THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 288, NO. 32, pp. 23447–23456, August 9, 2013

para-Aminosalicylic Acid Is a Prodrug Targeting Dihydrofolate Reductase in *Mycobacterium tuberculosis*[S][◆]

Received for publication, April 9, 2013, and in revised form, June 11, 2013 Published, JBC Papers in Press, June 18, 2013, DOI 10.1074/jbc.M113.475798

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Background: PAS is an antimycobacterial whose mechanism(s) of action remains elusive.
Results: PAS is incorporated into the folate pathway by DHPS-DHFS, generating an anti-metabolite. DHFS and RibD are associated with PAS resistance.
Conclusion: Hydroxyl dihydrofolate inhibits DHFR. *folC* and *ribD* are drug target genes for identification of clinical PAS resistance.
Significance: Metabolite analog incorporation into essential biosynthetic pathways is promising for developing antibacterials.

***para*-Aminosalicylic acid (PAS) is one of the antimycobacterial drugs currently used for multidrug-resistant tuberculosis. Although it has been in clinical use for over 60 years, its mechanism(s) of action remains elusive. Here we report that PAS is a prodrug targeting dihydrofolate reductase (DHFR) through an unusual and novel mechanism of action. We provide evidences that PAS is incorporated into the folate pathway by dihydropteroate synthase (DHPS) and dihydrofolate synthase (DHFS) to generate a hydroxyl dihydrofolate antimetabolite, which in turn inhibits DHFR enzymatic activity. Interestingly, PAS is recognized by DHPS as efficiently as its natural substrate *para*-amino benzoic acid. Chemical inhibition of DHPS or mutation in DHFS prevents the formation of the antimetabolite, thereby conferring resistance to PAS. In addition, we identified a bifunctional enzyme (riboflavin biosynthesis protein (RibD)), a putative functional analog of DHFR in a knock-out strain. This finding is further supported by the identification of PAS-resistant clinical isolates encoding a RibD overexpression mutation displaying cross-resistance to genuine DHFR inhibitors. Our findings reveal that a metabolite of PAS inhibits DHFR in the folate pathway. RibD was shown to act as a functional analog of DHFR, and as for DHFS, both were shown to be associated in PAS resistance in laboratory strains and clinical isolates.**

Mycobacterium tuberculosis, a human pathogen causing tuberculosis, claims 1.2–1.5 million lives each year, and it is estimated that one third of the global population is latently infected by the bacilli (1). With the alarming rise of multidrug-resistant tuberculosis, there is an urgent need for new antituberculosis agents to combat the disease (2). Understanding the molecular mechanism(s) of action and resistance of current drugs will provide new perspectives and approaches for drug discovery. *para*-Aminosalicylic acid (PAS)[5] is one of the first antituberculosis agents found to be effective in the 1940s (3). Although PAS has been widely used clinically, its precise mode of action remains largely speculative (4–6). The structural similarity between PAS and *para*-aminobenzoic acid (*p*ABA) has led to the speculation that it might inhibit dihydropteroate synthase (DHPS) in the folate biosynthetic pathway. This speculation is founded on the well studied mechanism of action of sulfonamides, another group of *p*ABA structural analogs. However, unlike the sulfonamides, PAS has not been shown to be an inhibitor of DHPS (4). Nevertheless, mutations within the thymidylate synthase gene *thyA* have been associated with resistance to PAS in *M. tuberculosis* (7), suggesting that PAS may interfere with the folate pathway through an unknown mechanism. Recently, Chakraborty *et al.* (8) demonstrated that PAS acts as an alternative substrate for folate metabolism in *M. tuberculosis*. However, the specific site of action in the folate pathway that results in growth inhibition remains to be elucidated (8). The folate pathway generates tetrahydrofolate in prokaryotes and eukaryotes, which is a ubiquitous one-carbon carrier involved in the biosynthesis of purine, thymidine, glycine, methionine, and pantothenate. In bacteria, tetrahydrofolate is also required for the synthesis of formylmethionyl $tRNA^{fMet}$,

◆ This article was selected as a Paper of the Week.
[S] This article contains supplemental Methods.
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[5] The abbreviations used are: PAS, *para*-aminosalicylic acid; *p*ABA, *para*-aminobenzoic acid; DHFR, dihydrofolate reductase; DHF, dihydrofolate; *r*DHFR, recombinant *M. tuberculosis* DHFR; DHPS, dihydropteroate synthase; DHFS, dihydrofolate synthase; RibD, riboflavin biosynthesis protein; ThyA, thymidylate synthase; DMSO, dimethyl sulfoxide; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt; MS/MS, tandem mass spectrometry; CLFT, cell lysate flow-through; H_2PtPP, 6-hydroxymethyl-7,8-dihydropterin-pyrophosphate; MIC, minimum inhibition concentration.

which is essential for the initiation of protein synthesis. In the folate pathway, DHPS catalyzes the condensation of *p*ABA and 6-hydroxymethyl-7,8-dihydropterin pyrophosphate into 7,8-dihydropteroate, which is then converted by the dihydrofolate synthase (DHFS) into dihydrofolate and reduced to generate the cofactor tetrahydrofolate by the enzyme dihydrofolate reductase (DHFR). DHFR is the target of several important anticancer and antibacterial drugs. For example, methotrexate targets human DHFR and is used as an anticancer drug, whereas both trimethoprim and pyrimethamine are inhibitors of bacterial and protozoal DHFR (9). WR99210 is another potent DHFR inhibitor and antimalarial whose clinical development was terminated due to unacceptable side effects (10). Interestingly, WR99210 is also a potent inhibitor of DHFR and a growth inhibitor in *M. tuberculosis* (11). Despite the pharmacological validation of DHFR as a drug target for malaria and cancer, it has not yet been fully exploited in the battle against *M. tuberculosis*. Here we provide evidence that PAS is a prodrug and targets DHFR in *M. tuberculosis*. DHFR in *M. tuberculosis* is encoded by *dfrA*. We show that the folate pathway is essential for the conversion of PAS into an active antimetabolite. Overexpression of *dfrA* was sufficient to confer resistance to PAS in *M. tuberculosis*. We demonstrated that PAS must be metabolized by an intact folate pathway before it can inhibit DHFR activity. We found that spontaneous mutants overexpressing a previously uncharacterized protein RibD are resistant to PAS or other DHFR inhibitors. When expressed at high level, RibD can act as an alternative dihydrofolate reductase and compensate for a genetic deletion of *dfrA* in *M. tuberculosis*, reinforcing the notion that DHFR is the target of PAS. Finally, we identified a similar polymorphism in *ribD* in *M. tuberculosis* clinical isolates that are associated with PAS resistance.

