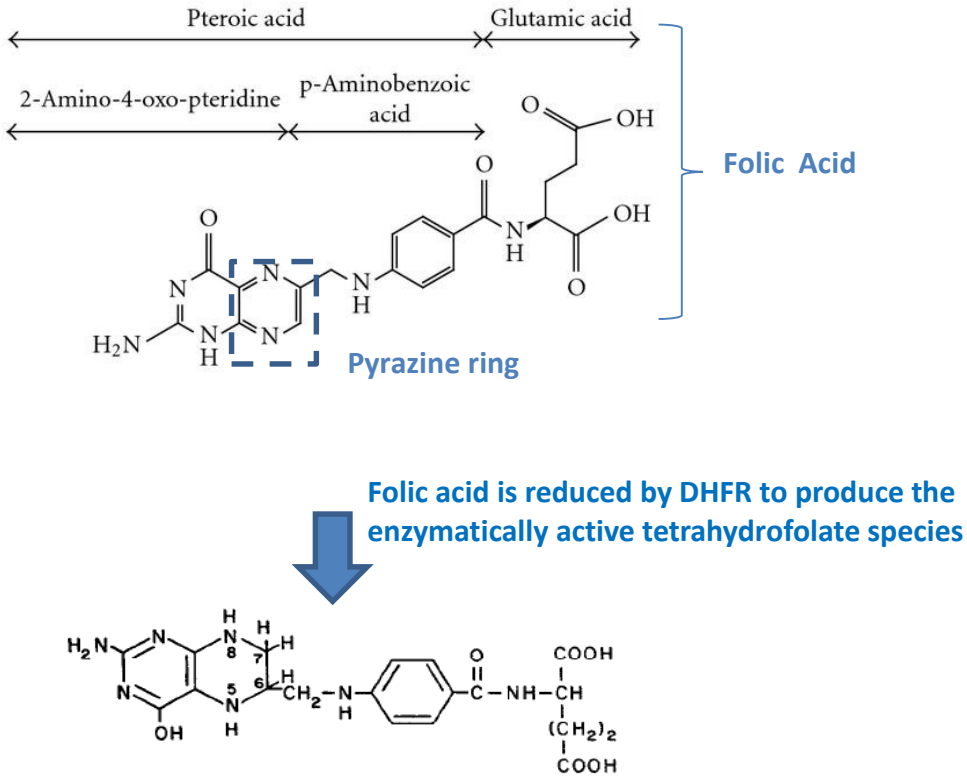

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Figure 1.1: The reduction of folic acid. Above is the structure of the folic acid, a synthetic derivative of naturally occurring forms found in legumes, green leafy vegetables and some fruits. Folic acid is comprised of three major groups including 1) pteridine 2) para benzoate and glutamic acid moieties. Folic acid enters the folate metabolism cycle at the site of dihydrofolate reductase, which acts to reduce the pyrazine ring of the pteridine group resulting in a dihydrofolate form. This dihydrofolate undergoes an additional reduction through the same enzyme to produce the active tetrahydrofolate co-enzyme that participates in one-carbon metabolism.

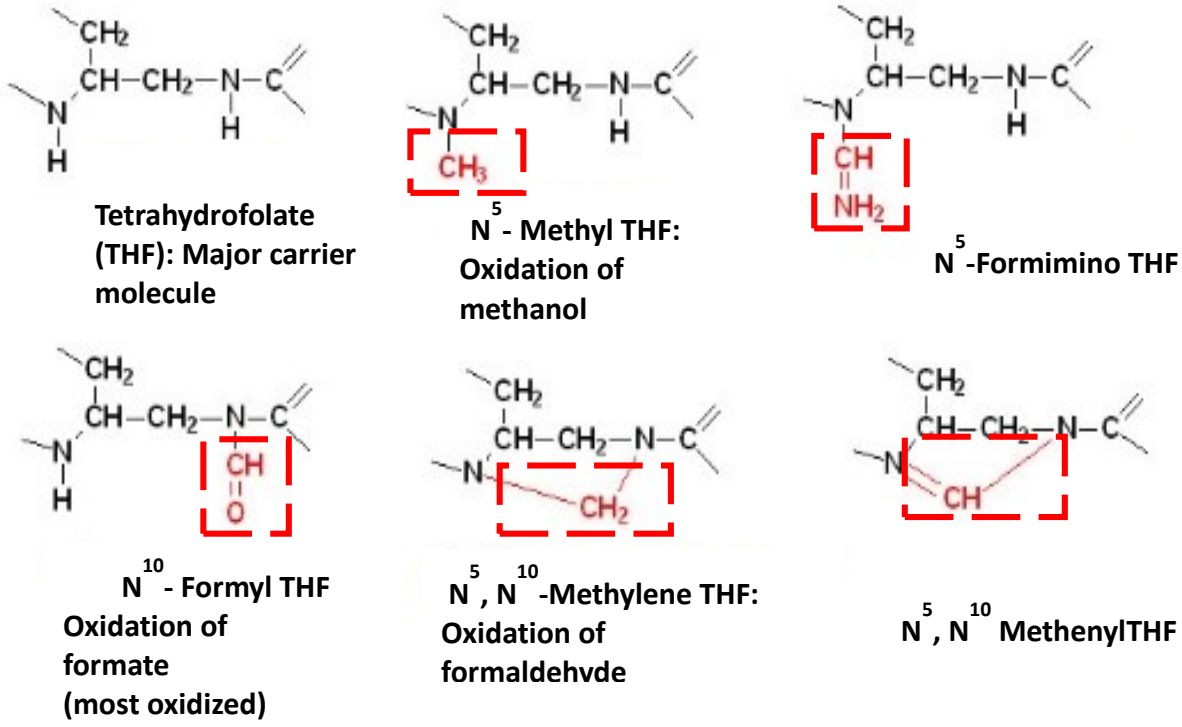

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Figure 1.2: Enzymatically active folate metabolites. This figure highlights multiple forms of active folate derivatives. Each metabolite differs in the oxidation state of the acquired substituent and the location of substituent. These metabolites play distinct roles in folate metabolism which result cellular stores of serine, glycine, purines, methionine and thymidylate.

There are multiple enzymes and binding proteins involved in folate metabolism that act to regulate folate homeostasis in tissues through a complex system of feedback loops, substrate specificities and allosteric regulation.[19] These reactions have a global impact on the cell by affecting both genomic and physiological aspects of function.[15,28] The epicenters of folate mediated one-carbon metabolism reside in the cytoplasm, the mitochondria and the nucleus (**Figure 1.5**).[17,29] Cytoplasmic one-carbon metabolism has been studied extensively with well-documented and detailed studies exploring the mechanics of folate metabolism in this compartment.[8,17,19] While our knowledge remains limited for both mitochondrial and nuclear folate metabolism, there is a growing surge of attention directed towards these two compartments with the hope of gaining a complete picture of the role of folates in cellular physiology. The primary source for carbon units used in cytoplasmic one-carbon metabolism is believed to originate from the one-carbon metabolism that occurs in the mitochondria which is estimated to contain 40% of the total intracellular folates.[20,25] While knowledge is limited on nuclear folate metabolism, studies with a radio-labeled folate precursor suggest that 10% of total cellular folates reside in the nucleus.[20]

Upon cellular entry, naturally occurring folates derived from leafy materials in foodstuff exist as 5-methyl THF polyglutamates, but begin their journey across the cell membrane in the monoglutamate transport form.[19,26] Mammalian serum/plasma contains folate conjugases/γ-glutamyl hydrolases, which strip the once polyglutamylated vitamin of additional glutamates at the gamma carboxyl end of the molecule, resulting in mono-glutamylated folates in the blood and urine.[19] Folates carry a slight negative charge due to ionization of the dicarboxcyclic acid tail, thereby requiring some form of transmembrane transport to gain intracellular entry.[19,25,26] Following intracellular translocation through a specified mechanism of folate transport, folates are

then subjected to polyglutamylation performed by the cytosolic enzyme folylpoly-gamma-glutamate synthetase (FPGS).[19] Polyglutamylation via FPGS was validated as an essential step in folate metabolism through the examination of the Chinese hamster ovary (CHO) cell lines that were auxotrophic for glycine, thymidine and purines, (CHO AUX B1 cells). [19,23] These cells were discovered to lack polyglutamylated folates due to the absence of FPGS activity, consequently resulting in a low intracellular folate pool.[19,23] This provided evidence of a dependency or requirement for these cells to obtain products of folate metabolism/one carbon metabolism from exogenous sources.[19,23] More recently, studies that investigated the anti-tumor activity of antifolates in cells with augmented human FPGS, reported that cells with increased expression of the FPGS protein showed enhanced sensitivities to antifolates.[30,31] Alternatively, the loss of FPGS expression and/ or activity has been elucidated as a probable mechanism in antifolate resistance.[32]

In addition to serving as a mechanism of cellular retention, polyglutamylation also aids in folate homeostasis by providing a layer of regulation by controlling substrate specificity.[17] The step-wise addition of glutamates at the tail of intracellular folates by FPGS has been well documented.[18,19] The extent of polyglutamylation that can differ among intracellular folates is thought to create competition with the lower or monoglutamylated species for which the affinity is higher when compared to extensively glutamylated species for which the Km has increased.[18,19] In general, increasing the length of the polyglutamate tail is associated with an increased affinity to enzymes involved in these pathways with the exception of DHFR.[17] There is a clear correlation between affinity and polyglutamylation. However there is a plateau or change in kinetics for the heptaglutamylated species, which is the predominant form in humans.[19,33] Curiously, some of these highly glutamylated folate metabolic products can act as inhibitors towards other metabolic

enzymes within this pathway.[17] This highlights the product-driven regulation of one-carbon metabolism that occurs within the folate cycle.

Another important feature of polyglutamate tail is the role it plays in the "channeling" of substrate.[17,34,35] Channeling is described as the sequential movement of substrate from one enzyme to another without exposure or release into the surrounding solvent.[29,36] Channeling is thought to add an additional layer of regulation in one-carbon metabolism due to the formation of enzyme complexes necessary to produce the end product.[20,34] Many investigators studying folate metabolism, more specifically the enzymology associated with this complex and sophisticated system, have proposed and supported the theory of the formation of a "metabolon" commonly referred to as the "purinosome".[29] The purinosome has been described as a dynamic multi–enzyme complex that forms in the cytoplasm during the G1 phase of the cell cycle that facilitates *de novo* purine synthesis.[29] While metabolic compartmentalization in organelles has been suggested to serve as a regulatory mechanism, the temporal and spatial assembly of such multi-enzyme complexes plays a role in directing the activation these pathways.[20] Folates with extended polyglutamyl tails are believed to participate in this distinct type of metabolic partitioning.[19,34,37,38] Experts postulate that such channeling evolved not only to increase the efficiency of this metabolic pathway but to also protect labile intermediates that would be rendered inactive due to extensive oxidation.[19,20,34,36] With this in mind, when surveying enzyme activity within this pathway, *in situ* methods are more likely to accurately recapitulate the physiological picture as the channeling process will be uninterrupted and the natural abundance of multiple polyglutamylated species would be present.

Intracellular polyglutamylated folate species are the primary molecules that are prepared to engage in cellular interactions with enzymes in one-carbon metabolism. [17] Many reviews on

one-carbon metabolism have separated these enzymes by their involvement in assorted pathways based on their functions and the organelles that house these reactions. Fox and Stover created four distinct functional categories for these enzymes which include (i) one carbon generating enzymes, (ii) THF interconverting enzymes, (iii) THF-dependent biosynthetic enzymes and (iv) the non-catalytic THF binding proteins.[17] These enzymes can be further distinguished based on the organelle in which they performed their specialized metabolic function. In general, the metabolic products formed in these reactions are not exchanged with other cellular compartments, which offers an explanation for the seemingly redundant existence of metabolic enzymes (or isozymes) in the mitochondria, cytoplasm and the nucleus (**Figure 1.5**).[8]

There are a number of enzymes involved in the folate-dependent one-carbon metabolic cycle. In this cycle, folate is used as a very versatile carrier molecule that can transfer activated one-carbon units of varying oxidation states from one metabolic reaction to the other. These enzymes may be present in multiple isoforms with very specific roles that are determined by where they are compartmentalized and when they are expressed; this may vary with the stage of cell cycle, cell type and most interestingly, in normal versus cancer cells.[17,34] The first enzyme that the naturally occurring folylpolyglutamate, 5-methyl THF, encounters is the cytoplasmic enzyme methionine synthase (MS). This enzyme is characterized as a THF biosynthetic enzyme and acts to transfer the methyl group from 5-methyl THF to participate in the remethylation of homocysteine and the production of methionine. The latter, upon adenylation, becomes the universal methyl donor, S-adenosylmethionine (SAM), which is responsible for a number of cellular methylation events. In addition, this reaction also leads to the regeneration of the THF cofactor, which can now act as a carrier molecule and serve as a substrate for cytoplasmic serine hydroxymethyltransferase (SHMT). Cytoplasmic SHMT is a one-carbon generating enzyme that

participates in the pyridoxal-5 phosphate- (PLP also known as vitamin B_6) dependent reversible conversion of serine to glycine.[17,39] In this reaction, the C3 of serine is transferred to THF, forming N^5,N^{10} methylene THF and the resulting glycine product. [19,23] SHMT is appropriately regarded as the most critical enzyme in folate metabolism due to the importance of the metabolic product it yields, N^5, N^{10}-methylene THF, which serves as a substrate for multiple enzymes in this pathway.

In the cell, SHMT exists as three isoforms with some overlapping functions. The function of each isoform appears to be dictated by the organelle that houses the enzyme. However, one feature that is common among the isoforms is the irreversible inhibition by 5-formyl THF (leucovorin) which acts to negatively regulate all isoforms, albeit to varying degrees.[40] Studies have shown that the primary role for the cytoplasmic isoform, SHMT1, is to direct the cycle in the direction of glycine, resulting in the production of N^5, N^{10}-methylene THF for the production of thymidylate or thymidine monophosphate.[41-43] The monophosphate is later converted into the tri-phosphate form that serves to support DNA replication and repair as the pyrimidine nucleotide, thymidine triphosphate (dTTP). However, depending on the cellular environment, N^5,N^{10}-methylene THF can be converted into other metabolic products that could lead either to the remethylation of homocysteine or the *de novo* production of purine nucleotides.

Limiting concentrations of N^5,N^{10}-methylene THF, in conjunction with the demand for this folate derivative in feeding multiple outputs in the metabolic pathways, creates competition and a requirement for intricate feedback regulation to prevent accumulation of metabolic products. The interconnectedness of three metabolic cycles is clearly exemplified by the fate of N^5,N^{10}-methylene THF and the importance of the SHMT enzyme. As previously mentioned, the folate derivative 5-methyl THF serves as the immediate substrate leading to the remethylation of homocysteine, resulting in methionine and consequently SAM production. Elevated SAM

concentrations can lead to the inhibition of 5,10-methylene tetrahydrofolate reductase (MTHR), the enzyme responsible for the reduction of N^5,N^{10}-methylene THF to 5-methyl THF.[8,17,19] In doing so, SAM regulates the folate cycle to allow cellular provisions for *de novo* nucleotide biosynthesis while maintaining homeostasis for the methionine cycle.[42] Cytoplasmic SHMT has also been implicated in the regulation of the flux of this pathway that preferentially supplies N^5, N^{10}-methylene THF to TS by two proposed mechanisms: (1) acting as a folate binding protein and sequestering 5-methyl THF from the enzymatic activity of MS; and, (2) as the SHMT reaction is reversible, it can act to deplete the N^5,N^{10}-methylene THF pool to synthesize serine with the use of glycine, therefore preventing its conversion to 5-methyl THF by MTHR.[17,42]

Additional validation of the important role of cytoplasmic SHMT role in the production of thymidylate is suggested by the binding of 5-formyl THF to cytosolic SHMT1. Studies have suggested that this binding acts a regulatory switch to drive the pathway towards thymidylate synthesis instead of the remethylation of homocysteine.[40,42] The mitochondrial SHMT2 isoform is believed to be involved in the conversion of serine to glycine and is ubiquitously expressed (thought to be responsible for the majority of the glycine content in cells). The cytoplasmic isoform is predominately found in the kidney and liver where it is not as active in the serine to glycine conversion. It facilitates the reversible interconversion of serine and glycine while forming 5,10 methylene THF.[44]

Serving as one of the most critical components of this pathway, 5, 10-methylene THF is principally involved in the methylation of dUMP to dTMP in thymidylate formation catalyzed by TS.[26] Dihydrofolate is the by-product of this reaction, which is converted to THF by dihydrofolate reductase (DHFR). THF can be metabolized to promote an additional revolution in this cycle.

Synthetic folic acid supplements also act to generate THF when reduced to the di- then tetrahydrofolate cofactor form by DHFR.

In addition to participating in thymidylate formation, 5, 10-methylene THF can be reduced to 5-methylTHF by MTHR. THF can be converted into 10-formyl THF by mitochondria-derived formate and cytosolic ATP. This enzyme is also responsible for the irreversible conversion of 5, 10-methenyl THF to 5- formyl-THF.

Both the mitochondrial isozyme SHMT2, and cytoplasmic enzyme SHMT1 play a critical roles in one-carbon metabolism through the production of active one-carbon units and the production of serine, glycine and formate that can also serve as carbon donors for other reactions. Interestingly, compartmentalization of these enzymes determines the fate of the newly formed product, 5,10-methyleneTHF (**Figure 1.5**).[20] Radioactive isotope tracing experiments provided evidence that cytoplasmic SHMT (SHMT1) produces this substrate for the transmethylation of dUMP, performed by the cytoplasmic and nuclear TS.[45]

Both *in vivo* and *in vitro* studies have identified the SHMT conversion of THF to 5, 10-methylene THF as the rate-limiting step in thymidylate synthesis. *In vitro* studies with SHMT -/- null mice show a near 75% reduction in TS activity in isolated nuclei.[46,47] The abundance of $NADPH/NADP^+$ supports a reductive cytoplasmic space that makes the conversion of 5,10-methylene THF to 10-formylTHF highly unlikely; therefore the cytoplasmic supply of 5,10-methylene THF does not support the purine biosynthetic pathway.[17] Rather, as demonstrated by previous observations of SHMT1, the cytoplasmic isozyme is principally involved in the production of 5,10-methylene THF to support thymidylate synthesis.[42] There is research that demonstrates the unique role of mitochondrial SHMT2 function, which is distinguishable from its cytoplasmic counter parts, SHMT1 and SHMT2α.[17,40]

Mitochodrial folate metabolism

Conjugated folates cannot be transported into the mitochondria, as polyglutamylated species are not substrates for the mitochondrial folate transporter (MFT), solute carrier family member SLC25A32.[19,20] However, the shared affinity for monoglutamylated folate forms between MFT and folypolyglutamate synthase creates competition among these two proteins in the cytoplasm.[19] Monoglutamate forms of folates that successfully enter the mitochondria generate a distinct folate pool that is maintained within the organelle.[20] The mitochondria serves as an important source of cytoplasmic carbon, glycine and formate.[48] Mitochondrial formate is a product of serine, glycine, dimethylglycine, sarcosine and choline catabolism.[8] SHMT2 provides carbon, glycine and formate to participate in cytoplasmic biosynthetic reactions offered by the mitochondrial metabolism of serine.[8] The glycine autotrophic CHO cell line clearly illustrates the independence of function of the cytoplasmic and mitochondrial SHMT isozymes.[19]

Additional sources of 5, 10-methylene THF include the glycine cleavage system, which is a multi-enzyme system that participates in the oxidation of glycine into 5, 10-methylene THF, CO_2 and ammonia.[8] This system is thought to produce 40% of glycine flux, with the one carbon units produced from this system contributing to purine and thymidylate biosyntheses that occur in the cytoplasm.[8]

***De novo* purine biosythesis**

The *de novo* purine nucleotide biosynthetic pathway is a multi-step enzymatic process that yields IMP from the initial starting product phosphoribosyl pyrophosphate (PRPP). The numbered reactions shown in **Figure 1.3** are catalyzed by the following monofunctional enzymes: **1**,

glutamine phosphoribosylpyrophosphate amidotransferase (GPAT); **4**, formylglycinamide ribonucleotide synthase (FGAM synthetase); and **8**, adenylosuccinate lyase (ASL). Reactions **2**, **3** and **5** are catalyzed by the trifunctional glycinamide ribonucleotide (GAR) formyltransferase (GARFTase) which contains GAR synthase (GARS; reaction **2**), GAR formyltransferase (GARFTase; reaction **3**) and 5-aminoimidazole ribonucleotide synthase (AIRS; reaction **5**) activities. Reactions **6** and **7** are catalyzed by the bifunctional phosphoribosylaminoimidazole carboxylase/phosphoribosylaminoimidazole succinocarboxamide synthetase (PAICS) enzyme, which contains carboxyaminoimidazole ribonucleotide synthase (CAIRS; reaction **6**) and 5-aminoimidazole-4-(*N*-succinylocarboxamide ribonucleotide synthase (SAICARS; reaction **7**) activities. Reactions **9** and **10** are catalyzed by a bifunctional enzyme, 5-aminoimidazole-4-carboxamide (AICA) ribonucleotide (AICAR) formyltransferase (AICARFTase)/IMP cyclohydrolase (ATIC) that catalyzes the last two steps in the pathway for *de novo* synthesis of IMP. Folate-dependent reactions (reactions **3** and **9**) in which 10-formyl THF serves as the one-carbon donor are catalyzed by GARFTase and AICARFTase. AICA and AICAR can be metabolized to AICAR monophosphate (ZMP) by either adenine phosphoribosyl transferase (APRT) or adenosine kinase (AK), thus circumventing the reaction catalyzed by GARFTase. The activation of AMPK that results in inhibition of mTOR is also depicted.

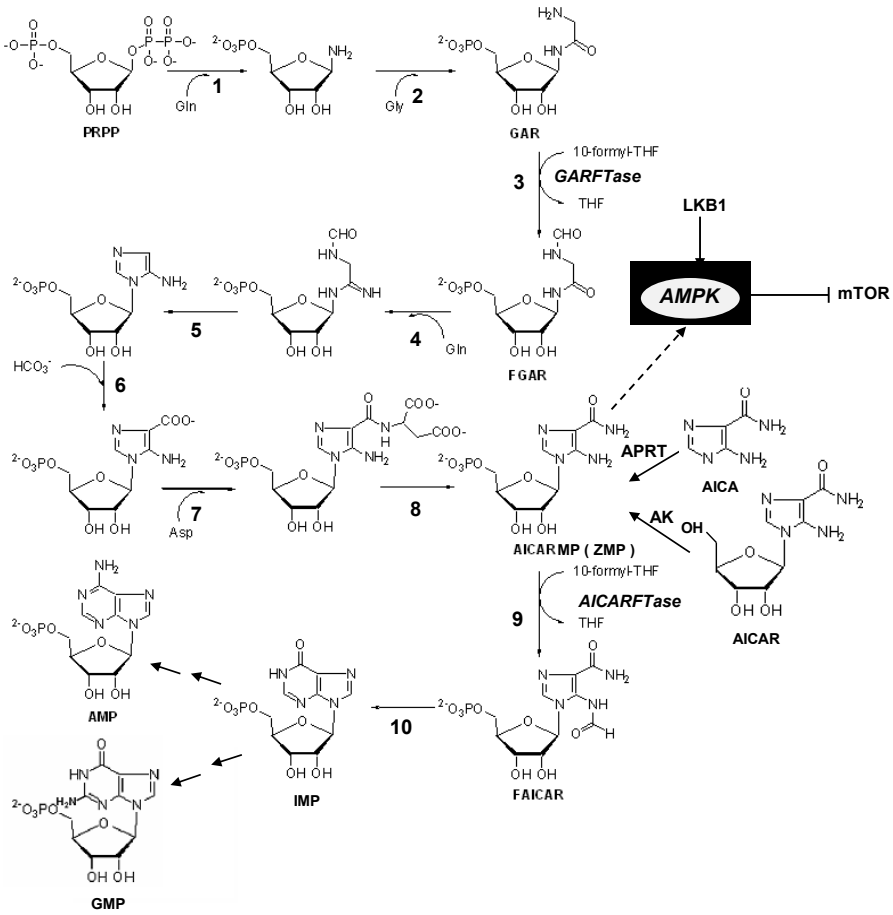

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Figure 1.3: *De novo* purine biosynthesis. The activated ribose PRPP, the phosphorylated product of the pentose phosphate pathway, is systematically converted into the purine precursor IMP in a 10 step process. Folate-dependent steps 3 and 9 are initiated by the enzymes GARFTase and AICARFTase, respectively. In addition to the *de novo* production of purine nucleotides, this pathway also provides metabolic intermediates (i.e., AICAR (ZMP)) that can activate signal transduction pathways that are responsive to cellular energetics which function to modulate cell survival via AMPK activation and mTOR inhibition.

