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Revitalizing antifolates through understanding mechanisms that govern susceptibility and resistance

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In prokaryotes and eukaryotes, folate (vitamin B₉) is an essential metabolic cofactor required for all actively growing cells. Specifically, folate serves as a one-carbon carrier in the synthesis of amino acids (such as methionine, serine, and glycine), *N*-formylmethionyl-tRNA, coenzyme A, purines and thymidine. Many microbes are unable to acquire folates from their environment and rely on *de novo* folate biosynthesis. In contrast, mammals lack the *de novo* folate biosynthesis pathway and must obtain folate from commensal microbiota or the environment using proton-coupled folate transporters. The essentiality and dichotomy between mammalian and bacterial folate biosynthesis and utilization pathways make it an ideal drug target for the development of antimicrobial agents and cancer chemotherapeutics. In this minireview, we discuss general aspects of folate biosynthesis and the underlying mechanisms that govern susceptibility and resistance of organisms to antifolate drugs.

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1. Introduction

De novo folate biosynthesis begins with synthesis of *para*-aminobenzoic acid (PABA, Fig. 1) and 6-hydroxymethyl-7,8-dihydropterin pyrophosphate (DHPPP, Fig. 2). Both pathways are absent in mammals and essential in bacteria, protozoa and fungi. There are no antimicrobial agents in clinical use that target PABA or DHPPP biosynthesis. As described below, recent efforts have revealed many challenges that must be overcome to target these pathways.

Later steps in folate synthesis involve the conversion of 7,8-dihydropteroate (DHP) to 5,6,7,8-tetrahydrofolate (THF) in three ordered steps by DHP synthase (FolP), dihydrofolate synthase (FolC), and dihydrofolate reductase (DHFR in mammals and Fola in bacteria) (Fig. 3). The only approved therapeutic agents in clinical use that target this portion of the pathway inhibit FolP and DHFR. In this review, we discuss the therapeutic agents that are currently used to target folate biosynthesis in bacteria, protozoa, fungi and mammals.

Antifolates, agents that impair folate biosynthesis, were the first class of antimetabolites to enter clinical use. They were used to treat a variety of cancers and inflammatory disorders in humans and were among the first antimicrobial agents used to treat bacterial infections. Historically, antifolates have targeted the enzymes FolP (sulfonamides and sulfones) and DHFR (methotrexate, proguanil and trimethoprim). Resistance

to antifolates was reported shortly after their introduction into clinical use. Since some antifolates can be associated with adverse reactions, some have been replaced with safer and more effective drugs. With the rise in resistance to many chemotherapeutics and antimicrobial agents and with the advent of better-tolerated antifolates, there has been a renewed interest in using this drug class to treat many diseases.

Sulfonamides were introduced into the clinic in 1935 to treat a variety of bacterial infections. However, some sulfonamides, such as sulfamethoxazole (SMX), were associated with rare adverse reactions such as blood dyscrasias, hypoglycemia, hyperkalemia, and inhibition of cytochrome P450.^{1–3} In 1962, TMP was introduced into the clinic as a bacterial Fola inhibitor. It was noted that SMX and TMP showed potent synergistic activity, permitting the use of lower dosages of SMX that resulted in fewer adverse reactions.⁴ Consequently, co-administration of SMX with TMP (co-trimoxazole) became standard of care for treating many infections. Since co-trimoxazole is generally well-tolerated, relatively inexpensive and can target a broad spectrum of microbes, it is commonly used for prophylactic treatment against opportunistic infections in immunocompromised individuals.

Herein, we primarily focus on antifolates that target microbes and will briefly cover inhibition of mammalian folate metabolism. Further, we examine PABA, DHPPP, and later steps in folate biosynthesis as drug targets and the most recent strategies used to target enzymes in the respective pathways. As all of the antifolates in clinical use are associated with multiple mechanisms of resistance, there is a need to understand the differences in susceptibility to each antifolate.

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Fig. 1 *para*-Aminobenzoic acid biosynthesis pathway in bacteria with known inhibitors.

2. Targeting *para*-aminobenzoic acid biosynthesis in microbes

In most bacteria, fungi, protozoans and plants, folates are synthesized from the convergence of the PABA and pterin biosynthesis pathways. The PABA biosynthesis pathway is absent in the metazoan lineage. The first step to synthesize PABA involves the conversion of chorismate to amino-deoxychorismate (ADC) (Fig. 1). Chorismate is the product of the shikimate pathway, from which aromatic amino acids, quinones and salicylic acid are also derived. In the synthesis of PABA, chorismate and glutamine are converted to ADC and glutamate by the action of ADC synthase. ADC synthase is comprised of two enzymatic subunits PabA and PabB encoded by *pabA* and *pabB*, respectively. Both enzymes require physical interaction for optimal catalysis. PabA catalyzes glutamine lyase activity and is typically only active in the presence of stoichiometric amounts of PabB.^{5,6} In the absence of PabA and glutamine, PabB is able to catalyze the amination of chorismate in the presence of vast molar excess ammonia.⁶ Both *pabA* and *pabB* are predicted to be essential in many bacterial species.

