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MINIREVIEW

Mycobacterium tuberculosis Folate Metabolism and the Mechanistic Basis for *para*-Aminosalicylic Acid Susceptibility and Resistance

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***para*-Aminosalicylic acid (PAS) entered clinical use in 1946 as the second exclusive drug for the treatment of tuberculosis (TB). While PAS was initially a first-line TB drug, the introduction of more potent antitubercular agents relegated PAS to the second-line tier of agents used for the treatment of drug-resistant *Mycobacterium tuberculosis* infections. Despite the long history of PAS usage, an understanding of the molecular and biochemical mechanisms governing the susceptibility and resistance of *M. tuberculosis* to this drug has lagged behind that of most other TB drugs. Herein, we discuss previous studies that demonstrate PAS-mediated disruption of iron acquisition, as well as recent genetic, biochemical, and metabolomic studies that have revealed that PAS is a prodrug that ultimately corrupts one-carbon metabolism through inhibition of the formation of reduced folate species. We also discuss findings from laboratory and clinical isolates that link alterations in folate metabolism to PAS resistance. These advancements in our understanding of the basis of the susceptibility and resistance of *M. tuberculosis* to PAS will enable the development of novel strategies to revitalize this and other antimicrobial agents for use in the global effort to eradicate TB.**

Mycobacterium tuberculosis is responsible for approximately 8.6 million new cases of active tuberculosis (TB) infection and 1.3 million deaths annually despite the existence of TB therapy (1). While this therapy has a high success rate in curing drug-susceptible TB infections, it is challenging, in part because it requires a minimum of 6 months of treatment with drugs that are associated with adverse reactions (2, 3). These factors contribute to treatment errors and noncompliance, which have been implicated in the emergence of drug-resistant strains of *M. tuberculosis* (4, 5). Further, subsequent relapse of the disease can occur and is associated with a high incidence of drug resistance (6). Together, these complications have enabled the emergent spread of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains of *M. tuberculosis* that require greater than 2 years of therapy with second-line drugs and threaten the efficacy of existing TB therapy (1, 7). Elucidating the mechanisms that govern the susceptibility and resistance of *M. tuberculosis* to existing antitubercular agents will facilitate the discovery of new therapeutic approaches to shorten treatment times and counter drug-resistant TB.

para-Aminosalicylic acid (PAS) entered clinical use as a bacteriostatic antitubercular agent in 1946 (8). Shortly before the introduction of PAS, the discovery of streptomycin as a therapeutic tool had dramatically improved TB survival rates (9). At that time, it was apparent that the rapid emergence of streptomycin-resistant *M. tuberculosis* strains posed a threat to this monotherapy strategy for TB infection (9). As PAS was effective against streptomycin-resistant strains of *M. tuberculosis* (10), it was soon recognized that combination therapy could reduce the emergence of drug resistance (11–13). In the early 1950s, isoniazid was found to be highly effective in treating *M. tuberculosis* infections and was often included in streptomycin-PAS treatment regimens (14). This three-drug combination was found to dramatically increase cure rates and further decrease the emergence of drug resistance (15, 16).

PAS treatment was commonly associated with gastrointestinal disturbance and was eventually replaced with a better-tolerated companion agent, ethambutol (17). Yet, with the development of improved formulations of PAS and the global spread of MDR and XDR strains of *M. tuberculosis*, this drug has re-entered antitubercular drug regimens as an important second-line agent (18). In response to the revitalization of PAS use in TB therapy, there have been recent critical advances in our understanding of the molecular details of susceptibility and resistance of *M. tuberculosis* to this drug.

In this minireview, we summarize the current understanding of the impact of PAS on *M. tuberculosis* metabolism. We focus much of this discussion on folate metabolism, as PAS activity is intimately associated with this essential metabolic pathway. We discuss the proposed modes of action of PAS, its bactericidal effects, and recently characterized resistance mechanisms. Finally, we summarize areas of investigation to further our understanding of PAS interaction with *M. tuberculosis*.

FOLATE METABOLISM AS A HIGH-VALUE DRUG TARGET.

In prokaryotes and eukaryotes, reduced folate species serve as essential cofactors in the transfer of one-carbon groups in pathways for the synthesis of methionine, *N*-formylmethionyl-tRNA, glycine, serine, pantothenate, purines, and thymidine (Fig. 1) (19, 20). While mammals lack the *de novo* folate biosynthesis pathway and must obtain this nutrient from their diet, many microbes are unable to acquire folates from the external environment and rely on *de novo* folate synthesis to support one-carbon metabolism