GARFTase and AICARFTase are two cytoplasmic enzymes involved in *de novo* purine synthesis. These folate-dependent enzymes work to generate inosine monophosphate (IMP), a purine precursor that can be further metabolized to guanosine and adenosine nucleotides. There are two pathways responsible for intracellular purines, the *de novo* purine pathway and salvage pathways (**Figures 1.3 and 1.4**). Most often, normal cells that possess intact machinery for the salvage pathway will undergo this energy efficient method that uses preformed bases from degraded and metabolized purines that are recycled into nucleotides for the use in DNA synthesis.[49] The process of cellular transformation introduces chromosomal abnormalities that include deletions in chromosomal segments that house tumor suppressor genes. One common deletion involves chromosome 9, and is reported to include the chromosome 9p21 locus of multifunctional tumors suppressors, p14ink4a, p15ink4b and p16ARF.[50,51] Methylthioadenosine phosphorylase (MTAP), an essential enzyme in the purine salvage pathway that is often co-deleted with other well-established aforementioned tumor suppressors located on chromosome 9.[46,47] Tumors that harbor this deletion lack the capacity to use this purine salvage pathway as a source of purines and are heavily dependent upon the *de novo* purine biosynthetic pathway to support the high demand for purine nucleotides and carbon backbones in aberrant cell division.[46,47]

There is some debate over the folate metabolite(s) that acts as a coenzyme for purine biosynthetic reactions. Earlier studies proposed 10-formyl THF as the sole cofactor associated with the conversion of GAR and AICAR to their respective formylated species formyl glycinamide ribonucleotide (FGAR) and formyl 5-aminoimidazole-4-carboxamide ribonucleotide of (FAICAR), driven by GARFTase and AICARFTase.[8,17] However, more recent studies suggest that 10-formyl dihydrofolate not only plays a part in the activation of these enzymes but is the preferred substrate for AICARFTase.[52,53]

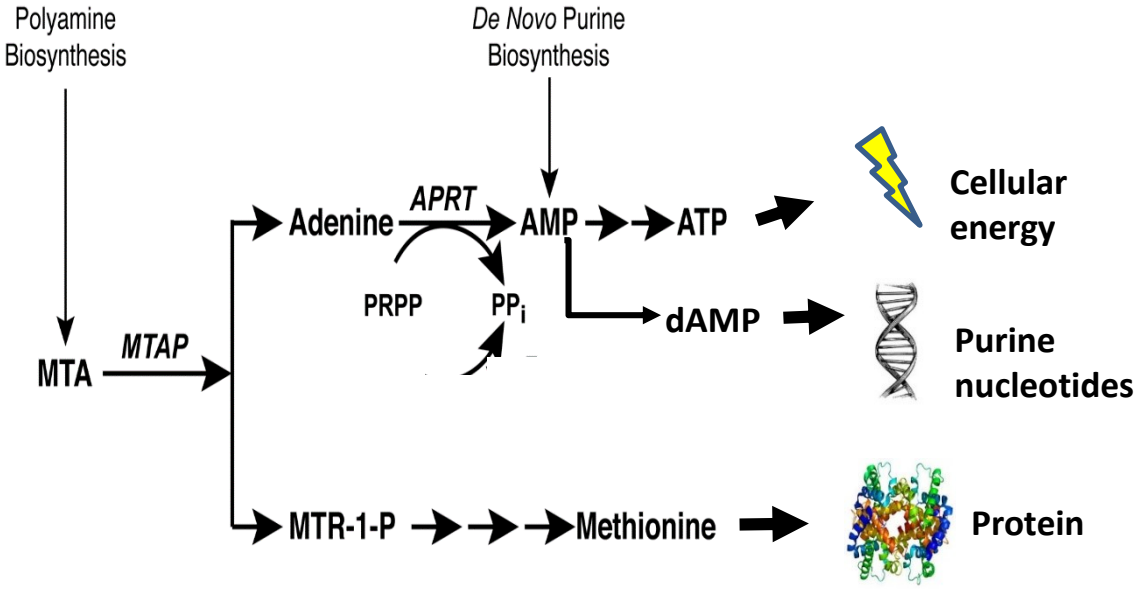

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Figure 1.4: The purine salvage pathway and cellular products. MTAP is an essential enzyme in the purine salvage pathway, producing both 5'-deoxymethylthioribose-1-phosphate (MTR-1-P) and the adenine free base. Through APRT, adenine can be converted into AMP and later into ATP following phosphorylation events, thereby aiding in cellular energetics. Deoxyribose AMP (dAMP) can be converted into the purine nucleotide adenosine, which can incorporated into DNA during replication and DNA damage repair. Through a multistep process, MTR-1-P is converted into Methionine, that can go on to assist in protein synthesis. The loss of MTAP would leave the cell dependent upon *de novo* purine synthesis for adenosine nucleotides, and therefore DNA replication.

Nuclear folate metabolism

An increasing number of studies have described a type of folate metabolism that occurs in the nucleus. This is thought to arise from the nuclear translocation of SHMT2α (which has a redundant role in both the cytoplasm and the nucleus), TS and DHFR, which form a multi-enzyme complex to facilitate nuclear thymidylate synthesis.[47] The purported significance of this physiological event is to maintain genomic integrity in DNA replication and repair (as this has been cited to occur during S, G2/M and during damage to DNA from UV exposure), in addition to preventing the mis-incorporation of uracil.[45,47] This also holds particular significance in the prevention of uracil accumulation, which also plays a part in compromising genomic integrity.[45] Further, *in vitro* studies have substantiated these observations by indicating that nuclear membrane disruption (via sonication) resulted in nuclei that failed to possess *de novo* TS activity.[45]

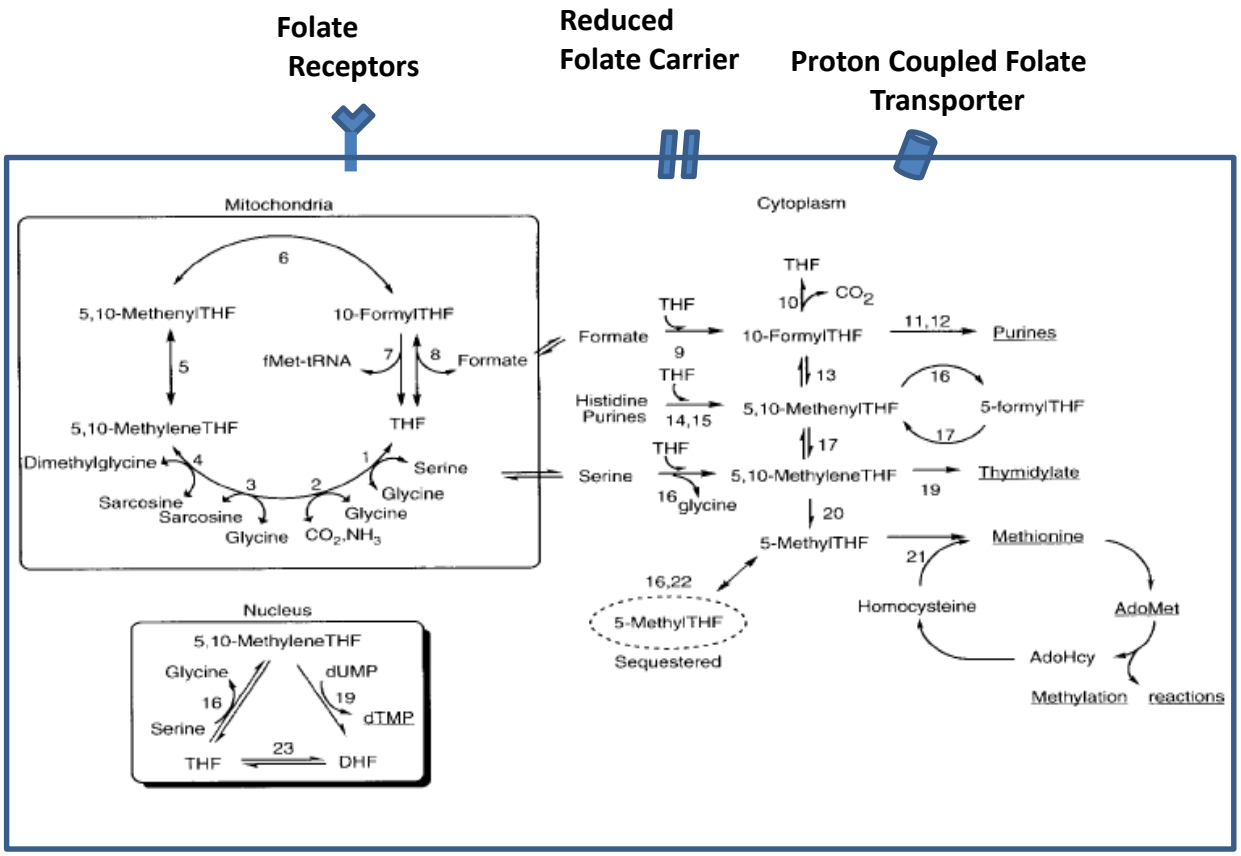

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Figure 1.5: The compartmentalization of folate metabolism. Folate metabolism can occur in three compartments with the cell, the mitochondria, the nucleus and the cytoplasm. Not much is known about nuclear folate metabolism, however, it is thought to play an important role in thymidylate synthesis. Folate pools in the cytoplasm and the mitochondrial are not interchangeable. The mitochondrial formate combines with cytoplasmic THF to produce the folate cofactor essential for *de novo* purine synthesis.

1.3 Folate tissue absorption

Mammals must acquire preformed folates from the diet due to an inability to synthesize these water–soluble vitamins *de novo*. Dark green leafy vegetables and select fruits are notable high sources of the vitamin. Intracellular accumulation of folates is predominately performed by transmembrane carriers/transporters and receptors. Tissue accumulation of folate is dictated by the expression of transporters/carriers that permit the influx of folates, polyglutamylation at the γ glutamate on the carboxyl end of the parent molecule and expression or ability for exporter proteins to bind folate and contribute to their efflux from cells.

The mechanisms of folate transport have been evolutionary conserved over many millions of years. The existence of the two solute carrier (SLC) family members that participate in folate transport, SLC19A1 (reduced folate carrier or RFC) and SLC46A1 (proton coupled folate transporter or PCFT) date back to the Vendian period (approximately 523-543 million years ago).[15] Genes of both transporters have been identified in *Pseudocoelomata*, more specifically *C.elegans*.[15] Folate receptor genes evolved much later and made their first appearance in chordates.[15] Salbaum and colleagues speculate that folate receptors (FRs), with their superior binding affinities, evolved to ensure genomic stability and to preserve DNA methylation patterns even when folate supplies were limited.[15] Folate availability is an important factor in modulating the expression levels for each of the three proteins. A number of studies have confirmed that low concentrations of folates lead to increased expression of intestinal RFC and PFCT.[54-57] Much like RFC and PCFT, FR alpha mRNA transcripts and protein expression is increased under folate-deficient conditions.[58-63] Cancer is one of many disease states that can result in a decrease in serum folate levels due to the increased demand for folate cofactors to participate in DNA replication for rapidly dividing cells.[64] Interestingly, patients with FRα positive tumors, like ovarian cancer, have

increased levels of FRα protein in their serum.[65-67] Due to this elevation, serum FRα levels have been considered as a plausible biomarker to detect epithelial ovarian cancer.[65,66]

The Reduced Folate Carrier (RFC;SLC19A1)

The RFC, as its name suggests, is a facilitative transmembrane transporter with a very high affinity for reduced folates (Km: 1-3μM), e.g 5-methyl-THF.[25] The RFC protein is comprised of 591 amino acids and has a molecular mass of approximately 65 kDa (**Figure 1.6**).[25,68] Due to pronounced glycosylation at residue Asn 58, the molecular weight of the modified protein is ~85kDa, a ~20 kDa difference from the original molecular weight of ~65 kDa.[25,69] The human RFC gene is located on chromosome 21q22.2 and is reported to be regulated by a host of regulatory proteins and transcriptional start sites.[70] The very complex transcriptional regulation of RFC is carried out by six non-coding exons with multiple promoters and with the aid of multiple transcription factors from various families.[71,72] RFC has been described by Whetstine and colleagues as a ubiquitously, but differentially expressed protein.[73] In this study they speculated that the differential expression and the utilization of multiple promoters and transcription factors could be the product of varying folate demands in various tissues.[73] The expansive expression of this protein, along with its ability to act as a "high capacity" carrier of reduced folates has led to the belief that RFC is responsible for the bulk influx of reduced folates into mammalian cells, thereby having the greatest impact on the intracellular folate pools.[69,74] As the major transporter of reduced folate derivatives, it is conceivable that both normal and malignant tissues would find expression of this protein advantageous for successful DNA replication and for the preservation of genomic integrity. In fact, quantitative real time PCR (RT-PCR) and multiple methods of protein detection have validated RFC expression in various human tumors and tissues. Human RFC mRNA transcripts have been identified in cells from various origins including the central

nervous, leukocytes, liver, placenta and the intestine.[73,75] The presence of RFC expression in normal tissues has also implicated the protein as the source of dose-limiting toxicities seen in patients treated with antifolates that are accumulated into normal tissues through this mechanism.[69]

The bidirectional transport of RFC is driven by the extrusion of organic phosphate which allows for the exchangeable influx of reduced folate derivatives and a select number of antifolates that are recognized by this protein.[25,69] While the exact mechanism of exchange is not fully understood, some speculate that it involves a trans-stimulation phenomenon whereby the movement of a molecule in one direction enhances or "stimulates" the movement of another molecule in the opposite direction.[76,77] Optimal transport generally occurs at a neutral pH (7.4), although some studies have demonstrated residual RFC transport at a low pH.[78,79] Interestingly, prior to the discovery of PCFT, RFC was thought to be responsible for low pH folate transport in the intestine where the protein is expressed.[80] However, more recent experiments have confirmed that RFC transport of leucovorin, a reduced folate derivative, was severely impaired at pH 6.5.[69,81] While it has been demonstrated that RFC plays little to no role in the intestinal absorption of dietary folates, folate depletion increases the intestinal expression of RFC and the role of RFC in this context has yet to be determined.[26,55,56] Studies suggest that RFC does not play a major role in the tissue absorption and or transport of folic acid (Km~200μM) which may partly explain the reduced transport efficiency seen with novel antifolates that closely resemble the chemical structure of folic acid.[25,75]

The principal route of cellular entry for many "classical" antifolates (i.e., AMT, methotrexate (MTX) and pemetrexed (PMX)) is through RFC. The pharmacological impact of RFC expression on the activity of a number of antifolates has been extensively studied. Many of these studies have demonstrated that the functional loss of RFC modulates drug sensitivity.[74,82] In

the case where drug influx is completely RFC-dependent, cells exhibit resistance. Alternatively and contrary to what one may expect, the loss of RFC increases drug sensitivity with agents that can undergo non-RFC-mediated cell transport, thought to be a consequence of a contracted intracellular folate pool, which diminishes competing substrates for targeted enzymes, therefore increasing drug potency.[82-84]

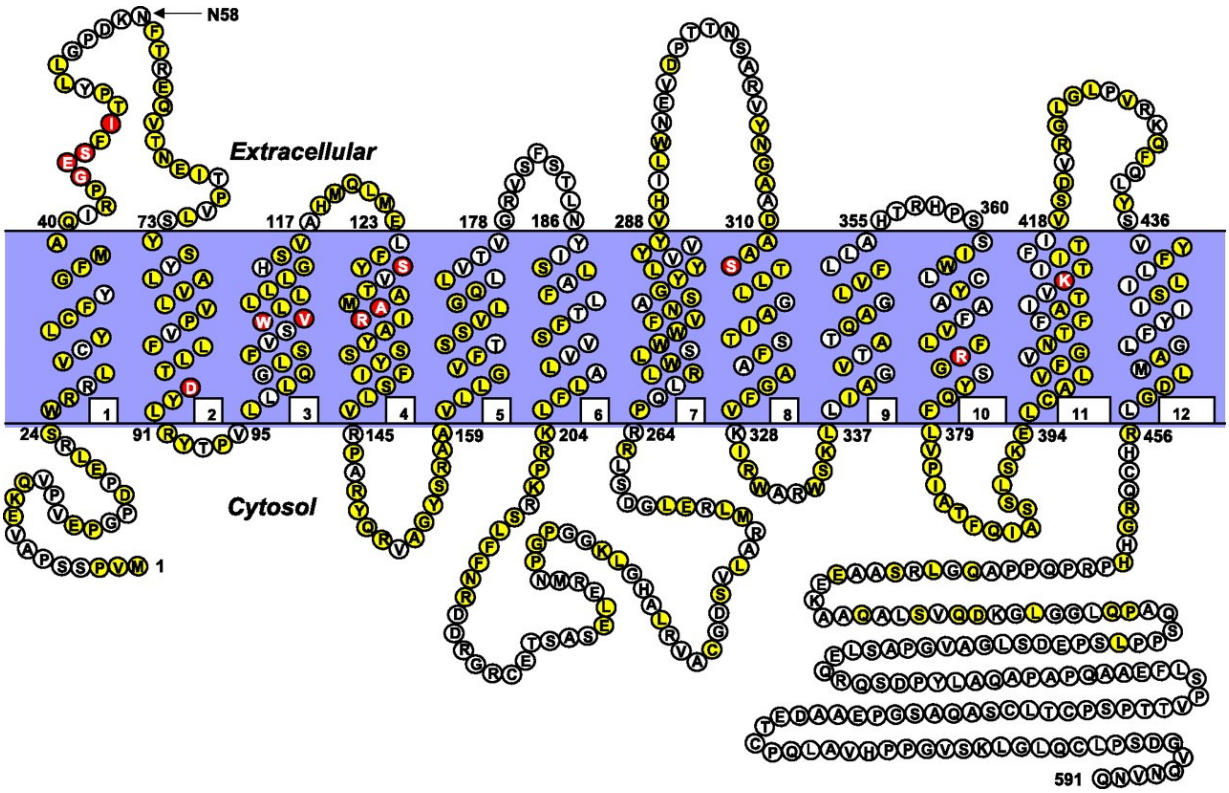

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Figure 1.6: Predicted topology map of human reduced folate carrier (hRFC). hRFC is an integral membrane protein consisting of 12 transmembrane domains and both intracellular and extracellular loops. The diagram attempts to designate specific amino acid sequences within the cell membrane, as well as those exposed to the extracellular and cytosolic space. Topology modeling allows for a greater understanding of how hRFC functions and encourages the generation of hypotheses surrounding the identification of critical amino acid residues involved in the activity of the protein.

The Proton-Coupled Folate Transporter (PCFT; SLC46A1)

The proton couple folate transporter (PCFT) is the most recently identified of the predominant mechanisms of folate transport.[86] Prior to receiving its current designation as a folate uptake mechanism, it was originally named the heme transporter protein-1, which describes its ability to effectively transport heme in the intestine (Km:125μM).[25,87] The dietary acquisition of folates begins in the acidic micro-environment of the duodenum and upper jejunum of the intestines.[88] The existence of a low pH folate transporter became evident as accumulating data ruled out RFC despite its expression in the intestinal brush boarder membrane. More specifically, early experiments using a RFC-null HeLa model validated the existence of a non-RFC low pH transport mechanism that was later determined to be PCFT.[89] The cloning of PCFT by Qui et al formally identified PCFT as the low-pH folate transporter.[86] This soon led to structural and biological studies to obtain more information about the protein and how it participates in folate homeostasis and more importantly what role if any it plays on the pharmacological activity of antifolates in clinical use.

Like RFC, PCFT is an integral membrane protein that participates in the facilitative transport of (anti)folates and is also a member of the major facilitator super family (MFS). However the transport profile and mechanism of transport is distinct from that of RFC.[73] The unidirectional transport of folates has been described as electrogenic and proton driven and occurs optimally at a low pH (5.5), hence the naming "proton coupled".[73] The charge carried by the proton creates an electrochemical gradient which classifies this proton driven mechanism as electrogenic.[80,90]

The PCFT chromosomal location is 17q11.2 which encodes a 55 kDa protein made up of 459 amino acids (**Figure 1.7**).[25,91,92] PCFT is encoded from 5 exons with a minimal transcriptional

regulatory region located -42 and +96 bases from the transcriptional start site.[75,91-93]. Transcription of this protein is thought to be governed by a number of factors. Kruppel like factor -4 (KLF-4), hepatocyte nuclear factor (HNF-1) and nuclear respiratory factor -1 (NRF-1) have all been reported to enhance PCFT promoter activity resulting in an increase in PCFT transcripts.[94,95] Vitamin D was also shown to increase PCFT expression via the transactivation of the PCFT promoter by the vitamin D receptor (VDR) when heterodimerized with retinoid X receptor –α (RXRα).[96] Promoter hypermethylation results in decreased transcriptional activity and therefore gene product.[93] While little is known about the intrinsic transcriptional regulation of PCFT, studies have shown that xenobiotics, more specifically, proton pump inhibitors, can decrease intestinal PCFT expression in patients administered this type of therapy.[97]

Compared to RFC, PCFT has restricted tissue expression that is limited to the duodenum, jejunum, liver, kidneys, and choroid plexus and comparably much lower expression in the bone marrow and colon.[88] A wide range of malignant cells express PCFT, including but not limited to tumors of the colon/rectum, lungs, liver and ovaries.[98] The expression of PCFT may be especially advantageous in tumors with high glycolytic activity or what had been described as "The Warburg effect", creating a proton-rich acidic microenvironment. In theory, the creation of such a microclimate would increase the activity of PCFT and provide a context in which RFC activity is reduced. This very observation is the basis of PCFT-targeted anti-tumor therapy.[75] A better understanding of the transcriptional regulation of PCFT will undoubtedly result in novel approaches and creative strategies to improve drug efficacy in PCFT-expressing tumors.

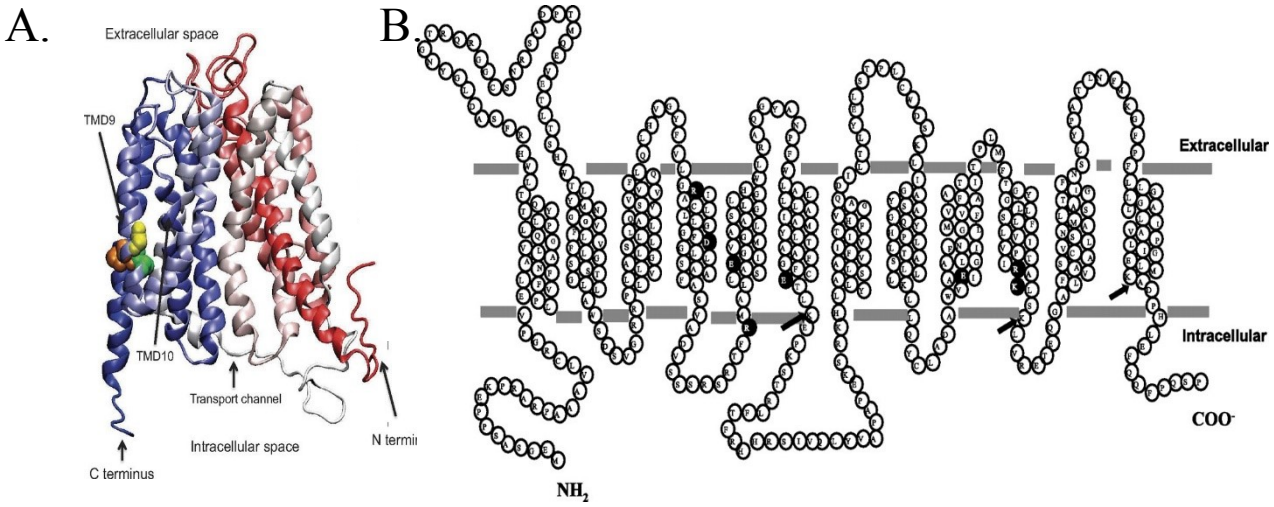

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Figure 1.7: Homology model and predicted membrane topology for hPCFT. (A) The cartooned homology model of hPCFT (generated from the bacterial glycerol-6-phosphate transporter) demonstrates a side view of the protein. Featured in this model the two residues (Ala 335 (red) and Gly 338 (yellow) that were found to be mutated in human folate malabsorption syndrome, which believed to play an important role in protein stability. In addition, the spatial arrangement of transmembrane domains (TMD) 9 and 10 and their proximity to the transport channel are also shown. Much like the predicated membrane topology (B), loops that are believed to reside in the intracellular or extracellular space are shown above. The topology model highlights conserved (black) and partially conserved residues (arrows) within the TMDS.

Folate Receptor alpha (FRα)

FRs are an extensively studied family of proteins that are recognized for their role in embryonic and fetal development where they act as an essential mechanism of folate transport, supporting rapid cell division during gestation. FRs, also known as folate binding proteins (FBP or folbp for murine homologs) were first identified in bovine milk in 1972.[101,102] Since the discovery of these high affinity/low capacity folate binding proteins, four isoforms and pseudo-genes have been identified. While each isoform shares some sequence homology (70-80%), they each possess distinct structures, patterns of tissue expression (with some overlap), binding affinity and stereospecificities.[103,104] Collectively, FR's have the highest affinities toward folic acid, physiological reduced folates and formylated THF (5-formylTHF) compared to PCFT and RFC.[25] These features allow FRs to successfully engage in cellular uptake when folate concentrations are low. These genes are all a part of a larger superfamily that includes the riboflavin binding protein and the retinbindin, a retinal binding protein.[103] Prior to the solving of the crystal structure of FRα, homology modeling using bovine riboflavin binding protein was employed to elucidate or to gather more insight on the structural characteristics of this protein and possibly other identified isoforms due to known similarities of each protein.[105]

FR genes, Folr 1-4, are localized to chromosome 11q13.3-14 and are responsible for the transcription for FRα, β, γ/γ' and δ.[62,103,104] Of the four isoforms, FRδ is the most recently discovered. While the mouse ortholog, folrbp3, is expressed in the mouse thymus and spleen and is thought to play a role in the proliferation of immune cells, expression of this isoform has not been detected in human adult or fetal cells.[104] The failure to detect FRδ in human tissues has been thought to arise from the temporal/spatial regulation and expression of the protein.[104] Others suggest that like FR pseudogene1 (Folr1p) identified by Raggoussis *et al*., the delta isoform is a

pseudogene in the human genome.[104,106] Due to an elusive pattern of expression in human tissues, the capacity of this isoform to bind folate is undetermined, as well as its putative role in folate metabolism and perhaps cell signaling.