Interestingly, some bacteria do not encode a defined PabA homolog and instead there is functional overlap with an analogous enzyme TrpG from the tryptophan biosynthetic pathway. In tryptophan biosynthesis TrpG functions in conjunction with TrpE to catalyze synthesis of anthranilate from chorismate and glutamine in a reaction similar to that for synthesis of ADC.^{7–11} As TrpG and PabA show a high degree of sequence conservation and have mechanistically identical enzymatic function,^{7,11} organisms such as *Bacillus subtilis*, *Acinetobacter calcoaceticus* and *Salmonella enterica* express a bi-functional (amphibolic) glutamine ammonium lyase that operates as a subunit of both anthranilate synthase and ADC synthase.

The last step in PABA biosynthesis is the conversion of ADC to PABA by ADC lyase encoded by *pabC* (Fig. 1).^{12,13} This reaction proceeds with the elimination of the pyruvyl moiety of ADC enabling subsequent aromatization to PABA. Since

elimination of pyruvate from ADC can occur spontaneously, *pabC* is not predicted to be essential in many bacteria.

In *Mycobacterium tuberculosis* disruption of PABA biosynthesis, specifically through mutagenesis of *pabB* or *pabC*, results in enhanced susceptibility to antifolates, including sulfa-drugs and the diaminophenylsulfone dapsone.¹⁴ There are multiple natural products that inhibit PabB including abyssomicin C and anthelminthycin C and their derivatives (Fig. 4).^{15–19} These compounds show inhibitory activity against *M. tuberculosis* and methicillin resistant *Staphylococcus aureus* (MRSA) in whole cell assays.^{17–21} Evaluation of their activity in animal models of infection has not been reported. Abyssomicin C and some of its derivatives display a high level of cytotoxicity against mammalian cells.¹⁸ Although, atrop-*O*-benzyl-desmethylabyssomicin C was found to have significantly reduced cytotoxicity whilst retaining activity against MRSA and may represent a promising lead (Fig. 4).¹⁹ In addition to these derivatized natural products, the synthetic dichloronitrophenyl propanone, MAC173979, was found to inhibit PABA biosynthesis in *E. coli* both enzymatically and in whole cell assays.²² MAC173979 exhibited time-dependent inhibition in a one-pot assay using purified recombinant PabA, PabB, and PabC, yet, the exact target of MAC173979 is not known (Fig. 4).²² MAC173979 also showed potent activity against *M. tuberculosis* that could be antagonized by exogenously supplied PABA.¹⁴ Consistent with genetic studies described above, synergistic inhibition of growth was observed when *M. tuberculosis* was treated with MAC173979 in combination with various sulfa-drugs or dapsone.¹⁴ Taken together, the PABA biosynthesis pathway remains a promising drug target especially in combination with sulfa-drugs.

3. Targeting pterin biosynthesis in prokaryotes and eukaryotes

Prokaryotes and eukaryotes synthesize pterins, albeit, the metabolic functions they serve can be quite distinct. For instance, in humans, guanine cyclohydrolase (encoded by

Fig. 2 Pterin biosynthesis and inhibitors in bacteria and mammals.

GCH1) catalyzes GTP hydrolysis, isomerization and cyclization to form 7,8-dihydroneopterin triphosphate (Fig. 3), a pre-

cursor in tetrahydrobiopterin synthesis. Tetrahydrobiopterin is a critical co-factor for the synthesis of aromatic amino

Fig. 3 Folate biosynthesis and inhibitors in bacteria and eukaryotes.

acids and multiple neurotransmitters.²³ Deficiencies in the production of tetrahydrobiopterin results in dystonia, or sustained muscle contractions. Bacteria, fungi, protozoans and plants also encode a guanine hydrolase (FolE, encoded by *folE*) that performs the same biochemical function as *GCH1*. However, in this context, 7,8-dihydroneopterin triphosphate is essential for folate biosynthesis (Fig. 2). FolE requires a zinc ion to be bound that allows for subsequent binding of the purine ring of GTP. Following binding, GTP is cleaved and isomerized to allow for the closure of the dihydropyrazine ring.^{24–26} Interestingly, 8-aminoguanosine tri-

phosphate and other structural analogs of GTP, can competitively inhibit bacterial FolE and *GCH1* by mimicking the transition state between GTP and 7,8-dihydroneopterin triphosphate (Fig. 5).^{24,27}

In addition to FolE, bacteria encode other distinct classes of GTP glycohydrolases, including *GCH1b* and *RibA*.²⁶ *GCH1b* is found in about 20% of eubacteria and lacks sequence similarity with FolE. Unlike FolE, *GCH1b* is not zinc-dependent and can utilize a variety of divalent cations for catalytic activity.²⁶ Interestingly, in eubacteria encoding both FolE and *GCH1b*, FolE is constitutively expressed while

Fig. 4 Chemical structures of inhibitors of PABA biosynthesis.

Fig. 5 Chemical structures of pterin biosynthesis inhibitors.

GCH1b is only expressed during zinc starvation.^{26,28} Since GCH1b is not present in humans, it represents a novel antibacterial target, specifically for *Staphylococcus aureus* and *Neisseria gonorrhoeae*, as both organisms lack GCH1.²⁸

RibA was first characterized in *E. coli* as a novel enzyme that catalyzed the release of 2,5-diamino-6- β -ribosyl-4(3H)-pyrimidinone 5'-phosphate (DARP) and formate from GTP.²⁹ DARP is required for the synthesis of riboflavin, an essential cellular cofactor required for growth of bacteria. RibA contains no amino acid sequence homology to FolE. Although, like FolE, RibA is also a zinc dependent enzyme that is responsible for the opening of the guanine ring.³⁰ In most bacteria, RibA catalyzes the first step in riboflavin biosynthesis, producing DARP.³¹ Many pathogenic bacteria lack the ability to transport riboflavin from the extracellular environment making RibA essential. Since RibA is absent in higher eukaryotes, it represents an attractive target for antimicrobial drug discovery.