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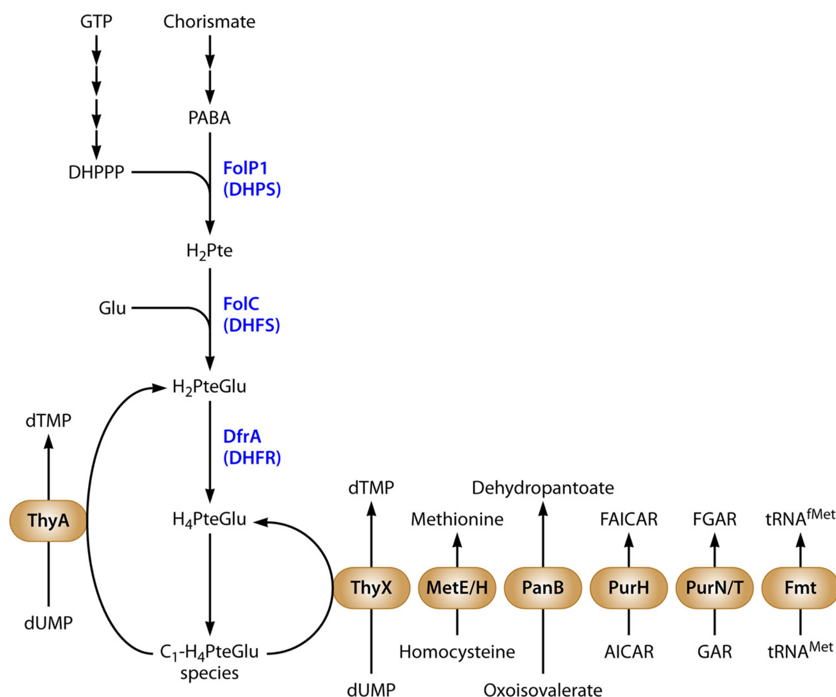

FIG 1 Schematic representation of *M. tuberculosis* folate metabolism. Enzymes of folate biosynthesis are blue, and enzymes of one-carbon metabolism are in beige ovals. Pathway intermediates are connected by black arrows. Abbreviations: GTP, guanosine-5′-triphosphate; DHPPP, 7,8-dihydropterin pyrophosphate; PABA, *para*-aminobenzoic acid; Glu, glutamate; H_2Pte, dihydropteroate; H_2PteGlu, dihydrofolate; H_4PteGlu, tetrahydrofolate; C_1-H_4PteGlu, various single-carbon-modified species of H_4PteGlu; DHPS, H_2Pte synthase; DHFS, dihydrofolate synthase; DHFR, dihydrofolate reductase; dTMP, deoxythymidine monophosphate; FAICAR, 5-formamidoimidazole-4-carboxamide ribotide; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; FGAR, 5′-phosphoribosyl-*N*-formylglycinamide; GAR, 5′-phosphoribosylglycinamide; $tRNA^{Met}$, methionyl-tRNA; $tRNA^{fMet}$, *N*-formylmethionyl-tRNA.

(21). The dichotomy in essentiality of this biosynthetic pathway in humans and microbial pathogens makes it an ideal target for the development of antimicrobial agents. There also exist structural differences in key enzymes of folate utilization that enable selective targeting of microbes (22). Indeed, antifolate drugs, such as sulfonamides and diaminopyrimidines (Fig. 2), have been widely used in the treatment of numerous bacterial and parasitic infections (20, 23–26).

While antifolates are not part of the current first-line TB drug regimen, they have a long history of use in TB therapy and are of interest in ongoing TB drug discovery efforts. Shortly after their discovery, sulfonamides were used with limited success in the treatment of TB until more effective antitubercular drugs were introduced (27, 28). The need for novel therapeutic agents to meet the challenge of MDR and XDR TB has renewed interest in these and other antifolates. Several reports indicate that the vast majority of clinical isolates of *M. tuberculosis*, including MDR and XDR strains, are susceptible to a combination of sulfamethoxazole and the diaminopyrimidine trimethoprim (29–33). In addition, PAS has recently been shown to be an antifolate prodrug (34–36). Moreover, a series of folate structural analogs have recently been developed that show potent disruption of *M. tuberculosis* one-carbon metabolism through inhibition of folate reduction (37, 38). Thus, drugs that target folate metabolism show promise for the future treatment of drug-susceptible and drug-resistant TB infections.

The folate biosynthesis pathway of *M. tuberculosis*. The folate biosynthetic pathway of *M. tuberculosis* begins with the synthesis of *para*-aminobenzoic acid (PABA) and 7,8-dihydropterin pyrophosphate (DHPPP) (Fig. 1). PABA is produced from chorismate by the concerted action of aminodeoxychorismate synthase (PabAB) and aminodeoxychorismate lyase (PabC) (39). While these enzymes have yet to be biochemically characterized in *M. tuberculosis*, genes corresponding to *pabB* and *pabC* were predicted to be essential for growth in high-throughput insertional transposon mutagenesis studies (40, 41). A gene corresponding to *pabA*, encoding chorismate-glutamine amidotransferase, is not predicted in the annotated complete genome sequence of strain H37Rv (42). Yet, it is possible that *M. tuberculosis* encodes an amphibolic glutamine amidotransferase (annotated as *trpG*) that is involved in the synthesis of both folate and tryptophan, as has been described for *Bacillus subtilis* (43). Similar to *pabB* and *pabC*, *trpG* has been predicted to be required for the growth of *M. tuberculosis* in a comprehensive assessment of gene essentiality (41).

