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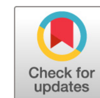

The Bewildering Antitubercular Action of Pyrazinamide

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SUMMARY Pyrazinamide (PZA) is a cornerstone antimicrobial drug used exclusively for the treatment of tuberculosis (TB). Due to its ability to shorten drug therapy by 3 months and reduce disease relapse rates, PZA is considered an irreplaceable component of standard first-line short-course therapy for drug-susceptible TB and second-line treatment regimens for multidrug-resistant TB. Despite over 60 years of research on PZA and its crucial role in current and future TB treatment regimens, the mode of action of this unique drug remains unclear. Defining the mode of action for PZA will open new avenues for rational design of novel therapeutic approaches for the treatment of TB. In this review, we discuss the four prevailing models for PZA action, recent developments in modulation of PZA susceptibility and resistance, and outlooks for future research and drug development.

KEYWORDS pyrazinamide, tuberculosis, drug resistance, drug susceptibility, mode of action, coenzyme A, antimicrobial activity, drug resistance mechanisms

INTRODUCTION

In a 1954 urgent call to scientific action (1), Floyd M. Feldmann, medical director at the National Tuberculosis Association in New York, argued the case for accelerated research into fundamental aspects of tuberculosis (TB) therapy. Feldmann described knowledge gaps concerning the newly discovered sterilizing drug pyrazinamide (PZA). In highlighting areas of need for rapid progress, he posed some basic questions such as "Does [PZA] work in other animal species [besides mice]?" "What is the optimum drug dosage?" and "How [does PZA] work?" More than 65 years later, we know that Feldmann's seemingly simple questions have rather complicated and currently incomplete answers.

Regarding mechanism of action, we know that PZA is a prodrug that is hydrolyzed to pyrazinoic acid (POA) in the mycobacterial cytoplasm by the *Mycobacterium tuberculosis* pyrazinamidase/nicotinamidase (PZase) (2, 3). This amidase, encoded by *pncA* (4), is involved in the salvage pathway for synthesis of the essential cofactor NAD. Since the NAD salvage pathway is nonessential for virulence of *M. tuberculosis* (5, 6), *pncA* loss-of-function mutations represent the most prevalent mechanism for PZA resistance in clinical isolates (4, 7–13). Mutations within *pncA*, including single-nucleotide poly-

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morphisms (SNPs), multinucleotide polymorphisms, and indels, have been mapped along the entire span of the 561-bp open reading frame in PZA-resistant clinical isolates (14–20). Mutations in *pncA* have been shown to confer resistance largely through the loss of PncA enzymatic activity and/or protein abundance (21, 22). While loss-of-function mutations in *pncA* represent the major mechanism of PZA resistance in *M. tuberculosis* clinical isolates, up to 30% of PZA-resistant isolates show PZase activity and possess a wild-type *pncA* gene (15, 23, 24). The latter class of PZA-resistant strains indicates the existence of additional resistance mechanisms that remain to be defined.

Regarding *in vivo* efficacy, it is now well known that PZA is a sterilizing drug that is exquisitely selective against *M. tuberculosis* in multiple animal species, including mice (25, 26), rabbits (27, 28), nonhuman primates (29), humans (30, 31), and guinea pigs (32–34). Through extensive clinical trials, PZA has been found to shorten the required duration of TB therapy by 3 months (35–37) and shows activity against both replicating and slow-growing and nongrowing populations of *M. tuberculosis* bacilli (38–40). Despite the important role of PZA in TB therapy, a significant proportion of those receiving PZA treatment might not achieve the necessary concentrations required for therapeutic benefit due to differences in drug metabolism between individuals (41–43). Furthermore, patient adherence or early cessation of PZA treatment is influenced by its large dosing regimen (25 mg/kg of body weight daily) and adverse side effects, such as liver inflammation, gastrointestinal distress, and joint pain (44–46). Thus, development of more tolerable or more potent PZA or POA analogs may be necessary to treat some populations. Regardless of these unknowns, due to its unparalleled sterilizing activity in the majority of individuals, PZA has become an irreplaceable component of the first-line standard short-course therapy for drug-susceptible TB (47–50) and second-line treatment regimens for multidrug-resistant TB (MDR-TB) (51–53). Further, PZA is anticipated to be a component of future TB therapies (54, 55) involving novel drugs such as bedaquiline (TMC207) (56), the bicyclic nitromidazole pretomanid (PA-824) (57), and moxifloxacin (56).