In the next step of DHPPP synthesis, 7,8-dihydroneopterin triphosphate is dephosphorylated by a cytoplasmic Nudix hydrolase to produce 7,8-dihydroneopterin (Fig. 2). Nudix hydrolases form a superfamily of pyrophosphatases that are often involved in detoxifying toxic metabolites. The first 7,8-dihydroneopterin triphosphate pyrophosphatase Nudix hydrolase enzyme to be described was YlgG, encoded by *ylgG*, in *Lactococcus lactis*.³² Since YlgG did not show sufficient sequence similarity to Nudix enzymes of bacteria with known genome sequences, identifying other Nudix hydrolases involved in pterin synthesis has been quite challenging. The product of the gene *orf17*, NudB (or NtpA), was the first dihydroneopterin triphosphate pyrophosphatase described in *E. coli*.^{33,34} Nudix enzymes hydrolyze substrates through the use of a nucleophilic substitution and require a metal ion for coordination of GTP, though the specific metal varies between Nudix enzymes.^{32–35} Since dihydroneopterin triphosphate pyrophosphatases have only been recently discovered, inhibitors of this enzyme have yet to be identified. Nevertheless, deletion of *nudB* in *E. coli* has been shown to dramatically enhance susceptibility to both SMX and TMP.^{36,37} Thus, these recent discoveries open avenues for novel therapeutic discovery.

Once formed, 7,8-dihydroneopterin is converted to 6-hydroxymethyl-7,8-dihydropterin by dihydroneopterin aldolase (FolB) yielding glyceraldehyde as a byproduct (Fig. 2). FolB, encoded by *folB*, is predicted to be essential in bacteria. Most aldolases require a Schiff base for the cleavage of the carbon-carbon double bond and a zinc ion for catalysis, both of these features are targets for selective aldolase inhibitors.³⁸ FolB is a unique aldolase as it requires neither a Schiff base for cleavage nor a zinc ion for catalysis.^{39,40} Due to its small active site binding pocket and unique catalytic mechanism, designing FolB inhibitors has been challenging. In some organisms, dihydroneopterin aldolase is a bi-, or in some parasites, a tri-functional enzyme. For instance, in *Streptococcus pneumoniae*, dihydroneopterin aldolase (SulD, encoded by *sulD*) is a bi-functional enzyme that encodes 6-hydroxymethyl-

7,8-dihydropterin pyrophosphokinase that can convert 7,8-dihydroneopterin to 6-hydroxymethyl-7,8-dihydropterin pyrophosphate.^{41,42} The opportunistic pathogen *Pneumocystis jiroveci* encodes a tri-functional enzyme that encodes a dihydroneopterin aldolase and 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase, as well as a dihydropteroate synthase.⁴³ These multi-functional enzymes have been extensively targeted for development of antimicrobial inhibitors.⁴⁴

Most FolB inhibitors mimic the pterin or pyrimidine moiety of 7,8-dihydroneopterin.⁴⁵ A high-throughput X-ray crystallography screen and structure-directed lead optimization discovered multiple potent inhibitors of dihydroneopterin aldolase from *S. aureus* including 9-methylguanine, 2-amino-5-carboxyethylpyrimidine-4-one, 8-amino-1,3-dimethyl-3,7-dihydropurine-2,6-dione, and 2-amino-5-bromo-6-phenylpyrimidine-4-one⁴⁶ (Fig. 5). Unfortunately these compounds do not possess whole cell inhibitory activity against *S. aureus*, *Haemophilus influenzae*, *E. coli*, *Enterococcus faecium* and *Streptococcus pneumoniae*.

The last step in pterin biosynthesis for folates requires the conversion of 6-hydroxymethyl-7,8-dihydropterin to DHPPP via the enzyme 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase (FolK, encoded by *folK* in bacteria) (Fig. 2). In bacteria, disruption of FolK results in a pronounced growth defect.⁴⁷ FolK is a unique pyrophosphokinase because it transfers a pyrophosphate from the beta phosphate of ATP rather than a more conical gamma phosphate yielding AMP.⁴⁸ FolK has been studied for the development of novel bisubstrate analog inhibitors allowing for selective inhibition of this target.^{49–51} Most of the bisubstrate inhibitors developed mimic the ATP phosphate donor and acceptor (serine, threonine, or tyrosine). Some such compounds include P1-(6-hydroxymethylpterin)-P4-(50-adenosyl)tetraphosphate (HP₄A) and 50-S-[1-(2-[(2-amino-7,7-dimethyl-4-oxo-3,4,7,8-tetrahydropteridin-6-yl)carbonyl]amino)ethyl]piperidin-4-yl]-50-thioadenosine (HP-18) (Fig. 5).⁵² Despite both compounds potently inhibiting purified FolK, they showed limited whole cell activity. 8-Mercaptoguanine (Fig. 5) has also been shown to inhibit *S. aureus* FolK by acting as a non-competitive inhibitor.^{53,54} Although 8-mercaptoguanine is a potential scaffold for discovery, its activity against *S. aureus* has not been reported and it is likely to show polypharmacology through interaction with other proteins with purine binding domains. In bacteria, the pterin biosynthetic pathway is highly conserved, however, the enzymes are structurally distinct and may ultimately allow for selective targeting.⁵⁵

