Aspartate decarboxylase (PanD) as a new target of pyrazinamide in *Mycobacterium tuberculosis*

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Pyrazinamide (PZA) is a frontline anti-tuberculosis drug that plays a crucial role in the treatment of both drug-susceptible and multidrug-resistant tuberculosis (MDR-TB). PZA is a prodrug that is converted to its active form, pyrazinoic acid (POA), by a nicotinamidase/pyrazinamidase encoded by the *pncA* gene, the mutation of which is the major cause of PZA resistance. Although RpsA (ribosomal protein S1, involved in trans-translation) has recently been shown to be a target of POA/PZA, whole-genome sequencing has identified mutations in the *panD* gene encoding aspartate decarboxylase in PZA-resistant strains lacking *pncA* and *rpsA* mutations. To gain more insight into a possible new target of PZA, we isolated 30 POA-resistant mutants lacking mutations in *pncA* and *rpsA* from *M. tuberculosis* in vitro, and whole-genome sequencing of 3 mutants identified various mutations in the *panD* gene. Additionally, sequencing analysis revealed that the remaining 27 POA-resistant mutants all harbored *panD* mutations affecting the C-terminus of the PanD protein, with PanD M117I being the most frequent mutation (24/30, 80%). Conditional overexpression of *panD* from *M. tuberculosis*, *M. smegmatis* or *E. coli*, or of *M. tuberculosis* mutant PanD M117I, all conferred resistance to POA and PZA in *M. tuberculosis*. β-alanine and pantothenate, which are downstream products of PanD, were found to antagonize the antituberculosis activity of POA. In addition, the activity of the *M. tuberculosis* PanD enzyme was inhibited by POA at therapeutically relevant concentrations in a concentration-dependent manner but was not inhibited by the prodrug PZA or the control compound nicotinamide. These findings suggest that PanD represents a new target of PZA/POA. These results have implications for a better understanding of this peculiar persister drug and for the design of new drugs targeting *M. tuberculosis* persisters for improved treatment.

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INTRODUCTION

Pyrazinamide (PZA) is an important first-line tuberculosis (TB) drug used in combination with other TB drugs for the treatment of both drug-susceptible TB and multidrug-resistant tuberculosis (MDR-TB).¹,² PZA is a peculiar persister drug that acts only on dormant non-growing persisters and has poor activity against growing *Mycobacterium tuberculosis*.³ Its high activity against persisters bacteria is responsible for PZA’s unique sterilizing activity, which shortens the TB treatment period from 9–12 months to 6 months.⁴ Because of its indispensable sterilizing activity, all new TB regimens in clinical development include PZA.⁵

PZA is a prodrug that requires activation to its active form, pyrazinoic acid (POA), by an *M. tuberculosis* pyrazinamidase/nicotinamidase enzyme encoded by the *pncA* gene.⁶ Mutations in *pncA* are the major mechanism of PZA resistance in *M. tuberculosis*.³,⁶ Recently, we identified ribosomal protein S1 (RpsA, Rv1630), a vital protein involved in trans-translation, as a target of PZA.⁷ Mutations in *rpsA* have been found in some PZA-resistant clinical isolates lacking *pncA* mutations.⁷–¹⁰ However, some PZA-resistant strains do not have mutations in either the *pncA* or *rpsA* genes,³,⁹,¹¹ indicating the presence of a possible new resistance mechanism or target of PZA.

Recently, we identified a new gene, *panD* encoding aspartate decarboxylase and involved in β-alanine biosynthesis, mutations in which are associated with PZA resistance in *M. tuberculosis*.¹² PanD is involved in the synthesis of pantothenate (vitamin B₅), which in turn is required for the synthesis of coenzyme A (CoA), a molecule that is at the center of all energy metabolism and allows carbohydrates, fats, and proteins to be burned as energy sources. However, how *panD* mutations cause PZA resistance and how PZA might interfere with pantothenate and CoA function are unclear. In an attempt to shed light on possible new targets of PZA, in this study, we isolated mutants of *M. tuberculosis* resistant to PZA, the active form of PZA, and characterized mutations potentially involved in PZA resistance. Whole-genome sequencing of select POA-resistant mutants without *pncA* or *rpsA* mutations together with targeted sequencing mapped all the mutations in the *panD* gene. Our biochemical and genetic studies suggest that PanD is a new target involved in PZA action and resistance.