Despite the indispensable role of PZA in modern TB drug therapy, the mechanistic basis for its action remains unresolved. Feldmann's question "How [does PZA] work?" both fascinates and torments geneticists, microbiologists, and biochemists alike. Advances in mycobacterial genetics, transcriptomics, metabolomics, antibiotic resistance surveillance, and whole-genome sequencing have enabled researchers to identify multiple PZA-linked metabolic pathways that potentially converge on a single cellular process (58). We discuss the merits and drawbacks of four proposed models for the mechanism of PZA action, recent developments in modulation of PZA susceptibility and resistance, and outlooks for future research and drug development.

MODEL 1: PYRAZINOIC ACID FUNCTIONS AS A PROTONOPHORE

The discovery and implementation of PZA as a TB drug are a fascinating story and are covered in depth by Zhang and Mitchison (59) and Murray (60). In brief, PZA was discovered in a screen for antitubercular structural analogs of nicotinamide (vitamin B_3) (61) following the unexpected observation that this vitamin had antitubercular activity in mice (62) and in humans (63, 64). Early experimental studies of the antitubercular action of PZA were largely restricted to mice infected with *M. tuberculosis* (25, 26, 61, 65) because PZA showed no inhibitory activity against the bacilli in standard mycobacterial culture medium (66). Reductionist bacterial culture-based approaches involving PZA were not possible until it was found that exposure of *M. tuberculosis* to mildly acidic conditions could induce PZA susceptibility (67). Dependence on an acidic environment to promote susceptibility to PZA was proposed as the major discrepancy between *in vitro* and *in vivo* environmental conditions. Consistent with this prediction, during initial infection, *M. tuberculosis* is engulfed by alveolar macrophages, in which the bacilli replicate within immature phagosomes with a pH of ~6.2 (68–70). Upon interferon gamma-mediated activation, phagosomal acidification ensues (pH 4.5 to 5.0) (70, 71), rendering this niche well within the pH requirements for induction of PZA susceptibility of *M. tuberculosis*. Consistent with acidic pH as a driver for PZA suscep-