EXPERIMENTAL PROCEDURES

Strains and Growth Conditions—M. tuberculosis H37Rv and *Mycobacterium bovis* BCG Pasteur strains and their derivatives were maintained in Middlebrook 7H9 broth medium supplemented with 0.2% glycerol, 0.05% Tween 80, and 10% ADS (albumin-dextrose-saline) supplement or Middlebrook 7H11 agar. Culture medium was supplemented with hygromycin (50 μg/ml) or kanamycin (25 μg/ml) when necessary.

Drug Susceptibility Testing—Bacterial drug susceptibility was determined in 7H9 medium as described previously (12). Briefly, compounds dissolved in 90% DMSO were 2-fold serial-diluted in duplicates and spotted in 96-well clear plates, resulting in 10–12 dilutions of each compound. A volume of 200 μl of *M. tuberculosis* or BCG culture (final $A_{600} = 0.02$) was added to each well, and the assay plates were incubated at 37 °C for 5 days. A_{600} values were recorded using a SpectraMax M2 spectrophotometer, and minimum inhibition concentration curves were plotted using GraphPad Prism 5 software.

Protein Purification and Analysis—M. tuberculosis H37Rv *dfrA*, *folP* (DHPS), and *ribD* were expressed as His_6-tagged proteins in *Escherichia coli* BL21 as described previously and purified by standard procedures (13). Proteins were analyzed with Western blot. Antibody against RibD was raised in rabbits against keyhole limpet hemocyanin-conjugated peptides (RibD, qrqhrqargqsevp) by GenScript. The antibodies were affinity-purified by using specific peptides as ligands. Antibody against ClpC1 (14) was used as an internal control.

DHFR Enzymatic Assay—The DHFR enzymatic assay utilizes a tetrazolium dye, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS). Briefly, 2 μl of test compound in 90% (v/v) DMSO was added into 96-well plates to incubate with 50 μl of 60 nM DHFR for 30 min at room temperature. Then 50 μl of 200 μM NADPH was added and incubated for another 10 min. The enzymatic reaction was started by adding 100 μl of a mixture containing 10 μM dihydrofolate (5 μM final concentration) and 300 μM MTS (150 μM final concentration) diluted in DHFR assay buffer (100 mM HEPES, pH 7.5, + 50 mM potassium chloride + 0.05% CHAPS). The reaction was monitored at an absorbance of 490 nm with kinetic reading for 10 min. For the DHFR enzymatic assay with cell lysate flow-through (CLFT), bacterial cultures were grown to an A_{600} of ~0.6 in 7H9 medium, and antibiotics were added at concentrations of 10-fold higher than the minimum inhibition concentration (MIC_{50}) achieved with drug susceptibility testing. Cultures were shaken at 60 rpm for varying periods as indicated. Bacterial cells were pelleted and washed once with PBS + 0.05% Tween 80. The pellet was resuspended with 1.5 ml of DHFR enzymatic assay buffer. The cells were then broken using 0.1-mm glass beads with Precellys 24 (Bertin Technologies), and cell lysates were collected after centrifugation. Samples were normalized by measuring protein concentration and then passed through a 10-kDa cut-off membrane to remove the native DHFR protein. The flow-through (CLFT) was then used in the DHFR enzymatic assay. To perform the assay, 10 μl of 300 nM DHFR was mixed with 90 μl of bacterial lysates and incubated at room temperature for 30 min, and 50 μl of 20 μM NADPH was then added and kept at room temperature for another 10 min. Fifty microliters of 20 μM DHF/600 μM MTS mixture in DHFR assay buffer was added to start the reaction.

DHPS Enzymatic Assay and Identification of Hydroxyl-7,8-dihydropteroate by LC/MS/MS—The incorporation of PAS into the folate pathway was examined by DHPS assay as described previously (15). Pyrophosphate released by the DHPS reaction is cleaved into monophosphate by the addition of inorganic pyrophosphatase to allow the formation of a green complex in the presence of malachite green and molybdate. Briefly, 6-hydroxymethyl-7,8-dihydropterin-pyrophosphate (H_2PtPP) (30 μM), purified recombinant *M. tuberculosis* DHPS (0.6 μg), and inorganic pyrophosphatase (0.1 units/ml) were incubated with various amounts of *p*ABA or PAS (0, 2, 4, 8, 16, 32, 64, and 128 μM) in 20 mM Tris-HCl (pH 8.8), 200 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol in 500 μl at 37 °C for 30 min. After reaction, 100 μl of sample was transferred to a 96-well plate, and 20 μl of malachite green assay solution was added. All further steps were carried out according to the manufacturer's protocol for the malachite green assay. Samples from the DHPS reaction were used for analysis by a triple quadruple Quattro Premier™ mass spectrometer (Waters, Milford, MA) with electrospray ionization. Instruments were controlled by MassLynx software (Version 4.0, Waters, Milford, MA). In brief, sample aliquots from the DHPS reaction were extracted with acetonitrile. After the removal of proteins by centrifugation at

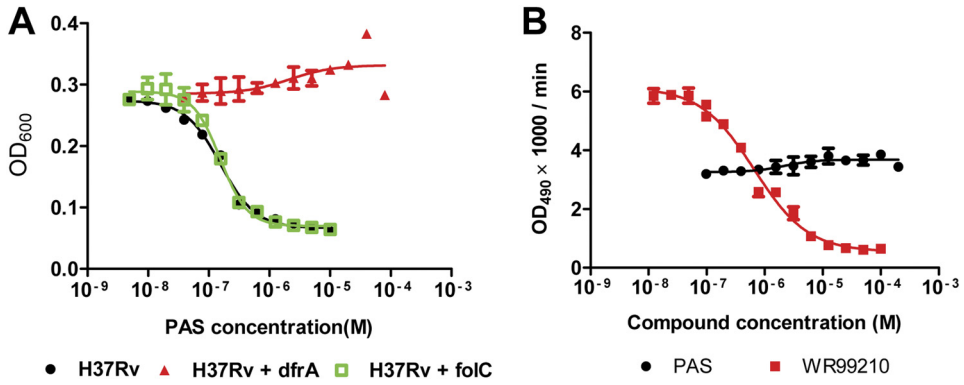

FIGURE 1. **dfrA contributes to PAS resistance in *M. tuberculosis*.** *A*, H37Rv overexpressing *dfrA* but not *folC* is resistant to PAS. *OD*, optical density. *B*, PAS does not inhibit *r*DHFR enzymatic activity as compared with the specific DHFR inhibitor WR99210 with the DHFR assay. The values represent the means ± S.D. from one representative experiment performed with triplicate samples.