4. Targeting folate biosynthesis in prokaryotes and eukaryotes

DHP is produced from the condensation of PABA and DHPPP with elimination of pyrophosphate via DHP synthase (DHPS, encoded by *folP*) (Fig. 3). FolP is a homodimer and is the a target of many antimicrobial agents including sulfonamides and diaminodiphenyl sulfones (Fig. 6). Both drug classes are analogs of PABA. Sulfonamides, such as sulfamethoxazole (SMX), are broad spectrum antimicrobial agents that inhibit FolP by creating the dead-end product sulfamethoxazole-dihydropterin.^{56–58}

Fig. 6 Chemical structures of folate biosynthesis inhibitors.

Another inhibitor of FolP is 8-mercaptopguanine that has been shown to have promiscuous activity by inhibiting many enzymes that have a substrate bearing a purine moiety.⁵⁹ Since FolP is an important drug target, there have been many structural and biochemical studies focused on designing more po-

tent FolP inhibitors^{60,61} during the catalytic cycle DHPPP binds first, and the pyrophosphate is removed in a magnesium dependent reaction.^{57,58,61,62} Once the pyrophosphate is eliminated, the PABA binding pocket opens and allows for binding of PABA, sulfonamides or diaminodiphenyl sulfones.^{57,61,63,64}

Sulfonamides and sulfones bind the PABA binding pocket that is separate from the pterin binding pocket. To develop novel classes of inhibitors, there is an increased interest in targeting the pterin binding pocket. Recently, a series of novel 4,5-dioxo-1,4,5,6-tetrahydropyrimido[4,5-*c*]pyridazine compounds were designed to inhibit the pterin binding pocket of purified recombinant *Bacillus anthracis* FolP (Fig. 6).⁶⁵ These compounds potently inhibited through binding within the pterin binding pocket and provided structure activity relationship information for designing new inhibitors. Furthermore, pterin-sulfonamide conjugates have been found to competitively inhibit FolP and inhibition was enhanced in the presence of pyrophosphate.⁶⁶ The pterin-sulfonamide conjugates displayed antimicrobial activity against *E. coli* that could be antagonized by PABA, and to a lesser extent by methionine.⁶⁶

One challenge in designing small molecules that inhibit the pterin binding pocket results in pterin-like molecules that have poor solubility.⁶⁵ Therefore, there is an interest in inhibitors that bind outside of the active site. Hammoudeh and colleagues performed a fragment based lead discovery and found fragments bound at the dimer interface.⁶⁷ Specifically, *N*-(4-(trifluoromethyl)-benzylidene)-1-(4-(trifluoromethyl)-benzylamine) inhibited multiple FolP enzymes from *B. anthracis*, *Yersinia pestis*, and *S. aureus*; however, the compound lacked antimicrobial activity.⁶⁷

Many sulfonamides and sulfonylurea-based drugs, such as sulfasalazine, sulfathiazole, sulfapyridine, sulfamethoxazole, and chlorpropamide, are associated with adverse reactions, most commonly allergies, that can limit the usage in HIV patients. Furthermore, sulfa-drugs can cause toxicity through off target effects in humans. These compounds have been found to inhibit the NADPH dependent enzyme sepiapterin reductase.^{68,69} Sepiapterin reductase is an essential enzyme that is required for synthesis of tetrahydropterin, a critical co-factor for neurotransmitter synthesis in mammals.

DHP is converted to 7,8 dihydrofolate (DHF) by the ligation of L-glutamate using the ATP-dependent enzyme DHF-synthase (FolC, encoded by *folC*) (Fig. 3). In many organisms, such as *E. coli*, *Corynebacterium* sp., and *P. falciparum*, this enzyme is bi-functional and can catalyze the gamma linkage of additional L-glutamate residues to fully reduced folate species.⁷⁰⁻⁷² Polyglutamates are important in bacteria for methionine synthesis.⁷³ In humans, folates are polyglutamylated by the enzyme downstream of dihydrofolate reductase, folypolyglutamate synthase (FPGS). In eukaryotes, polyglutamylation is important for retaining reduced folates in subcellular compartments. The role of polyglutamylation has not been extensively studied in bacteria, but, it is conceivable that the negatively charged polyglutamate tail may prevent folate species from diffusing across the cytoplasmic membrane.