DHPPP is produced from GTP via a multistep process (Fig. 1). The first step is the conversion of GTP to dihydroneopterin phosphate and formate by GTP cyclohydrolase (FolE) (44). Dihydroneopterin phosphate is then dephosphorylated to dihydroneopterin by a nonspecific cytoplasmic phosphatase (45). Next, dihydroneopterin aldolase (FolB) converts dihydroneopterin to 6-hydroxymethy-7,8-dihydropterin and glycolaldehyde (46). The structure of *M. tuberculosis* FolB has been solved and was shown to form a novel tetramer that undergoes substrate-induced octamerization, which is essential for its catalytic activity (47). Finally, 6-hydroxymethy-7,8-dihydropterin is converted to DHPPP by the diphosphotransferase FolK (48, 49). While *folE* and *folB* have been predicted to be essential in *M. tuberculosis* (40, 41), disruption of *folK* is associated with a strong *in vitro* growth defect (41).

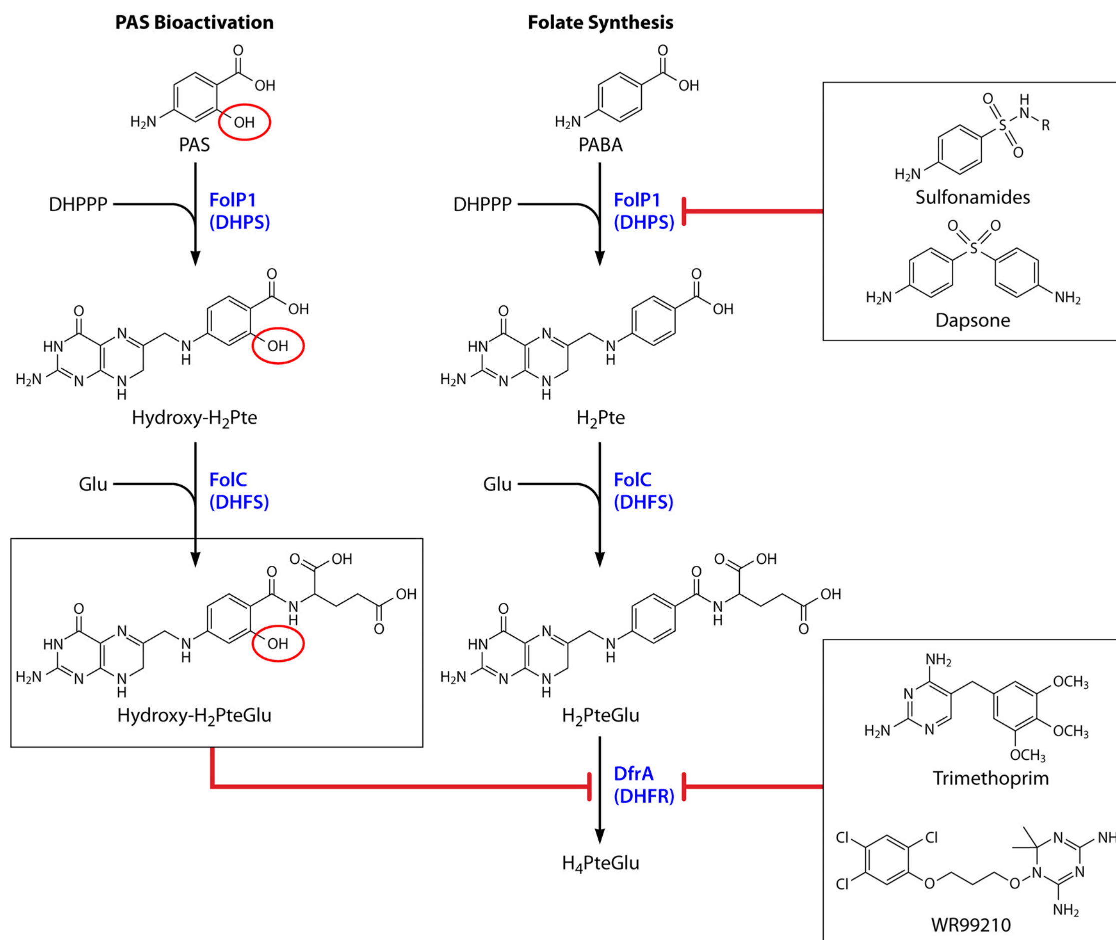

FIG 2 Targets of antifolate drugs. As indicated by red blunted arrows, bioactivated PAS (hydroxy-H_2PteGlu), trimethoprim, and WR99210 inhibit DfrA; 4-aminobenzene sulfonamides and dapsone inhibit FolP1. PAS is a prodrug that is activated through the folate biosynthetic pathway (shown on the left). PAS differs from PABA by the presence of a hydroxyl group in the *ortho* position (circled in red). The native folate synthesis pathway is represented on the right. During bioactivation, PAS serves as an alternate substrate to PABA and is sequentially converted to hydroxy-H_2Pte and hydroxy-H_2PteGlu by FolP1 and FolC, respectively. Relevant enzymes are blue. Abbreviations: DHPPP, 7,8-dihydropterin pyrophosphate; PABA, *para*-aminobenzoic acid; Glu, glutamate; H_2Pte, dihydropteroate; H_2PteGlu, dihydrofolate; H_4PteGlu, tetrahydrofolate; DHPS, H_2Pte synthase; DHFS, dihydrofolate synthase; DHFR, dihydrofolate reductase.

The genes of the DHPPP pathway are organized in an apparent operon that includes one other gene involved in folate synthesis (*folP1*), as well as *ftsH* (involved in cell division) and a gene of unknown function (*Rv3605c*) (50, 51).

Production of dihydropteroate (H_2Pte) from PABA and DHPPP is catalyzed by H_2Pte synthase (DHPS; Fig. 1) (48, 49). While the *M. tuberculosis* genome contains two putative genes for DHPS, *folP1* and *folP2*, biochemical analysis has revealed that only FolP1 is catalytically active in the production of H_2Pte (52, 53). The physiological role of FolP2 has yet to be defined (52).