13,200 rpm for 10 min at 4 °, the supernatant was subjected to HPLC separation with tandem mass spectrometric detection. The mobile phases were (A) water with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid at a flow rate of 0.4 ml/min. The LC conditions were 5% B at 0 min, a linear gradient from 5 to 50% B over 0.5 min, held at 50% for 0.25 min and then ramped from 50 to 95% over 0.25 min followed by 95% B for 0.75 min and back to 5% B over 0.25 min and then held at 5% B for the remaining 0.75 min.

Whole Genome Sequencing—Whole genome sequencing was performed as described previously (16).

Mutant Construction and Gene Overexpression—Disruption of mycobacterial *dfrA* gene was obtained by allelic exchange as described previously (17). The mycobacterial mutant was constructed by using a modified plasmid pYUB854. Briefly, a *sacB-lacZ* cassette was excised from the pGOAL17 plasmid (18) and ligated into the PacI site of pYUB854. About 1-kb flanking sequences of the gene of interest were cloned into the derivative plasmid on each side of the hygromycin cassette. The resulting plasmid was introduced into mycobacterial cells by electroporation. Deletion mutants were confirmed by PCR or Southern blotting. For gene overexpression in mycobacterium, the genes of interest were amplified and cloned into plasmid pMV262 (19). The constructs were then electroporated into mycobacterial strains.

Southern Blot Analysis—Bacterial genomic DNA was digested with PvuII with restriction endonuclease. Southern blotting was performed with the digoxigenin DNA labeling kit, digoxigenin wash, and block buffer set and nitroblue tetrazolium salt (NBT)/5-bromo-4-chloro-3-indolyl phosphate, toluidinium salt (BCIP) stock solution (Roche Diagnostics). Transfers of DNA to nylon membranes, hybridization, and visualization were as recommended by the manufacturer's protocol.

RESULTS

Overexpression of dfrA Leads to PAS Resistance in M. tuberculosis—Because the folate pathway is known to be associated with the mechanism of PAS resistance, we tested whether the overexpression of the key enzymes DHFS (encoded by *folC*) and DHFR (*dfrA*) would result in PAS resistance. *M. tuberculosis* H37Rv growth was inhibited by PAS with an MIC_{50} of 0.4 μM. H37Rv overexpressing *dfrA* was fully resistant to PAS, with no inhibition observed at PAS concentrations up to 100 μM. Conversely, overexpression of *folC* had no impact on the susceptibility of H37Rv to PAS (Fig. 1*A*). These results suggested that DHFR is a potential target of PAS. However, in an *in vitro* enzymatic assay, PAS did not show any inhibition of recombinant *M. tuberculosis* DHFR (*r*DHFR), whereas the known DHFR inhibitor WR99210 inhibited *r*DHFR as expected (Fig. 1*B*).

DHFR Is Inhibited by PAS-treated Mycobacterial Cell Lysates—The observation that PAS did not inhibit DHFR *in vitro* led us to speculate that PAS might be a prodrug that requires metabolic activation to manifest its inhibitory effect. To test this hypothesis, we made an attempt to bioactivate PAS in mycobacteria. *M. bovis* BCG was treated with PAS for 3 h, and the cell lysate was isolated, normalized to the protein concentration, and passed through a 10-kDa cut-off membrane to remove any DHFR protein produced by mycobacterial cells. The CLFT was then used as a potential source of bioactivated PAS to examine the inhibition of *r*DHFR enzymatic activity. CLFT from PAS-treated cells significantly inhibited *r*DHFR activity. In contrast, CLFT from streptomycin-treated cells did not show any inhibitory effect (Fig. 2*A*). Similar results were obtained in *M. tuberculosis* H37Rv (Fig. 2*B*). Intracellular activation of PAS was proven to be time-dependent, with maximum *r*DHFR inhibition (~60%) observed following 6 h of PAS treatment prior to CLFT isolation and testing in the enzymatic assay (Fig. 2*C*). These results suggest that PAS is activated within mycobacterial cells and that its bioconversion product inhibits DHFR.

Because PAS is structurally similar to *p*ABA, the natural substrate of DHPS, we hypothesized that PAS might be incorporated into the folate pathway by competing with *p*ABA in the reaction catalyzed by DHPS, the product of which is further processed by DHFS to generate hydroxyl dihydrofolate (Fig. 3). This derivative could be the active form of PAS, which would subsequently interact with DHFR to inhibit its enzymatic activity and eventually bacterial growth (Fig. 3). A prediction based on this hypothesis is that the exogenous addition of *p*ABA should reduce PAS-mediated growth inhibition of *Mycobacterium* and the formation of the PAS-derived antimetabolite.