Much like the delta isoform, little is known about FRγ, when compared to the more prominent and widely studied β and α isoforms. Unlike FRδ, the gamma isoform has been identified in human tissue. The protein was first discovered in 1993 and was identified as an overexpressed high affinity folate binding protein in chronic myelogenous leukemia (CML) and acute myelogenous leukemia (AML) patient samples.[107] Since the discovery of the γ isoform, it has been identified in normal hematopoetic cells, bone marrow, spleen and thymus.[103] Of the three functional forms of FR expressed in human tissues, FRγ is to date, the only identified polymorphic isoform of this family. A genetic polymorphism results in a mutation that codes for a premature stop codon leading to the translation of a truncated non-functional protein, FRγ'.[108] Despite multiple N-glycosylation sites (3) which are shared among all isoforms and are thought to contribute to the cell surface expression of the protein, there is an additional anomaly that distinguishes FRγ from its counterparts- this protein is constitutively secreted due to a lack a hydrophobic amino acid sequence that serves as a signal for glycosylphosphatidylinositol (GPI) modification that anchors the protein to the cell surface.[103,109]

FRs are known to exist in membrane bound and soluble states. Membrane bound receptors (α and β) are anchored to the membrane by glycolipids known as GPI anchors.[103,110] This GPI modification is signaled by the hydrophobic carboxyl terminal segments in the α and β isoforms which allow for a post-translational transamidase reaction, resulting in the cleavage of the present C-terminus while creating an amide linkage to the GPI and a newly formed C-terminus.[103,110] Soluble folate binding proteins (sFBP) arise from the membrane-bound forms that undergo well-

defined cleavage events. They are generally present in extracellular fluids, which include milk, cord blood, urine, and cerebrospinal and amniotic fluids.[103] The soluble FR α isoforms are thought to be the result of (1.) the phospholipase cleavage of the GPI anchor or (2.) proteolysis by a Mg^{+2} dependent protease.[103] These mechanisms are thought to be responsible for soluble α isoforms detected in KB nasopharyngeal carcinoma cells in culture and those detected from placental cells. In contrast, the soluble β isoform is the product of two independent pathways that either result in GPI anchor attachment or secretion.[103] All soluble forms of the protein have been shown to bind and stabilize folates; therefore these soluble proteins may have a functional role in normal physiology.[103] For instance, the sFBP in milk are thought to play a role in the intestinal absorption of folates.[103] These soluble proteins may also serve a diagnostic purpose in detecting disease as many pathological conditions, including cancer, results in the aberrant shedding of these membrane proteins into the circulation where they are not generally detected in healthy individuals.[103,111]

The FR β has increasingly become the subject of interest due to its role in a number of human pathologies, most notably cancer and inflammation.[103,112-116] The expression of this isoform is confined to hematopoietic cells of the myelomonocytic lineage and the protein is routinely used as a differentiation marker in normal hematopoiesis.[103] Protein expression extends to the placenta and to mature neutrophils with elevated expression seen in activated blood monocytes and the expression status remains consistent during their transformation to macrophages.[103] Macrophages are known to play a critical role in the initiation, maintenance and resolution of the inflammatory process.[113] Rheumatoid arthritis (RA) is a disease characterized by the infiltration of lymphocytes and macrophages to synovial joints, causing proliferation of local fibroblasts and severe joint damage. Activated macrophages in synovial tissues from RA patients

were shown to have significant expression of the β isoform.[116] The selective expression of FR β on this population of macrophages makes this isoform an excellent target for therapies specific to this protein isoform. Presently, the antifolate MTX is the preferred treatment option in patients suffering from RA.[117] However, MTX substrate promiscuity among other mechanisms of folate transport has created a demand for novel therapeutics that have selective uptake by FR β.[117-119] These therapeutics may not only prove useful in the treatment in RA, but they may also show efficacy in FR β-expressing malignancies such as leukemia (CML and AML). Tumor-associated macrophages (TAM's), which also express FRβ, have been implicated in the initiation and progression of a number of malignancies.[120-122] Folate-based therapies targeted to FR β may extend the survival of patients diagnosed with difficult to treat tumors like pancreatic cancer which are reported to have significant TAM infiltration.[123,124] An additional and perhaps more practical application in exploiting the population of TAMs in cancer is the use of fluorescent conjugates linked to FR β, which may serve as an optical tool that can aid in detection of relapse (in the case of hematopoietic disease), primary tumors and their metastasis.[122]

FRα is, by far, the most extensively studied of the four FR isoforms. While this receptor has been well documented as a mechanism of folate transport, additional physiological roles of FRα continue to be the subject of many ongoing studies. Much intrigue surrounds the functional role of FRα especially when co-expressed with other high capacity folate uptake mechanisms such as PCFT or RFC, which has led to speculation on alternative roles for this receptor. Undoubtedly, FRα has a clear role in the reabsorption of folates from kidney proximal tubules and in embryonic development, including the development of the neural tube and transplacental folate transport from mother to fetus. What remains unclear is the utility of this receptor when expressed in other epithelial tissues with limited or no access to circulating folates. One popular line of reasoning

suggests that FRα, when expressed in tissues with limited access to circulation, acts as a folate scavenger and participates in the transcellular transport of folates, as described in the transport of folates across the retinal pigment epithelia, the luminal uterine epithelia during pregnancy and in lung epithelia to prevent bacterial growth.[125,126] It has been proposed that FRα, like other GPI anchored proteins clustered by lipid rafts, participates in signal transduction pathways that are involved in regulation, survival and growth.[127,128] More studies are required to determine and validate the exact signaling pathways that are involved with ligand-bound FRα.

There are a number of disorders associated with mutations or the functional loss of FRα. The homocysteinylation of FRα, a consequence of excess/elevated homocysteine, may lead to the development of FRα autoantibodies which are thought to contribute a significant decrease in folate uptake, resulting in defects in the developing embryo.[129,130] Such deficiencies that arise from deleted, dysfunctional or blocked FRα have been linked to NTDs, orofacial cleft, congenital heart defects, autism spectrum disorders and cerebral folate deficiency (CFD).[130-132] CFD is thought to arise from the presence of FRα autoantibodies or a loss of function mutation in the folr1 gene, causing a neurological syndrome that presents in children as young as 4 months with cognitive and neuromuscular deficits.[133,134] Studies have shown that maternal FR autoantibody concentrations are linked to NTDs, further highlighting the important role of FRα in embryonic development.[130,131,135] An incomplete understanding of FRα's role in signal transduction makes it difficult to delineate if folate deficiency or a disruption in signal transduction pathways play a causal role in the development of such malformations. More work in this area may lead to a greater understanding of the tissue distribution of this protein and the global importance of its expression, in addition its acknowledged role in folate metabolism.

As previously mentioned, FRα has a unique and distinct pattern of tissue expression. In adult tissues, FRα is confined to the apical surface (luminal) of polarized (non-transformed) cells of epithelial origin, including the choroid plexus, proximal renal tubules, fallopian tubes, uterus, epididymus, acinar cells of the breast, submandibular salivary and bronchial glands, type 1 and 2 pnuemocytes in the lung and placental trophoblasts.[103] The only known basolateral surface expression of this protein has been identified in the epithelia of retinal pigment cells.[103] The luminal expression of FRα, by virtue of its physical location, makes this receptor inaccessible to circulating folates in the blood.[136-138] The role that FRα plays in the reabsorption of folates from the kidneys creates an opportunity for renal FR to encounter serum folates.[103] Interestingly, it has been reported that glomerular filtration allows for the separation of low molecular weight FRα-targeted drugs that are transcytosed, thereby preventing renal accumulation of cytotoxic agents and circumventing neurotoxicity.[136]

Many epithelial based tumors also express FRα, however, ovarian and endometrial cancers are among the most recognized partly due to their consistent and elevated expression of the protein. One classic feature of the transformed cell is the absence of cellular organization (loss of polarity) and a disruption of cellular architecture. These structural changes that occur during tumorigenesis lead to the altered spatial orientation of the receptor such that it is expressed on the basolateral surface. The newly acquired basolateral surface expression of the receptor on tumors permits the sequestration of circulating (anti)folates due to a change in orientation that leads to accessibility. We argue that this feature, in addition to the very narrow and restricted tissue expression exhibited on normal tissues, makes FRα a rational and attractive target for drug delivery to promote the selective killing of FRα-positive tumors.

There are many theories surrounding the elevated expression of FRα in select epithelial malignancies. Many of these encouraged thorough investigations of the regulation of *folr1* gene and gene products in both normal and transformed cells. The complex regulation of FR genes is governed by a variety of factors which include serum folate concentrations, nuclear receptors and their respective ligands, and post transcriptional/translational modifications. Although FR isoforms share significant sequence homology, the organization of the gene, the use of alternative promoters and alternative splicing distinguishes each isoform from the other while also accounting for the differences observed in tissue distribution. [96]

The *folr1* gene has a reported length between 6.7 [139] and 7.5 kb [140] and is composed of 7 exons and 6 introns that encodes a 257 residue polypeptide and a 38-42 kDa protein.[141,142] Multiple transcripts of folr1 have been identified and appear to be the product the use of alternative promoters, as well as alternative splicing that occurs at exons1-4.[141-143] The promoters responsible for the diverse FRα mRNAs are TATA-less and designated P1 and P4, located upstream of exons 1 and exon 4, respectively (**Figure 1.8**).[103,140] Although they produce divergent transcripts, the protein product derived from each promoter is identical.[142] The regulation of transcription, the efficiency of translation and the length and sequence of the resulting transcripts are where these two promoters diverge.[82,111,142] Despite these differences, the open reading frame (ORF), 3' untranslated region (UTR) and mRNA stabilities are the same for transcripts derived from both promoters in FRα-expressing cells.[142] The P1 and P4 promoters are reported to be under tissue-specific regulation which is thought to account for the abundance of specific transcripts in specific tissues. [82,142]

Relative to P4, not much is known P1 promoter, but this promoter is understood to exhibit functional and structural differences from the P4 promoter.[141] Transcripts produced by the P1

promoter have been identified in the normal human kidney, the testis and the cerebellum of the brain.[103,141] The multiple initiation sites combined with the alternative splicing of upstream exons results in heterogeneity of the 5' UTR in the transcripts generated and explains the varying lengths and sequences of the 5' UTR among the transcripts.[142] The regulation of the P1 promoter in certain ovarian cancer cells differs from that which is seen in normal human cells. Bagnoli and colleagues described an inverse relationship between caveolin-1 protein, known to negatively regulate intermediates in signal transduction involved with cell proliferation, and FRα expression in human ovarian cancer cells.[139,144] The result of their efforts determined that caveolin-1 negatively regulated FRα expression through repression of the P1 promoter.[144] Additional studies examining P1 regulation in ovarian carcinomas concluded that the promoter was under the control of a variant hepatocyte nuclear protein that enhanced promoter activity via the interaction of a DNA-binding site located in the untranslated region of exon1.[139] Exon 1 is believed to include the elements required for optimal transcriptional activity for the P1-derived FRα transcript in ovarian cancer cells.[139] For reasons not fully understood, P1 transcripts display a lower rate of translation efficiency when compared transcripts generated from the P4 promoter.[142] Whether or not translation efficiency corresponds to surface expression density, is yet to be determined. Additional research is required to validate if similar factors modulate P1 promoter activity in normal cells and to determine if there is a direct relationship between promoter activity and overall protein expression.

A number of FRα expressing cells transcribe FRα under the control of the P4 promoter including the KB nasopharyngeal carcinoma cells and normal epithelial tissues of the lung, placenta, salivary gland, uterus breast and stomach.[103] This promoter's activity is influenced by three GC rich sequences that allow for non-canonical SP1 binding, with each of the three sites

making a significant contribution to promoter activity.[103,143] Multiple sites of potential transcription factor binding were reported to be contained within this promoter, including sequence motifs for AP2, E-box and the aforementioned SP1 (**Figure 1.8**).[101,140]

mRNA stabilities in FRα-expressing cells have been discussed; irrespective of the promoter of origin, all transcripts are reported to have similar stabilities.[103,140,142] However a novel nuclear post transcriptional mechanism of FRα regulation has been described. Residing in the ORF is a discrete 60 base pair mRNA element that is reported to control the stability of FRα mRNA.[140] This structure ultimately results in the selective nuclear degradation of FRα transcripts in FRα null cells and may be responsible for the restricted tissue expression seen with this protein.[140]

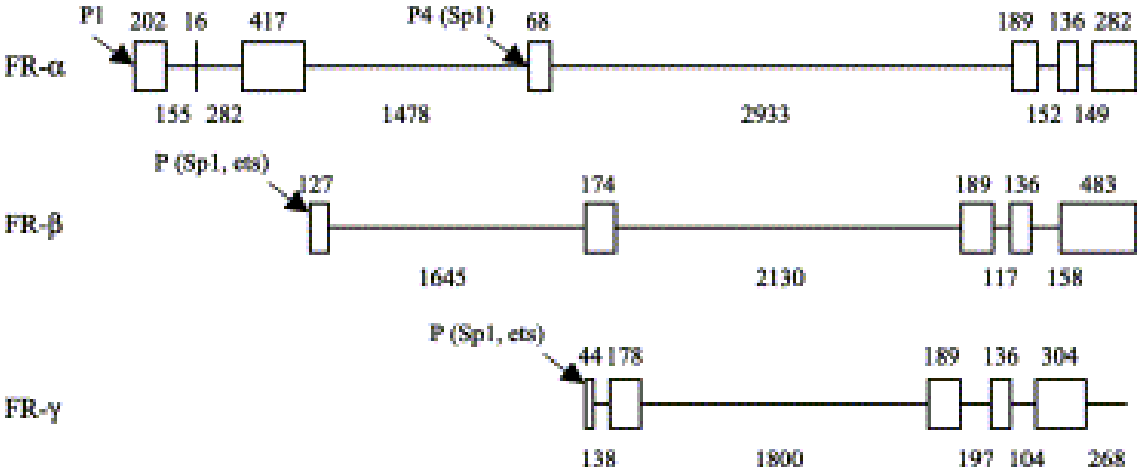

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Figure 1.8: Folate receptors gene organization. Above is a representation of human folate receptors α, β and γ gene organization. Both the β and γ isoforms are thought to be under the transcriptional control of a single promoter (indicated here with the letter P) and cis regulatory elements Sp1 and ets. The alpha isoform shows two promoter regions, P1 and P4. The P4 promoter includes a Sp1 cis regulatory element. The length of the exon and intron present are featured with numbers above each segment. An examination of the gene organization includes a visual comparing the genomic similarities of each isoform, while offering an explanation in the divergence of distribution, expression and function.

The inverse relationship between FRα expression levels and serum folate concentrations has been well documented.[59,145-147] Both *in vivo* and *in vitro* models have validated the regulation of this isoform by serum and media folate levels, using both folate-deplete and folate-replete conditions, the latter including multiple folate metabolites.[58,59,148-152] In each instance, restricted or reduced folate levels accompanied an increase in FRα expression levels, while increasing folate levels reversed the phenotype resulting in reduced expression levels of the protein. In support of these studies, an examination of serum folate levels in patients with FRα-positive tumors recapitulated these experimental results, suggesting that this regulation by folate is a naturally occurring phenomenon.[64,153-156] A consequence of reduced or depleted serum folates is a rise in homocysteine levels due to inactivity of MS, which is responsible for the conversion of homocysteine to methionine. Elevated homocysteine levels were shown to encourage or increase the interaction between the heterogeneous nuclear ribonucleoprotein E1 (hnRNP E1) and the *cis*-element in FRα transcripts, which is believed to result in unregulated translation.[157] Other studies have reported an alteration in the level of mRNA transcripts or amplification of the FR gene.[103]

The use of antifolates, more specifically MTX, has also been shown to increase FRα expression.[81] MTX resistance is thought to be a result of a number of factors including the functional loss of RFC or a reduction or silencing of protein expression via gene hypermethylation or other mechanisms.[25,158,159] Experts suggest that MTX resistant cells experience an increase of FRα expression as an attempt to meet the folate requirements of a malignant cell through the use of a transport mechanism not affected by drug exposure.[81] The exact transcriptional mechanism is unknown; however, it has been postulated that DHFR inhibition by MTX may mimic the conditions of folate depletion and may trigger the same regulatory pathways that lead to the enhancement of FRα expression.[81]

Steroid receptors have also been implicated in the regulation of FRα. Activation of androgen[160], glucocorticoid[161], and progesterone[162] receptors have been associated with increased transcriptional activity of the P4 FRα promoter and an increase in cell surface FRα protein expression. Steroid hormone regulation of the FRα P4 promoter by the progesterone and glucocorticoid receptors is indirect but it is direct by androgen receptors. In contrast, the estrogen receptor has been identified to negatively regulate FRα through transcriptional repression.[163] Estrogen exerts FRα transcriptional repression by interacting with GC-rich Sp1 binding site(s) of the P4 promoter and recruiting the co-repressor SMRT which prevents the cluster of transcriptional machinery.[163] This finding was thought to help explain the lack of FRα in ER-positive tumors and the increased expression in anti-estrogen (tamoxifen) treated tumors.

Many suspect the physiological role of FRα extends beyond the role it plays in folate transport and metabolism.[164,165] Studies have shown that cells that are transfected with FRα undergo a rapid increase in cellular proliferation and alternatively, cells treated with antibodies, sense/antisense particles or oligonucelotides directed to this receptor showed a delay in growth and in some cases reversion of the malignant phenotype.[127,164-166] Folate metabolism alone cannot account for a robust proliferative response but rather alterations in signaling pathways may be involved. Notch and Hes-1 were both implicated in FRα directed cell growth in alpha T3-1 cells transfected with FRα.[164] In addition to cellular proliferation, FRα has also been linked to cell motility, resistance to apoptosis, migration and invasion.[167,168] This is somewhat consistent with the correlation made between FRα overexpression and a poor prognosis/aggressive phenotype and decreased survival in ovarian cancer patients.[169,170] FRα expression has been linked to many changes seen in malignant transformation which has led some investigators to think the protein plays a significant role in normal to malignant transition.[168]

Membrane bound FRs translocate extracellular folates through receptor mediated endocytosis. After endocytosis, the ligand bound receptor is enclosed in an endosome, which subsequently acidifies, releasing the ligand from the receptor, after which the free ligand is translocated to the cytosol, through some uncharacterized mechanism. There have been two competing theories surrounding the fate of the endocytosed ligand-bound receptor: (1) clathrin-coated vesicles/pits are involved in intracellular transport; and (2) a clathrin-independent mechanism that occurs in lipids rafts which involves the development of early endosomes that mature and acidify resulting in the release folates in the intracellular accumulation o. Many studies support the clathrin- independent mechanism as this model appears to be congruent with observed absorption, localization and trafficking that has been described for the receptor.[110,171,172] FRs are proposed to associate with lipid rafts which have been shown to play a role in signal transductions;[173] this offers some plausibility to the growing hypothesis that FRα is also involved in signaling. [128,174]

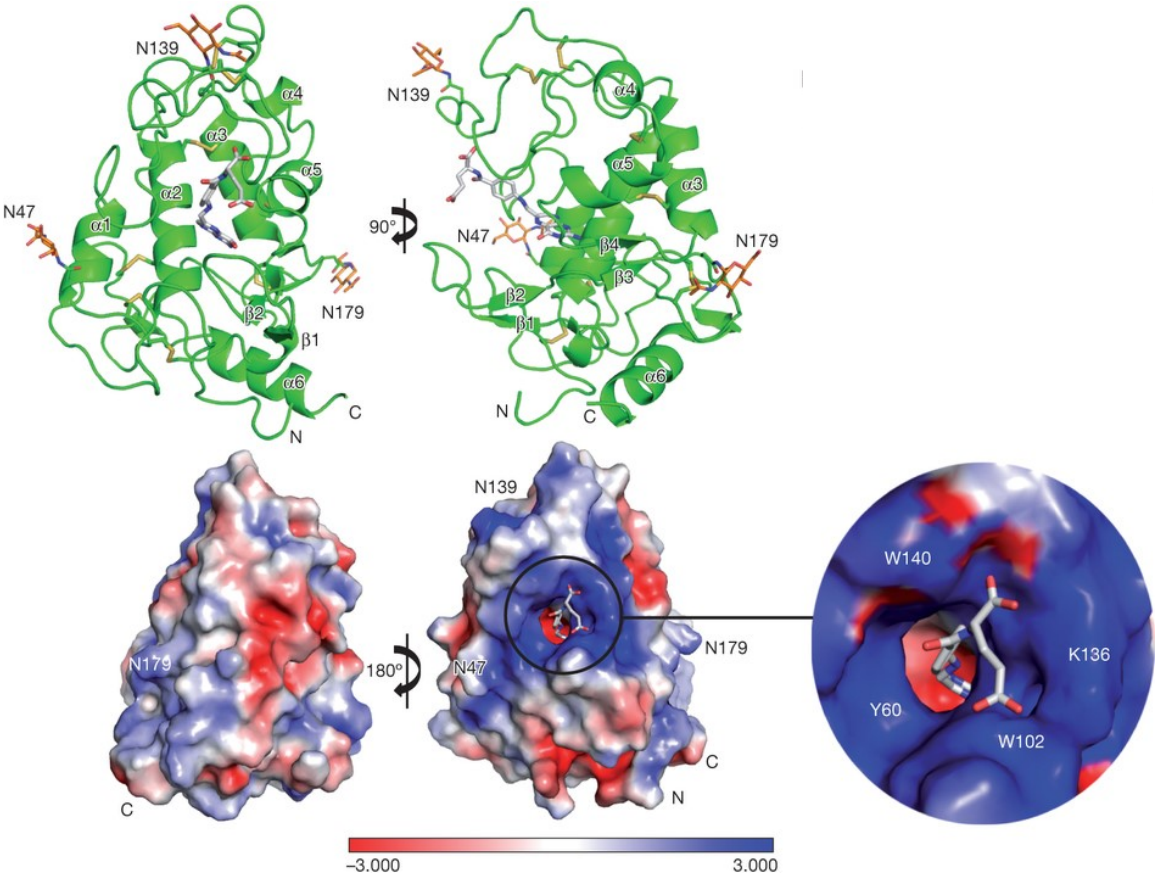

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Figure 1.9: Ribbon and charge distribution surface model of FRα complex with folic acid substrate. (Above) The alpha and beta helices (shown in green) demonstrate how the protein interacts with the endogenous ligand, folic acid. (Top-Right) A 90 degree rotation of the protein offers additional information about the protein-substrate interaction and the key amino acids involved in the folic acid recognition/binding process. (Bottom) The charge distribution surface model illustrates folic acid contained within the binding pocket and the charge of the amino acids surrounding the binding surface.

1.4 Folate based targeted therapy

Shortly following the discovery and first synthetic synthesis of folic acid, folate analogs termed antifolates were the subject of heightened attention resulting from their roles as effective agents against hematopoietic malignancies.[176,177] More specifically, the development of the first clinically used antifolate, AMT, fundamentally changed the treatment of pediatric leukemia and ultimately served as a platform for the development of many novel antifolates.[176,178,179] The vast and rapidly accumulating knowledge of folate metabolism also played an integral role in understanding the pharmacology of these analogs in addition to providing direction in the development of less toxic congeners.

Antifolates describe a class of agents that are structural analogs of folate and exert their antiproliferative actions through inhibition of enzymes in folate metabolism, resulting in the depletion of nucleotides required for DNA replication and DNA repair, and RNA synthesis.[180] Today antifolates remain the most well-studied chemotherapy agents with well-defined and well-characterized mechanisms of action. The recent discovery of the molecular structure of FRα and growing structural information about the β isoform will encourage the synthesis and development of more sophisticated agents with the promise of achieving successful and precise tumor targeting.[175,181] The identification of malignancies that over-express ideal targetable folate transport systems (FR's and PCFT) are steadily growing. Recent studies that examined patient tumor samples reveal that *in vitro* tumor models may inaccurately reflect the amount of folate transporter proteins that are actually expressed in *in vivo* patient tumors (personal communication; Folate Receptor Conference, Couzumel (2013)). This overexpression not only increases target area and points of entry for FR targeted drugs, but this may also have dramatic impact on tumor response and the concentration of drug required for efficacy.

There has been tremendous progress in the area of antifolate development since the introduction AMT and MTX. While these agents were the first ground breaking therapies of their kind, there are currently a number of novel agents under investigation that target different folate pathway enzyme and inhibit growth of neoplastic cells through the disruption of DNA synthesis. This diverse array of compounds not only includes new and improved DHFR inhibitors that differ from the first generation agents, AMT and MTX, such as pralatrexate, but also PMX which inhibits TS.[25,182,183] GARFTase inhibitors are under development as well.[25] These novel compounds can be grouped based on their intended enzyme target, their chemical structure and their ability to undergo polyglutamylation.