The final enzyme of the *de novo* folate synthesis pathway is dihydrofolate synthase (DHFS), which catalyzes the ATP-dependent addition of L-glutamate to H_2Pte to generate dihydrofolate (H_2PteGlu) (Fig. 1) (49). In *M. tuberculosis*, DHFS activity is encoded by *folC* (54) and has been predicted to be essential for *in vitro* growth (40, 41). In many species, DHFS is a bifunctional enzyme that also catalyzes the gamma linkage of additional L-glutamate residues to the fully reduced folate species tetrahydrofolate (H_4PteGlu), producing polyglutamylated folates (H_4PteGlu$_n$, where n refers to the number of glutamate residues) (55). In mammalian cells, it has recently been shown that polyglutamylation is important for the retention of folate species in subcellular compartments (56). Polyglutamylation of folates in bacterial systems is widespread, but its physiologic role has not been exhaustively studied (55). One important role for bacterial polyglutamylated folates lies in methionine synthesis. In contrast to most bacterial folate-dependent enzymes that can utilize H_4PteGlu species, it has been demonstrated that the cobalamin-independent methionine synthase MetE preferentially utilizes 5-methyl-H_4PteGlu$_3$ for catalysis (57).

H_2PteGlu is reduced to H_4PteGlu by dihydrofolate reductase (DHFR) (58). H_4PteGlu serves as a cofactor for serine hydroxylase in the synthesis of glycine and is the essential precursor for various one-carbon-carrying folate species (C_1-H_4PteGlu) used in one-carbon metabolism (Fig. 1). Since DHFR is essential in many organisms, therapeutic agents targeting DHFR have been developed for cancer, malaria, and toxoplasmosis, in addition to bacterial infections. In *M. tuberculosis*, DHFR is encoded by *dfrA* (22) and is essential for growth (41). Significant structural differences between the active sites of *M. tuberculosis* DHFR and human DHFR

make it an attractive target for antitubercular drug development (22, 59).

Folate metabolism as a target for antitubercular agents. Among the various enzymes of folate metabolism, only two, DHPS and DHFR, are currently used as targets for antimicrobial agents (Fig. 2). Many sulfonamides and the related compound dapsone (Fig. 2), are structurally similar to PABA and have been found to be competitive inhibitors of DHPS in various pathogens (23, 24, 60–65). Sulfonamides inhibit *M. tuberculosis* FolP1 enzymatic activity in cell-free assays and show variable antitubercular activity in culture (53, 60, 66). Dapsone, a first-line leprosy drug (67), also inhibits *M. tuberculosis* FolP1 enzymatic activity in cell-free assays (53, 60). Like some sulfonamides, dapsone lacks significant activity against whole cells because of the expression of an uncharacterized inactivation pathway (34).