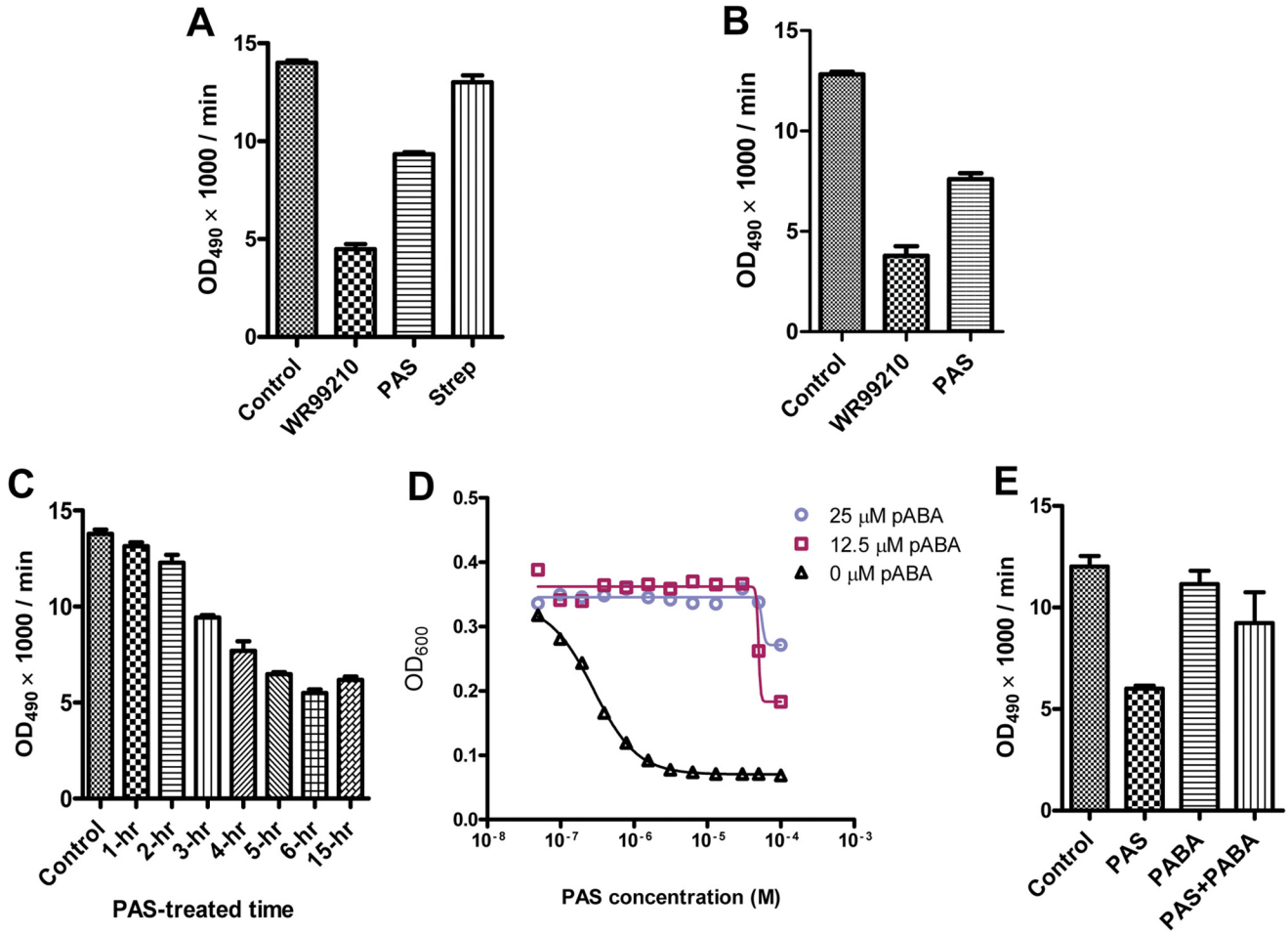

FIGURE 2. **PAS is activated intracellularly to target DHFR.** *A* and *B*, CLFTs from PAS-treated *M. bovis* BCG (*A*) and *M. tuberculosis* (*B*) inhibit *r*DHFR activity. WR99210 and streptomycin (*Strep*) were used as positive and negative controls, respectively. *OD*, optical density. *C*, PAS intracellular activation is time-dependent. *D*, PAS inhibition was decreased upon the addition of *p*ABA. *E*, providing *p*ABA in PAS-treated mycobacterial cells neutralized the inhibition of CLFT on *r*DHFR. The values represent the means ± S.D. from one representative experiment performed with triplicate samples. The experiments were carried out in three independent biological replicates resulting in the same conclusion.

Indeed, the PAS-mediated mycobacterial growth inhibition in *M. tuberculosis* (20) and *M. bovis* BCG was alleviated in the presence of *p*ABA (Fig. 2*D*). In addition, the formation of the PAS-derived antimetabolite was significantly reduced in the presence of *p*ABA (Fig. 2*E*). Thus these results indicate that PAS is activated within mycobacterial cells and competes with *p*ABA for its bioactivation, the product of which targets DHFR.

PAS Acts as a Substrate of DHPS and Is Incorporated into the Folate Pathway—We next sought to test whether PAS is recognized as a direct substrate by DHPS. A nonradioactive enzymatic assay for the recombinant mycobacterial DHPS revealed that PAS is indeed recognized as a substrate by DHPS, and the affinity of PAS to DHPS was comparable with that of *p*ABA in the presence of the cofactor H_2PtPP. Under our experimental conditions, *p*ABA had a Michaelis-Menten constant (K_m) of 11.4 ± 0.1 for DHPS, whereas PAS had a K_m of 17.7 ± 0.1 (Fig. 4, *A* and *B*). The condensation of PAS with the DHPS cofactor H_2PtPP should theoretically lead to the synthesis of hydroxyl dihydropteroate. The mixture reactions were then analyzed by LC/MS/MS. A peak at 329 (*m*/*z*) corresponding to hydroxyl dihydropteroate was identified from the reaction mixture by the Q1 full scan (Fig. 4*C*), which is not observed with the reaction lacking DHPS. The ion was further fragmented into two peaks at 176 (*m*/*z*) and 151 (*m*/*z*) corresponding to H_2PtPP and PAS, respectively, in the Q3 scan (Fig. 4*D*). The chromatograms monitoring this ion demonstrated a single peak (Fig. 4*E*), suggesting that hydroxyl dihydropteroate was indeed produced by DHPS with PAS and H_2PtPP as substrate. These data showed that PAS can be incorporated into the folate pathway and bioactivated into an antimetabolite at the level of DHPS.

Enzymes from the Folate Pathway Are Required for PAS Bioactivation—According to our proposed antimetabolite model for the PAS mechanism of action (Fig. 3), the activation of PAS requires functional DHPS and DHFS. Any interruption of their function should therefore impair hydroxyl dihydrofolate synthesis and thus give rise to PAS resistance. To test this hypothesis, we first examined whether inhibition of DHPS would result in PAS resistance. Depletion of DHPS fails to phenocopy treatment with sulfonamides, a presumed inhibitor of the enzyme, probably because DHPS is present in considerable excess or the activity of the enzyme has to be reduced by ~99% to prevent growth (21–24). We thus treated mycobacterium with PAS in the presence of different concentrations of sulfonamide, and bacterial growth was measured after 5 days of treat-