Both FolC and FPGS are members of the Mur superfamily of enzymes and both contain an ATPase domain.^{55,74} ATP is positioned in a narrow channel and is stabilized by two magnesium ions.⁷⁵ In the crystal structure of many apo-FolC enzymes the pterin binding site is not well resolved, however, the active site becomes more ordered upon the binding of

DHP.⁷⁴⁻⁷⁶ Interestingly, in bacteria, FolC has an induced fit pterin binding pocket to allow mono- and poly-glutamylated folates to bind.⁷⁷⁻⁷⁹ The conversion of DHP to DHF generates a tetrahedral intermediate through the transfer of a phosphate from ATP to DHP.^{80,81} Glutamate will react with the intermediate to produce DHF. Attempts to target the tetrahedral transition state have yielded initial hits against purified FolC but none have shown whole cell activity.^{82,83}

The last step in folate biosynthesis is the reduction of DHF to THF by the NADPH dependent enzyme dihydrofolate reductase (FolA in bacteria, DHFR in eukaryotes) (Fig. 3). Although most enzymes in the folate biosynthesis pathway are essential, FolA in bacteria can be conditionally essential. In *E. coli*, *folA* can be deleted following disruption of the gene for thymidylate synthase,⁸⁴ a folate-dependent enzyme required for thymidine synthesis. Unlike other folate-dependent enzymes thymidylate synthase yields DHF rather than THF. Thus, while elimination of this activity confers thymidine auxotrophy, it decreases the demand for folate reduction which can be inefficiently catalyzed by FolM.⁸⁵ In *E. coli*, FolM functions as a dihydromonapterin reductase. FolA reduces DHF using NADPH as an electron donor to THF and with the release of NADP⁺. Under steady state reaction conditions, the catalytic mechanism of DHFR is stepwise.^{86,87} The reaction begins with NADPH bound to the enzyme (E: NADPH), next DHF binds which creates the Michaelis complex (E: NADPH: DHF). Once DHF binds, the hydride transfer occurs (E: NADP⁺: THF). The exact biochemical mechanism of the hydride transfer is not well understood. To date there are two proposed mechanisms, the first states the hydride transfer is pH dependent and occurs by an ionizable side chain in the active site.⁸⁸ The second mechanism states the pH dependent hydride transfer is solvent dependent.⁸⁹ NADP⁺ is released (E: THF), another NADPH binds (E: NADPH: THF), and finally THF is released in the rate determining step to prime the enzyme for another catalytic cycle.

There are many drugs that selectively inhibit DHFR in humans, bacteria, fungi, and protozoa. DHFR inhibitors in humans and protozoans generally mimic the substrate folate. One class of inhibitors, the diaminopteridines, include methotrexate (MTX) and aminopterin (Fig. 6), both are used to treat cancers and autoimmune disorders.⁹⁰ Diaminopteridines are transported into human cells by folate receptors. Since, folate receptors are highly upregulated by as much as 100-fold in cancer cells, MTX and other diaminopteridines potently target these cells compared to non-cancerous cells.⁹¹ Not only do folate receptors recognize folate and folate analogs but they also recognize polyglutamylated folate species. As described above, polyglutamylated species are retained within cellular compartments. Thus, this mechanism for retention of folates has been co-opted to promote accumulation DHFR inhibitors, such as pralatrexate (Fig. 6), within target cells.⁹²

Aminopterin (Fig. 6) was the first human DHFR inhibitor to enter clinical use for the treatment of childhood lymphocytic leukemia. Aminopterin was associated with high toxicity and was quickly replaced with MTX (Fig. 6) which was found

to be as effective, but less toxic. MTX is known to inhibit all purified DHFRs, including those from bacteria, fungi and protozoans, and is characterized as a slow, time-dependent, tight-binding competitive inhibitor.^{93,94} Binding of MTX results in a slow conformational change in the enzyme active site and is known to bind almost identically to folate by forming similar hydrogen bonding within the active site. One notable difference occurs when the pterin ring of MTX is flipped 180° with respect to DHF. This flip allows hydrogen bonding with the ionizable group in the active site.⁹⁵ From a mechanistic perspective, this flip within the active site provided key insight that a keto-enol equilibrium pertaining to the transition state of the enzyme.⁹⁶ Furthermore, this flip suggests that THF release is assisted by NADPH binding *versus* NADP⁺ binding in NADP⁺ rich environments of bacteria.⁹⁷

Since most pathogenic bacteria lack folate receptors and cannot actively take up this cofactor, antibacterial Foa inhibitors are not close structural analogs of folate. In general, bacterial Foa inhibitors must diffuse across the cell envelope or require folate independent transport.^{98,99} The structure of many of these inhibitors leaves them vulnerable to numerous efflux pumps.^{98,99} One notable bacterial Foa inhibitor, trimethoprim (TMP, Fig. 6), contains a 2,4-diaminopyrimidine ring that allows for critical hydrogen bonding in the active site. TMP binds much more tightly (about 10⁵) to bacterial Foa than mammalian DHFR.¹⁰⁰ The benzyl ring of TMP undergoes a thermodynamically unfavorable flip in the apo-enzyme. However, in the presence of NADPH the flipping of the benzyl ring is decreased. Therefore, in bacteria, TMP is a competitive inhibitor with respect to DHF and a non-competitive inhibitor with respect to NADPH. Conversely, mammalian DHFR can bind TMP but cannot form key hydrogen bond contacts within the active site.¹⁰¹

TMP was first introduced into the clinic in 1962. Resistance was described as early as 1968, therefore, TMP derivatives were synthesized and explored for antimicrobial activity. Two notable TMP derivatives are tetroxoprim used for the treatment of *Pneumocystis pneumonia* and iclaprim for treatment of methicillin and vancomycin resistant strains of *Staphylococcus aureus* as well as drug resistant strains of *Streptococcus* species (Fig. 6).^{102,103} Trimetrexate is a quinazoline derivative and is a chimera of pterin moiety of methotrexate and contains the benzyl ring of TMP. Iclaprim, like TMP, is a diaminopyrimidine that contains a chromene ring in place of the benzyl ring of TMP that favors more hydrophobic interactions in the active site.^{102,104}