GARFTase has become a metabolic target of increasing relevance since the inception of lometrexol (LMTX) by the Eli Lilly Corporation in 1985.[91,184] Much of the interest surrounding LMTX and subsequent GARFTase inhibitors reflects their ability to disable the rate-limiting step in the *de novo* biosynthetic pathway of purines which involves the transfer of the formyl group from 10-formyl-THF to the primary amine of β GAR which provides the C8 carbon in the purine skeleton.[185,186] The disruption of purine production in tumor cells is believed to be a favorable feature of a chemotherapeutic drug, as these drugs not only interfere with DNA replication but they also disturb the cellular energy balance via ATP depletion, thereby interfering with energy requiring processes necessary for cell viability.[187] Another interesting and advantageous aspect of many of the prototypical GARFTase inhibitors is that they were designed based on the crystal structure of either human or *E. coli* GARFTase, which share homology at the folate co-factor binding site.[185] The structure-based drug design approach used for these inhibitors may account for their characterization as "clean" drugs, in that they have a clear mechanism of action unlike nucleoside analogs or purine based drugs that may have some influence on purine synthesis but

also possess the capacity to be incorporated into nucleic acids, making their primary function indeterminate.[185]

LMTX was the first folate-based antipurine that possessed potent inhibitory actions against GARFTase.[184] Remarkable preclinical data in xenografts of human tumors and murine models of solid tumors aided in making LMTX the first GARFTase inhibitor that was clinically investigated.[184,188] Further development of LMTX was halted during clinical trials due to delayed and cumulative side effects, which include severe and unpredictable myelosupression and neuropathy that was thought to be a function of a long terminal half-life and liver accumulation.[91,184,189] RFC-directed transport of LMTX may have been an additional contributing factor to toxicity seen in patients.[189] Folic acid supplementation was shown to curtail these adverse side effects and allowed patients to tolerate higher concentrations of drug.[184] In light of this, new dosing schedules with co-administration of folic acid were implemented in additional studies using this drug.[190,191] However, further clinical development of LMTX was brought to a standstill, reportedly for economic reasons.[192]

The LMTX studies validated GARFTase as an important target in *de novo* purine biosynthesis. In efforts to create novel GARFTase inhibitors that maintained the potent biological activity of LMTX, but with reduced untoward toxicities, computer assisted drug design was used to create AG2034 and AG2037. The GRID program, a computer generated method for systematically investigating an array of chemical groups and how they interact with suggested protein binding sites, revealed two hotspots at the GARFTase binding site that possessed an affinity for a thioester probe.[185] The structure of human GARFTase was the model for drug design.[193] The sulfur containing AG2034 was designed to complement the two identified hotspots at the GARFTase active site identified by the GRID program.[185] AG2034 possessed promising

characteristics of an ideal GARFTase inhibitor, with a K_i of 28 nM against the human enzyme,[185] and the ability to undergo polyglutamylation.[185,193] Together, these attributes are thought to be responsible for the potent antiproliferative activity seen in L1210 cells (IC50:4 nM) and CCRF-CEM cells (IC50:2.9 nM).[193] Cells with a mutant p53 and/or those lacking a functional G1 checkpoint were particularly sensitive to purine deprivation elicited by this drug (A549 and MCF-7).[194] After being selected for preclinical trials and tested against a range of murine tumors and human xenograft models, AG2034 was taken to clinical trials. These initial trials were suspended to investigate the newer analog AG2037. AG2037, like AG2034, is a sulfur containing GARFTase inhibitor. However unlike, AG2034 and LMTX, which can achieve cellular entry through both RFC and FR, AG2037 is transported by RFC exclusively.[185] The selective transport by RFC was thought to be ideal at the time. This was supported by a belief that efficacy of drugs that undergo FR-mediated transport would be modified based on the nutritional status (folate intake) of the patients.[185] A clinical trial with AG2037 in colorectal cancer patients that failed treatment was completed in 2004, however this study has no posted or published results to date.

Attempts to develop improved GARFTase inhibitors continued with the creation of Eli Lilly's GARFT II, also referred to as LY309887. When compared to LMTX, LY309887 had a 9-fold greater potency but produced metabolites with reduced polyglutamylation.[188] To address the issue of polyglutamylation, two monoglutamate GARFTase inhibitors were introduced, LY254155 and LY222306. LY254155 and LY222306 are 2', 5 thiophene and 2', 5 furan derivatives of LMTX, respectively. These compounds exhibited greater inhibition against GARFTase when compared to the parental compound LMTX and both possessed reduced affinity towards FRα (LY254155: 6 fold, LY222306: 350 fold).[195] These agents would presumably be valuable in the treatment of tumors with inherently low FPGS activity or tumors that have acquired

resistance to antifolates through reduced expression and or function of FPGS or increased gamma glutamyl hydrolase activity.[195] The search for an efficacious but well tolerated GARFTase inhibitor is ongoing and recent developments in folate transport proteins may produce a generation of equally as effective but targeted agents.

Currently, there is no antifolate that uses AICARFTase as its primary target. While the multi-targeted antifolate, PMX, inhibits AICARFTase as a secondary target, the extent of enzyme inhibition by this drug has not been directly quantified.[196,197] The rising and alarming acquired resistance to current antifolate therapies through multiple mechanisms, including overexpression of enzyme targets and decreased expression of essential transport proteins. The need to circumvent antifolate resistance encouraged the design of novel AICARFTase and GARFTase inhibitors (among the most infrequently targeted enzymes in folate metabolism) that use an alternative mechanism of cellular entry.[158]

FR targeted drugs

Experimental evidence from the Matherly lab and a host of others have shown that classical antifolates like MTX, LMTX and PMX bind, at varying degrees, to FRα and elicit a cytotoxic effect in FRα-expressing cells, albeit at different concentrations.[136,198] Increasing concentrations of these agents may introduce off target effects, making it difficult to ascertain if the toxic effects were FRα-mediated or otherwise. Most of the drugs in this class are transported by the ubiquitously-expressed RFC, which is thought to lead to intestinal and bone marrow toxicities as these are highly proliferative tissues with an increased dependence on the very enzymes these drugs inhibit.[136]

The adverse effects of these drugs were thought to arise, at least in part, from their RFC transport. This created demand for agents that would reduce undesirable toxicities in normal cells.

Ovarian and endometrial cancers were recognized for their expression of FRα and were considered difficult to treat with available therapies. These factors may have led to the quest for FRα-targeted therapies. In the 1980s, efforts to design a FR-based antifolate culminated in the development of the TS inhibitor CB3717.[199] This agent showed preferential transport by FRα over RFC and could be metabolized to polyglutamates, but further development of this compound was halted due to nephrotoxicity (thought to be a product of insolubility associated with high dose administration rather than FRα transport) and myelosupression.[200]

The cyclopenta(g)quinazoline based drug, CB300638, became the first validated FRα targeted TS inhibitor.[136] This agent showed high selectivity for FRα when compared to related analogs, and showed efficacy in a physiological folate concentration (20 nM) and inhibited TS in KB cells but did not compromise normal tissue.[201] BCG 945, like CB300638, also a cycoplenta(g)quinazoline based compound, showed inhibitory effects against TS in FRα-expressing cells.[202] Due to its *in vitro* and *in vivo* efficacy and FRα selectivity, BCG 945 was selected as a candidate for phase I clinical trials that are currently on going.[202]

De novo purine synthesis has become a rather attractive target in non-RFC based therapies with the development of novel GARFTase inhibitors that possess selective membrane transport for FRs and PCFT over RFC. The circumvention of RFC membrane transport by a collection of agents within this category can be seen as an improvement upon earlier agents such as LMT which was reported to induce severe myelosuppression in clinical trials, likely due to RFC transport. Both FR- and PCFT- targeting have distinct advantages that include narrow tissue expression (PCFT and FR), optimal performance in tumor acidic microenvironment (PCFT) and basolateral expression (circulation accessible) expression only seen in tumor cells (FRα). These pyrrolo[*2,3-d*]pyrimidine compounds are currently undergoing development, but show extraordinary potency

toward PCFT- and FRα- positive engineered cell lines, as well as towards PCFT- and FRα-expressing tumors in both *in vivo* and *in vitro* models.[198,203-207]

Monoclonal antibodies

FRα-specific monoclonal antibodies have been developed for both therapeutic and diagnostic applications.[208] One of the first antibodies directed to FRα was the murine LK26 whose further development was impeded by the inability to create a humanized version that maintained a suitable affinity towards FRα.[208] Farletuzumab (MORab -003), the improved humanized "offspring" of LK26, is a composite of murine complementary determining regions into the human IG-1 Kappa backbone.[125,208,209] This improved congener retained its high affinity to FRα with limited reactivity to normal tissue. Farletuzumab's proposed mechanism of action is through complement-dependent cytotoxicity (CDC) and antibody-dependent cell mediated cytotoxicity (ADCC).[125] The antibody is also thought to inhibit cell proliferation through suggested interactions with Lyn kinase and membrane signaling complexes that act to inhibit growth.[208,209] The binding of farletuzumab does not interfere with FRα binding to its ligands and was reported to marginally reduce the rate of folate delivery, which may offer opportunities for use in combination treatment with other FR directed therapies.

Farletuzumab is reported to be a well-tolerated drug with adverse effects that are well managed with antihistamine treatment.[208] When used in combination with carboplatin/taxane treatment, there was reported clinical activity and improved duration of response when treatment was followed with the single agent maintenance with farletuzumab.[125] No safety concerns or dose-limiting toxicities have been reported for farletuzumab and a maximum tolerated dose has not yet been identified.[208]

Folic acid-conjugated therapies

Folic acid conjugated therapies present a platform to repurpose potent but non- specific cytotoxins for delivery in a context that allows for target specificity. The γ-carboxylic group on the folic acid permits linkage of a "warhead" (potent cytotoxin) without disturbing the affinity of the ligand to the target.[208] Like monocolonal antibodies, folic acid conjugates can be used for imaging/diagnostic purposes or therapy, all which is dictated by the conjugate compound. An excellent example of this is seen in vintafolide (EC145) and etarfolatide (EC20) developed by Endoycote. Vintafolide is a folic acid conjugate comprised of a potent vinca alkaloid, desaceytlvinblastine hydrazine "warhead" and etarfolatide is a folic acid conjugate containing the medical radioisotope technetinum 99m.[210-212] Vintafolide was shown to be most effective (~80% overall response rate) in ovarian cancer patients who showed high detection of FRα positive tumors by etarfolatide.[213] While 90% of ovarian cancers overexpress FRα, there is much variation in the level of expression among individuals and subtypes of the disease. Imaging with etarfolatide will aid in the identification of patients who would most benefit from this form of FR-targeted treatment. While these agents performed well in phase I and phase II clinical trials, a phase III clinical trial in platinum resistant ovarian cancer was halted due to the failure to produce proper efficacy (an improvement in progression free survival).

Cytotoxic warheads are not limited to the vinca alkaloids. Promising pre-clinical data were obtained from EC0746, a modified folic acid ligand linked to a γ hydrazide analog of AMT. The compound was directed towards FRβ expressing activated macrophages in murine models of autoimmune uveitis and encephalomyelitis and showed significant activity through macrophage modulation.[214] The concept of re-directing RFC-transported cytotoxic antifolates to alternative routes for uptake, like FRα, is a very attractive idea. FRα-mediated uptake by a multi-targeted

antifolate, like PMX, may allow ovarian cancer patients to benefit from the activity of this drug without the consequence of its debilitating side effects.

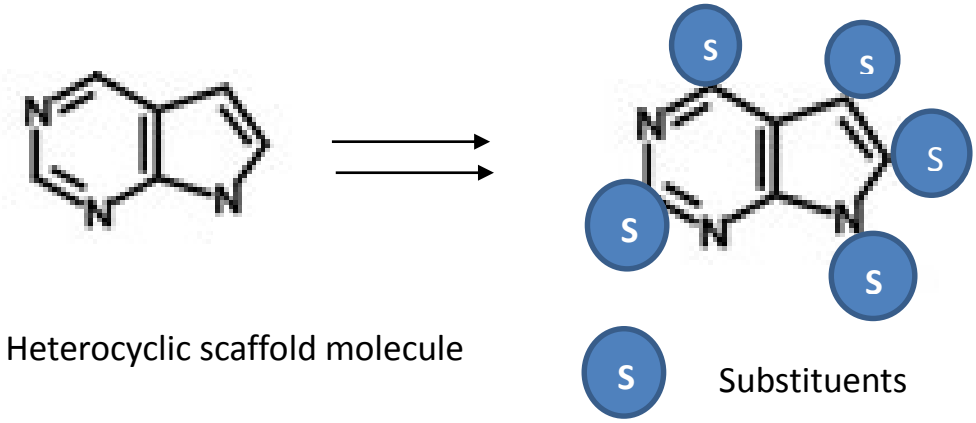
Novel pyrrolo[2,3-d]pyrimidine antifolates

Pyrrolo[2,3-*d*]pyrimidines define a collection of heterocyclic compounds that share a common heterocyclic fused-ring structure. These compounds may vary in respect to their physiochemical properties as many of them participate in a wide range of biological activities, that include functioning as kinase inhibitors[215-217], metabolic inhibitors[204,205,207,218] and modulators of efflux pumps that are often referred to as multi-drug resistance proteins, MRP1s.[219] The diverse actions of pyrrolo[2,3-*d*]pyrimidine compounds are thought to be dictated by the addition of functional groups (R-group or substituents) at the C4-C7 positions on the molecule[218] (**Figure 1.10a**). Studies performed in this body of work as well as earlier studies published by Matherly and colleagues[203-205,207,220,221] suggest that the chemical and structural properties of the R-groups have a tremendous impact on pharmacology of these molecules.

Pyrrolo[2,3-*d*]pyrimidines are naturally occurring compounds that can be synthesized by variety of organisms including marine and terrestrial bacteria[222]. The pyrrolo[2,3-*d*]pyrimidine compounds synthesized by these organisms (also referred to 7-deazapurines) are described as secondary metabolites because of their non-essential role in primary metabolism; however these compounds are thought to provide an advantage to the organism when present in a host.[222] 7-deazapurines are found to be involved in the disruption of cellular process that involve adenine nucleosides and nucleotides and were also discovered to perturb cellular metabolism. These compounds are suitable substrates for phosphorylation and can be converted into di-, tri-phosphorylated forms which can then be incorporated into DNA and or RNA as modified bases.[222] Together, these features have made these compounds useful tools against bacterial[223], fungal and

viral infections as well as neoplasms.[224,225] Toyocamycin, tubercidine and sangivamycin are all soil derived 7-deazapurines which contain the pyrrolopyrimidine scaffold with different substituents making the action of each compound distinct from the other (**Figure 1.10b**).

A.



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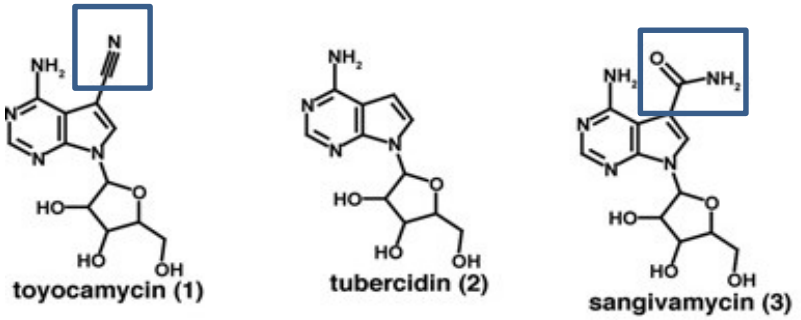

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Figure 1.10: Pyrrolo[2,3-d]pyrimidine scaffold molecule. (A) The pyrrolo[2,3-*d*]pyrimidine scaffold can be substituted at multiple carbons sites (C4-C7). The addition of these R-groups have been reported to confer selective physiological properties[157]. Highly saturated scaffolds demonstrate greater biological activity.[218] The diversity in function derived from the addition of functional groups is beautifully illustrated by toyocamycin, tubercin and sangivamycin (B), a few of many naturally occurring 7-deazapurines, which have unique cytotoxic, antibacterial and antifungal properties while possessing one distinctive R-group.

Pyrrolo[2,3-*d*]pyrimidine antifolate compounds, like the naturally occurring soil metabolites, can be functionally malleable based on the functional group added and the position at which the functional group is added. The lack of crystallographic information for all three forms of folate transport proteins necessitated high throughput screening of pyrrolo[2,3-*d*]pyrimidine compounds that showed activity against cell lines engineered to express one of the three transport mechanisms. From such studies, rational drug designs, based on both the biological activity of the compounds studied and the known structure of folate metabolism proteins GARFTase and TS, created a number of pharmacologically interesting compounds. The recent determination of the FRα crystal structure offers promise to structure-based drug design which will prove invaluable to FR-directed therapies and imaging modalities (**Figure 1.9**).

Studies performed by the Matherly lab using novel pyrrolo[2,3-d]pyrimidines synthesized by Dr. Aleem Gangjee and colleagues at Duquesne University (Pittsburgh PA), demonstrated qualities consistent with that of a classical antifolate, in that membrane transport by RFC, PCFT or FR could be identified and their antiproliferative capacities could be linked to the inhibition of enzymes involved in folate metabolism. The position of the substitution on the fused heterocyclic ring appears to play an important role in determining the substrate specificity of enzymes in folate metabolism, as well as the route of intracellular transport.[198,203-207,220,226] Earlier studies performed by Gangjee and colleagues suggested that a novel 2-amino-4 oxo-5-substituted pyrrolopyrimidine compound possessed antitumor activity against human head and neck squamous carcinomas and CCRF-CEM human leukemic cell lines.[227] The antitumor effect was thought to be a function of TS suppression, indicated by purified enzyme assays and metabolite protection assays.[227] Alternatively, collaborative studies with the Matherly lab using 6-substituted pyrrolo[2,3-*d*]pyrimidines antifolates active against both engineered CHO cell lines and human tumor cell

lines expressing folate transporters, demonstrated inhibition towards GARFTase. GARFTase suppression was demonstrated via metabolic nucleoside protection assays as well as radioactive metabolic incorporation assays and isolated enzyme assays.[198,205,220] Further, these studies also revealed that drug activity can be dictated by the length of the carbon bridge region which connects to either a thienoyl or benzoyl ring system. These studies provided greater insight into the structural components required for folate-based targeted drug activity.

The most potent, tumor-targeted analogs from these studies were lead compounds AG23, AG71, and AG94. These three compounds represent three distinct series of novel pyrrolo[2,3-*d*]pyrimidine antifolates. While structurally unique, each of the lead compounds was found to have profound antitumor activity against FRα and PCFT expressing cell lines (nanomaolar/subnanomolar range) with minimal or greatly reduced activity towards RFC expressing cell lines.[198,203-206] These antipurines are effective inhibitors of *de novo* purine synthesis as the studies cited above demonstrated remarkable dose-dependent inhibition of GARFTase. Mindful of the impressive targeted antitumor activity of these leads, congeners of these compounds are thoroughly investigated in this body of work to determine how structural modifications impact membrane transport, inhibition of targeted enzymes and ultimately cell death.

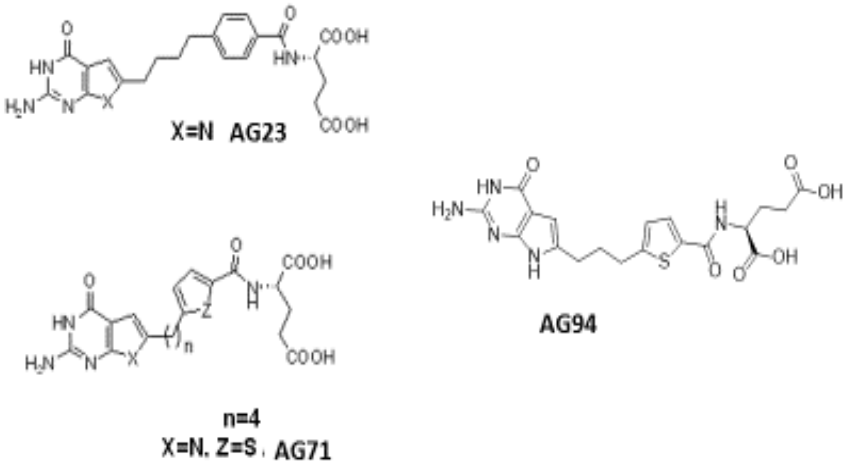

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Figure 1.11: Structure of lead compounds from three distinct series of novel antifolates. Each compound depicted in this figure contains the 2-amino-4-oxo fused hetercyclic ring structure (pyrrolo [2,3-*d*]pyrimidine). Both **AG71** and **AG94** conclude their carbon bridge with thienoyl ring system unlike **AG23** that contains a benzoyl ring in its second ring system. Each compound is substituted at the 6 position. All novel pyrrolo[2,3-*d*]pyrimidine compounds presented in this work were synthesized by the lab of collaborating medicinal chemist Dr. Aleem Gangjee.

1.5 Ovarian cancer

Gonadal tissues in the female reproductive tract originate from mesoderm layer of embryonic tissue. A vast majority of the female reproductive tracts is developed from the paramesonephric duct, also known as Mullierian ducts, which is responsible for the formation for approximately two thirds of the female reproductive tract.[228] The ovaries, however, are descendants from the genital ridge and are involved in the production of eggs and the hormones that help to nurture and prepare eggs for ovulation.[229] During ovulation, the eggs travel through the Fallopian tube to the uterus where they await fertilization. There are a host of hormones, cytokines and cells that are involved in the cyclical transition of ovulation and menstruation, some of which are thought to be influential in ovarian pathogenesis.[230-232]

Ovarian cancer is a rare but very lethal gynecological pathology in the United States. While the lifetime risk of developing ovarian cancer is 1.6%, the majority of the women diagnosed will ultimately die from the disease, as it is the fifth leading cause of cancer death in American women.[232,233] The American Cancer Society 2014 statistics estimates that there will 21,980 new cases of ovarian which is a negligible decrease from the 2013 estimate of 22,240 cases.[233,234] While there has been a slight decline in the estimated rate of incidence, the mortality rates remain staggering with the 2014 estimate of 14,270, which is a slight increase from the 2013 estimate of 14,030 deaths.[233,234] A vast majority of women diagnosed present at late stage with aggressive disease that is often inclined to recur as a fatal treatment resistant disease. There are a number of factors that contribute to late stage (stage III/IV) diagnosis including the lack of appropriate and reliable screening modalities and the mismanagement of primary disease. The mismanagement of ovarian cancer, which generally entails the failure to direct patients to experienced gynecological oncologists for optimal cytoreduction strategies, has a direct impact on the mortality disparities

that exist between populations within this disease.[235,236] The molecular profiles of ovarian cancer are identical among African-American and Caucasian women, suggesting that there are no genetic factors leading to a more aggressive phenotype for African-American women.[236,237] However, the mortality rates are higher among African-American women, which some believe is a function of mismanagement of disease, unequal application of existing treatments and the perceived socio-economic status of the patient.[235,236,238]

Late stage ovarian cancer presents a host of treatment challenges. An overwhelming majority of patients are diagnosed with late-stage ovarian cancer which is no longer confined to the ovary. At this time, treatment options are limited and are generally ineffective with gross toxicities to the patients. Depending on the extent of tumor burden, surgical reduction no longer exists as an option.[236] An additional determinant in choosing a candidate for debulking surgery is the presence of comorbidities that are contraindications for the procedure.[239,240] Independent of these conundrums, approximately 90% of patients that show initial response to first-line platinum/taxane therapy will experience a recurrence of treatment resistant disease.[240]

The term ovarian cancer serves an umbrella to describe a collection of malignant tissues that are distinguishable by cell type and more recently, by specific molecular events. These subtypes correspond to different types of epithelia and organs within the female reproductive system. Traditionally, ovarian cancers were separated in to three major subtypes based on their histology, including: (1) surface epithelium which includes serous, mucinous, endometroid, clear cell and Brenner tumors; (2) sex cord-stroma which includes granulosa cell, sertoli cell , sertoli leydig , thecoma, fibroma and steroid tumors; and (3) germ cell tumors that include dysgerminoma, yolk sac, embryonal carcinoma, choriocarcinoma and teratomas.[241] In line with the initial system

of categorizing these tumors, they were further characterized based on clinical behavior as benign, malignant and intermediate.[242]

Congruent with the general principles of carcinogenesis, ovarian cancer is typically a disease of aging with the median age of diagnosis at 63 years.[243] Epithelial ovarian cancer (EOC) is the most common form of this disease that accounts for 90% of ovarian cancers.[213] Historically this subtype of ovarian cancer was thought to arise from ovarian surface epithelium (OSE).[244-247] This early perspective on ovarian cancer suggests that OSE is capable of phenotypic plasticity which is derived from the numerous histological transitions that begin with a stromal ovarian epithelial inclusion cyst (OEI).[247] Through metaplasia, this cyst acquires a Mullerian phenotype which eventually evolves into a malignant transformation resulting in the multiple subtypes of EOC.[247] Critics of this theory express skepticism due to (1) the failure to identify an ovarian precursor lesion and (2) there are no reported observations of the transition of an OEI to multiple EOC subtypes.[247] Further, OSE shows no resemblance to EOC and is distinct from Mullerian epithelia.[247] However proponents of this theory offer a theory based on experimental models and histopathology that suggests that tumor promoting agents have the ability to transform OSE to malignant tumors.[246]

A growing number of reports suggest that the ovaries serve only as a secondary site and that the premalignant lesion is formed at a primary site adjacent to the ovaries.[232,247-256] The "Secondary Mullerian System Theory" was the first suggestion that ovarian cancer originates from an alternate tissue of origin.[257] This theory suggest that pelvic tumors with a mullerian phenotype (like many ovarian cancers) originate or are derived from mullerian type epithelium (uterus, cervix, Fallopian tubes) directly by a metaplastic process.[247] This theory fostered the hypothesis that most EOC begin as precursor lesion in the distal Fallopian tube.