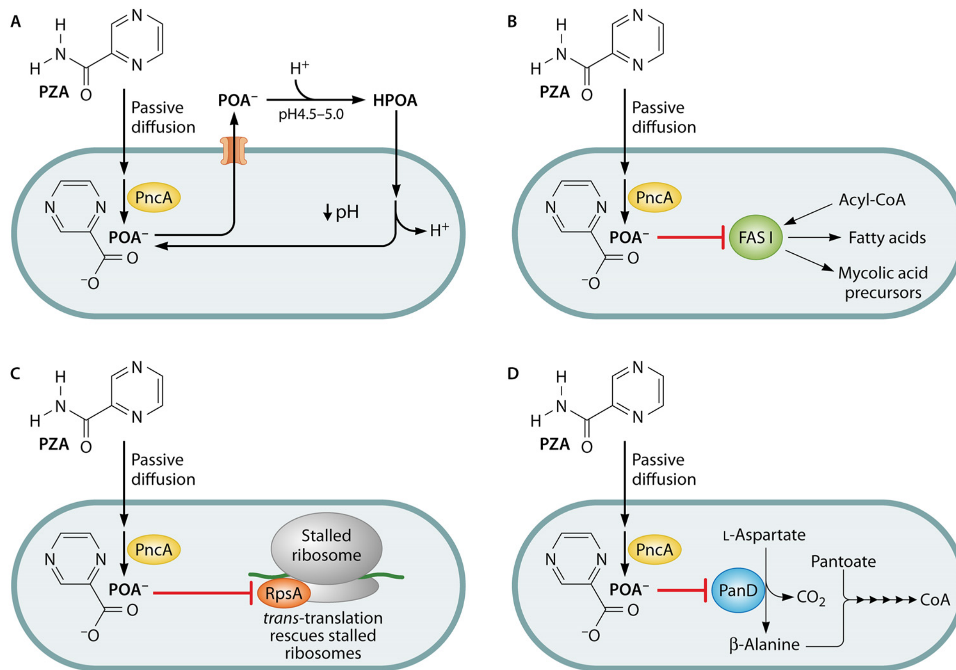

FIG 1 Proposed modes of antitubercular action of pyrazinamide. Pyrazinamide enters the cell by diffusion and is activated by the cytoplasmic pyrazinamidase/nicotinamidase PncA. Pyrazinoic acid has been proposed to act as a protonophore leading to the acidification of the bacterial cytoplasm (A), an inhibitor of fatty acid synthase I (B), an inhibitor of *trans*-translation (C), and/or an inhibitor of coenzyme A biosynthesis (D).

tibility of *M. tuberculosis in vivo*, mice that produce tubercle lesions with alkaline pH respond poorly to treatment with PZA (72, 73).

Acidic pH-driven susceptibility was the initial inspiration for the first mechanistic model for PZA action proposed by Zhang and colleagues (74–76) and has been extensively reviewed (77–79) (Fig. 1A). Under this model, PZA enters *M. tuberculosis* by passive diffusion across the cellular envelope to the cytoplasm (pH 7.2), where it is converted to the weak acid POA (pK_a of 2.9) by PZase (76). POA anion is then exported from the bacillus through an unidentified weak efflux mechanism. In an acidic environment (e.g., activated phagosome or acidified culture medium), a small fraction of POA becomes protonated to form HPOA, which can permeate back across the bacterial envelope into the cytoplasm. By the Henderson-Hasselbalch equation {$pH = pK_a + \log_{10}([A^-]/[HA])$}, the theoretical amounts of protonated POA would be 0.1% at pH 5.8 and 0.008% at pH 7.0 (74). Once in the cytoplasm, HPOA dissociates to H^+ and POA, and this cycle continues, resulting in cytoplasmic accumulation of protons, collapse of the cellular membrane potential, and acidification of the cytoplasm.

Consistent with this model for POA as a protonophore, it has been shown that PZA treatment is associated with disruption of intrabacterial pH (pH_{IB}) from 7.2 to below 6.5 within 48 h of treatment at pH 4.5 (80). Further, compounds that interfere with oxidative phosphorylation, such as the membrane potential uncoupling ionophore carbonyl cyanide 3-chlorophenylhydrazone (CCCP; pK_a, 4.8) and the F_oF_1-type ATP synthase inhibitor *N,N′*-dicyclohexylcarbodiimide (DCCD), have been reported to be synergistic with PZA when used in combination against *M. tuberculosis* in culture (74). Moreover, treatment of *Mycobacterium bovis* BCG with POA resulted in a progressive depletion in bacterial levels of ATP (81). While the protonophore model is widely cited within the literature, the studies described above did not directly address whether these physiologically relevant effects were the direct result of the proposed proton shuttling mechanism or a downstream effect of a yet-to-be-defined activity of POA. Consistent with the latter, collective evidence demonstrates that acidic pH is not strictly required for PZA action. Indeed, under near neutral culture conditions, PZA suscepti-

bility can be promoted by overexpression of *pncA* (82, 83), inhibition of efflux pumps to prevent POA export from the bacilli (74, 84, 85), exposure of bacilli to conditions such as alkaline pH (67), nutrient limitation (83, 86), decreased temperature (87), and hypoxia (88–90), and replacement of PZA with POA (29). The nonessentiality of acidic pH for PZA and POA susceptibility of *M. tuberculosis* challenges the protonophore model as the principal basis for action of this drug.