MATERIALS AND METHODS

Isolation of spontaneous POA-resistant mutants of *M. tuberculosis*

A single colony of *M. tuberculosis* strain H37Rv was cultured at 37 °C in 7H9 medium. At 2–3 weeks, the culture reached an optical density

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panD conditional overexpression and POA susceptibility testing

The panD gene was amplified by PCR from the M. tuberculosis parent strain H37Ra, the POA-resistant mutants, M. smegmatis and E. coli using the primers (panD_F: 5’-GCG CTG TAT CTC CAA GCT GAA CTG TAT CAC ACC CTT-3’ and panD_R: 5’-TAT CCG GCA CTG CAA CTG CCA TGC TCA CTA CTA CTA CTA-3’) were used to amplify a 650-bp PCR product that contains the whole panD gene from POA-resistant M. tuberculosis mutants as described previously. The nucleotide sequences were analyzed by using Sequencer software (Gene Codes Corporation, Ann Arbor, MI, USA) to identify possible mutations in panD.

RESULTS

POA-resistant M. tuberculosis mutants had PanD mutations

To identify possible new targets of POA, we plated M. tuberculosis H37Ra on 7H11 plates containing POA (25–200 μg/mL at pH 5.7 or 200–700 μg/mL at pH 6.8). Through two more rounds of screening, we were able to isolate 30 mutants resistant to both POA and PZA. DNA sequencing revealed that these 30 POA-resistant mutants did not
panD overexpression caused POA/PZA resistance in M. tuberculosis

To confirm PanD as a target of PZA, we overexpressed panD genes from M. tuberculosis in M. tuberculosis strain H37Ra and tested their PZA and POA susceptibility. Overexpression of the panD gene from wild-type M. tuberculosis, mutant POA-35 (nucleotide change G351A; amino acid change M117I), mutant POA-140 (nucleotide change T431C; amino acid change V138A), or the panD genes from M. smegmatis or E. coli caused POA resistance in M. tuberculosis H37Ra using the tetracycline-inducible vector pUV15tetORs in the presence of ATc (50 ng/mL) inducing agent (Figure 2). In contrast, the recombinant strains were sensitive to POA (<300 μg/mL, pH 6.8) without the inducing agent ATc (Figures 2A–2C). POA-resistant M. tuberculosis mutants with the M117I mutation grew in the absence or presence of ATc induction (Figure 2D) because this is the mutant alone and is not transformed with a panD expression construct. It is interesting to note that expression of either wild-type H37Rv panD or mutant panD (G351A, M117I and T431C, V138A) from M. tuberculosis conferred POA resistance to M. tuberculosis (POA>700 μg/mL, pH 6.8) (Figures 2B and 2C), as did panD from M. smegmatis and E. coli (Figures 2E and 2F). The vector control remained susceptible to POA with or without ATc induction (Figure 2G). In addition, conditional overexpression of the above constructs caused a three-fold increase in the MIC of PZA for M. tuberculosis from 25 μg/mL in the uninduced and control strains to 75 μg/mL (pH 5.7) in the ATc-induced strains.

Pantothenate and β-alanine antagonized POA/PZA activity against M. tuberculosis

Because PanD (aspartate decarboxylase) is involved in converting L-aspartic acid to β-alanine, a precursor for pantothenate and coenzyme A biosynthesis, we wondered if supplementation with β-alanine or pantothenate might render M. tuberculosis resistant to POA. To test this, pantothenate, β-alanine, and different amino acids that served as controls, were incorporated into 7H11 agar containing different concentrations of POA (200–800 μg/mL, pH 6.8) followed by inoculation with M. tuberculosis H37Ra. The results showed that the parent strain