The first anti-malarial antifolate drug discovered was the biguanide proguanil which is one of the components of the widely used malaria prophylactic drug malarone. Shortly after its discovery, proguanil was found to be a pro-drug that is metabolized by the host CYP2C19 to cycloguanil (Fig. 6).^{105,106} This activated form was found to inhibit *Plasmodium falciparum*, the most prevalent causative agent of malaria, through targeting of DHFR. Many proguanil derivatives, including the cycloguanil structural analog pyrimethamine, have since been developed to counter drug resistant strains of

P. falciparum.¹⁰⁷ Pyrimethamine (Fig. 6) showed excellent efficacy in humans because of the low dosage required for treatment and paucity of side effects.¹⁰⁸ However, pyrimethamine resistance quickly emerged which diminished its use in treatment of malaria. Pyrimethamine has been repurposed to treat toxoplasmosis and is used in combination with dapsone to treat *Pneumocystis pneumonia* in HIV infected individuals.

The dihydrotriazine, WR99210, is an anti-malarial DHFR inhibitor that was developed after emergence of resistance to pyrimethamine and cycloguanil (Fig. 6). WR99210 potently inhibits DHFR at very low concentrations, in the nano- to picomolar range. Unfortunately, in human clinical trials, daily administration of 200 mg of WR99210 for three days resulted in severe gastrointestinal distress. As a result, further clinical investigation into WR99210 stalled. To counter WR99210 toxicity, the biguanide pro-form PS-15 (*N*-(3-(2,4,5-trichlorophenoxy)propyloxy)-*N'*-(1-methylethyl)-imidocarbonimidic diamide hydrochloride) was synthesized based on proguanil that could be activated *in vivo* (Fig. 6).¹⁰⁹ PS-15 showed improved antimalarial activity and it is anticipated that it will be better tolerated than WR99210. Importantly, PS-15 was found to selectively inhibit *P. falciparum* DHFR and had little activity against mammalian DHFR. Unfortunately, large-scale synthesis of PS-15 is challenging for drug production and has caused its development to lag. However, with this scaffold, other WR99210 analogs have been explored as novel DHFR inhibitors not only in *Plasmodia* species, but also in *M. tuberculosis*, *Mycobacterium avium* and *P. jiroveci*.^{110,111}

The last Foa inhibitor that we will discuss is *para*-aminosalicylic acid (PAS, Fig. 6). PAS is an antitubercular agent used to treat drug resistant strains of *M. tuberculosis*.¹¹² PAS is a structural analog of PABA, possessing a hydroxyl-group in the *ortho*-position to the carboxylic acid. Soon after its discovery, PAS was thought to disrupt folate biosynthesis based on the observation that exogenous PABA could antagonize its inhibition of *M. tuberculosis* growth.¹¹³ Initially, PAS was thought to act as a Foa inhibitor similar to sulfonamides. However, no cross-resistance between PAS and sulfonamides in *M. tuberculosis* was observed.¹¹⁴ Further, in contrast to sulfonamides, PAS was found to only weakly inhibit purified Foa enzymatic activity and was later demonstrated to be converted to a hydroxylated version of DHP, hydroxy-DHP.^{115–117} Foa was found to catalyze the glutamylation of hydroxy-DHP to hydroxy-DHF (Fig. 3 and 6), which suggested that Foa is not the target of bioactivated PAS and the target was likely downstream within the folate pathway.^{115–117} It was predicted and later confirmed that hydroxy-DHF could potentially inhibit Foa.^{115,117,118} Interestingly, PAS has been shown to only have activity against *M. tuberculosis*, although the mechanistic basis for this high level of selectivity is not known.^{119–123}

5. Antifolate synergy

Currently, TMP and SMX are rarely given in monotherapy. When used in combination, TMP and SMX (co-trimoxazole) exhibit highly synergistic antimicrobial activity. Due to differences in drug metabolism rates, they are given in a 1:5

(TMP:SMX) ratio to achieve serum concentrations that are typically observed to be synergistic. Co-trimoxazole is used to treat a variety of microbial infections including respiratory tract infections, otitis media, urinary tract infections, MRSA infections, and cholera. With the emergence of HIV, co-trimoxazole is often given as lifetime prophylactic therapy to treat and prevent opportunistic infections, such as *Pneumocystis pneumonia* and toxoplasmosis, in individuals with acquired immunodeficiency syndrome. When SMX is not well-tolerated, dapsone is used as a surrogate. Given the success and global availability of highly active antiretroviral therapy for HIV, there is ongoing debate regarding the continued need for lifetime co-trimoxazole prophylactic therapy.

For several decades it was presumed that the basis for synergy between SMX and TMP was exclusively due to the ability of SMX to prevent DHF synthesis and enhance the ability of TMP to inhibit Fola.^{37,124,125} While this model explains how SMX is able to enhance microbial susceptibility to TMP, it is not sufficient to explain the ability of TMP to enhance susceptibility of microbes to SMX.¹²⁶ It was recently demonstrated that TMP treatment results in depletion of DHPPP through impairment of GTP synthesis and is the basis for potentiation of SMX susceptibility.³⁷ Thus, it is the cyclic nature of the folate biosynthetic pathway that enables the potent synergy between SMX and TMP.³⁷

6. Antifolate resistance

While folate metabolism remains a powerful target for drug discovery and development, there has been a surge in resistance to many anti-folates. This section will focus on the broad resistance mechanisms to sulfonamides, TMP, PAS and MTX.