High grade serous carcinoma (HGSC) is the most common and lethal subtype of EOC, accounting for 70-80% of the cancers within this category (clear cell carcinoma and endometroid carcinoma accounts for 10 %).[213,232,247] HGSC frequently displays p53 mutations and genomic instability.[213,247] This is in stark contrast to type I low grade carcinomas, which include low grade serous carcinoma, low grade endometroid carcinoma, clear cell carcinoma, mucinous carcinoma and Brenner tumors.[211] Type I tumors are described as a clinically non-aggressive disease that generally present at early stage.[209,243] Type I tumors rarely contain p53 mutations and are typified by mutations involving KRAS, BRAF, ERBB2, PTEN, CTNNB1, PIK3CA, ARID1A and PPP2R1A.[213,247] Type I and Type II tumors are distinguishable not only by molecular markers, but they differ in presentation, metastatic potential, response to therapy and prognosis.[232]

There have been a number of observations that support Fallopian tube involvement in ovarian cancer, many of which include the identification of putative precursor lesions. BRCA1/2 mutant carriers have a 30-60% increased risk of EOC.[232] In one study that examined the Fallopian tubes of asymptomatic BRCA1/2 mutation carriers along with women from the general population diagnosed with pelvic serous carcinoma, identified at least one of three putative precancerous lesions; they include: (1) serous tubal intraepithelial carcinoma (STIC) in the Fallopian tubal frimbria; (2) p53 signatures; or (3) frimbrial secretory outgrowth.[247] STIC are generally located at the distal end of the Fallopian tube and are characterized by frequent p53 mutations, increased cell proliferation, DNA damage and a secretory phenotype.[252,253,258,259] The multiple shared characteristics of STIC to HGSC are believed to be suggestive of a histological relation between the two. Similarities in copy number changes, tubal involvement in HGSC cases, a shared secretory phenotype of STIC and HGSC all serve to support the hypothesis that HGSC is the clonal outgrowth of STIC lesions.[247,260,261] Further, there is a dramatic 98% reduction of ovarian,

Fallopian tube and primary peritoneal cancer risk in BRCA1/2 carriers that underwent prophylactic bilateral salphingo-oophorectomy which involves the removal of both the ovaries and the Fallopian tubes.[232] The Sectioning and Extensively Examining the FIMbria protocol (SEE-FIM) which was put in place to maximize the detection of precursor lesions in early Fallopian and ovarian cancer, demonstrated that women diagnosed with ovarian or primary peritoneal HGSC present with STIC (40-60%) that have identical mutational status observed in tumors and also show evidence of tubal involvement (70%).[232] Altogether, these studies provide a convincing link to HGSC and the Fallopian tubes, and point to STIC as the initiating lesion that leads to this aggressive cancer.

The ovaries are believed to be the secondary tumor site for disease that originates in the Fallopian tubes. The proposed mechanism by which this occurs is thought to involve the shedding of STIC cells from the distal Fallopian tubes onto the nearby ovaries during ovulation.[247] While trapped in the ovary, these pre-malignant Fallopian tube-derived cells thrive in the ovulatory environment where they are exposed to hormones, pro-inflammatory cytokines and reactive oxygen species (ROS) which all may aid in the clonal expansion or re-initiation of cell proliferation in the ovary.[230,247] This proposed mechanism has helped to construct a new model for the development of HGSC which differs from the step-wise development observed in type I low grade carcinomas.[247]

An additional feature of EOC (including 90% of HGSC) is the overexpression of FRα. In 1991, amino acid sequencing analysis determined that the protein enriched on the surface of non-mucinous ovarian cancer was FRα.[213] This identification led to the emergence of the FR protein as a tumor marker for this cancer.[213] The surface expression of the FRα protein corresponded with grade and stage of the tumor.[170,262,263] Interestingly, tumors (with some degree of FRα expression)

treated with chemotherapy (i.e., MTX, tamoxifen and cisplatin/carboplatin) experienced increased (MTX, Tamoxfien) or stable (cisplatin/carboplatin) expression of FRα.[264] Knowing this, drugs that selectively target FRα can be used as adjuvant therapy in situations where first-line therapies have failed.

Currently, there is no widely accepted comprehensive tumor progression model for ovarian cancer, which presents a myriad of challenges in studying this cancer.[265] Cell line models (**Table 1.1**) and mouse models of ovarian cancer, which are the existing experimental tools, have their limitations and in some cases fail to accurately represent the *in vivo* (patient) tumor. Molecular profiling of ovarian cancers has allowed research to distinguish between high grade (type II) and low grade (type I) tumors.[266,267] This categorization of tumors based on molecular markers has called into question the true histopathology of many of the human ovarian cancer cell lines currently used in research.[268] Cell line contamination may also be a factor contributing to the conflicting reports and misidentification concerning the histopathology of these experimental cell lines.[269,270] Mouse models of human ovarian cancer make the assumption that the ovary is the primary site of disease. The current models attempt to create tumors in the ovary through the use of promoters, many of which are described as leaky.[271] *Ex vivo* implants and use of transgenes arguably do not mimic the natural evolution of human EOC and are not representative disease models.[271] The limitations inherent in both *in vivo* and *in vitro* models present an opportunity for the development or the investigation of a new model that incorporates an identified and validated precursor lesion.

While there may be continued debate over the etiology of ovarian cancer and the utility of the models currently used to study the disease, reproductive, genetic and environmental factors continue to be implicated as risk factors in this multifactorial malignancy. Like most other cancers,

increasing age, inherited genetic cancer syndromes and family history remain important determinants of ovarian cancer risk.[272]

Cell line	Clinical pathology Clinical relevance	References
KB	**Nasopharyngeal carcinoma**	Round et al. 1970,[273] Kou et al. 2003,[274] Jhaveri et al. 2004
IGROV-1	**HGSC** (Kulbe, Domcke) **Serous** (Gloss, Dejardins) **Polymorphous adenocarcinoma**: endometroid,clear cell, serous and undifferentiated foci (Benard)	Kulbe et al 2012,[275] Dejarins et al 2014,[276] Gloss et al 2014,[277] Benard et 1985[278] Domcke et al. 2013[268]
SKOV-3	**HGSC**	Ueda et al 2010,[279] Anglesio et al. 2013,[280] Tsofade et al. 2013,[281] Jiao et al 2011[282] Domcke et al. 2013[268]
OVCAR-3	**HGSC**	Ueda et al 2010,[279] Tsofade et al. 2013,[281] Berger et al. 2001,[283] Domcke et al. 2013[268] Gloss et al 2014[277]
CAOV-3	**Serous carcinoma** **HGSC** (Berger, Domcke)	Berger et al. 2001,[283] Gloss et al. 2014, Domcke et al. 2013[268]
TOV-112D	Aggressive stage III endometroid tumor	Gagne et al 2007,[284]

Table 1.1: A Commonly used FRα positive cell line model with commonly used ovarian cell line models. The table above contains human ovarian cancer cells lines (and one FR model, KB) commonly referenced in research literature.

Reproductive risk factors

Ovulation has been thought to contribute to malignant transformation of the epithelium due to proliferation that occurs during this process. This proliferation is believed to promote carcinogenesis via the accumulation of genetic mutations in a repetitive "trauma-and-repair" cycle.[265,272] Therefore, agents that reduce ovulation should have a protective effect against the development of disease.[285-287] Indeed, oral contraceptive use has been shown to have an inverse relationship with ovarian cancer risk in a number of epidemiological studies.[285-287] Gestation also temporarily suppresses ovulation; therefore parity, number of pregnancies and long term breast feeding have all been reported to have protective effects against the development of ovarian cancer.[265,272,288]

Genetic risk factors

Women with a family history of ovarian cancer have an increased risk of cancer development. Women with one or more first-degree relatives with ovarian cancer have an increased life-time risk of ovarian cancer development of 5-9%.[272] Hereditary cancer syndromes, like BRCA1/2 mutations, also dramatically increases the risk of developing ovarian cancer; however, this only accounts for approximately 5-12% of cases.[289] Most ovarian cancer cases arise from sporadic mutations that may not be associated with genes found in hereditary breast and ovarian cancer (HBOC) and hereditary nonpolyposis colorectal cancer (HNPCC) syndromes.[265,272] Aberrations in TP53, HER2, EGFR, DAB2, AKT, RAS, MYC, PI3K and AR have been suggested to play some role in sporadic cases[129,130] , yet mechanistic details of their involvement in carcinogenesis have not been thoroughly investigated.

Environmental risk factors

Obesity is a common modifier of cancer risk in nearly all types of cancer. Obesity has been linked to inflammation and there is mounting evidence that links both obesity and inflammation to cancer risk. [129,130] Pelvic inflammatory disease and endometriosis exposes the ovaries and other reproductive organs to prolonged inflammation that may increase ovarian cancer risk.[290-294]

There have been conflicting reports on the role that genital talcum powder exposure plays in the development of ovarian cancer.[295] The source surrounding much of this controversy lies in the fact that prior to 1976 talc was contaminated with asbestos and much of the data was thought to be confounded due to the presence of cancer causing substance.[295] However, it has been postulated that ovarian cancers can arise from exposure to hydrous magnesium silicates (e.g., talcum powder) and recent findings are not tainted by the asbestos association.[295,296] Cancerous ovaries removed from patients have been shown to contain traces of talc.[272]

Early detection and prevention

To date, there is no reliable screening method to detect early stage ovarian cancers. Transvaginal ultrasounds, CA (Cancer Antigen)-125 serum levels and pelvic exams are the primary detection/screening tools.[129,130,297] Together, these methods are deemed inefficient or inadequate due to their inability to capture subtle changes that may occur during tumor initiation and early cancer progression in ovarian cancer.[297] These modalities also fail to possess the specificity and sensitivity required for practical positive predictive values.[297] The absence of a defined and validated precursor lesion only adds to the challenge in developing a proper screening modality.[129,130] There are currently efforts to identify new biomarkers to replace or to use in conjunction with tools that are currently available.[298]

Current treatments for ovarian cancer

Ideally, patients who are declared good surgical candidates are recommended for cytoreductive surgery followed by adjuvant chemotherapy.[129,138,139] Unfortunately, at stages I-II of disease, patients with EOC are asymptomatic or symptoms are easily mistaken for innocuous conditions.[299] This, in conjunction with the lack of adequate early detection screening, results in infrequent early stage-diagnosis.[129, 297,139] Vaginal bleeding/discharge, frequent urination and constipation are typical symptoms of early stage ovarian cancer but are often ignored because they are common symptoms of less severe minor gynecological abnormalities.[129] Approximately 1.5% of patients present with early stage disease for whom complete surgical removal of the tumor can be successful and survival is greater than 90% (when cancer is confined to the ovaries); therefore, chemotherapy is the primary treatment option for a vast majority of cases.[129, 130, 297,300] Over the past 40 years, standard therapies used to treat EOC have not significantly extended the median overall survival beyond 25-30% in women diagnosed with advanced disease.[130,137] Approximately 75-80% of ovarian cancers exhibit initial chemotherapy sensitivity to first line taxane- and platinum-based agents (paclitaxel/docetaxel and cisplatin/carboplatin). However, most of these patients will experience relapse within a year.[258,301] Due to the chronic recurring nature of EOC, treatment strategies should allow for prolonged treatment by using agents with low toxicity profiles, to ensure reasonable patient comfort and minimal disruption to the quality of life for the patient.

There are well recognized cumulative and sometimes irreversible toxicities associated with these standard agents (regardless of method of delivery) which makes additional treatment with these classes of agents intolerable for patients, thereby reducing the available options in treatment and creating a precipitous decline in patients' quality of life.[300-302] Hematological and neurological toxicities are of greatest concern as they may impede the patient's ability to carry out everyday

activities. With this in mind, there is growing support to include "improved quality of life" along with "duration of survival" as major considerations in the therapy of advanced cancers.[300]

CHAPTER 2: DISCOVERY OF 5-SUBSTITUTED PYRROLO[2,3-*D*]PYRIMIDINE ANTIFOLATES AS DUAL ACTING INHIBITORS OF GARFTASE AND AICARFTASE IN *DE NOVO* PURINE NUCLEOTIDE BIOSYNTHESIS: IMPLICATIONS OF INHIBITING AICARFTASE TO AMPK ACTIVATION AND ANTI-TUMOR ACTIVITY

2.1 Introduction

The antifolates are widely recognized for their inhibition of folate metabolism that leads to growth suppression, tumor reduction and remission in a number of malignancies and the reduction of inflammation in pro-inflammatory conditions.[38,303,304] Major enzyme targets of this particular class of agents include TS and DHFR. Inhibition of these enzymes suppress *de novo* nucleotide biosynthesis, resulting in an imbalance of purine and pyrimidine precursors, rendering cells incapable of undergoing accurate DNA replication, ultimately resulting in cell death. Clinically relevant TS and DHFR inhibitors, typified by PMX, and MTX (**Figure 2.1**) and pralatrexate, respectively, continue to play important roles in treating hematologic malignancies and solid tumors.[25,303]

Antifolates targeting *de novo* purine nucleotide biosynthesis were also described and include LMTX [(6R)5,10-dideazatetrahydrofolate] (**Figure 2.1**), (2S)-2-[[5-[2-[(6R)-2-amino-4-oxo-5,6,7,8-tetrahydro-1H-pyrido[2,3-*d*]pyrimidin-6-yl]ethyl]thiophene-2-carbonyl]amino]pentanedioic acid (LY309887)[188,305] and (2S)-2-[[5-[2-[(6S)-2-amino-4-oxo-1,6,7,8-tetrahydropyrimido[5,4-bate][1,4]thiazin-6-yl]ethyl]thiophene-2-carbonyl]amino]pentanedioic acid (AG2034).[183] These drugs inhibit the first folate-dependent step in purine biosynthesis, catalyzed by GARFTase, and have progressed to clinical trials.[69,306,307] However, their toxicities were dose-limiting, most likely a consequence of their cellular uptake and metabolism to polyglutamates in normal tissues.

The RFC is one of three principal mechanisms of (anti)folate uptake into mammalian cells.[308] Cellular requirements for folate cofactors for DNA replication provide a plausible

explanation for the high levels of RFC in most tumors. However, demands for folates, and consequently RFC, are also shared by normal tissues, such that RFC may not be the optimal mechanism for tumor-selective uptake of cytotoxic folate analogs. Other cellular uptake mechanisms, notably the PCFT and FRs α and β, are also expressed in tumors, while showing more restricted expression in normal tissues.[92] Furthermore, PCFT is a proton symporter such that in the acidic microenvironment generated by glycolytic tumors, membrane transport and selective tumor targeting by this mechanism is enhanced.[75,88] For FRα, the apical spatial orientation in normal epithelial tissues is often disrupted in tumors such that its basolateral membrane expression in tumors results in exposure to the circulation.[88,92] These features provide compelling rationale for developing folate-based therapeutics which target PCFT and FRα for cancer therapy. Examples of FRα-targeted therapies include a monoclonal antibody [Farletuzumab (Morphotech)][138], a cytotoxic folate conjugate, N-[4-[[(2-amino-1, 4-dihydro-4-oxo-6pteridinyl)methyl]amino]benzoyl]-L-(-glutamyl-L-α-aspartyl-L-α-aspartyl-L-cysteine [e.g., Vintafolide (EC145; Endocyte)],[211] and a classical antifolate which is selectively transported into cells by FRs over RFC and inhibits *de novo* thymidylate biosynthesis, [(2R)-2-((4R)-4-carboxy-5-(4-((2-(hydroxymethyl)-4-oxo-4,6,7,8-tetrahydro-*3H*-cyclopenta[*g*]quinazolin-6-yl)(prop-2-yn-1-yl)amino)phenyl)-5-oxopentanamido)pentanedioic acid] (ONX0801).[202]

PMX is a 5-substituted pyrrolo[2,3-*d*]pyrimidine antifolate with a 2 carbon bridge linked to a *p*-aminobenzoyl glutamate. PMX is a good transport substrate for RFC and among the best PCFT substrates,[69,75,92] yet it is a modest substrate for FRs.[198,205-207,220] We previously described 6-substituted pyrrolo[2,3-*d*]pyrimidine benzoyl antifolates related to PMX (compounds AG17, AG23, AG71 and AG94 (**Figure 2.2**).[198,204] Whereas the 6-regioisomer of PMX was pharmacologically inert, the 3- and 4-bridge carbon analogs of this series (compounds AG17 and

AG23, respectively) were selectively transported by FRα- and PCFT over RFC and were potently inhibitory toward FRα- and PCFT-expressing tumor cells.[113,117] Subsequent generation 6-substituted pyrrolo[2,3-*d*]pyrimidine thienonyl antifolates (compounds AG71 and AG94) with 3- and 4- carbon bridge lengths, respectively, were even more potent as inhibitors of cell proliferation then that of the corresponding benzoyl analogs[203,207,226]. Compounds AG17, AG23, AG71 and AG94 all showed substantial PCFT membrane transport activity but not for RFC.

PMX was originally described as a "multi-targeted" antifolate with a primary inhibition at TS and secondarily at GARFTase and DHFR.[309] However, recent studies suggested that although TS was the primary cellular target of PMX, AICARFTase could be a pharmacologically important target in the presence of excess thymidine (circumvents TS) at sufficiently high PMX concentrations.[196,197] Hence, in the presence of thymidine, inhibition of proliferation of CCRF-CEM leukemia cells and solid tumor cell lines was circumvented by hypoxanthine but only partially by AICA which is metabolized to ZMP, the AICARFTase substrate.[170,171] Further, PMX resulted in marked accumulations of ZMP. By contrast, the 6-substituted pyrrolo[2,3-*d*]pyrimidine analogs AG17, AG23 AG71 and AG94 are inhibitors of GARFTase whose inhibitory effects are completely circumvented by AICA.[113,117,207,226]

The accumulation of ZMP in PMX-treated cells is intriguing as ZMP is an AMP mimetic that activates AMP-activated protein kinase (AMPK).[310] AMPK negatively regulates mTOR, a critical pro-survival pathway that is activated in many tumor cells along with PI3K/AKT, secondary to loss or mutation of PTEN.[310-312] This may provide a possible explanation for the inhibitory effects of PMX in the presence of thymidine, as purine nucleotides are not depleted.[170,171] However, this has not been directly tested, as no AICARFTase-targeted drugs without TS inhibition have been described.

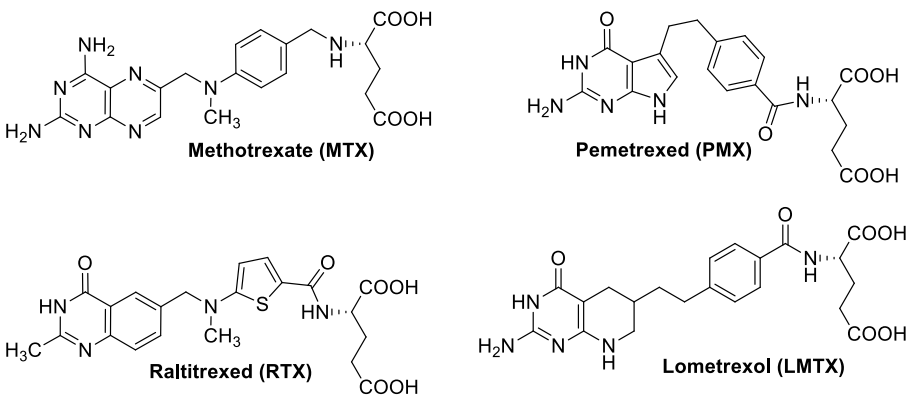

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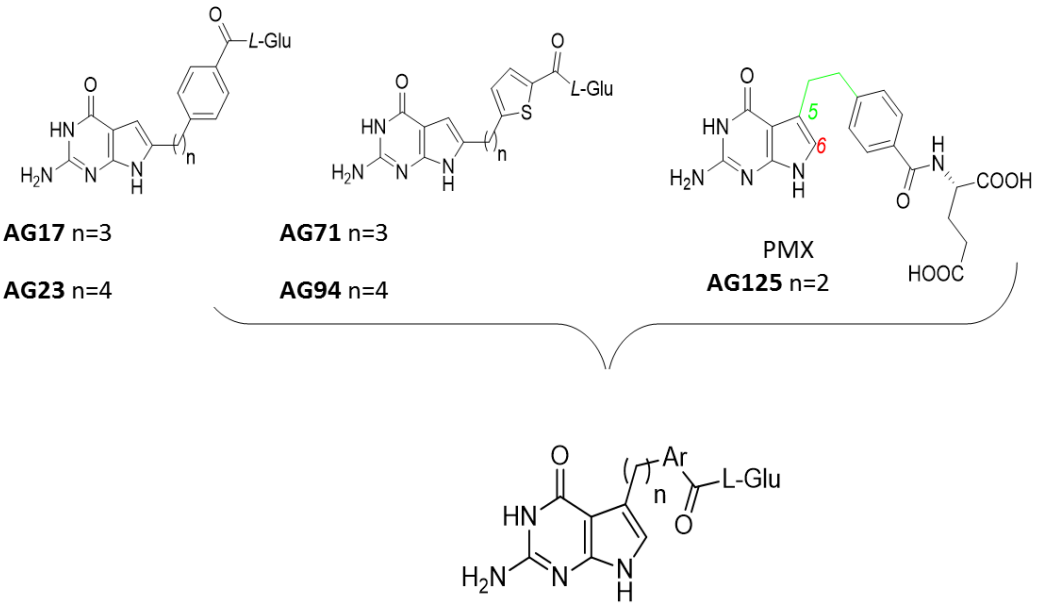
Figure 2.1: Structures of classical antifolates including MTX, PMX, raltitrexed (RTX) and LMTX.



AG124= Phenyl (1,4) n=1	**AG133**=Thiophene (2,5) n=1
AG125= Phenyl (1,4) n=2	**AG134**=Thiophene (2,5) n=2
AG126= Phenyl (1,4) n=3	**AG135**=Thiophene (2,5) n=3
AG127= Phenyl (1,4) n=4	**AG136**=Thiophene (2,5) n=4
AG128= Phenyl (1,4) n=5	**AG137**=Thiophene (2,5) n=5
AG129= Phenyl (1,4) n=6	**AG138**=Thiophene (2,5) n=6

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Figure 2.2: Design of 5-substituted pyrrolo[2,3-*d*]pyrimidines based on structures of 6-substituted analogs 1-4 and pemetrexed.

In this chapter, we describe, synthesize and perform a systematical characterization of the *in vitro* anti-proliferative activities and cellular mechanisms of novel 5-substituted pyrrolo[2,3-*d*]pyrimidine antifolates (including PMX) with a benzoyl (AG124-AG129) or thienoyl (AG133-AG138) rings in the side chain and one-to-six bridge carbons closely related to the 6-substituted pyrrolo[2,3-*d*]pyrimidine analogs AG17, AG23, AG71 and AG94[113,117207,226] (**Figure 2.2**). We reasoned that the 5-substituted analogs might inhibit AICARFTase, analogous to PMX, albeit without TS inhibition. Further, if this was accompanied by FRα and/or PCFT transport selectivity over RFC, this would be especially appealing for targeting tumors that express high levels of these systems.

We describe in this chapter the impact of these structural modifications on (i) transporter specificity and (ii) the targeted pathway (TS versus *de novo* purine nucleotide biosynthesis), including (iii) the extent of cellular GARFTase and AICARFTase inhibition. Our results document an emerging structure–activity-relationship (SAR) for the pyrrolo[2,3-*d*]pyrimidine antifolates, accompanying translocation of the 6-pyrrolo[2,3-*d*]pyrimidine substituent to the regio 5-position, including sustained FRα cellular uptake accompanying restoration of RFC and loss of PCFT transport activities. The 4 carbon bridge analog of the benzoyl series (compound AG127) is unique in that it provides the strongest evidence of dual inhibition of both AICARFTase and GARFTase exclusive of TS in tumor cells, resulting in depletion of purine nucleotides. Finally, our results directly examine the possibility of whether AMPK activation secondary to AICARFTase inhibition contributes to decreased proliferation of KB human tumor cells.