Peterson et al. (83) recently compared the activity of bona fide ionophores with those of PZA and POA for the ability to disrupt membrane potential and pH_{IB} under conditions that mediate susceptibility. pH_{IB} homeostasis was assessed in an *M. tuberculosis* strain harboring a plasmid encoding a pH-sensitive ratiometric green fluorescent protein (pH-GFP) (91). This strain was treated with PZA, POA, CCCP (80, 92, 93), or monensin (93–95) under standard conditions used for PZA susceptibility testing (pH 5.8). While both CCCP and monensin led to rapid and dose-dependent intrabacterial acidification, there was no significant change in pH_{IB} associated with POA and PZA treatment within the first 3 h (83). Since POA has a low pK_a relative to those of CCCP (pK_a, 4.8) and monensin (pK_a, 6.6), it is not surprising that POA does not have robust protonophore activity under the experimental conditions that were used. Furthermore, PZA treatment of *M. tuberculosis* overexpressing *pncA* at neutral pH did not lead to a measurable decrease in pH_{IB} despite full inhibition of bacterial growth (83). Membrane potential was also measured using a membrane-permeable fluorescent dye ($DiOC_2$) in *M. tuberculosis* cells treated with CCCP or with POA at concentrations up to 10-fold over the MIC (83). As expected, CCCP treatment resulted in a dramatic loss of membrane potential. However, even at concentrations 10-fold above the MIC, POA failed to alter the membrane potential of *M. tuberculosis* in medium at pH 5.8 over the period that was evaluated (83).

The dispensability of acidic pH for PZA and POA action, lack of cytoplasmic acidification following exposure to inhibitory concentrations of PZA and POA, and lack of impact on membrane potential in treated and control cultures significantly undermine the protonophore model as the principal basis for POA action. It is likely that acidic conditions within the phagosomal compartment of activated macrophages provide the initial salvo for host-mediated potentiation of PZA action, but POA-dependent disruption of membrane potential and pH_{IB} seem unlikely to be the driving forces behind the sterilizing activity of this drug.

MODEL 2: PYRAZINOIC ACID INHIBITS MYCOBACTERIAL FATTY ACID SYNTHASE I

In a second model for the mechanism of PZA action, it was proposed that POA selectively inhibits mycobacterial fatty acid synthase I (FAS-I) (96), a large multifunctional enzyme required for synthesis of C_{16} to C_{26} fatty acids (97–99) (Fig. 1B). Like its eukaryotic homolog, this enzyme contains all domains necessary for synthesis of fatty acids using acetyl coenzyme A (acetyl-CoA) as the primer unit and malonyl-CoA as two-carbon extender units (100, 101). In each round of extension, two molecules of NADPH are oxidized via the enoyl and beta-ketoacyl reductase activities of FAS-I (102). In mycobacterial species, FAS-I products can either be utilized for synthesis of cytoplasmic membrane lipids or be transferred to the fatty acid synthase II complex for synthesis of a diverse array of mycolic acids (101).

The FAS-I inhibition model for PZA action emerged from a study in which genomic DNA libraries from *M. bovis* BCG and *Mycobacterium avium* were expressed from multicopy cosmids in *Mycobacterium smegmatis* to screen for loci that conferred resistance to a structural analog of PZA, 5-chloropyrazinamide (5-Cl-PZA) (96). Subcloning analysis demonstrated that 5-Cl-PZA resistance was associated with *fas-I* overexpression (96). Likewise, overexpression of the *M. tuberculosis fas-I* gene in *M. smegmatis* also conferred resistance to 5-Cl-PZA (96). Since *M. smegmatis* is intrinsically resistant to PZA and POA, and overexpression of FAS-I was found to be toxic for *M. tuberculosis*, it was not possible to determine whether FAS-I overexpression could confer resistance to PZA or POA in mycobacteria (96). Through the use of [^{14}C]acetate labeling studies, it