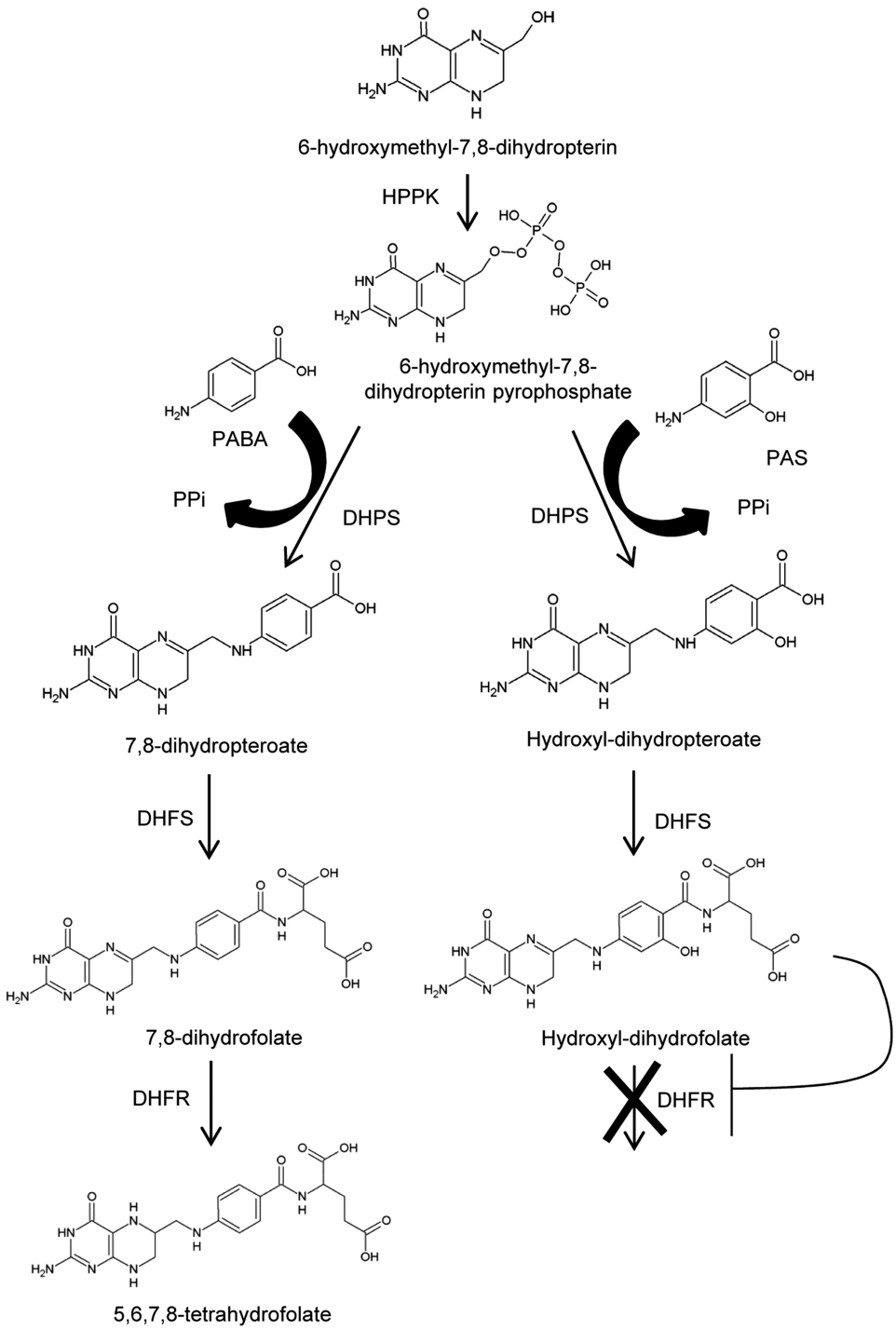

FIGURE 3. **A model of PAS mechanism of action in *Mycobacterium*.** The normal folate pathway is depicted on the *left side*. As a *p*ABA analog, PAS is incorporated into the folate pathway by competing with *p*ABA in the reaction catalyzed by DHPS, the product of which is further processed by DHFS to generate hydroxyl dihydrofolate (*right side*). This antimetabolite in turn inhibits DHFR activity (denoted by the *T bar*) and thus blocks the folate pathway (denoted by the *cross*).

ment. In agreement with our hypothesis, the addition of sulfathiazole to bacterial cultures indeed decreased *M. tuberculosis* susceptibility to PAS, with complete neutralization of PAS-mediated growth inhibition at sulfathiazole concentrations above 40 μM (Fig. 5*A*). Similar results were observed with other sulfonamides (data not shown). Furthermore, the addition of sulfathiazole to PAS-treated mycobacterial cultures suppressed the inhibition of *r*DHFR by CLFT (Fig. 5*B*).

The other enzyme predicted to be required for generating the proposed hydroxyl dihydrofolate is DHFS (*folC*). Because there are no known DHFS inhibitors, we resorted to a genetic approach to demonstrate the requirement of DHFS for PAS activation. By screening for spontaneous PAS-resistant mutants, we isolated one *M. tuberculosis* mutant (*folCE40A*) displaying high level resistance to PAS (Fig. 5*C*). This mutant harbored a mutation in *folC* resulting in a substitution of DHFS