2.2 Biological evaluation

Transporter-selectivity for inhibition of cell proliferation by 5- and 6-substituted pyrrolo[2,3-*d*]pyrimidine regioisomers. Our initial goal was to better define the SARs for

membrane transport of pyrrolo[2,3-*d*]pyrimidine antifolates relating to the impact of 5- versus 6-substitutions on the pyrrole ring, based on the established antitumor activities of the 6-pyrrolo[2,3-*d*]pyrimidine-substituted compounds AG17, AG23, AG71 and AG94 with 3- and 4-bridge carbons, respectively, [113,117,156,228] in addition to the preclinical and clinical efficacy of PMX,[196] a 5-substituted pyrrolo[2,3-*d*]pyrimidine with a 2-carbon bridge (**Figure 2.2**). In our previous studies,[113,117,207,226] compounds AG17, AG23, AG71 and AG94 were efficiently internalized by tumor cells via FRα and PCFT but not by RFC, whereas PMX was a substrate by all three systems.[113,117]

Transporter specificities are exemplified by patterns of inhibition of cell proliferation toward isogenic Chinese hamster ovary (CHO) cell models, engineered to express human RFC (PC43-10), PCFT (R2/PCFT4), or FRα (RT16),[198,205-207,220] treated with compounds AG17, AG23, AG71 and AG94 (**Table 2.1**). Results are compared to those for RFC-, PCFT-, and FR-null MTXRIIOua^{R}2-4 (R2) CHO cells. As previously reported, [113,117,207,226] compounds AG17, AG23, AG71 and AG94 are potent inhibitors of FR-expressing RT16 and D4 cells, and to a lesser (albeit still potent) extent, R2/PCFT4 cells. However, compounds AG17, AG23, AG71 and AG94 were poorly inhibitory toward RFC-expressing PC43-10 and R2 cells. Compounds AG17, AG23, AG71 and AG94 were also highly active toward KB nasopharengeal carcinoma cells and IGROV-1 ovarian carcinoma cells, both of which express elevated FRα, along with RFC and PCFT.[220] Complete reversal of antiproliferative effects of compounds AG17, AG23, AG71 and AG94 toward the FRα-expressing cell lines was seen in the presence of excess (200 nM) folic acid, further demonstrating that cellular uptake of these analogs are mediated primarily via FRα, as previously reported (**Table 2.1**).[198,207]

5-Substituted pyrrolo[2,3-*d*]pyrimidine analogs with a side-chain benzoyl group (analogs of compounds AG17and AG23) and thienoyl group (analogs of compounds AG71 and AG94) with bridge lengths from 1 to 6 carbons (compounds AG124-129 and AG133-138) were synthesized and tested for their antiproliferative activities toward the CHO and human tumor models (**Table 2.1**). For the benzoyl series, the 2 carbon 5-pyrrolo[2,3-*d*]pyrimidine analog (compound AG125) is structurally identical to PMX. 5-Substitutions restored RFC-directed anti-proliferative activity, as reflected in dramatically increased anti-proliferative effects of this series toward PC43-10 cells compared to RFC-, PCFT-, and FR-null R2 cells, in the order (numbers of bridge carbons noted in parentheses), AG125 (2C) > AG127 (4C) ~AG126 (3C) > AG128 (5C) >> AG124 (1C) ~AG129 (6C) (benzoyl series), and AG134 (2C) ~AG136 (4C) > AG135 (3C) > AG137 (5C) >> AG133 (1C) ~ AG138 (6C) (thiophene series). Conversely, for PCFT-expressing R2/PCFT4 CHO cells, with the exception of compound AG125 and (to a lesser extent) the thiophene analogs AG134 and AG137, the antiproliferative effects for the 5-substituted analogs were attenuated compared to the 6-substituted analogs (e.g., for direct comparisons between paired 5- and 6-substituted analogs, compare IC_{50}s for compounds AG17 and AG126 and for compounds AG71 and AG135, respectively in **Table 1.1**).

With the FRα-expressing sublines, potencies were also reduced for the 5-substituted compounds, compared to 6-substituted analogs. These differences range from slight (compare AG23 to AG127**,** and AG71 to AG28) to substantial (compare AG17 to AG126, AG71 to AG135, and AG94 to AG136**)** (**Table 2.1**). In contrast to results for PCFT- and RFC-expressing cells, the impacts of bridge lengths were distinctly different between the benzoyl and thienoyl series, with anti-proliferative activities toward FRα-expressing RT16 CHO cells in rank order, AG127 (4C) > AG125 (2C) > AG128 (5C) > AG126 (3C) > > AG124 (1C) ~ AG129 (6C) (benzoyl series), and

AG136 (4C) ~ AG137 (5C) > AG135 (3C) > AG134 (2C) > AG133 (1C) ~ AG138 (6C) (thioenoyl series). An analogous pattern of sensitivities was obtained with KB human tumor cells, and for both the KB and RT16 cell lines, inhibitory effects were substantially reversed by 200 nM folic acid, confirming FRα as the major uptake mechanism. While PCFT and RFC likely mediate cellular uptake of certain analogs (e.g., AG127 and AG136), uptake by FRα appears to predominate.

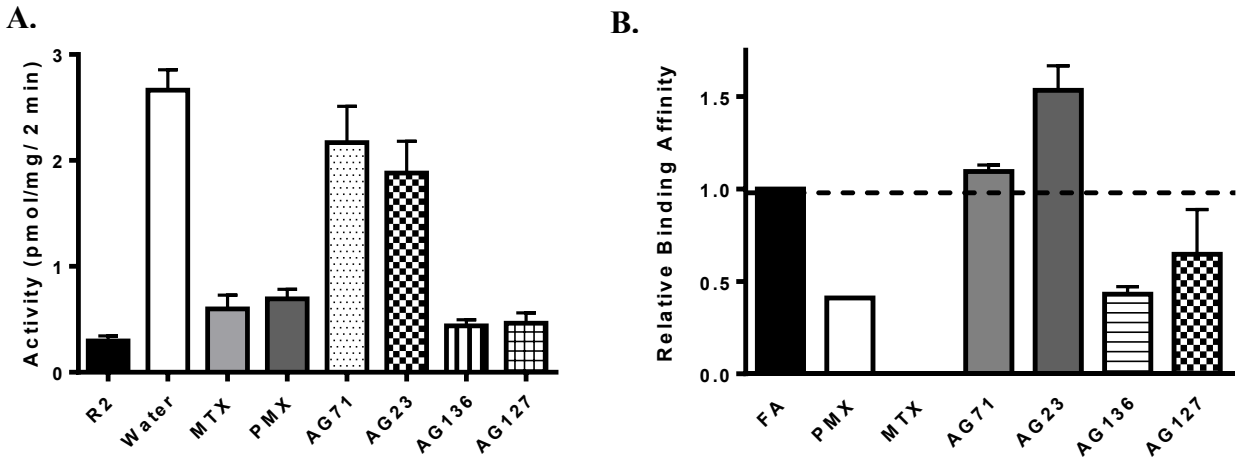

Figure 2.3: Inhibition of RFC-mediated transport and relative FRα-binding affinities by 6- (compound 2) and 5- (compound 8) substituted pyrrolo[2,3-*d*]pyrimidine antifolates. Panel A: To determine if novel antifolates were able to bind human RFC and inhibit transport of [^{3}H]MTX, reflecting transport of the novel analogs by RFC, we used PC43-10 CHO cells ectopically expressing human RFC but not FRs or PCFT. Uptake of 0.5 μM [^{3}H]MTX was measured at 37˚ C for 2 min in Hepes-buffered saline (pH 7.2), in the absence or presence of 10 μM of non-radioactive inhibitors, including compound (Cpd) AG23**,** AG71**,** AG127 or AG136, or the classical agents, PMX and MTX. Experimental details are described in the Methods and Materials. Internalized [^{3}H]MTX was normalized to total cell protein and expressed as pmol/mg protein. Transport results are compared to those for RFC-null R2 cells. Panel B: To assess the relative binding affinities of the novel antifolates to FRα, we performed direct competitive binding assays using [^{3}H]folic acid (FA) in the FRα-engineered CHO cell line, RT16. RT16 cells were washed, then incubated with Hank's balanced salts solution containing 50 nM [^{3}H]folic acid in the absence or presence of unlabeled 6-pyrrolo[2,3-d]pyrimidine compounds AG23, AG71, AG127or AG136, or folic acid, PMX, or MTX at various concentrations. Relative binding affinities were calculated as the inverse molar ratios of unlabeled ligands required to inhibit [^{3}H]folic acid binding by 50%. By definition, the relative affinity of folic acid for FRα is 1. Further, details for the transport and binding assays are provided in the Methods and Materials. For both series of experiments, results are presented as mean values plus/minus standard errors from 3 experiments.

To complement results showing anti-proliferative effects of the most potent 5-substituted analogs, AG127 and AG136, toward PC43-10 CHO cells expressing human RFC, we measured competitive inhibitions of [^{3}H]MTX (0.5 μM) transport in this model over 2 minutes at 37° C (**Figure 2.3A**). MTX is a well-characterized surrogate substrate for RFC and the extent of [^{3}H]MTX transport inhibition by competitive unlabeled (anti)folate ligands correlates with their membrane transport by RFC.[9] For our study, we compared inhibitory effects of 10 μM of compound AG127 and AG136, to those for the corresponding 6-substituted compound AG23 and AG71, and to PMX and MTX. Transporter-null R2 cells were used to as a measure of baseline [^{3}H]MTX uptake. Whereas AG23 and AG71 were poor inhibitors of [^{3}H]MTX uptake (< 25% inhibition), compound AG127 and AG136 were a reasonably good inhibitors (>90%), with a inhibitory potency approximating that for PMX and net transport only slightly greater than the very low levels recorded for R2 cells. Clearly, unlike the 6-substituted pyrrolo[2,3-*d*]pyrimidine analogs, the 5-substituted compounds lack specificity for FRα and/or PCFT over RFC.

Compounds AG127 and AG136 showed anti-proliferative effects toward FRα-expressing RT16 CHO cells and KB human tumor cells (**Table 2.1**). To directly demonstrate drug binding to FRα, a prerequisite of cellular uptake by this endocytotic mechanism, we used competitive binding assays with [^{3}H]folic acid with RT16 cells, treated with unlabeled ligands which compete for FRα binding[16-20] (**Figure 2.3B**). Relative FRα binding affinities for the 5- (compounds AG127 and AG136) versus 6-substituted (compounds AG23 and AG71) analogs paralleled their anti-proliferative activities. However, this correlation did not quite extend to PMX (compound AG125) or to MTX, indicating that multiple factors in addition to FRα binding affinities contribute to net growth inhibition of FRα-expressing cells for these antifolates.

A.

Compound	Structure	hRFC		hFRα		hPCFT	
		PC43-10	R2	RT16	RT16 (+FA)	R2/hPCFT4	R2(VC)
1-AG17	**6-pyr/3C/Benzoyl**	648.6(38.1)	>1000	4.1(1.6)	>1000	23.0(3.3)	>1000
2-AG23	**6-pyr/4C/Benzoyl**	>1000	>1000	6.3 (1.6)	>1000	213 (28)	>1000
3-AG71	**6-pyr/3C/Thieno**	>1000	>1000	1.82(0.28)	>1000	43.4(4.1)	>1000
4-AG94	**6-pyr/4C/Thieno**	101.0(16.6)	273.5(49.1)	0.31(0.14)	>1000	3.34(0.26)	288(12)
5-AG124	**5-pyr/1C/Benzoyl**	>1000	>1000	>1000	>1000	>1000	>1000
6-AG125	**5-pyr/2C/Benzoyl**	30.6 (6.2)	>1000	18.2(3.8)	>1000	22.3(8.6)	>1000
7-AG126	**5-pyr/3C/Benzoyl**	68.8(21.2)	>1000	72.0(27.1)	>1000	329(61)	>1000
8-AG127	**5-pyr/4C/Benzoyl**	56.6(5.8)	>1000	8.6(2.1)	>1000	840(90)	>1000
9-AG128	**5-pyr/5C/Benzoyl**	196.4 (55.0)	>1000	33.5(2.5)	>1000	>1000	>1000
10-AG129	**5-pyr/6C/Benzoyl**	>1000	>1000	>1000	>1000	>1000	>1000
11-AG133	**5-pyr/1C/Thieno**	>1000	>1000	>1000	>1000	468 (112)	>1000
12-AG134	**5-pyr/2C/Thieno**	59.8(11.1)	>1000	550 (50)	>1000	80.2(5.5)	>1000
13-AG135	**5-pyr/3C/Thieno**	116.0(22.5)	>1000	109 (44)	>1000	312 (90)	>1000
14-AG136	**5-pyr/4C/Thieno**	38.3(6.6)	>1000	49.3(11.5)	>1000	141(40)	>1000
15-AG137	**5-pyr/5C/Thieno**	243.2(49.9)	>1000	45.5(22.8)	>1000	57.4(21.4)	>1000
16-AG138	**5-pyr/6C/Thieno**	>1000	>1000	>1000	>1000	>1000	>1000
MTX	-	12(1.1)	216(8.7)	114(31)	461(62)	120.5(16.8)	>1000
RTX	-	6.3(1.3)	>1000	15(5)	>1000	99.5(11.4)	>1000
LMTX	-	12(2.3)	>1000	12(8)	188(41)	38.0(5.3)	>1000

B.

Compound	Structure	hRFC/ FRα/hPCFT				
		KB	KB (+FA)	KB (+Thd/Ade/ AICA)	IGROV-1	IGROV-1 (+FA)
1-AG17	**6-pyr/3C/Benzoyl**	1.7(0.4)	>1000	Ade/AICA	--	--
2-AG23	**6-pyr/4C/Benzoyl**	1.9 (0.7)	>1000	Ade/AICA	3.9 (.98)	>1000
3-AG71	**6-pyr/3C/Thieno**	0.026(0.03)	>1000	Ade/AICA	.98 (2.0)	>1000
4-AG94	**6-pyr/4C/Thieno**	0.55(0.10)	>1000	Ade/AICA	--	--
5-AG124	**5-pyr/1C/Benzoyl**	>1000	>1000	--	--	--
6-AG125	**5-pyr/2C/Benzoyl**	9.94(3.11)	690 (310)	Thd/Ade	60.7 (7.1)	>1000
7-AG126	**5-pyr/3C/Benzoyl**	49.5(13.2)	533(233)	Ade	100 (55)	>1000
8-AG127	**5-pyr/4C/Benzoyl**	12.7(5.4)	700 (300)	Ade	94.3 (7.6)	>1000
9-AG128	**5-pyr/5C/Benzoyl**	17.3(8.9)	898(102)	Ade/AICA	--	--
10-AG129	**5-pyr/6C/Benzoyl**	>1000	>1000	--	--	--
11-AG133	**5-pyr/1C/Thieno**	>1000	>1000	--	--	--
12-AG134	**5-pyr/2C/Thieno**	875 (125)	>1000	--	--	--
13-AG135	**5-pyr/3C/Thieno**	211 (58)	825(175)	Ade/AICA	--	--
14-AG136	**5-pyr/4C/Thieno**	66.0(14.4)	900 (100)	Ade/AICA	29.5 (14.9)	>1000
15-AG137	**5-pyr/5C/Thieno**	41.7(10.1)	>1000	Ade	--	--
16-AG138	**5-pyr/6C/Thieno**	>1000	>1000	-	--	--
MTX	-	6.0(0.6)	20(2.4)	--	--	--
RTX	-	5.9(2.2)	22(5)	--	--	--
LMTX	-	1.2(0.6)	31(7)	--	--	--

Table 2.1: IC_{50}s (in nM) for 5- and 6-substituted pyrrrolo[2,3-*d*]pyrimidine antifolates and classical antifolates in RFC-, PCFT-, and FR-expressing cell lines. Growth inhibition assays were performed for CHO sublines engineered to express human RFC (PC43-10), FRα (RT16), or PCFT (R2/PCFT4), for comparison with transporter-null [R2, R2(VC)] CHO cells (A), and for the KB human tumor subline (express RFC, FRα, and PCFT) (B), as described in the Methods and Materials. For the FRα experiments, growth inhibition assays were performed in the presence and the absence of 200 nM folic acid (FA). The data shown are mean values from 3-10 experiments (plus/minus SEM in parentheses). Results are presented as IC_{50} values, corresponding to the concentrations that inhibit growth by 50% relative to cells incubated without drug. Data for MTX, RTX, LMTX, and compounds AG17, AG23, AG71, and AG94 (**Figure 2.1**) were previously published (structures of these compounds are in Figure 1 whereas the structures for compounds AG133-AG138 are shown in **Figure 2.2**. For KB cells, data are shown for the protective effects of nucleoside additions including adenosine (60 μM), thymidine (10 μM), or AICA (320 μM). Examples of these data are graphed in **Figure 2.4**. For the 5- and 6-pyrrolo-2,3-*d*)pyrimidine compounds AG17-AG138, the structural motifs are documented, i.e., the pyrrole substitution/bridge length/B-ring structure. The most potent compounds in the 5-substituted series

(AG127 and AG136), along with their 6- substituted analogs (AG23 and AG71) and PMX (AG125) were evaluated for anti-tumor activity in the human ovarian cancer cell line IGROV-1. Undefined abbreviations: Cpd, compound; ND, not determined. The results with compounds AG17**,** AG23**,** AG71 and AG94 were previously reported.[198,204,207,226]

Inhibition of *de novo* purine nucleotide biosynthesis by both 5- and 6-pyrrolo[2,3-*d*]pyrimidine compounds leads to a cytotoxic response via purine nucleotide depletion. *De novo* purine nucleotide biosynthesis includes 10 reactions by which phosphoribosyl pyrophosphate (PRPP) is converted into inosine monophosphate (IMP), the precursor of AMP and GMP (refer to **Figure 1.3** in chapter 1). There are two folate-dependent enzymes in the pathway which are possible targets for folate-based therapies i.e., GARFTase (catalyzes steps 2, 3 and 5) and AICARFTase (catalyzes steps 9 and 10). Previous studies established that GARFTase was the intracellular enzyme target for LMTX[183,313] and for compounds AG17, AG23, AG71 and AG94.[198,207] For PMX, TS is the primary intracellular target, although modest inhibitions of GARFTase and DHFR were also reported.[309] Most recently, AICARFTase was implicated as a potentially important secondary enzyme target for PMX (in the presence of excess thymidine to circumvent TS inhibition) by nucleoside protection experiments and metabolic assays.[196,197]

To identify the targeted pathway for 6-substituted compounds AG23 and AG71, we previously used nucleoside protection experiments with adenosine (60 μM) and thymidine (10 μM), to distinguish *de novo* purine nucleotide from thymidylate biosynthesis, respectively.[198,204-207,220,313] To further identify the likely folate metabolizing enzyme targets in purine nucleotide biosynthesis (GARFTase versus AICARFTase), cells were treated with the antifolates in the presence of AICA (320 μM), which is metabolized to AICA ribonucleotide (ZMP), the substrate for AICARFTase substrate, thus bypassing the step catalyzed by GARFTase[198,204-207,220,313] (**Figure 1.3**).

We used this approach for KB cells treated with compounds AG125-128 and AG135-AG137, with results compared to those for compounds AG23 and AG71, and to PMX (compound AG125) (**Table 2.1**; **Figure 2.4** shows the nucleoside protection results for PMX and compounds

AG23 and AG71 compared to those for compounds AG127 and AG136). With compounds AG23 and AG71, both adenosine and AICA were completely protective, establishing *de novo* purine biosynthesis and GARFTase as the principal targets.[198,204,207] With PMX, thymidine, adenosine, and AICA were all partially protective, albeit to different extents. Combined thymidine and adenosine completely protected KB cells from the growth inhibitory effects of PMX (not shown). The growth inhibitory effects of the 5-substituted compounds AG126-AG128, AG135 and 137 with KB cells were unaffected by excess thymidine but were completely reversed in the presence of adenosine alone, indicating that exclusively *de novo* purine nucleotide synthesis was being targeted (rather than combined thymidylate and purine nucleotide synthesis). Whereas AICA alone completely protected KB cells from the inhibitory effects of compound AG128 andAG135, AICA alone was only partially protective with compounds AG126, AG127 and AG137.

Thus, inhibition of purine nucleotide biosynthesis by compounds AG126, AG127 and AG136 is likely a composite effect of dual inhibitions involving both GARFTase and AICARFTase. These results strongly imply that shifting the substituent from the 6- to the 5-position on the pyrrolo[2,3-*d*]pyrimidine ring system results in an altered inhibition of the targeted enzymes.

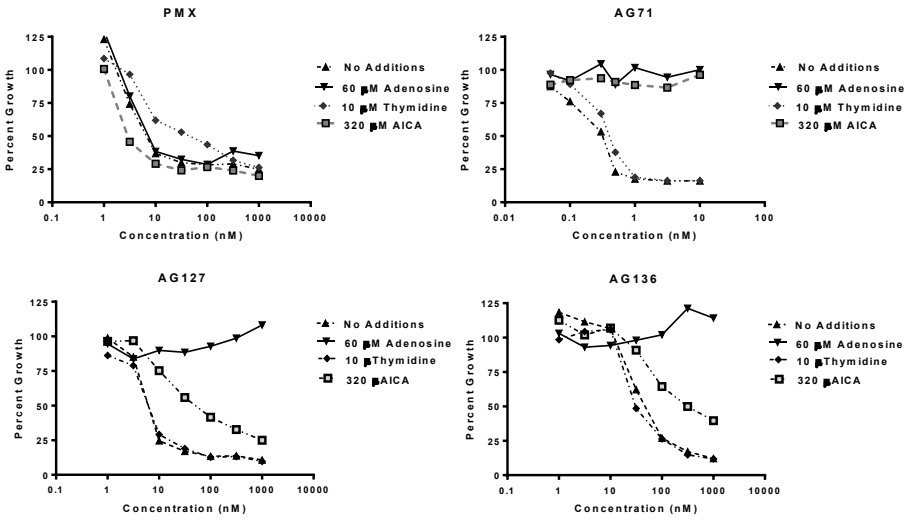

Figure 2.4: Cell proliferation assays with protection by nucleosides including thymidine and adenosine and 5-amino-imidazole-4-carboxamide (AICA) to identify intracellular targets of compounds AG127 and AG136. To identify the targeted pathways and the folate-dependent intracellular enzymes in KB cells treated with compounds AG127 and AG136 (1-1000 nM), cell proliferation assays were performed in the presence of 10 µM thymidine, 60 µM adenosine or 320 µM AICA. Relative cell numbers were measured with a fluorescence-based viability assays (CellTiterBlue™). Results were normalized to those for untreated cells (no drug) and for compounds AG127 and AG136, results are shown in comparison to those for PMX and compound AG71. Details are provided in the Methods and Materials. Results shown are representative of triplicate experiments. Analogous experiments were performed for compounds AG126 and AG128 and the results are summarized in **Table 2.1**.

Decreased purine nucleotide biosynthesis resulting from inhibition of folate-dependent enzymes can be monitored by following changes in ATP pools, a surrogate measure of total purine nucleotides (**Figure 2.5**). KB tumor cells were treated for 24 h with the novel 5-substituted analog AG127 and AG136, or the corresponding 6-subsituted regioisomers, compounds AG23 and AG71, respectively. A parallel incubation was performed with PMX. ATP pools were extracted and measured by an HPLC ion-pairing method.[204,314] In KB cells, following a 24 h incubation, compounds AG127 and AG136, like AG23 and AG71, potently depleted ATP pools (>90%), whereas PMX was much less effective (~60% inhibition).

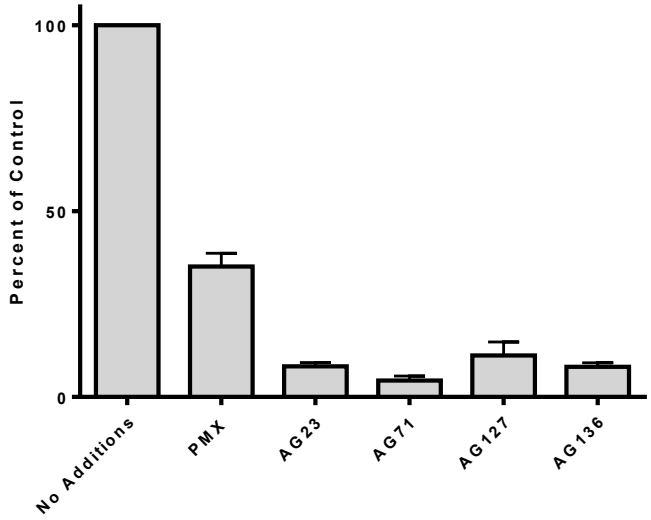

Figure 2.5: Compounds AG23, AG71 AG127and AG136 deplete ATP pools in KB cells. KB cells were treated with 1 μM of the novel 5- and 6-substituted pyrrolo[2,3-*d*]pyrimidine antifolates AG23, AG71 AG127 and AG136 , or with PMX for 24 h. Nucleotides were extracted and analyzed by ion-pair HPLC using a C18 column. Experimental details are in the Methods and Materials. Results are presented as mean values +/- standard errors.

Inhibition of *de novo* purine biosynthesis by compound 8 results in cytotoxicity and apoptosis. To confirm that depletion of purine nucleotides by the most potent 5-substituted analog, compound AG127, results in cytotoxicity toward KB cells, we performed colony-forming assays. In these experiments, KB tumor cells were treated with various concentrations (1-10 μM) for 48 h, then washed and plated without drug. Colonies were enumerated after 10 days (**Figure 2.6A**). Following drug exposures, inhibitory effects of compound AG127 were irreversible with complete loss of colony formation at the highest drug concentrations, thereby establishing compound AG127 as cytotoxic.

Programmed cell death type I (mitochondrial pathway) requires ATP to form the death complex with cytochrome C and caspase 9 and to carry out other energy-requiring processes for enzymatic degradation and modification of cellular components.[315] Previous studies suggested that the 6-substituted pyrrolo[2,3-*d*]pyrimidine antipurine antifolate AG17 was capable of inducing apoptosis, albeit less than that indicated by daunorubicin over 24 h.[198] Additional experiments were performed with KB cells treated with compound AG127 to assess its effects on cell cycle progression and induction of apoptosis, with comparisons to PMX and compound AG23. Cells were exposed to 1 μM drugs for 48 or 96 h. Cultures were then divided, with one fraction fixed and stained with propidium iodide (PI) for flow cytometry analysis of cell cycle distribution (**Figure 2.6B**). An additional fraction was assayed for apoptosis by flow cytometry with annexin V/fluorescein isothiocyanate (FITC) /7-amino actinomycin D (AAD) staining (**Figure 2.6C**).