was found that susceptible mycobacteria treated with PZA (82, 96, 103), 5-Cl-PZA (82, 96, 99, 103), and other PZA derivatives (103) showed a significant reduction in C_{16} to C_{26} fatty acid biosynthesis. Despite these findings, a direct association between PZA action and FAS-I inhibition was questioned by Boshoff et al. (104). Although 5-Cl-PZA was confirmed as a potent and irreversible FAS-I inhibitor, POA did not inhibit purified mycobacterial FAS-I at physiologically relevant concentrations (104). In a subsequent study, inhibition of recombinant *M. tuberculosis* FAS-I was confirmed, yet more than 9 mM POA was required to achieve 50% inhibition, in contrast to just 15 μM for 5-Cl-PZA (105). Since an intrabacterial concentration of 0.5 mM POA is sufficient for *M. tuberculosis* growth arrest (74), it seems unlikely that direct inhibition of FAS-I via POA is sufficient to explain mode of action. Enzymology and ligand interaction studies involving saturation transfer difference nuclear magnetic resonance (NMR) showed that PZA, 5-Cl-PZA, and other PZA analogs are competitive inhibitors of NADPH binding to purified mycobacterial FAS-I (105–107). In contrast, while POA was found to interact with FAS-I, it did not compete with NADPH for binding (107), indicating that association of 5-Cl-PZA and POA with FAS-I is mechanistically distinct. Together, these observations suggest that POA does not directly inhibit FAS-I, and inhibition of fatty acid synthesis by POA may be due to a linked metabolic disruption or inhibition of FAS-I by an as-yet-unidentified mycobacterial metabolite of POA.

MODEL 3: PYRAZINOIC ACID BINDS TO RpsA AND INHIBITS *TRANS*-TRANSLATION

In a third model for the mechanism of PZA action, it was suggested that POA selectively disrupts the process of *trans*-translation (108) (Fig. 1C). *trans*-Translation, discovered by Keiler et al. (109), is a ribosome salvage pathway used by nearly all bacterial species to free ribosomes that cannot disengage from the 3′ end of an mRNA lacking an in-frame stop codon (non-stop mRNA) (110). Without ribosome rescue, ribosomes can become sequestered by non-stop mRNAs, ultimately resulting in cell death due to arrest of protein synthesis (110). In the *trans*-translation pathway, SmpB and elongation factor Tu recruit tmRNA, a specialized RNA that has both tRNA and mRNA properties, to stalled ribosomes that lack an mRNA codon at the A site (111–113). Once recruited, alanine-charged tmRNA acts as a codon-independent tRNA and becomes linked to the nascent peptide through transpeptidation (109). Cotranslational switching then results in release of the non-stop mRNA with replacement by a loop of the tmRNA which encodes a degradation tag (109, 113). Following translation of this tag, the nascent peptide is released and targeted for proteolysis, and the ribosome disengages from tmRNA and is free to initiate translation of other available mRNA (109). This pathway is essential for viability of *M. tuberculosis* and many other bacterial pathogens and represents an outstanding novel target for drug discovery (114–117). Indeed, structurally related families of oxadiazole and tetrazole-based compounds have recently been identified that inhibit *trans*-translation in a large number of bacterial species, including Gram-negative, Gram-positive, and mycobacterial species (114–117).