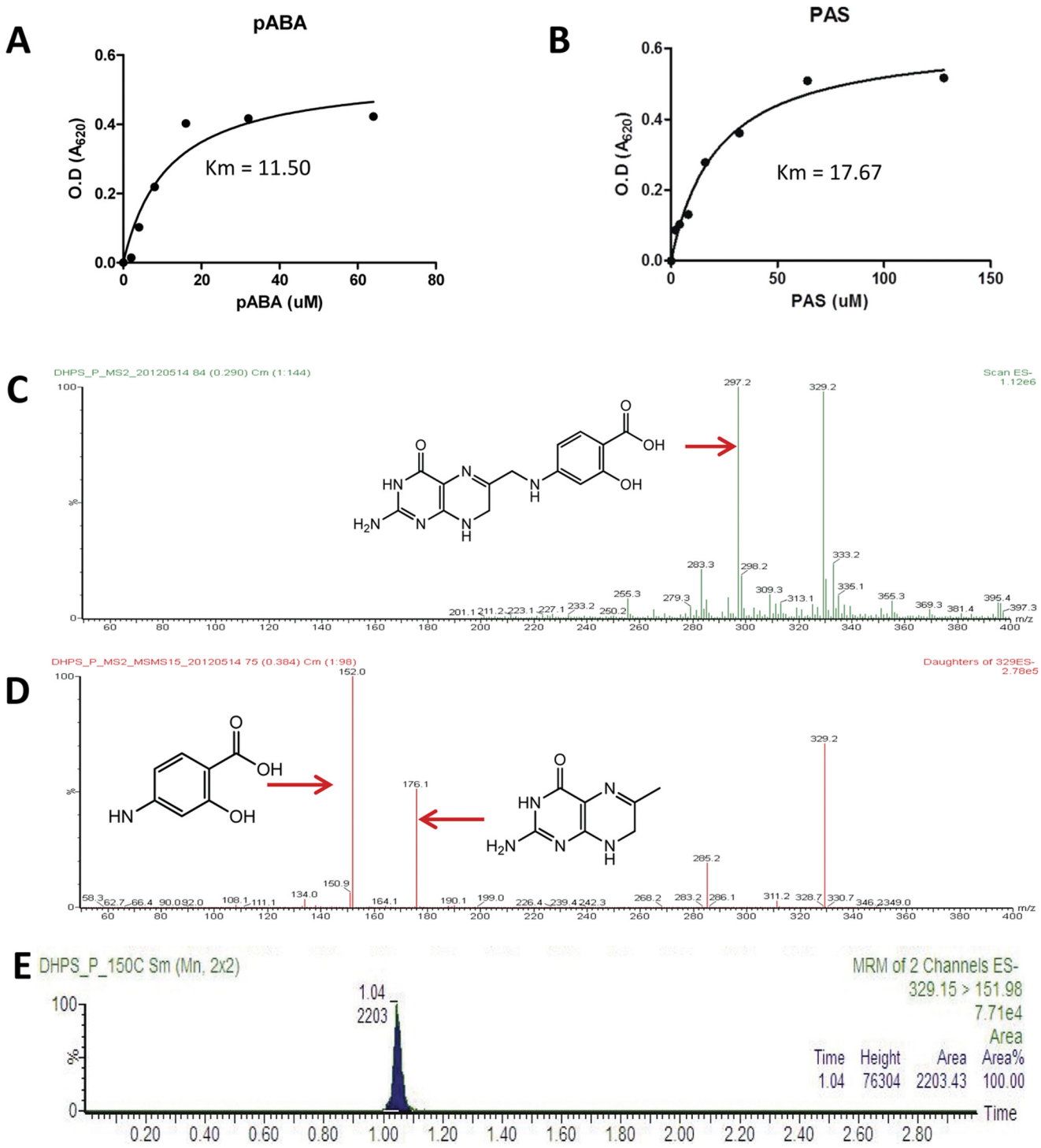

FIGURE 4. **PAS is incorporated into the folate pathway and bioactivated into an antimetabolite at the level of DHPS.** *A* and *B*, DHPS catalyzes the reaction of H_2PtPP and its natural substrate *p*ABA (*A*) and PAS (*B*) with a similar affinity. The Michaelis-Menten values (K_m values) of *p*ABA and PAS to DHPS are labeled. *O.D.*, optical density. *C*, LC/MS/MS chromatography revealed a peak at 329 (*m*/*z*) corresponding to hydroxyl dihydropteroate identified from the reaction mixture by the Q1 full scan. *D*, the ion was further fragmented into two peaks at 176 (*m*/*z*) and 151 (*m*/*z*) corresponding to H_2PtPP and PAS, respectively, in the Q3 scan. *E*, the chromatograms monitoring this ion demonstrated a single peak, suggesting that hydroxyl dihydropteroate was produced by DHPS with PAS and H_2PtPP as substrate.

residue Lys-40 by Ala. Lys-40 is located in a mobile loop of both *M. tuberculosis* and *E. coli* DHFS and has been shown to be important for pteroate binding in *E. coli* (25, 26). Complementation of *folCE40A* with wild-type *folC* restored PAS susceptibility to wild-type levels (Fig. 5*C*), confirming that a mutation in the *folC* gene is associated with resistance to PAS. Collectively, these results indicate that PAS-mediated growth inhibition is DHPS- and DHFS-dependent.

RibD Is a Putative Functional Analog of DHFR—To date, the isolation of spontaneous PAS-resistant mutants has not revealed mutations in DHFR (7). This may suggest that the fitness cost of a mutation in DHFR is higher than for any other

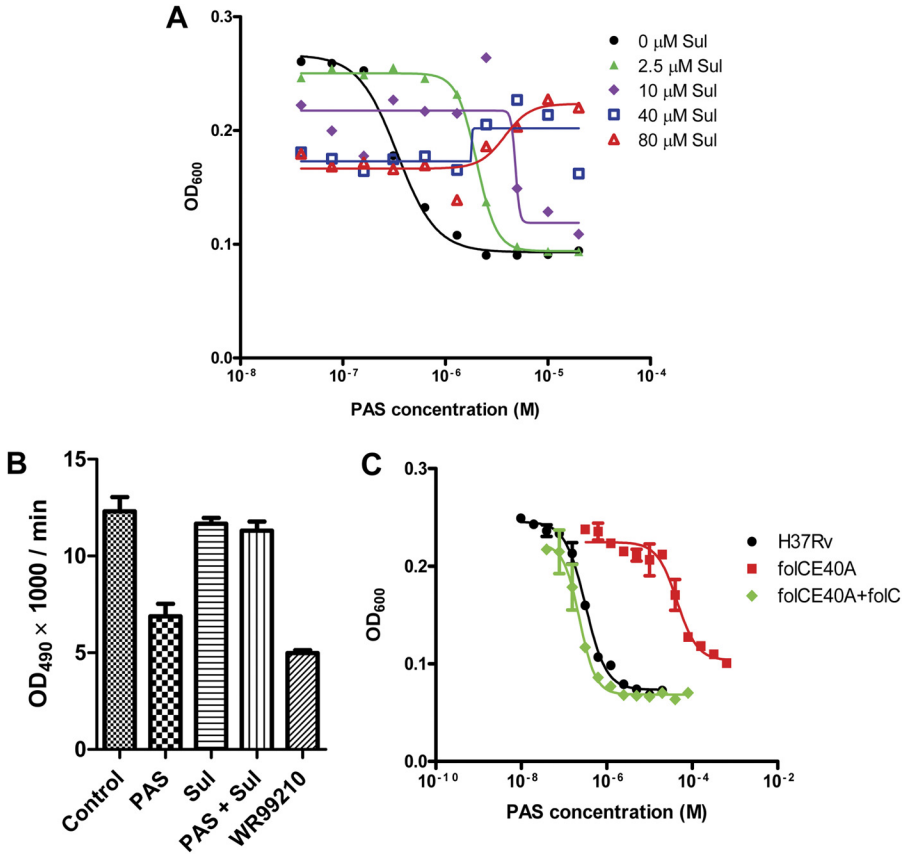

FIGURE 5. **Enzymes in the folate pathway are required for PAS activation.** *A*, inhibition of DHPS antagonizes PAS-mediated inhibition, as shown by the growth inhibition curves of PAS in the presence of increasing sulfathiazole (*Sul*) concentrations. *OD*, optical density. *B*, the addition of sulfathiazole to PAS-treated mycobacterial cultures suppresses the inhibition of rDHFR activity by CLFT. *C*, mutation in *folC* (*folCE40A*) reduces *M. tuberculosis* susceptibility to PAS, and complementation with wild-type copy of *folC* (in pMV262) restores its susceptibility to wild-type level. The values in *B* and *C* represent the means ± S.D. from one representative experiment performed with triplicate samples.

genes of the folate pathway, at least *in vitro*. We thus set out to select spontaneous *M. tuberculosis* mutants resistant to a specific DHFR inhibitor. In the context of a target-based drug discovery project, we synthesized a series of WR99210 analogs, one of which (NITD344) inhibited both DHFR enzymatic activity and *M. tuberculosis* growth (Fig. 6, *A* and *B*). Analysis of 20 spontaneous *M. tuberculosis* mutants resistant to the DHFR inhibitor NITD344 demonstrated no mutations in *dfrA*. To get an insight into the genetic determinant of the resistance to NITD344, the whole genome of the NITD344-resistant mutant *M. tuberculosis* R7 was sequenced. The results revealed a Gly to Ala substitution located 11 bp upstream of the translational start codon of RibD (*Rv2671*), causing increased RibD expression (Fig. 6*C*). Although RibD is annotated as a riboflavin biosynthesis protein, its C-terminal reductase domain shares strong sequence similarities with dihydrofolate reductases (27, 28) (data not shown). Because overexpression of RibD caused resistance to a specific DHFR inhibitor, we tested whether it also conferred resistance to PAS. Interestingly, *M. tuberculosis* R7 was cross-resistant to PAS (Fig. 6, *D* and *E*). Moreover, overexpression of RibD into *M. tuberculosis* also conferred resistance to PAS (Fig. 6, *B* and *C*). In contrast, overexpression of another classical riboflavin biosynthesis protein RibG did not affect mycobacterial growth inhibition by NITD344 or PAS (data not shown). Importantly, 3 out of 30 distinct *M. tuberculosis* clinical isolates (7) resistant to PAS harbored the same regulatory mutation found in the strain *M. tuberculosis* R7, showing that this mutation is associated with PAS resistance in humans.