Trimethoprim is a bacteriostatic antimicrobial agent that potently inhibits DHFR in various bacterial species. For example, the K_i of trimethoprim for *Escherichia coli* DHFR is in the low nanomolar range (68). Trimethoprim is frequently used in combination with sulfonamides because of the synergistic impact on the disruption of folate metabolism. Unlike that which is observed in many other bacteria, trimethoprim only weakly inhibits *M. tuberculosis* DHFR enzymatic activity in cell-free assays (50% inhibitory concentration of 16.5 μM) (69). Accordingly, *M. tuberculosis* is not regarded as being highly susceptible to trimethoprim alone (MIC, >128 μg/ml) (31, 70, 71). A recent structural study suggested that the Tyr100 residue in *M. tuberculosis* DHFR may be responsible for the weak binding of trimethoprim to *M. tuberculosis* DHFR and showed that a variant, Y100F, had increased affinity for trimethoprim (59). Despite this limited antitubercular activity of trimethoprim, several studies have demonstrated that combinations of trimethoprim and sulfamethoxazole are effective against drug-susceptible and MDR strains of *M. tuberculosis* (29–33). An evaluation of synergy between these drugs showed that subinhibitory concentrations of sulfamethoxazole conferred a significant reduction of the MIC of trimethoprim (fractional inhibitory concentration index of 0.5) (31). Yet, a parallel study reported that while sulfamethoxazole alone showed measurable activity against various *M. tuberculosis* isolates, no apparent improvement of trimethoprim activity was observed (70). Interestingly, it has been demonstrated that specific disruptions in the folate interconversion pathway can modulate the susceptibility of *M. smegmatis* to various trimethoprim-sulfonamide combinations (72), suggesting opportunities for potentiation of antifolate action in mycobacteria (73).

Several studies have focused on the identification of new *M. tuberculosis* DHFR inhibitors that have more potent antitubercular activity than trimethoprim (37, 71, 74, 75). Suling et al. screened a series of lipophilic deazapteridine derivatives with structural similarity to trimethoprim and identified several *M. tuberculosis* DHFR inhibitors with improved activity relative to that of trimethoprim in cell-free and whole-cell assays (71). In addition, the antimalarial lead compound WR99210 (76) was found to be effective against several species of mycobacteria (77, 78), including *M. tuberculosis* (37). The crystal structure of *M. tuberculosis* DHFR and the brominated analog of WR99210 revealed that Br-WR99210 binds within the active site of *M. tuberculosis* DHFR (22). Using a novel screening approach with a yeast strain expressing *M. tuberculosis dfrA*, Gerum et al. identified several promising WR99210 analogs that also showed improved antitubercular activity (37). The ability of these compounds to target tubercle bacilli in animal models of infection awaits further study.

MODE OF ACTION OF PAS

Since the discovery of PAS as an antitubercular agent, there have been multiple hypotheses regarding its antitubercular mode of action. In 1940 and 1941, Bernheim observed that salicylate stimulated increased oxygen consumption in *M. tuberculosis* (79), while some structural analogs of salicylate had a negative impact on oxygen uptake (80). Following up on these studies, Lehmann screened a panel of salicylate analogs and identified PAS as a potent antitubercular agent (8). These initial observations implied a role for PAS in the disruption of a salicylate-linked metabolic pathway; however, recent studies have revealed that the principal antitubercular action of PAS occurs through poisoning of folate metabolism.

PAS is a prodrug targeting folate metabolism. Initial hints toward an interaction of PAS with folate metabolism came from the observation that its antitubercular activity could be antagonized by supplementation with exogenous PABA (10) or methionine (81, 82). This link to folate metabolism was further solidified by the observation that loss-of-function mutations in *thyA*, encoding a folate-dependent thymidylate synthase, conferred resistance to PAS (83). On the basis of both the structural similarity of PAS to many antimicrobial sulfonamides and the ability of PABA to antagonize the antitubercular activity of these drugs, it was predicted that these compounds possess a conserved mode of action (10). However, despite these structural and functional similarities, there was no measurable cross-resistance between PAS and sulfonamides in *M. tuberculosis* (84). Further, it was shown that, in contrast to sulfonamides, PAS imposed only weak inhibition of purified recombinant *M. tuberculosis* FolP1 enzymatic activity (60). Thus, as described below, for PAS to disrupt *M. tuberculosis* folate metabolism, it must do so downstream of FolP1.

Recently, Chakraborty et al. used a metabolomic approach to investigate the impact of PAS treatment on *M. tuberculosis* folate metabolism (34). In this innovative study, it was revealed that PAS could be bioconverted to the folate intermediate analogs hydroxy-H_2Pte and hydroxydihydrofolate (hydroxy-H_2PteGlu) via the *M. tuberculosis* folate synthesis pathway (34). Further, by using cell-free assays with purified recombinant proteins, it was demonstrated that PAS and hydroxy-H_2Pte were competent substrates for *M. tuberculosis* DHPS and DHFS, respectively (Fig. 2) (34). These data suggested for the first time that PAS was likely a prodrug that required activation via the folate synthesis pathway. This concerted conversion of PAS to hydroxy-H_2Pte and hydroxy-H_2PteGlu was confirmed by two subsequent studies (35, 36). Taken together, these findings suggested that PAS is bioactivated to a hydroxylated folate species analog that is disruptive for a downstream target(s) in folate metabolism.