Table 1 panD mutations identified in POA-resistant mutants of M. tuberculosis

| Strain                        | Nucleotide change | Amino acid change |
|-------------------------------|-------------------|------------------|
| Mycobacterium tuberculosis H37Ra POA-37 | G351A             | M117I            |
| Mycobacterium tuberculosis H37Ra POA-38 | G351A             | M117I            |
| Mycobacterium tuberculosis H37Ra POA-48 | G351A             | M117I            |
| Mycobacterium tuberculosis H37Ra POA-54 | G351A             | M117I            |
| Mycobacterium tuberculosis H37Ra POA-55 | G351A             | M117I            |
| Mycobacterium tuberculosis H37Ra POA-56 | T407G             | L136R            |
| Mycobacterium tuberculosis H37Ra POA-57 | G351A             | M117I            |
| Mycobacterium tuberculosis H37Ra POA-59 | G351A             | M117I            |
| Mycobacterium tuberculosis H37Ra POA-64 | G351A             | M117I            |
| Mycobacterium tuberculosis H37Ra POA-68 | Del 393G          | 131 codon shift  |
| Mycobacterium tuberculosis H37Ra POA-71 | G351A             | M117I            |
| Mycobacterium tuberculosis H37Ra POA-72 | G351A             | M117I            |
| Mycobacterium tuberculosis H37Ra POA-73 | G351A             | M117I            |
| Mycobacterium tuberculosis H37Ra POA-76 | G351A             | M117I            |
| Mycobacterium tuberculosis H37Ra POA-88 | G351A             | M117I            |
| Mycobacterium tuberculosis H37Ra POA-89 | G351A             | M117I            |
| Mycobacterium tuberculosis H37Ra POA-90 | G351A             | M117I            |
| Mycobacterium tuberculosis H37Ra POA-92 | G351A             | M117I            |
| Mycobacterium tuberculosis H37Ra POA-102 | G351A             | M117I            |
| Mycobacterium tuberculosis H37Ra POA-106 | G351A             | M117I            |
| Mycobacterium tuberculosis H37Ra POA-108 | G351A             | M117I            |
| Mycobacterium tuberculosis H37Ra POA-125 | G351A             | M117I            |
| Mycobacterium tuberculosis H37Ra POA-126 | G351A             | M117I            |
| Mycobacterium tuberculosis H37Ra POA-133 | G351A             | M117I            |
| Mycobacterium tuberculosis H37Ra POA-135 | T413G             | V138A            |
| Mycobacterium tuberculosis H37Ra POA-136 | G376T             | E126*            |
| Mycobacterium tuberculosis H37Ra POA-137 | T413A             | V138A            |
| Mycobacterium tuberculosis H37Ra POA-140 | T413C             | V138A            |
| Mycobacterium tuberculosis H37Ra POA-147 | G351A             | M117I            |
| Mycobacterium tuberculosis H37Ra POA-156 | G351A             | M117I            |

* indicates the three POA-resistant mutants that were sequenced by whole-genome sequencing.
* indicates a stop codon.
and the mutant strain were sensitive and resistant to POA, respectively, on 7H11 plates containing different concentrations of POA (Figures 3A and 3B), but susceptibility to POA dramatically decreased when pantothenate or β-alanine (Figures 3C and 3D) was added to the medium at 0.1 mM. However, other amino acids such as L-alanine, L-aspartate, L-valine, and L-glutamate had no effect on POA activity (Figures 3E–3H). This finding suggests that pantothenate and β-alanine, which are the downstream products of PanD, specifically antagonize the antituberculosis activity of POA and that POA inhibits pantothenate or β-alanine synthesis by blocking PanD activity in M. tuberculosis.

Effects of different concentrations of β-alanine or pantothenate on POA susceptibility

To identify the minimum concentration of β-alanine or pantothenate that antagonizes POA activity, M. tuberculosis strain H37Ra was plated on 7H11 plates containing various concentrations of β-alanine or pantothenate (0, 0.1, 1, 5, 10, and 100 μM). The results showed that the parent strain H37Ra was susceptible to 200 μg/mL POA (Figure 4A) and 0.1 μM β-alanine did not appear to antagonize POA activity (Figure 4B). The minimum concentration of β-alanine that showed obvious POA antagonism was 1 μM (Figure 4C), and the antagonism increased with the concentration of β-alanine (Figures 4C–4F). Interestingly, there was not much variation in the level of β-alanine antagonism across the range of 1–100 μM as all concentrations caused resistance to 600–800 μg/mL POA (Figures 4C–4F). Similarly, we evaluated the effect of pantothenate on POA susceptibility and found that concentration required to cause resistance to POA was an order of magnitude higher for pantothenate (10 μM) than β-alanine (1 μM) (Figure 5).