There are multiple mechanisms that can confer broad resistance to sulfonamides and were found to emerge shortly after entrance of this drug class into clinical use. One such resistance mechanism involves single amino acid point mutations in the chromosomal *folP* (Fig. 7). Among clinically resistant isolates, many have been characterized that have mutations that result in alteration of the ternary structure and prevent productive interaction with sulfonamides.¹²⁷ In the case of the pathogen *S. pyogenes*, clinical resistance to sulfonamides emerged much faster than in other most organisms and was the result of horizontal acquisition of a plasmid encoding a sulfonamide resistant FolP variant.¹²⁸ Similarly, in many Gram-negative bacteria, resistance to sulfonamides has been traced to mobile genetic elements bearing *sul* genes that encode fully functional FolP variants that fail to interact with sulfonamides (Fig. 7).¹²⁹ In early reports of this resistance mechanism, *E. coli* was found to transfer sulfonamide resistance to *Shigella* species via a Tn21 family transposon encoding *sul1* or *sul2*.¹³⁰ *Sul1* and *Sul2* both have dihydropteroate synthase activity.¹³¹ While *Sul1* and *Sul2* were found to bind PABA with similar affinity as FolP, their affinity for sulfonamides was 10 000 times lower.¹³¹ Tn21 family transposons have since been found to be broadly distributed amongst enteric bacteria that are resistant to sulfonamides.¹³² Recent evidence suggests that the *sul* genes originated in the

Gram-negative bacterial orders *Rhodobiaceae* and *Leptospiraceae* prior to the development of sulfonamides and were subsequently distributed amongst human associated microbes with broad clinical use of sulfonamides.¹³³

Another factor that limits the use of sulfonamides is intrinsic resistance through methionine-associated metabolism. Shortly after discovery of the sulfonamides, it was found that exogenously supplied methionine could potentially antagonize the antimicrobial action of these drugs in numerous bacterial species.¹³⁴ While the mechanism governing this antagonism has not been fully elucidated, it has been presumed to occur through methylation-dependent inactivation. In the context of *M. tuberculosis*, sulfonamides are known to inhibit purified recombinant FolP, yet, they show poor activity against intact bacilli.¹³⁵ The related drug dapsone was initially developed as an antitubercular agent and also inhibits purified recombinant *M. tuberculosis* FolP, but, like sulfonamides shows limited activity against whole cells.¹³⁵ In support of a model for methyl inactivation, it has been demonstrated that *M. tuberculosis* rapidly metabolizes sulfonamides and dapsone to *N*-methyl and *N,N*-dimethyl species.¹¹⁷ In addition to methyl inactivation, it has recently been demonstrated in *E. coli* and *M. tuberculosis* that methionine also mediates antagonism of antifolates through affecting synthesis of folate precursors.^{37,136} Beyond the problem of intrinsic resistance, sulfonamides show limited activity against intracellular bacilli, suggesting that it may be difficult to achieve therapeutically relevant concentrations needed to target organisms like *M. tuberculosis* in their *in vivo* niche.¹³⁷

Like SMX, TMP resistance can occur by a variety of mechanisms including intrinsic resistance, target modification or overexpression, and acquisition of mobile genetic elements (Fig. 7). Many bacterial species are naturally resistant to therapeutically relevant concentrations of TMP including *M. tuberculosis*, *Bacteroides* species, *Clostridium* species, and *Neisseria* species.^{138,139} In the case of *M. tuberculosis*, it was found that the native Fola Tyr100 residue was responsible for the weak binding of TMP.¹⁴⁰ Modification of Tyr100 to phenylalanine resulted in increased affinity for TMP.¹⁴⁰ It has been reported that some strains of *E. coli*, *S. aureus*, and *H. influenzae* harbor spontaneous mutations in the promoter region of *folA* that confer resistance through increased expression of Fola.^{138,141,142} In addition, it has been shown that TMP resistance in *S. aureus*, *S. pneumoniae* and *H. influenzae* can arise from mutations that result in a single amino acid substitutions in Fola.^{141,143,144} The mechanism that is primarily associated with TMP resistance in both Gram-positive and Gram-negative bacteria involves acquisition of mobile genetic elements encoding variants of Fola (encoded by *dhfr* or more recently *dfr*).^{145,146} To date there are over 30 different mobile genetic elements encoding unique *dfr* genes and are classified as either type I or type II variants.¹⁴⁷ The type I variants share sequence similarity with bacterial *folA* and are typically located on the site-specific integron Tn7.¹⁴⁸ In contrast, type II variants are often found on conjugal R plasmids and that lack discernable sequence similarity to *folA*.¹⁴⁹

Another common PAS resistance mechanism involves loss-of-function mutations in *thyA*, encoding a non-essential thymidylate synthase (ThyA) in *M. tuberculosis* that is responsible for the 5,10-methylene-tetrahydrofolate dependent conversion of dUMP to dTMP.^{116,150,151} When functional, ThyA releases DHF that must be re-reduced to be utilized in folate metabolism. *M. tuberculosis* encodes an alternate thymidylate synthase (ThyX, encoded by *thyX*) that regenerates THF from 5,10-methylene-THF following catalysis.¹⁵² Thus, in contrast to ThyA, ThyX places limited demand of FoaA for maintaining reduced folate pools. In contrast to other pathogens that do not encode a ThyX ortholog and are attenuated upon mutational inactivation of *thyA*,^{153,154} *M. tuberculosis* *thyA* loss-of-

function mutants are not compromised for fitness growth and survival *in vivo*.