The observation that RibD overexpression confers resistance to a specific DHFR inhibitor led us to speculate that RibD might function as a dihydrofolate reductase when expressed at high levels. Through genetic studies, we showed that *dfrA* is essential (29). However, the gene could successfully be knocked out in *M. tuberculosis* when RibD was overexpressed from a multicopy plasmid (Fig. 7, *A* and *B*). These results indicate that RibD, when expressed at high levels, can provide sufficient one-carbon carriers to sustain bacterial survival in the absence of *dfrA*.

DISCUSSION

The folate biosynthetic pathway is essential in the production of tetrahydrofolate and derivatives required as cofactors in the biosynthesis of purines, thymidylate, serine, and methionine (30). Unlike vertebrates, which utilize exogenous sources of folates, many prokaryotes and protozoa must synthesize these essential cofactors *de novo*. The folate pathway thus has been targeted for the development of new antibiotics. Nevertheless, none of the enzymes in the folate pathway has conclusively been shown to be the target of current antimycobacterials. Recently, PAS, an antimycobacterial drug that has been in clinical use for more than 60 years, has been linked to the folate pathway (5). However, its exact molecular target and mecha-

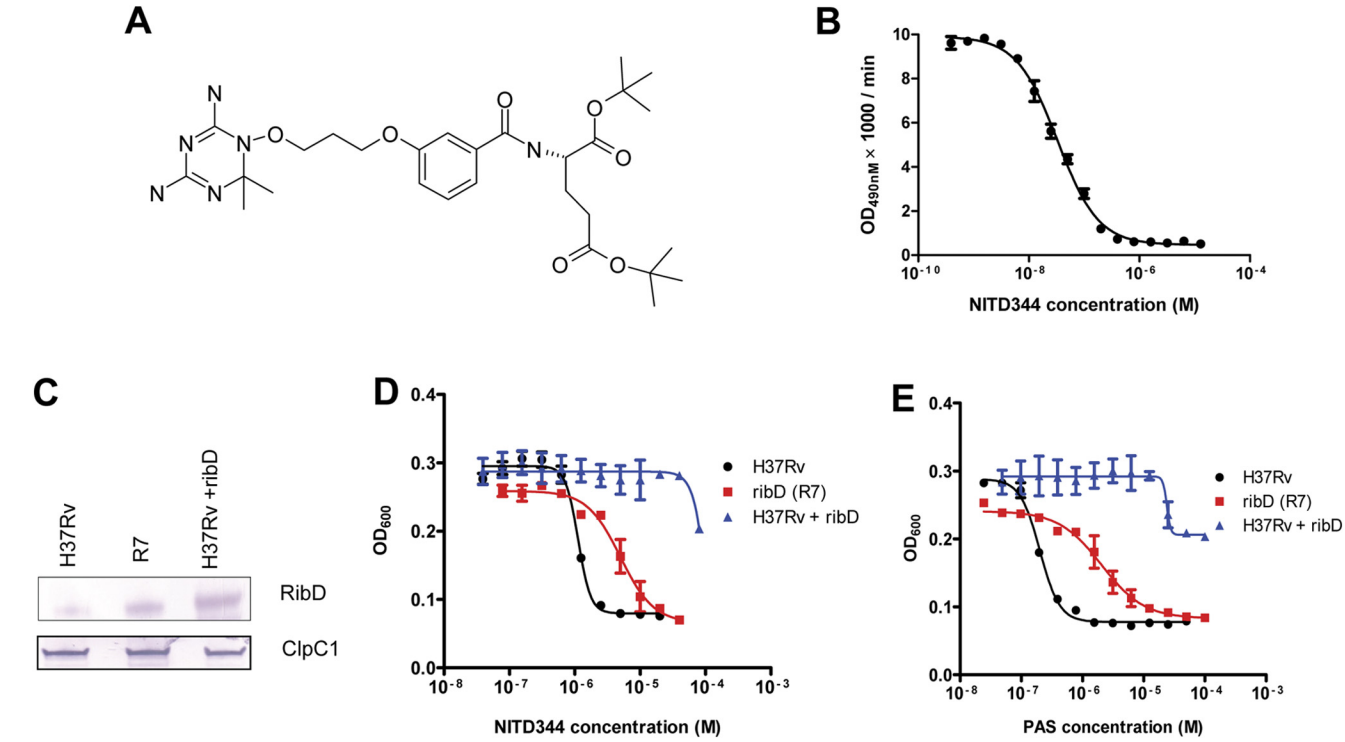

FIGURE 6. **RibD is responsible for *M. tuberculosis* PAS resistance.** *A*, chemical structure of NITD344. *B*, NITD344 inhibits *M. tuberculosis* *r*DHFR enzymatic activity demonstrated in the DHFR enzyme inhibition assay. *OD*, optical density. *C*, Western blot showing increased RibD expression in spontaneous NITD344-resistant mutant *M. tuberculosis* R7. *D* and *E*, both *M. tuberculosis* R7 (*red squares*) and H37Rv overexpressing *ribD* in pM262 (*blue triangles*) are resistant to NITD344 (*D*) and PAS (*E*). The values in *B*, *D*, and *E* represent the means ± S.D. from one representative experiment performed with triplicate samples.