At 48 h, minimal effects on the cell cycle were observed, except for PMX, which induced a G_1/G_o-phase arrest relative to the untreated (DMSO) control (data not shown**)**. At 96 h, all compounds tested increased the percentages of cells in S-phase (albeit to different extents), while reducing those in the G_1/G_o- and G_2/M-phases of the cell cycle. The histograms display a change

in DNA content in cells treated with AG23 and AG127, suggesting aneuploidy. A prominent increase in the sub-G_1 population at 96 h was also seen (**Figure 2.6B**; most notable for compounds AG23 and AG127), indicating apoptosis was induced.

Annexin V-FITC staining was used to further monitor induction of apoptosis. For compounds AG23 and AG127, as well as PMX, positive annexin V-FITC staining increased over time (results for 96 h are shown in **Figure 2.6C**, with distinct increases in both early (annexin-V-FITC^{high}/7-AAD^{low}) and late apoptotic fractions (annexin-V-FITC^{high}/7-AAD^{high}), compared to those for the untreated (DMSO) control (*i.e.*, the fractions of cells in early apoptosis were 33.8% and 0.49% for compound AG127-treated and control cells, respectively, whereas the fractions in late apoptosis at 96 h were 26.7% and 2.1%, respectively). Nearly identical results were obtained with compound AG71, although for PMX, the distribution between the early and late apoptotic fractions was somewhat different. Thus, the 5- and 6-substituted pyrrolo[2,3-*d*]pyrimidines AG23 and AG127 can induce apoptosis, resulting in cell death in spite of depleted ATP pools.

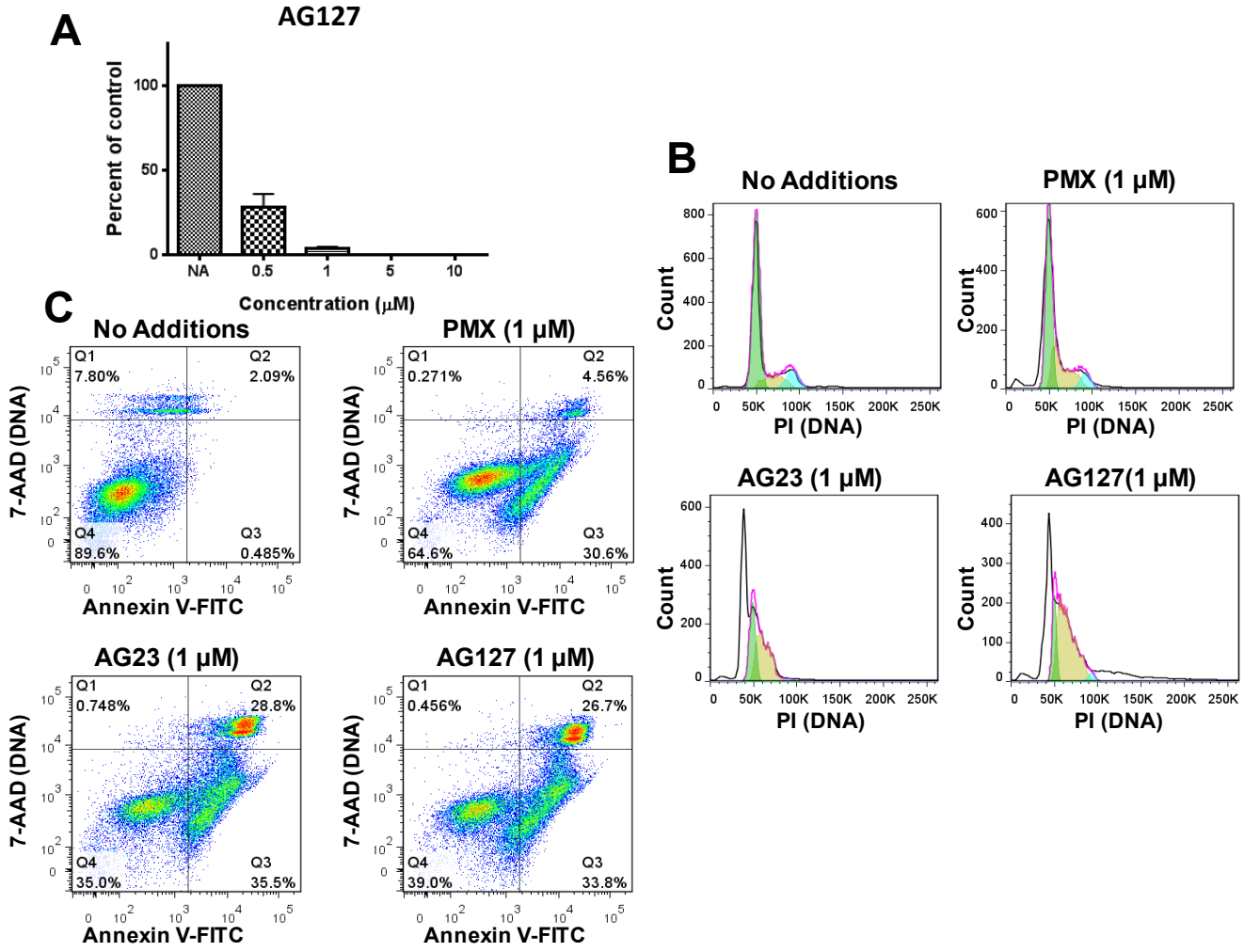

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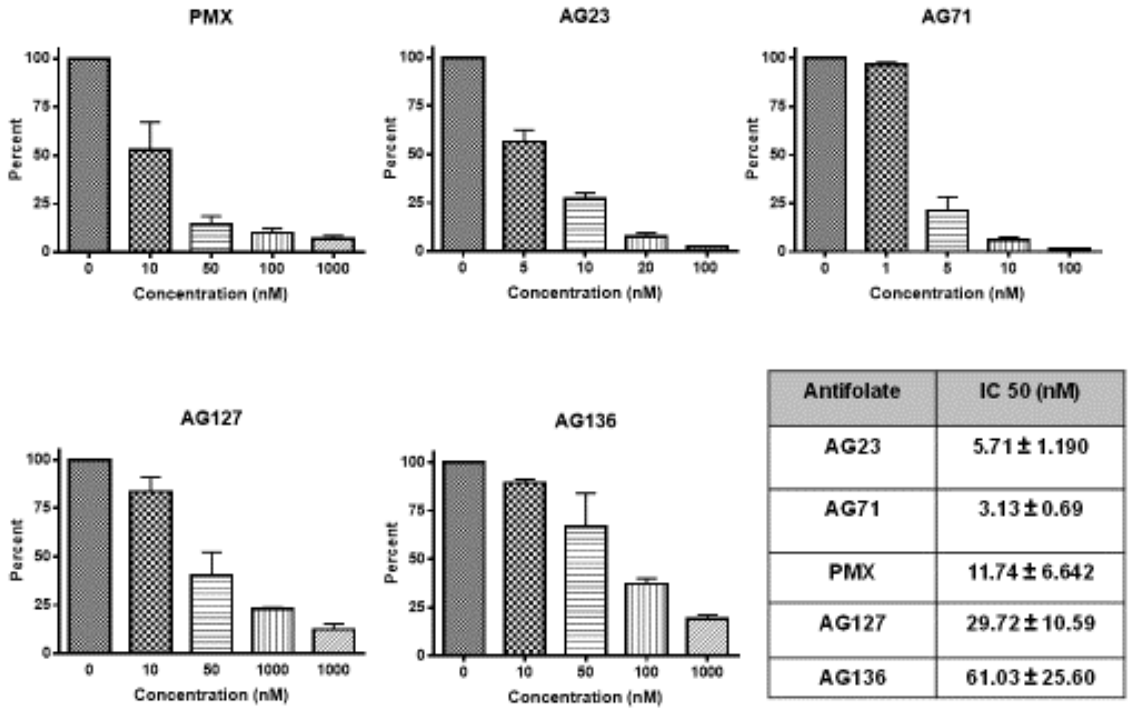
Figure 2.6: Inhibition of *de novo* purine biosynthesis by compound AG127 results in cytotoxicity and apoptosis. Panel A. KB cells (1000 cells) were treated with compound **AG127** (0.5 to 10 μM) for 48 h, after which cells were washed (3x) and complete drug-free medium was added. Colonies were allowed to grow for 10 days, at which time they were stained with methylene blue and counted. Results are presented as mean values +/- standard errors from 3 experiments. Panel B: Cell cycle analysis. KB cells were treated with 1 μM antifolates (PMX, compound **AG23**, compound **AG127**) for 96 h, washed, then fixed and stained with PI. Cell cycle distributions were analyzed by flow cytometry. The sub-G_1 fraction (white fill) increased from 3.52% in the absence of drugs upon treatment with PMX (17.3%) or compounds **AG23** (45.1%) or **AG127** (36.7%). For the non-sub-G_1 population, the cell cycle distributions [colors are green for G_1/G_0, yellow for S, and blue for G_2/M] are as follows: no additions, 67.4% G_1/G_0, 15.9% S, 13.2% G_2/M; PMX, 47.8% G_1/G_0, 27.5% S, 7.46% G_2/M; compound **AG23**, 22.3% G_1/G_0, 31.4% S, 1.18% G_2/M; and compound **AG127**, 12.9% G_1/G_0, 48.2% S, 2.31% G_2/M. Panel C: Analysis of apoptosis by annexin V-FITC/7-AAD staining and flow cytometry are shown for KB cells treated with PMX, or with compounds **AG23** or **AG127** for 96 h. Results are compared to those for controls treated with DMSO *in lieu* of drug. The percentages of cells in each quadrant [viable

cells (annexin V-FITC^{low}/7-AAD^{low};Q4), early apoptosis (annexin V-FITC^{high}/7-AAD^{low};Q3), and late apoptosis/necrosis (annexin V-FITC^{high}/7-AAD^{high};Q2) are summarized. These experiments were ran with the assistance of Dr Eric Hales who performed the data analysis.

Dual Inhibition of GARFTase and AICARFTase by 5-substituted pyrrolo[2,3-*d*]pyrimidine antifolates and downstream effects of accumulated ZMP on AMPK as mechanisms resulting in cytotoxicity. Experiments were performed to measure cellular GARFTase activity in KB tumor cells treated with the 6- (compounds AG23 and AG71) and 5-substituted (compounds AG127 and AG136, and PMX) pyrrolo[2,3-*d*]pyrimidines. For this purpose, we used an *in situ* activity assay for GARFTase which measures incorporation of [^{14}C]glycine into [^{14}C]formyl GAR in the presence of azaserine over 16 h.[147,153-156,168,233] Cells were harvested, washed with PBS and treated with trichloroacetic acid (TCA). Following deproteinization, acid-soluble metabolites were ether-extracted and fractionated by ion-exchange chromatography, permitting isolation and quantitation of [^{14}C]formyl GAR. Both 5- and 6-substituted analogs exhibited a dose-dependent decrease in [^{14}C]formyl GAR. Compound AG71 was the most potent of the analogs tested in contrast to its 5-substituted analog, compound AG136, which was the least. However, compound AG23 was more potent than either PMX or compound AG127, with an IC_{50} for this compound exceeding those for 5-substituted compounds by 2- to 5-fold (**Figure 2.7)**.



Antifolate	IC 50 (nM)
AG23	5.71 ± 1.190
AG71	3.13 ± 0.69
PMX	11.74 ± 6.642
AG127	29.72 ± 10.59
AG136	61.03 ± 25.60

Figure 2.7: *In situ* GARFT assay. GARFT activity and inhibition by 5- and 6-substituted pyrrolo[2,3-*d*]pyrimidine analogs, AG23, AG71, AG127, AG136 and PMX were evaluated *in situ* with KB cells. KB cells were treated with drugs in the presence of 4 μM azaserine. [^{14}C]glycine and *L*-glutamine were added and cells were incubated for 16 h at 37^{o}C. Radioactive metabolites were extracted and fractionated on 1 cm columns of AG1 x 8 (Cl^-); the fractions were collected and determined for radioactivity. Accumulation of [^{14}C]formyl GAR was calculated as a percent of vehicle control over a range of antifolate concentrations. Results are presented as mean values ± standard errors from 3 experiments. IC_{50}s are summarized in the table. Details are described in the Methods and Materials.

The anti-proliferative effects of PMX independent of its impact on TS were previously attributed to its inhibition of AICARFTase in addition to GARFTase, as reflected in incomplete protection by AICA in the presence of thymidine, and the accumulation of the AICARFTase substrate ZMP in tumor cell lines treated with PMX, as measured by anion-exchange HPLC analysis.[196,197] ZMP accumulation showed both dose- and time-dependence for PMX and resulted in activation of AMPK via its role as an AMP mimetic and suppression of mTOR.

To validate the findings from our AICA protection experiments with lead 5-substituted analog, compound AG127 (**Figure 2.4**), and to explore whether AICARFTase is a *bona fide* target for the novel 5-substituted pyrrolo[2,3-*d*]pyrimidine AG127, analogous experiments were performed to measure accumulation of ZMP in KB cells treated with 1 μM of compound AG127 or PMX, as a measure of AICARFTase inhibition. ZMP accumulated in a linear fashion for up to 48 h in KB tumor cells for both compounds (not shown), with PMX showing approximately 2-fold increased levels over compound AG127 (**Figure 2.8A**).

To verify the impact of ZMP accumulation on AMPK phosphorylation in KB cells treated with compound AG127 or with PMX, western blot analysis was performed of total and phosphorylated AMPK in KB cells treated with these antifolates for 48 h, compared to levels of phospho-AMPK resulting from treatment with established AMPK activators including AICA, AICA ribonucleoside, and metformin[316] (**Figure 2.8B**). Although KB cells are LKB1-null (the principal upstream kinase responsible for phosphorylating AMPK), AMPK is still activated upon treatment with known AMPK activators. While AMPK is activated by PMX, compound AG127 was not significantly activating despite the accumulation of ZMP. Interestingly, neither AICA (not shown) nor metformin (data not shown) at concentrations found to activate AMPK were growth inhibitory toward KB cells.

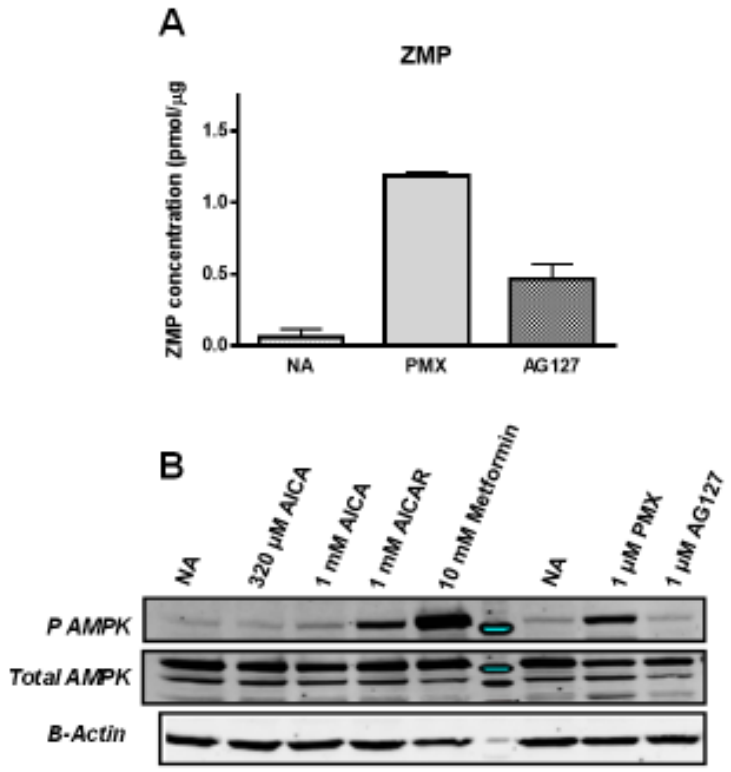


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Figure 2.8: Accumulation of ZMP by 5-substituted pyrrolo[2,3-*d*]pyrimidines PMX, and compound AG127. Panel A: ZMP (AICA ribonucleotide) accumulation was measured in KB cells treated for 48 h with the 5-substituted pyrrolo[2,3-*d*]pyrimidine antifolate AG127, or PMX. Methods for extracting nucleotide metabolites and for measuring ZMP levels by ion-exchange HPLC are detailed in the Methods and Materials. Panel B: KB cells were treated at indicated for 48 h, followed by western analysis of phosphorylated AMPK on a 4-20% gradient gel. Blots were probed with antibodies to phospho- and total AMPK, and to β-actin.

2.3 Discussion

The development of rationally designed cytotoxic antifolates remains an area of significant interest to anticancer drug development, as exemplified by the relatively recent approval of PMX[317] and pralatrexate[318] for cancer, more than 50 years since introduction of MTX.[319] Unfortunately, the ubiquitous presence of RFC may contribute to unwanted toxicities of these classical antifolates toward normal tissues. Based on this, growing attention has focused on developing novel folate-based compounds with tumor targeting premised on transport selectivity for FRs or PCFT over RFC,[75,198,204-207,220] a pursuit that until recently[175] continued to be challenged by the lack of structural information on the major folate transport systems.

The antitumor efficacy for the 5-substituted 2-carbon bridge pyrrolo[2,3-*d*]pyrimidine PMX reflects its cellular uptake by all the major folate transport systems.[147,153-156] While the corresponding 6-substituted analog of PMX was pharmacologically inert, with increasing bridge lengths, 6-substituted pyrrolo[2,3-*d*]pyrimidines were surprisingly potent inhibitors of cell proliferation, although membrane transport by RFC was lost.[204,320] The 6-pyrrolo[2,3-*d*]pyrimidine benzoyl analogs with 3 and 4 bridge carbons, compounds AG17 and AG23, respectively, were the most active of this series, reflecting their FR and PCFT cellular uptake, with little to no transport by RFC, and their potent inhibitions of GARFTase, the first folate-dependent step leading to the synthesis of IMP.[153,237] Inhibition of *de novo* synthesis of purine nucleotides resulted in ATP depletion, leading to cytotoxicity and apoptosis.[153,237]

In this report, our goal was to further establish a comprehensive SAR for the 5- versus 6-substituted pyrrolo[2,3-*d*]pyrimidine ring system in relation to folate transporter specificity and inhibition of intracellular targets, based in part on findings of transporter promiscuity for PMX,[198,204-207] and reports that PMX may in part derive its antitumor effects by inhibiting

AICARTase, resulting in accumulation of ZMP and activation of AMPK in addition to inhibiting TS. [145,146] Compared to the 6-substituted analogs, we found that the 5-substituted compounds in general: (i) were better substrates for RFC with the highest antiproliferative activities for compounds with 2-4 bridge carbons independent of the nature of the side chain ring system; (ii) were poorer substrates for PCFT with the exception of the 2-carbon bridge compound, PMX (AG125); and (iii) preserved FR cellular uptake, with the 4-carbon bridge analog AG127 showing the greatest FRα selectivity and potency in this expanded 5-substituted series of analogs without TS inhibitory activity.

Compound AG127 was cytotoxic, as reflected in loss of clonogenicity in KB cells, and effected S-phase accumulation and apoptosis over 96 h. Like its 6-substituted counterpart compound AG71,[204] compound AG127 resulted in ATP depletion, although this appeared to be only partly due to inhibition of GARFTase. Rather, a unique feature of the 5-substituted analog AG127 relates to its dual inhibition of both the GARFTase and AICARFTase reactions in *de novo* purine biosynthesis. While PMX also inhibits GARFTase, the finding that the AICARFTase substrate ZMP accumulates in the presence of this drug suggested that GARFTase inhibition must be incomplete and that AICARFTase must also be a target as previously described, [145,146] although for PMX, total purine nucleotides, as reflected in ATP pools, were significantly preserved. Compound AG127, also inhibited AICARFTase, as reflected in incomplete protection in cell proliferation assays by AICA and demonstrated accumulation of the AICARFTase substrate ZMP, albeit less than for a comparable concentration of PMX.

Previous studies suggested that AMPK activation secondary to ZMP accumulation and AICARFTase inhibition may result in inhibition of mTOR and contribute to the antitumor effects of high levels of PMX in the presence of thymidine.[196,197] In the present study, we used KB human

tumor cells treated with PMX or with compound AG127. In this LKB1-deficient tumor cell line, ZMP accumulation was accompanied by AMPK activation in the presence of PMX but not compound AG127. Robust AMPK activation was also seen upon treatment with AICAR, or metformin. However, ZMP accumulation and AMPK activation did not appear likely to contribute to the anti-proliferative effects of PMX in KB cells, as neither AICA nor metformin inhibited cell proliferation. The antiproliferative and cytotoxic effects of compound AG127 toward KB cells must derive from a depletion of ATP pools, secondary to inhibition of both GARFTase and AICARFTase, rather than from activation of AMPK.

In summary, a series of classical 5-substituted pyrrolo[2,3-*d*]pyrimidine antifolates AG124-AG129 were designed and synthesized as a hybrid of PMX and 6-substituted pyrrolo[2,3-*d*]pyrimidines AG17, AG23, AG71 and AG94. Compound AG127, the lead compound of this series, has an IC_{50} =12.7 nM against KB tumor cells in culture. Rather than only targeting GARFTase, as with AG17 and AG23, both GARFTase and AICARFTase were identified as the intracellular targets of AG127. The unique dual GARFTase and AICARFTase inhibition of compound AG127 makes it an excellent lead compound for further study including the design of additional antitumor analogs to optimize cellular folate uptake selectivity by FRs and inhibition of folate metabolizing enzymes, leading to a new generation of potent tumor-targeted agents. The dual inhibition of both GARFTase and AICARFTase results in potent inhibition of purine biosynthesis and could preserve antitumor activity in the event that tumors become resistant due to alterations in one or the other targeted enzyme. Such dual targeted inhibitors would seem to have substantial advantages over currently used agents that principally target only one intracellular enzyme target.

2.4 Materials and methods

Reagents for biological studies. [3', 5', 7-^{3}H]MTX (20 Ci/mmol), [3', 5', 7, 9-^{3}H] folic acid (25 Ci/mmol), and [^{14}C(U)]-glycine (87mCi/mmol) were purchased from Moravek Biochemicals (Brea, CA). Unlabeled folic acid was purchased from the Sigma Chemical Co. (St. Louis, MO). LCV [(6R,S) 5-formyl tetrahydrofolate] was provided by the Drug Development Branch, National Cancer Institute, Bethesda, MD. The sources of the antifolate drugs were as follows: MTX, Drug Development Branch, National Cancer Institute (Bethesda, MD); and PMX (Alimta) (LC Laboratories, Woburn, MA). Other chemicals were obtained from commercial sources in the highest available purity. Novel antifolates (denoted by AG17-138 (see **Table 2.1**))[221] were synthesized by Dr. Aleem Gangjee and colleuges at Duequesne University, Pittsburg Pennsylvania.

Cell culture. MTXRIIOua^{R}2-4 (referred to as R2) is a RFC, PCFT and FRα null Chinese Hamster (CHO) cell line that was a gift from Dr. Wayne Flintoff (University of Western Ontario).[321] From this parental cell line, RFC, PCFT and FRα were transfected in to give rise to isogenic CHO cell lines designated PC43-10 (expresses RFC but not PCFT or FRα), R2/PCFT4 (expresses PCFT) and RT16 (expresses FRα).[198,204-207,220,322] The CHO sublines were all maintained in α-minimal essential medium (α-MEM) supplemented with 5% penicillin-streptomycin solution, 2 mM L-glutamate and 10% heat-treated bovine calf serum (BCS) (Invitrogen). Transfected cell lines were cultured in the presence of 1.5 mg/ml G418. CHO cell lines were cultured prior to cell viability experiments in folate-free RPMI (FF-RPMI) (Invitrogen) with dialyzed fetal bovine serum (DFBS) (Invitrogen) supplemented with 5% penicillin-streptomycin and L-glutamate. Human FRα-expressing KB (nasopharyngeal) and IGROV-1

(ovarian) carcinoma cells were continuously maintained in FF-RPMI complete with 10% fetal bovine serum (FBS) and 5% penicillin-streptomycin and L-glutamate.

Cell viability assays were performed exactly as previously described.[198, 193-196] Cell lines were treated with a range of concentrations of standard or novel antifolates (0-1 μM), metformin (0-10 mM) or AICA (0-2 mM) in a 96 well plate with in FF-RPMI complete with DFBS, and 2 nM (RT-16, KB, IGROV1) or 25 nM LCV (R2/PCFT4), penicillin –streptomycin and L-glutamate over a 96 h incubation period at 37◦C with 5% CO_2. To confirm FRα-mediated drug uptake and growth inhibition, 200 nM folic acid was added to the incubations with drug. For proliferation assays with PC43-10 cells, cells were cultured in standard RPMI1640 with DFBS, penicillin-streptomycin and L-glutamate for 96 h. To quantitative viable cells, media was removed and Cell Titer-blue™ fluorescent reagent (Promega) was added. Relative cell numbers were determined by relative fluorescence units measured with a fluorescence plate reader at 590 nm emission and 560 nm excitation with Softmax Pro plate reader software. IC_{50} values, corresponding to the drug concentrations that resulted in 50% loss of cell growth, were calculated from dose-response curves using Sigma Plot (v.12) software.