To further probe into this metabolic disruption, Chakraborty et al. then profiled the change in abundance of folate-linked metabolites in PAS-treated *M. tuberculosis*. It was found that PAS treatment resulted in the rapid, dose-responsive accumulation of precursors of folate-dependent metabolites such as 5-amino-1-(5-phospho-D-ribosyl)imidazole-4-carboxamide, dUMP, homocysteine, and serine (34). This metabolic disruption could be antagonized by exogenously supplied PABA and mirrored metabolic responses to bona fide folate antagonists (34, 38). These data indicated that the ultimate bioactivation product of PAS was

broadly disruptive of one-carbon metabolism, likely at an early step in folate activation (34).

Subsequently, Zheng et al. provided the instrumental observation that PAS-mediated growth inhibition of *M. tuberculosis* could be circumvented by overexpression of DHFR or by expression of a structurally distinct enzyme (RibD) with DHFR activity (35). Further, the authors demonstrated that small-molecule extracts from PAS-treated *M. tuberculosis* contained an inhibitory activity against purified recombinant *M. tuberculosis* DHFR (35). Synthesis of this inhibitory activity could be blocked by the treatment of bacilli with the DHPS inhibitor sulfathiazole (35). Collectively, these data strongly support a model in which PAS is bioactivated within the folate synthesis pathway to the H_2PteGlu analog hydroxy-H_2PteGlu, which then disrupts folate metabolism through potent inhibition of DHFR (Fig. 2).

Bactericidal effects of PAS. PAS is generally regarded as a bacteriostatic agent for *M. tuberculosis* (8), yet bactericidal effects on metabolically active populations of bacilli have been noted (85). While antifolate drugs disrupt multiple biosynthetic pathways, limitation for most of these metabolites results in stasis. However, in metabolically active populations, limitation for dTMP typically imposes a unique microbicidal effect known as thymineless death (86–91). In organisms that express a thymine salvage pathway, thymineless death can be circumvented via supplementation with exogenous thymine or thymidine. Although the molecular mechanism leading to this loss of cell viability is still under investigation, several cellular changes have been causally linked with loss of dTMP and include an increase in single- and double-stranded DNA breaks, impaired Okazaki fragment assembly, and loss of origin-of-replication integrity (92–94). Accumulation of dUMP in *M. tuberculosis* cells treated with PAS was observed (34), indicating that PAS treatment interferes with dTMP synthesis. Further, induction of several DNA repair genes was observed in *M. tuberculosis* during treatment with other DHFR-inhibiting antifolates (38). These data suggest that thymineless death may contribute to loss of cell viability of *M. tuberculosis* during PAS treatment.

Folate metabolism is also intimately tied to the activated methyl cycle, which is involved in the biosynthesis of *S*-adenosylmethionine (SAM). SAM-dependent methyltransferases are essential for many cellular functions in *M. tuberculosis*, including DNA methylation, biotin synthesis, modification of mycolic acids, and methylation of rRNA (95–97). Loss of the ability to regenerate SAM has been shown to be bactericidal in *Borrelia burgdorferi* (98). Antifolate treatment has been demonstrated to reduce the intracellular abundance of SAM in *M. tuberculosis* (38). It was also recently shown that SAM can antagonize the antitubercular activity of WR99210. This finding suggests that WR99210 promotes cell death in *M. tuberculosis* through the depletion of SAM pools (38). Since WR99210 and bioactivated PAS act on the same cellular target, it is likely that PAS-mediated cell death is also linked to depletion of SAM abundance.

Direct evidence of the contribution of dTMP and SAM limitation to the bactericidal activity of PAS has yet to be described. Thymineless death is difficult to assess directly because of the lack of a thymidine salvage pathway in *M. tuberculosis* (99). Without a salvage pathway, supplementation with exogenous salvage pathway intermediates such as thymine or thymidine will not provide a source of dTMP. Further, it is unlikely that dTMP can be taken up directly by *M. tuberculosis* because of the limited permeability of the cell wall (100). Effects stemming from disruption of the activated methyl cycle have yet to be fully characterized in *M. tuberculosis*. Thus, further studies are essential to elucidate the mechanism of cell death in *M. tuberculosis* caused by PAS.

Other biological impacts of PAS. While folate metabolism is a principal target of PAS action in *M. tuberculosis*, there is compelling evidence that PAS can also interfere with mycobacterial iron acquisition (101–103). Salicylate is a structural analog of PAS and is an essential moiety of the mycobacterial siderophores mycobactin and carboxymycobactin (104–106). It has been proposed that PAS noncompetitively inhibits the incorporation of salicylate into both mycobactin and carboxymycobactin, thereby disrupting high-affinity iron scavenging of *M. tuberculosis* (101, 102). This model stems from the observation that PAS inhibited mycobactin production in *Mycobacterium smegmatis* and *Mycobacterium bovis* under iron-restricted growth conditions (102, 107). Interestingly, *M. smegmatis* mutant strains with the salicylate synthesis pathway genes *trpE2*, *entC*, and *entD* deleted were found to have enhanced susceptibility to PAS (101). In addition, it was found that treatment of *M. smegmatis* with PAS led to a reduction of the specific activity of some iron-containing enzymes, such as aconitase, glycerol dehydrogenase, and NADH oxidase (107). Further, *Mycobacterium avium* showed a modest enhancement of PAS susceptibility when cultivated under iron-limiting conditions (108). Interestingly, in contrast to the robust antagonistic activity of PABA on PAS-mediated growth inhibition, salicylate was found to be a relatively weak antagonist (109), and mycobactin did not show measurable antagonism of PAS action (107). Given the dispensability of mycobactins for the growth of *M. tuberculosis* under iron-replete culture conditions, yet the essentiality of mycobactins for growth in iron-restricted niches of the mammalian host (110), it will be important to evaluate the impact of PAS on *M. tuberculosis* fitness in the context of iron restriction.