Figure 2 M. tuberculosis became resistant to POA when panD expression was induced by Atc. Each strain was printed in triplicate with a replicator at a density of ~10⁵ CFU/mL. (A) WT/pLpUV15tetORs-panD(F) (expressing panD from M. tuberculosis H37Rv) without (−) sign and with (+) sign Atc. (B) WT/pLpUV15tetORs-panD(M) (expressing panD mutant) without and with Atc. (C) WT/pLpUV15tetORs-panD(M) (expressing panD mutant) without and with Atc. (D) POA (156 (M117I)) without and with Atc. (E) WT/pLpUV15tetORs-panD(M) (expressing panD from M. smegmatis) without and with Atc. (F) WT/pLpUV15tetORs-panD(E) (expressing panD from E. coli) without and with Atc. (G) WT/pLpUV15tetORs vector control without and with Atc.
DISCUSSION

PZA resistance in *M. tuberculosis* is most commonly caused either by mutations in the *pncA* gene encoding the PZase required to convert the PZA prodrug to POA or (occasionally) by mutations in the target gene *rpsA* encoding ribosomal protein S1 involved in trans-translation. We recently identified mutations in a third gene, *panD*, (encoding an aspartate decarboxylase involved in pantothenate [vitamin B5] synthesis) in five PZA-resistant strains when PZA was used to select resistant mutants. In this study, to shed new light on possible new targets of PZA, we isolated mutants of *M. tuberculosis* (Figure 1 and Table 1). This surprising finding suggests that POA resistance. Because POA is the active form of the drug PZA, the finding that all the POA-resistant mutants had *panD* mutations (Table 1) suggests that PanD is most likely to be a target of POA/PZA.

Further evidence that PanD is a target of POA/PZA was provided by the PanD overexpression experiment. Target overexpression is a well-known drug resistance mechanism in bacteria. Our results showed that inducible overexpression of wild-type *M. tuberculosis* PanD caused an *M. tuberculosis* strain to be resistant to POA (Figure 2C). In contrast, uninduced strains containing various PanD constructs and the strain that harbored the empty vector were still susceptible to POA, indicating that the induced PanD expression is responsible for the POA resistance. Intriguingly, overexpressing the *M. tuberculosis* PanD M117I mutant or PanD from either *M. smegmatis* or *E. coli* also increased the resistance of *M. tuberculosis* to POA (Figures 2D–2F). These findings suggest that elevated levels of active PanD enzyme, irrespective of the source, confer POA resistance.

The most direct evidence that PanD is a target of POA/PZA was provided by the finding that POA inhibits the enzymatic activity of PanD (Figure 7A) while the prodrug PZA and the structural analog nicotinamide both fail to do so (Figures 7B and 7C). In addition, the observation that all the PanD mutations from the 30 POA-resistant mutants are localized to the C-terminus of the PanD protein (Table 1) suggests that PanD is most likely to be a target of POA/PZA.

![Figure 3](image.png)  
**Figure 3** Antagonism of POA’s activity against *M. tuberculosis* by pantothenate and β-alanine. (A) *M. tuberculosis* parent strain H37Ra on 7H11 agar, (B) a POA-resistant mutant with a PanD mutation (M117I), (C) parent strain on 7H11 agar containing 0.1 mM calcium pantothenate, (D) parent strain with 0.1 mM β-alanine, (E) parent strain with 0.1 mM L-alanine, (F) parent strain with 5 mM calcium L-aspartate, (G) parent strain with 10 mM L-valine, (H) parent strain with 10 mM glutamate.