In order to identify the targeted pathways/folate-dependent enzyme by the classic and targeted antifolates, proliferation assays were performed in the presence of adenosine (60 μM), thymidine (10 μM), or AICA hydrochloride (320 μM) and results compared to incubations in parallel without drug additions.[198,205,220]

Colony forming assays were performed with compound AG127 to verify a cytotoxic (as opposed to cytostatic) response. KB cells (~500) were plated in 60 mm dishes in FFRPMI/DFBS supplemented with 2 nM LCV for a 24 h incubation period at 37◦C in the presence of 5% CO_2. Concentrations ranging from 0-10 μM of compound AG127 were added to media for an additional

24 h, after which the media was aspirated and replaced with FFRPMI/DFBS supplemented with 2 nM LCV for 8-10 days. To visualize colonies, the cells were washed (2x) with room temperature Dulbecco's phosphate-buffered saline (PBS) (Sigma), followed by a wash of 5% trichloroactetic acid (TCA). The TCA was removed, 10 mM borate buffer (pH 8.8) was added, followed by a 30 min incubation with 1% methylene blue in borate buffer at room temperature. The cells were was three times with borate buffer and the stained colonies were counted.

Competitive MTX transport assays. To determine if novel antifolates bound to RFC, reflecting membrane transport by this mechanism, competition with [^{3}H]MTX for cellular uptake was measured in PC43-10 CHO cells.[198,207] PC43-10 cells (~1.5x10^6 cells) were seeded in 60mm dishes. After 48 h, cells were washed with PBS and uptake of [^{3}H]MTX (0.5 μM) was measured in Hank's Balanced Salts Solution for 2 minutes at 37◦C. Cold (~4◦C) PBS was used to quench the reactions. Cells were washed with ice-cold PBS (3X), then solubilized with 0.5N NaOH. The cellular homogenates were assayed for radioactivity with a scintillation counter; protein concentrations were measured with Folin-phenol reagent.[203] Levels of drug uptake were expressed as pmol/mg cell protein.

FRα affinity binding assay. To determine the binding affinity of novel antifolates to FRα,[198] FRα-expressing RT16 CHO cells (~1.5 x10^6) were seeded in a monolayer with complete α-MEM media. Cells were washed with ice-cold PBS, followed by an cold acetate buffer (10 mM sodium acetate, 150 mM NaCl, pH 3.5) to release FRα-bound folates, then neutralized with ice-cold Hepes-buffered saline (HBS) (20 mM Hepes, 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 5 mM glucose, pH 7.4). Cells were then incubated at 0◦C for 15 min in a cocktail containing [3', 5', 7',9- ^{3}H]folic acid, in the absence or presence of unlabled folic acid or novel antifolate compounds (0-1 μM). Cells were washed with cold HBS and solubilized with 0.5 N NaOH. The cell

homogenates were collected and analyzed for radioactivity using a liquid scintillation counter. Protein concentrations were measured with the Folin-phenol reagent. Bound [^{3}H]folic acid was expressed as pmol/mg protein. Using Sigma plot (v.12) software, dose-response curves were created and IC_{50}'s were calculated. Relative binding affinities were calculated as the inverse molar ratios of unlabeled ligands require to inhibit [^{3}H]folic acid by 50% as determined graphically with Graphpad (v. 6.0) software. By definition, the relative affinity of folic acid is 1.

HPLC detection of ATP. Intracellular ATP concentrations were determined using nucleotide extractions and an HPLC method, exactly as previously described.[204,314] Briefly, KB cells were seeded in a T75 flask with FF-RPMI with 10% DFBS, 5% penicillin-streptomycin, 2 mM L-glutamine and 2nM LCV. Cells were treated with 1 μM of PMX, or compounds AG23, AG71, AG127 or AG136 for 24 h. After the incubations cells were trypsinized and washed with cold PBS. Nucleotide pools were fractionated on by reversed-phase HPLC on a C18 column for determination of ATP pools.

Apoptosis and cell cycle analysis. KB cells were treated with 1 μM of PMX of compounds AG23 or AG127 24, 48, 72 and 96 h in FF-RPMI media complete with 10% DFBS and 5% penicillin-streptomycin and 2 nM LCV. Harvested cells were trypsinized and washed with cold PBS. Samples were equally divided so cells could be stained with PI for cell cycle analysis and with Annexin V/7-AAD (Beckman) for apoptosis assays. For cell cycle analysis, cells were fixed with cold ethanol (added drop-wise), then resuspended and stained in PBS containing 50 μg/mL PI and 100 μg/mL RNAse (type I-A) (Sigma). Cell populations were analyzed by flow cytometry and Flowjo software. Flow cytometry and Flowjo software were also used to discriminate positively stained Annexin V/7-AAD populations including viable (annexin V and 7-

AAD negative), early apoptotic (annexin V positive/ PI negative or 7-AAD low), and late apoptotic/necrotic (annexin V positive/PI positive or 7-AAD high).

***In situ* GARFT inhibition assays.** To measure the extent of intracellular GARFT inhibition in the presence of antifolate inhibitors, we examined the metabolic incorporation of [^{14}C(U)] glycine into [^{14}C]formyl GAR (**Figure 2.4**) in the presence of azaserine. Using previously published methods, [147,153-156,168,233] KB cells were seeded in complete media (above) and allowed to adhere to substratum for 24 h. Cell were then washed with FF-RPMI, L- glutamine free with 10% DFBS, 5% penicillin-streptomycin and 2 nM LCV. Cells were incubated at 37^{0}C with 5% CO_2 for 1 h with folate- and L-glutamine-depleted media containing 2 nM LCV, with and without PMX and compounds AG23, AG71, AG127, or AG136 (in DMSO) over a range of concentrations. Control cells were treated with DMSO of equal volume. Azaserine (4 μM final concentration) was added to the cells and allowed to incubate for 30 minutes. This was followed by the addition of L-glutamine (2 mM) and [^{14}C(U)]glycine (final specific activity 0.1 mCi/L). Cells were incubated 16 h. Cells were washed with ice-cold PBS, trypsinized and collected. Cells were washed with cold PBS, then treated with 5% TCA at 0^{0}C. Samples were centrifuged (4^{o}C, 14,000 rpm) and the protein precipitants were solubilized with 0.5 N NaOH for quantitation of protein concentrations with the Folin-phenol protein method.[323] The supernatants were collected and extracted with cold ether. After evaporation of ether, the remaining aqueous layer was gravity filtered through a 1 cm polypropylene chromatography column (Bio-rad) of AG1x8 (chloride form). The columns were washed with 10 mL 0.5 N formic acid, followed by 4 N formic acid (10 ml), and finally eluted with 8 ml of 1 N HCL collected in 8 fractions. The eluate was measured for radioactivity and based on the percentages of radioactivity in the [^{14}C]formyl GAR and non-

specific radioactivity, the results calculated as pmol/mg protein. Data were graphed using Sigmaplot (v.12) and IC_{50} were calculated.

HPLC detection of ZMP pools. Methods for detecting ZMP in KB cells treated with drugs were based on those described by Rascanelli et al.[196] Briefly, KB cells were treated with assorted drugs (PMX, compound AG127 or drug free) for 48 h in FF-RPMI with 10% DFBS, 5% penicillin-streptomycin, 2 mM L-glutamine, and 2 nM LCV. Cells were washed with PBS, treated with ice-cold 5% TCA and proteins were precipitated by centrifugation (1600 rpm, 10 min). The soluble fractions were extracted with ether (2x) and fractionated by ion-exchange HPLC on a SAX 250 x 2 mm column (Phenomenex) with a linear gradient from 5 mM $NH_4H_2PO_4$ (pH 2.8) to 750 mM $NH_4H_2PO_4$ (pH 3.9) over 25 min at a flow rate of 0.2 mL/min. Absorbance was monitored at 280 nm and the intracellular ZMP concentrations were established by fitting the peak absorbances to a standard curve with commercial ZMP. AraCMP was used as an internal control.

Western blot analysis. KB cells were treated for 48 h with PMX or compound AG127 in the presence of thymidine (5.6 μM). Controls include vehicle control (water or DMS0; results were identical), or known activators of AMPK including metformin (10 mM), AICA (320 μM or 1 mM), or AICA ribonucleoside (1 mM). Cells were washed, suspended in 10 mM Tris-HCl (pH 7.0) plus proteolytic and phosphatase (Phosphostop) inhibitors (Roche Applied Science) and disrupted by sonication. Samples were centrifuged (14,000 rpm, 10 min) and 90 μg protein fractionated by SDS polyacrylamide gel electrophoresis on Laemmli gradient gels (4-20%).[324] Proteins were electrotransferred to polyvinylidene difluoride (PVDF) membranes (Pierce).[325] Detection of immunoreactive AMPK was with rabbit total and phospho-AMPK antibodies (Cell Signaling) and IRDye800-conjugated secondary anti-rabbit antibody (LI-COR, Lincoln, NE)

using an Odyssey® infrared imaging system (LI-COR, Lincoln, NE). β actin was detected with anti-β actin mouse antibody and IRDye800-conjugated secondary anti-mouse antibody (LI-COR).

CHAPTER 3: DISCUSSION

We are upon a major paradigm shift that has altered our view of ovarian cancer. With this shift in thinking, changing the strategies in how we treat this disease is not only appropriate but the next logical step in disease intervention. Currently, the treatment of this disease remains unaltered from the standard agents, irrespective of the diverse molecular biology of the tumor. The identified molecular markers should be used to determine appropriate targeted therapy to address the driving oncogenic mechanism of the tumor. Armed with the knowledge of the precise molecular pathways involved in the 2 categories of EOC (type I and type II), we can customize and design novel therapeutic strategies that may prove to be more beneficial than the non-specific chemotherapy that is currently in use.

Combination therapy has proven to be effective in the treatment of many malignancies.[326] Mathematical models predict that combination therapy will be more successful at disease eradication than single agent or adjuvant treatment because combination treatment is more likely to prevent drug resistance.[327] A treatment with a reduced propensity to drug resistance is highly desirable in ovarian cancer, as the recurrent drug resistant tumors are difficult to treat and are principally responsible for the patient's demise.[328] A recent study examined the effectiveness of combining PMX with simvastatin (a statin) in mesothelioma and lung cancers and reported an enhanced apoptotic response with combination treatment than with single agent therapy.[329] There is a heighten interest in the use of statins as anticancer agents which stems from their ability to disrupt the mevalonate pathway.[329-335] This pathway has been associated with cell signaling, cell-cycle progression, protein synthesis and membrane integrity.[330] This study may serve as a platform to encourage more studies examining the effectiveness of antifolates in conjunction with other well established anticancer therapies (in addition to statins which are currently in their experimental

infancy as antineoplastics) that possess a different mechanism of action. Combination therapy may have great significance in malignancies like ovarian cancers that harbor a number of mutations in kinases, GTPases and receptors for which there are experimental and well establish targeted therapies. For example a FRα targeted therapy may pair with inhibitors of AKT, K-RAS, or HER2, as many ovarian tumors have activating mutations or overexpression of these kinases GTPases and receptors.[336]

Overwhelming evidence suggests that FRα confers a growth advantage to malignant cells.[60,62,128,174] There is speculation that the use of DNA damaging agents, like platinum-based therapies, may enhance FRα expression to increase intracellular folate levels to encourage an up regulation of nucleotide production to assist in DNA repair to address drug induced damage.[263] These identified molecular events in ovarian cancer offer an opportunity for the continued growth of the next generation of FRα-targeted treatments. To date, classic antifolates have not made a notable appearance in the treatment of ovarian cancer. In light of the enhanced expression of FRα driven by the use DNA damaging agents, like those used in ovarian cancer, FRα targeted antifolates can be useful as a second line therapeutic in this disease. PMX, which shows some affinity to FR is currently being evaluated as a treatment option in platinum resistant ovarian cancer.[337,338]

3.1 Targeting the AMPK/mTOR pathway

We have characterized a novel class of agents that are effective against a cell culture model of HGSC, endometroid carcinoma and clear cell carcinoma. Prior to obtaining crystallographic information pertaining to FRα, rational drug design aided in determining structural elements required for FRα-mediated drug uptake. One unique feature of the agents under investigation is their ability to act as dual inhibitors of GARFTase and AICARFTase. This ability to inhibit

AICARFTase, led to an accumulation of a purine intermediate (ZMP) but not AMPK activation. Therefore this study also allowed us to review the potential significance of AMPK activation in ovarian tumors and the potential of indirectly targeting AMPK and mTOR signaling for therapeutic advantage. In recent years, AMPK-targeting in cancer treatment has become en vogue. Much of its popularity can be attributed to the antidiabetics metformin and phenformin and their association with antitumor activity and reports suggesting that type 2 diabetics taking metformin had better cancer treatment outcomes than those taking other antidiabetics.[339] While there has been much excitement surrounding the antitumor effects metformin has displayed in breast and ovarian cancer studies, the mechanism of action of this drug remains unclear.[339-346] Most of this uncertainty arises from metformin's ability to induce these antiproliferative and anti-tumor effects in an AMPK-dependent or -independent manner.[345,347] This leaves one to question if the effect on the cellular energy balance (AMPK/mTOR) has greater impact on cell viability than the antidiabetic is affecting the insulin metabolism of the tumor which is causing growth suppression and or apoptosis.

There are a number of studies that imply that AMPK activation may assist in the survival and growth of select tumors (e.g., prostate cancer) through the activation of macroautophagy, which in certain cellular contexts can lead to cell death or cell survival.[348] Autophagy is a common pathway for cell survival in many malignancies. The modulation of macroautophagy in autophagy competent cells may result in no significant consequence for such cells. A recent study showed that an apoptotic response to pemetrexed in combination with simvastatin can be enhanced in MPM and lung cancer cells when autophagy is inhibited.[332] This demonstrates that autophagy inhibition can be an effective strategy to further potentiate cell death. In the case where FRα is the sole means of drug transport, the method in which autophagy is inhibited becomes critical. Folate

receptors require acidification of the lysosome to release substrate. Many pharmacologic tools used to inhibit autophagy involve agents that prevent acidification of lysosomes and similar organelles, thus also making FRα-mediated uptake ineffective. ATG siRNAs are useful molecular tools in an experimental setting; however, the use of such tools in a clinical setting, to date, may be impractical. With the growing number of chemotherapeutics that are now being identified as AMPK activators, a closer examination of the effects of this activation should be conducted on the tumor of question prior to the selection of therapy.

3.2 Targeting AICARFTase

This study sought to describe the significance of AICARFTase inhibition in *de novo* purine synthesis in FRα-positive tumor cells. Eukaryotic AICARFTase is a bifunctional protein that participates in formylation and cyclization to produce the purine precursor molecule IMP.[349-352] Like GARFTase, AICARFTase is a critical player in the *de novo* production of purine nucleotides and therefore has been suggested as a viable target to disrupt the purine biosynthetic pathway. Although both GARFTase and AICARFTase are transformylases in the same pathway and can use the same folate co-factor to perform their activities, they share no sequence homology.[351,353] This divergence in sequence would make a drug directed to target one enzyme highly unlikely to behave as a substrate for the other.[351] For this reason, MTAs like MTX (primary target DHFR)and PMX (primary target TS) are rather unique because they can inhibit both enzymes.[349]

There are no known clinically investigated antifolates that primarily target AICARFTase. Burroughs and Wellcome designed and produced two antifolates, BW1540 and BW2315, to selectively target AICARFTase. These two novel agents are sulfamido bridged 5,8 dideazafolate analogs, that were designed to create a stronger interaction between the protein and the small molecule.[351] Both analogs were reported to exhibit strong inhibition towards AICARFTase

(BW1540, 8nM; BW2315, 6nM) as well as antitumor activity against human colon cancer cells (BW1540 0.7-3.0μM; BW2315 1.0-5.0μM).[351] Other non-folate based AICARFTase inhibitors, 326203-A (2, -[5 hydroxy-3-methyl-1-1(2 methyl-4-sulfophenyl0-H-pyrazol)-4 sulfo-benzoic acid) have been explored, however none of these novel analog have progressed to clinical studies.[353]

Experiments performed by the Moran lab with PMX prompted our own investigation using 5-substituted pyrrolo[2,3-*d*]pyrimidines that indirectly suggested inhibition of the enzyme. Direct quantitation of enzyme inhibition may help to elucidate a role in AICARFTase targeting for the disruption of *de novo* purine synthesis. Using the structural information for both FRα and the AICARFTase enzyme may bring us closer to designing an FR directed inhibitor.

This body of work includes the characterization of novel potent 5- and 6-substituted pyrrolo [2,3-*d*] pyrimidines that are effective against *in vitro* model of HGSC, endometroid carcinoma and clear cell carcinoma (IGROV-1). Approximately 90% of advanced stage ovarian cancers are HGSC as this subtype generally presents at an advanced stage.[232,247] Patients who present in stages III-IV have a 5 year survival rate of 15-30% and generally succumb to complications associated with this disease. The grim statistics of ovarian cancer prompt the clinical development of highly potent compounds with reduced toxicity to allow for treatment at longer intervals to achieve maximum efficacy. We can learn from the failure of the folic acid-conjugated toxin, vintafolide, and design improved folate-based cytotoxins akin to the novel compounds outlined in the study.

The novel compounds examined in these studies are excellent candidates for further development. Pharmacokinetic data and orthotropic mouse models of EOC may enhance and aid in greater support of these analogs as lead compound for more extensive pre-clinical and clinical investigations. All together, we have developed agents with tumor selectivity based on an inherent

loss of purine salvage abilities due to the deletion of MTAP and their overexpression of FRα. FRα-positive tumors treated with novel 6- substituted pyrrolo[2,3-*d*]pyrimidines will undergo cell death due to purine nucleotide depletion via GARFTase inhibition. Such depletion results in a reduction of cellular ATP pools, an induction of cell cycle arrest and eventually apoptosis. The 5- substituted pyrrolo[2,3-*d*]pyrimidines share the unique characteristic of acting as a dual inhibitor of both AICARFtase and GARFTase, albeit the potency of inhibition towards GARFTase is reduced. While the 5-substituted analogs were able to induce ATP depletion, cell cycle aberrations and apoptosis, despite ZMP accumulation (likely resulting from AICARFTase inhibition) they were unable to activate AMPK. The accumulation of ZMP alone, independent of AMPK activation may have been sufficient to initiate a cytotoxic response. There are a number of studies that suggest that ZMP is a toxic metabolite with many cellular targets in addition to AMPK.[354-356] ZMP accumulation has been linked to mitochondrial distress, increased apoptosis in malignant cells in an AMPK-independent manner and a depletion in pyrimidine nucleotide pools.[356] While substitution at the 5th position allows for the addition of an enzyme target, selective FRα-directed membrane transport is compromised and RFC-transport is acquired (**Figure 3.1**). These studies highlight the SAR of novel pyrrolo[2,3-*d*]pyrimidine antifolates . The success of PMX and the expansion of its therapeutic range beyond lung cancer, warrants further investigation of novel pyrrolo[2,3-*d*]pyrimidine antifolates. Using structure based drug design that incorporates the recently discovered crystal structure of FRα along with the structural information of AICARFTase, we will perhaps come closer to designing a potent FRα targeted AICARFTase inhibitor to provide a less toxic, yet efficacious treatment option for ovarian cancer.

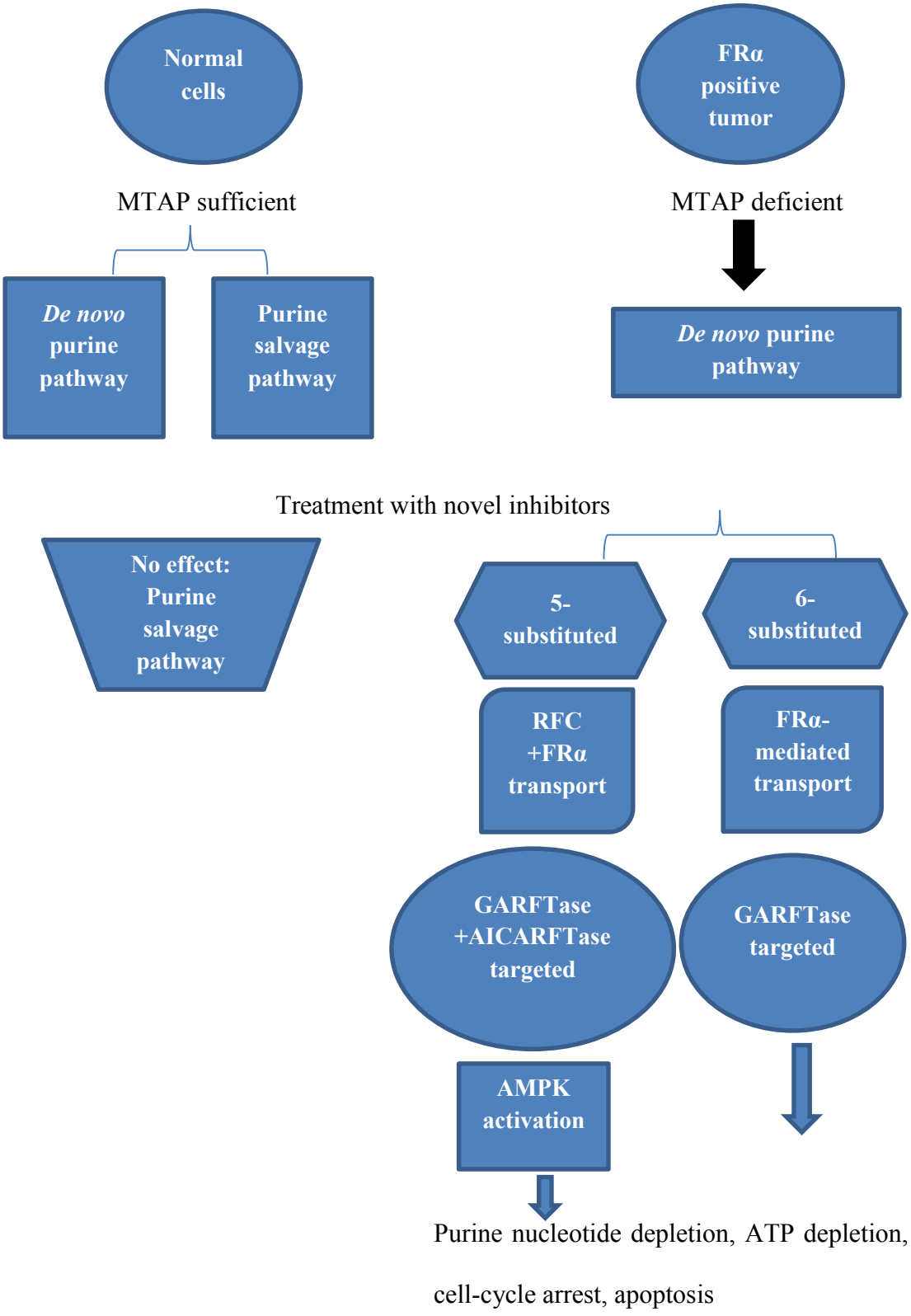

Figure 3.1: "Big picture" schematic: The following schematic highlight the key concepts in this body of work.

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ABSTRACT

THE THERAPEUTIC TARGETING OF FOLATE RECPTOR ALPHA POSITIVE TUMORS VIA FOLATE RECEPTOR-SELECTIVE NOVEL 5-AND 6- SUBSTITUTED PYRROLO [2,3-*D*] PYRIMIDINE ANTIFOLATES

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Ovarian Cancer is the fifth leading cause of cancer-related death of women in the United States. Epithelial Ovarian Cancer (EOC) constitutes 85-90% of malignancies within the ovary, with an alarming majority of these cases diagnosed at advanced stage. While most patients are initially highly responsive to the current treatment standard, there is a very high probability that they will recur with a drug resistant fatal disease. Currently there is no validated comprehensive model of disease progression for ovarian cancer, although tremendous progress has been made in understanding the origin of this disease and a putative precursor lesion has been identified via molecular profiling. This progress has led to the identification of molecular signatures that not only distinguish high grade from low grade tumors, but it also highlights mutations that are unique to each histological type. Even though we are armed with this information and are well within the age of molecular targeting, the treatment of ovarian cancer has remained the same for over 40 years, when the use of platinum-based therapies and taxanes were introduced.

Approximately 90 percent of EOC are folate receptor alpha (FRα) positive with the extent of receptor over-expression corresponding with stage and grade of disease. FRα based therapies

are a subject of increasing interest warranted by a growing number malignancies, from various tissue types, showing FRα expression. Due to this increase of receptor expression in advanced disease in EOC, designing FRα-targeted agents will enhance the therapeutic window in a population of patients where most treatments fail. The clinically approved antifolates, methotrexate (MTX) and pemetrexed (PMX), have greatly improved treatment outcomes in a number of malignancies, however they have been implicated in dose-limiting toxicities that are thought to arise from uptake mediated by the ubiquitously express protein, the reduced folate carrier (RFC). This encourages the development of novel antifolates that utilize other available folate transport mechanisms over RFC.

In this study we explored the impact of the addition of various structural components on a pyrrolo[2,3-*d*]pyrimidine scaffold and demonstrated: (1) the significance of a 5 vs 6 position substitution on the scaffold and how it impacts the pharmacology of the compound; (2) that novel 6-substituted pyrrolo[2,3-*d*]pyrimidines show remarkable potencies that are mediated via FRα membrane transport; and (3) the impact of purine salvage on the efficacy of novel antifolates.

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