MECHANISMS OF PAS RESISTANCE

Resistance to PAS was described shortly after its introduction into clinical use and was most prevalent when it was used in monotherapy (111). Recent findings have demonstrated that PAS resistance in *M. tuberculosis* can emerge via multiple mechanisms that include preventing sufficient bioactivation within the folate synthesis pathway, mitigating the impact of target inhibition, and limiting drug accumulation within the bacilli.

Mitigating the impact of target inhibition. The first genetic evidence of the involvement of folate metabolism in the antitubercular action of PAS was that loss-of-function mutations in one of the genes for thymidylate synthase conferred resistance on *M. tuberculosis* and *M. bovis* (83). In biological systems, thymidylate synthase is essential for the 5,10-methylene-H_4PteGlu-dependent conversion of dUMP to dTMP. In most organisms, this reaction is performed by a ThyA-type thymidylate synthase that releases H_2PteGlu following catalysis. This H_2PteGlu must be reduced by DHFR to re-enter folate metabolism (Fig. 3). Some organisms encode a ThyX-type thymidylate synthase. Like most other folate-dependent enzymes, ThyX regenerates H_4PteGlu following catalysis (Fig. 3) (112, 113). In contrast to ThyX utilization, ThyA utilization results in an increased demand for DHFR activity to provide sufficient levels of H_4PteGlu for one-carbon metabolism and is critical for the susceptibility of many organisms to DHFR inhibitors. Loss-of-function mutations in *thyA* significantly decrease the demand for DHFR activity and are commonly associated with resistance to DHFR inhibitors (89). In many pathogens,

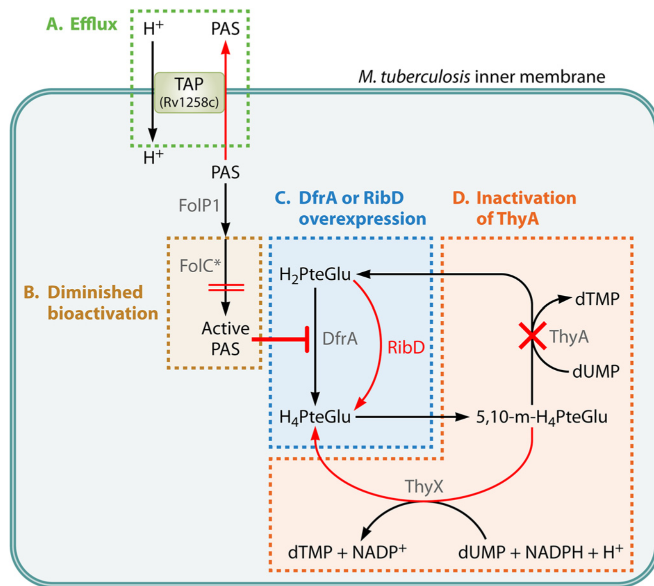

FIG 3 Mechanisms of PAS resistance. The four characterized mechanisms of PAS resistance are shown. (A) Efflux. Intracellular PAS is excluded by the efflux antiporter TAP of *M. tuberculosis* encoded by Rv1258c (green). (B) Diminished bioactivation. FolC variants (FolC*) with an altered substrate binding pocket show decreased bioactivation of PAS (gold). (C) DfrA or RibD overexpression. Inhibition of dihydrofolate reduction can be negated by overexpression of the target DfrA or by overexpression of the alternative reductase RibD (blue). (D) Inactivation of the thymidylate synthase ThyA. Loss of ThyA function is tolerated because of the alternate thymidylate synthase ThyX and confers resistance to PAS by decreasing the catalytic demand on DHFR (orange).

thyA loss-of-function mutations lead to thymine auxotrophy and loss of fitness during infection. Thus, *thyA*-mediated antifolate drug resistance is rarely clinically relevant (114, 115). In contrast to most bacterial pathogens, *M. tuberculosis* encodes both ThyA- and ThyX-type thymidylate synthases (99). As bioactivated PAS inhibits DHFR in *M. tuberculosis*, *thyA* loss-of-function mutations confer up to 100-fold resistance to PAS (83, 99). Since ThyX can support the cellular dTMP requirement, *M. tuberculosis thyA* mutants are not attenuated and are associated with clinical resistance to PAS (83, 116–118).