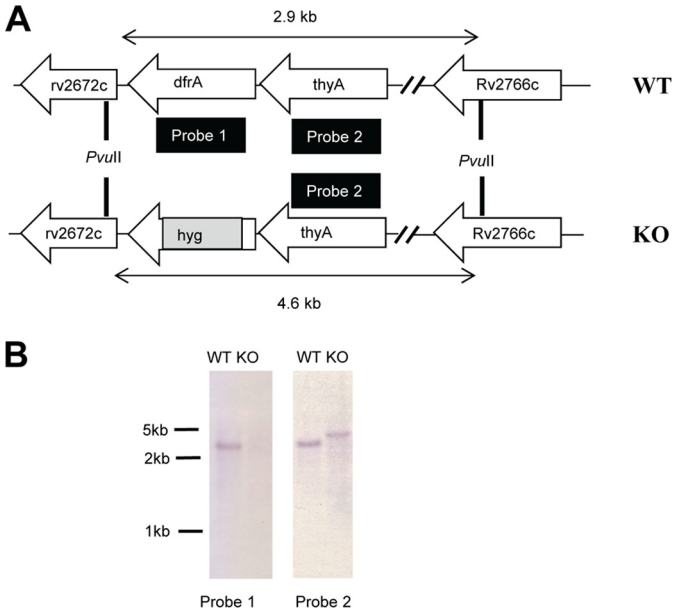

FIGURE 7. **Overexpression of RibD compensates for the knock-out of DHFR in *Mycobacterium*.** *A*, schematic representation of the genome region of the H37Rv wild type (*WT*) and knock-out mutant (*KO*) showing the relative gene organization of *dfrA* and its flanking genes, the locations of the probes for Southern blot analyses and PvuII restriction sites, and the corresponding fragment lengths. *B*, *dfrA* could be successfully knocked out only while overexpressing RibD in *M. tuberculosis*. The knock-out mutant of *dfrA* was confirmed by Southern blot using probes 1 and 2.

nism(s) of action are still not clear. Here our results indicate that PAS acts as a metabolic precursor that, when incorporated in the folate pathway by DHPS and DHFS, generates a toxic dihydrofolate analog that subsequently poisons the folate pathway.

During the synthesis of tetrahydrofolate, DHPS in the folate pathway catalyzes the condensation of *p*ABA and 6-hydroxymethyl-7,8-dihydropterin pyrophosphate to form 7,8-dihydropteroate (31), which is then used as a substrate of DHFS in the synthesis of DHF. Reduction of dihydrofolate to tetrahydrofolate is carried out in most bacteria and eukaryotes by the enzyme DHFR. Our results support that PAS is misincorporated into the folate pathway as a *p*ABA surrogate. Rather than directly inhibiting DHPS as speculated previously (4), PAS is recognized by DHPS as a substrate and converted into hydroxyl dihydropteroate. However, the hydroxyl dihydropteroate does not block the folate pathway itself. Instead, it functions as a substrate of DHFS in the synthesis of hydroxyl dihydrofolate through the incorporation of glutamate. The close structural analogy between hydroxyl dihydrofolate and dihydrofolate suggests that these compounds possibly compete for the active site of DHFR (Fig. 3). The binding of hydroxyl dihydrofolate to DHFR likely blocks its enzymatic activity. This leads to a depletion of tetrahydrofolates essential for protein synthesis, resulting in inhibition of bacterial growth and death. However, the specific effects of this interaction remain elusive. A proof of principle for this mechanism is to assess the activity of hydroxyl dihydrofolate in the *r*DHFR enzyme assay. However, our attempt to synthesize hydroxyl dihydrofolate was impaired by the production of unstable intermediaries. Note that consistent with our hypothesis, we demonstrated that PAS was bioactivated into an antimetabolite at the level of DHPS to generate its precursor hydroxyl dihydropteroate. Moreover, the fact

that CLFT from PAS-treated cells inhibits *r*DHFR as well as the fact that inhibition of DHPS or mutation in DHFS (*folC*) decreases *M. tuberculosis* susceptibility to PAS further supports our antimetabolite model for the mechanism of action of PAS.

Although we have been unable to identify spontaneous mutants resistant to PAS or NITD344 encoding mutations in DHFR (*dfrA*), polymorphisms in other enzymes of the folate pathway, notably ThyA (thymidylate synthase), are well known to contribute to PAS resistance (7) in addition to the contributions of DHFS and RibD identified in this study. These observations are not at odds with our model of DHFR being a target of a PAS-derived antimetabolite. Treatment of bacteria with PAS inhibits DHFR, resulting in reduction of tetrahydrofolate production. As thymidylate synthase is a major consumer of reduced folates, mutations in *thyA* are likely to cause a decrease in the utilization of tetrahydrofolate derivatives. Thus more reduced folates become available for other essential one-carbon addition reactions, leading to increased bacterial survival (32). Mutations in the *thyA* are permissible likely due to the fact that *M. tuberculosis* encodes ThyX, a highly active functional analog of *thyA* that utilizes tetrahydrofolate as a carrier of single-carbon groups but not as a cofactor. Mutations in the *thyA* are found in clinical PAS-resistant isolates but not in the *dfrA* (7). Interestingly, RibD provides an additional gene target for PAS resistance of clinical relevance. We found that RibD overexpression replaced the functional activity of *dfrA* in a knock-out *dfrA* recombinant strain, clearly indicating that RibD either possesses DHFR-like activity or is able to catalyze an alternative reaction that complements the classical DHFR. However, RibD is ordinarily present in low concentrations or has poor DHFR activity, and thus it cannot completely replace the essential function of *dfrA* unless overexpressed. Our findings suggest that in addition to *thyA*, *folC* and *ribD* are candidates for the rapid identification of PAS resistance in clinical studies.

PAS or its metabolite acts as substrate for an essential enzyme. Although it does seem possible to have mutations in the activating enzyme DHFS, which no longer catalyzes the bioconversion of PAS, most resistance in clinical isolates seems to result from metabolic remodeling of the cell. In other bacteria, drug synergy can occur by inhibition of multiple steps in the folate synthetic pathway, *e.g.* through the combined use of trimethoprim and sulfonamide drugs (33). Our results suggest that there are at least two mechanisms to evade killing even by such a combination, either through decreased utilization of folates (*thyA* mutations) or through up-regulation of an alternate pathway (RibD overexpression mutations). On the other hand, such adaptations could well incur significant fitness costs yet to be explored, particularly when growing in the hostile environment of the host.

In conclusion, our results indicate that PAS is a prodrug and that it acts as a metabolic precursor that, when incorporated in the folate pathway by DHPS and DHFS, generates a toxic dihydrofolate analog that subsequently inhibits DHFR activity. In this model, the antibiotic is converted to an active form intracellularly by a metabolic pathway, but unlike classical prodrugs, it mimics the substrate of an essential enzyme, leading to poisoning of the metabolic pathway itself by its bioactivation product.

Acknowledgments—We acknowledge several scientists at Novartis: Bryan Yeung, Ravinder Kondreddi, and Ranga Rao for providing NITD344; Boon Heng Lee and Koh Sin for technical help with MIC assays and LC/MS analysis; Whitney Barnes for whole genome sequencing; Veronique Dartois for critical review of the manuscript; and Paul Smith for support and stimulating discussions. The Institute Pasteur-Korea receives support from the Korean Ministry of Education, Science and Technology.

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