To date, few *folA* missense mutations associated with PAS resistance have been reported.¹⁵² Numerous studies have reported that alterations in the Fola active site can be highly deleterious to the overall enzymatic function. Thus, it could be rare for *folA* point mutations to confer resistance to PAS without compromising Fola enzymatic activity. One paper recently described five independent clinical PAS resistant isolates containing a deletion of the *folA-thyA* coding sequence.¹⁵⁵ These strains were 26 times more resistant to PAS than susceptible control strains. Unlike many bacteria, *M. tuberculosis* does not encode a FolM ortholog that would be expected to compensate for loss of Fola activity. Instead, this compensation likely comes from weak DHF reductase activity that can be catalyzed by the 5-amino-6-(5-phosphoribosylamino)uracil reductase RibD that normally functions in riboflavin synthesis.^{72,116} Consistent with this model, *ribD* promoter mutations have been described that confer PAS resistance and were shown to render *folA* as non-essential in *M. tuberculosis*.^{116,150}

Like TMP, PAS is prone to exclusion from the cytoplasm by the action of efflux pumps (Fig. 7). Overexpression of the major facilitator superfamily protein Tap (Rv1258c) in *Mycobacterium bovis* BCG was found to confer resistance to PAS.¹⁵⁶ Furthermore, overexpression of Tap in *M. tuberculosis*, *M. fortuitum* and *M. bovis* BCG was found to confer resistance to a variety of other antimicrobial agents that do not target folate biosynthesis including streptomycin, vancomycin, and tetracycline.^{157–159}

In humans resistance to MTX can arise and can lead to treatment failure. Several MTX resistance mechanisms have been described to date. One common mechanism for acquired MTX resistance stems from a defect in transport of the drug into target cells and is frequently observed in the context of acute lymphocytic leukemia.¹⁶⁰ One of the most frequently observed MTX resistance mechanism associated with acute non-lymphoblastic leukemia involves impaired polyglutamylation, a process that is normally associated with cytosolic retention of folates.^{92,161–163} To overcome this resistance mechanism, analogs of MTX that show better intracellular retention independent of polyglutamylation, such as pralatrexate, have been employed.¹⁶⁴ Other mammalian antifolate resistance mechanisms involve overexpression and missense mutations in DHFR.^{165,166} Interestingly, increased DHFR expression has been linked to mutations in *p53* (a tumor suppressor gene), *E2F* (a transcription factor in cell cycle regulation), and genes that are involved in regulation of thymidylate synthase.^{166–168} Similar to that which is observed for PAS resistance in *M. tuberculosis*, DHFR active site mutations that confer MTX resistance are rare but have been observed in the laboratory.^{169,170}

7. Conclusions and prospective

Antimicrobial agents and cancer chemotherapeutics have transformed treatment of disease in humans. With the criti-

cal role of folate as a cofactor in synthesis of DNA, RNA, protein and other essential cellular metabolites, folate biosynthesis is a highly attractive target for drug discovery. There has been a renewed interest in targeting the folate biosynthesis pathway for treating infectious diseases and cancer due to resistance to other chemotherapeutics. Selective inhibition of mammalian or bacterial enzymes is an achievable goal due to fundamental differences in microbial and mammalian pathways for synthesis and utilization of this cofactor. Even structural differences in bacterial enzymes within the folate biosynthesis pathway allow for selective bacterial targeting and facilitate development of inhibitors with fewer off target effects on the microbiome and the human host.

Some outstanding questions exist regarding the selectivity of antifolates. Why are some bacteria naturally resistant to certain Fola inhibitors? For instance, shortly after introduction of TMP into clinic use it was noted that several bacteria, especially Gram-negative enteric bacteria, showed intrinsic resistance. Further, the antitubercular Fola inhibitor PAS, is exquisitely selective for *M. tuberculosis*. Since TMP and PAS are not naturally occurring molecules what was the basis for emergence of distinct Fola structures and can these distinct features be exploited for selective targeting of microbes? Understanding the mechanisms that drive susceptibility and resistance could allow for design of highly selective next generation inhibitors.

With the increasing interest in the association between commensal microbiota and human health, it would be interesting to understand the consequences of antifolate treatment on the gut microbiota. For instance, in humans, bacteria of the large intestine are an important source of folate. During antifolate treatment, what are the consequences of decreased folate production on colonic enterocytes? Recent studies have found that both an increase and decrease in folate production in the colon are linked to colorectal cancer.^{171,172} Conversely, since there is interplay between bacteria found in the gastrointestinal tract, mucosal immunity and intestinal enterocytes, what are the consequences of antifolate treatment on this dynamic relationship?

Rapid evolution of antimicrobial drug resistance continues to threaten global healthcare. Resistance to antifolates has been broadly observed during cancer chemotherapy and in treating microbial infections. Our review highlights the recent understanding of the mechanisms that govern susceptibility and resistance. Despite the increasing scourge of resistance the folate biosynthesis pathway provides novel selective avenues of chemotherapeutic drug discovery.

Conflicts of interest

The authors declare no competing interest.

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