![Figure 4](image.png)  
**Figure 4** Effect of β-alanine concentration on POA susceptibility. *M. tuberculosis* H37Ra on 7H11 agar with no β-alanine (A), 0.1 μM β-alanine (B), 1 μM β-alanine (C), 5 μM β-alanine (D), 10 μM β-alanine (E), and 100 μM β-alanine (F).
and Figure 1) suggests that POA may bind to this region, and in particular to residue M117 as the most frequent site that is altered in POA-resistant mutants, to inhibit the enzymatic activity of PanD. Moreover, the finding that M117I overexpression confers POA/PZA resistance to M. tuberculosis and that the M117I mutation retains the enzymatic activity of PanD (Figure 7A) suggest that the C-terminal mutations in PanD may affect the binding of POA without eliminating PanD’s enzymatic activity. It is interesting to note that the C-terminal region spanning amino acid residues 114–139 of M. tuberculosis PanD, where all the PanD mutations are mapped (Figure 1), could not be determined in the crystal structure of the M. tuberculosis PanD protein. It is likely that the C-terminal region of M. tuberculosis PanD, where it shows the most sequence variability (Figure 1), is flexible and dispensable for enzymatic activity but is required for POA binding. Future studies are needed to confirm this possibility.

As a peculiar persister drug that plays a crucial role in shortening TB therapy, PZA’s mode of action is unusual and complex and has attracted considerable recent attention due to increasing interest in developing persister drugs not only for TB but also for other persistent infections. PZA clearly interferes with multiple targets in M. tuberculosis, with activities including membrane potential disruption, internal pH acidification, and trans-translation inhibition. In this study, we found new evidence that PanD may represent a target of PZA/POA in M. tuberculosis. PanD converts L-aspartate to β-alanine, which is in turn needed for the synthesis of pantothenate (vitamin B5) and CoA. CoA is a ubiquitous cofactor found in all domains of life that plays a central role in energy metabolism. In addition, M. tuberculosis PanD is a virulence factor that could be involved in persistence in vivo. Our finding that POA inhibits PanD’s enzymatic activity (Figure 7A) involved in energy metabolism and virulence could help to explain the unique sterilizing activity of PZA in vivo.

An interesting observation of this study was that both β-alanine and pantothenate were able to antagonize POA’s activity against M. tuberculosis. This finding suggests that POA inhibits PanD’s activity and that supplementation of downstream products such as β-alanine or pantothenate could rescue or antagonize the lethal action of PZA. This raises the intriguing possibility that variations in the levels of β-alanine or pantothenate in the diet of the host might affect the activity of PZA in vivo. Future studies are needed to address this possibility using animal models. Because humans lack the pathway to synthesize pantothenate that is present in various bacteria including M. tuberculosis, PanD could represent an attractive drug target for the development of new drugs targeting persister bacteria. Indeed, there is recent interest in developing new drugs targeting the pantothenate synthesis pathway in M. tuberculosis.

In summary, we found that panD mutations are closely associated with POA resistance in POA-resistant mutants lacking pncA and rpsA mutations. Inducible PanD overexpression caused significant resistance to POA in M. tuberculosis. In addition, the antituberculosis activity of POA could be antagonized by β-alanine or pantothenate. POA, but not the prodrug PZA or nicotinamide, inhibited the enzymatic activity of PanD in a concentration-dependent manner. These findings suggest that PanD is a new target of POA/PZA. These results have implications for the development of new drugs targeting pantothenate or CoA biosynthesis to improve TB treatment. Future studies are needed to further our understanding of the genetic, biochemical, and structural basis of PanD inhibition by POA and to assess the potential for food-derived β-alanine and pantothenate to antagonize PZA activity in vivo.
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Figure 7 Concentration-dependent inhibition of M. tuberculosis PanD by POA but not by PZA or nicotinamide. The rightmost lane is a 14C-aspartic acid control without PanD or POA addition. (A) POA at 25, 50, or 100 μg/mL significantly inhibited the enzymatic activity of both wild-type and M117I PanD in a concentration-dependent manner as shown by the reduced conversion of aspartate (Asp) to β-alanine (β-Ala). At 200 μg/mL, POA completely inhibited the enzymatic activity of wild-type PanD (panels B and C, first lane from left). In contrast, neither PZA as a prodrug (B) nor nicotinamide (NIC) (C) as a structural analog control at 100 and 200 μg/mL had an apparent effect on PanD’s enzymatic activity.

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