In addition to *thyA* loss-of-function mutations, artificial overexpression of *dfrA* has been shown to confer PAS resistance on *M. tuberculosis* (35). This observation is consistent with the idea that the active form of PAS inhibits DfrA. Interestingly, it was also found that a mutation 11 bp upstream of the *ribD* translational start site increased *ribD* expression and gave rise to PAS resistance in *M. tuberculosis*. *ribD* encodes a riboflavin biosynthesis protein that contains a C-terminal oxidoreductase domain with 43% amino acid sequence similarity to DfrA. It was suggested that overexpression of *ribD* confers PAS resistance by compensating for the inhibited DfrA function (Fig. 3). While *ribD* promoter mutations have been identified in PAS-resistant clinical isolates of *M. tuberculosis*, *dfrA* mutations have yet to be reported in such isolates (35, 118).

Reduced bioactivation of PAS. As described above, PAS is a prodrug that requires bioactivation within the folate biosynthetic pathway by the concerted action of DHPS and DHFS. By selecting for spontaneous PAS-resistant mutant strains of *M. tuberculosis* H37Rv and H37Ra and *M. bovis* BCG, it was found that mutations within *folC* (encoding DHFS) conferred resistance to PAS (35, 36). Mutations in *folC* were also identified in PAS-resistant *M. tuberculosis* clinical isolates and were associated with an up to 64-fold increase in PAS resistance (36, 118). A recent study tested clinically isolated PAS-resistant *M. tuberculosis* strains from northern China and identified *folC* mutations as the most predominant mutations among the PAS-resistant strains (118). In three recent studies, the reported *folC* mutations mapped within positions corresponding to substrate binding and nucleoside binding domains that are essential for DHFS activity (35, 36, 118). Indeed, when the DHFS activity of several of these FolC variants was evaluated, reduced conversion of H_2Pte to H_2PteGlu (10 to 20% of wild-type activity) was observed (36). Importantly, conversion of hydroxy-H_2Pte to hydroxy-H_2PteGlu was below the limit of detection (36). Further, while a metabolite extract from PAS-treated wild-type *M. tuberculosis* contained DHFR inhibitory activity, this activity was absent from a metabolite extract from a PAS-treated FolC variant strain of *M. tuberculosis* (35). These observations suggest that the reported FolC variants confer resistance by precluding sufficient bioactivation of PAS.

Active efflux. Efflux pumps play a major role in bacterial drug resistance (119). The *M. tuberculosis* genome encodes at least 46 putative drug efflux systems, and 22 drug efflux pumps have been shown to confer drug resistance (120). Among the *M. tuberculosis* drug efflux pumps, it was recently found that a major facilitator superfamily drug efflux pump, Tap (Rv1258c), confers resistance to PAS on *M. bovis* BCG (121). Overexpression of *tap* in *M. bovis* BCG increased the MICs of PAS, gentamicin, streptomycin, spectinomycin, tetracycline, triclosan, and vancomycin by more than 4-fold (121). In *M. tuberculosis*, expression of *tap* is induced in the presence of rifampin and ofloxacin *in vitro* (122) and *tap* expression levels have been found to be elevated in some clinical isolates of *M. tuberculosis* (123).

CLOSING REMARKS

In this review, we have summarized our current understanding of the basis of the susceptibility and resistance of *M. tuberculosis* to PAS. After nearly 70 years of clinical use of this drug in treating TB, recent findings clearly establish a role for perturbation of one-carbon metabolism as a major consequence of PAS incorporation in and disruption of folate metabolism. These findings are consistent with the observation that PAS-resistant clinical isolates show alterations in three distinct nodes in folate metabolism. Despite these incontrovertible findings, many standing questions remain regarding the action of this drug against *M. tuberculosis*. One major question is whether folate disruption is the exclusive antitubercular action of PAS, as there is compelling evidence that this drug disrupts iron assimilation in other species of mycobacteria. Such an effect on iron assimilation is in no way mutually exclusive with the impact on folate metabolism and warrants further investigation. In addition to gaps in our understanding of the mode of action of PAS, it is also clear that additional resistance mechanisms have yet to be described. Studies with cultured bacilli implicate PAS efflux via TAP as a potential resistance mechanism (121), yet a role for TAP in the limitation of PAS efficacy has yet to be established in infection models or in a clinical setting. Further, a PAS inactivation pathway involving the uncharacterized SAM-dependent methyltransferase Rv0560c has been suggested (34, 109, 124, 125) and may be the basis for methionine-linked antag-

onism of PAS activity (81, 82). Yet, whether this pathway limits the full potential action of PAS remains to be demonstrated. By interfering with pathways that antagonize PAS activity, it may be possible to potentiate the action of this drug and perhaps restore susceptibility in the context of PAS resistance.

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