The model for inhibition of *trans*-translation by POA emerged from a study focused on a presumed interaction between POA and the *M. tuberculosis* 30S ribosomal subunit protein S1 (108). In an attempt to identify interaction partners and putative targets of POA, Shi et al. (108) performed affinity chromatography studies in which the POA derivative 5-hydroxyl-2-pyrazinecarboxylic acid was covalently linked to a Sepharose column and used as a binding matrix for proteins from a whole-cell lysate of *M. tuberculosis* strain H37Ra. Nonspecific stripping of all proteins that had bound to the column using 25% ethylene glycol resulted in isolation of multiple proteins, of which RpsA (30S ribosomal protein subunit S1), Rv2783, Rv2731, and Rv3169 were identified by mass spectrometry (108). Consistent with a role for RpsA in PZA action, the authors stated that overexpression of *rpsA* conferred 5-fold resistance to PZA (108). In addition, the clinical isolate *M. tuberculosis* strain DHMH444, which shows 2-fold resistance to PZA and carries a wild-type *pncA* allele (12, 118), was found to harbor deletion of an alanine codon at position 438 (ΔA438) within the C-terminal region of the *rpsA* product (108). Isothermal titration calorimetry (ITC), an approach that can be used to determine

ligand binding affinities through monitoring changes in free energy, was employed to evaluate a possible interaction between POA and purified recombinant RpsA (108). Titration of a saturated solution of POA (~70 mM) into a solution of 10 μM wild-type *M. tuberculosis* RpsA showed a robust exothermic signal (108). When 100 μM POA was titrated into solutions of 10 μM *M. smegmatis* RpsA and *M. tuberculosis* RpsAΔA438, no signal was observed (108). While use of starkly different concentrations of POA in these assays makes it impossible to interpret these findings, it was concluded that POA bound wild-type *M. tuberculosis* RpsA with high affinity and failed to interact with *M. smegmatis* RpsA and *M. tuberculosis* RpsAΔA438 (108).

To evaluate whether POA could disrupt *trans*-translation, cell-free *in vitro* translation assays were conducted in reaction mixtures containing ribosomes isolated from *M. tuberculosis*, *M. smegmatis*, or *Escherichia coli* and supplemented with a charged tRNA mixture, *M. tuberculosis* SmpB, and unprocessed pre-tmRNA (108). Translation was assessed by detecting incorporation of [^{35}S]methionine into dihydrofolate reductase (DHFR) expressed from an mRNA containing an in-frame stop codon (wild-type DHFR), or a similar message with the DHFR coding sequence followed by 8 rare AGG codons, 18 additional downstream codons, and an in-frame stop codon (DHFR 8×AGG) designed to induce translational stalling (108, 119). It is important to note that for rare codon-mediated translational stalling to trigger *trans*-translation, the culprit mRNA must be cleaved by an RNase in order to permit interaction between the ribosome and aminoacyl-tmRNA/SmpB complex (111, 119). In the assays reported by Shi et al. (108), if *trans*-translation were to ensue from stalling on the DHFR 8×AGG message, the resulting peptide would be extended by 13 amino acids corresponding to the tmRNA degradation tag. Since DHFR 8×AGG produced by standard translation would be extended by 26 amino acids, it would be critical to characterize the C-terminal residues of the resulting peptide. As expected, POA treatment had no impact on translation of wild-type DHFR by *M. tuberculosis* ribosomes or of DHFR 8×AGG with ribosomes from *M. smegmatis* and *E. coli* (108). In contrast, translation of DHFR 8×AGG by *M. tuberculosis* ribosomes was fully inhibited by the addition of POA at concentrations of 200 μM and greater (108). Unfortunately, the authors did not determine whether the shifted DHFR contained the tmRNA degradation tag or simply the 26 additional amino acids introduced by standard translation of DHFR 8×AGG (108). It is curious that these data differ from those in an earlier version of the manuscript that was deposited in the NCBI database (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3502614/), where signal for translation of DHFR 8×AGG is visible in the presence of as much as 800 μM POA. Regardless, it is of fundamental importance that since translation precedes *trans*-translation, inhibition of *trans*-translation would have resulted in synthesis of a nontagged DHFR. Thus, the reported results showing full inhibition of signal for protein synthesis are consistent with inhibition of translation, not inhibition of *trans*-translation.

Inspired by the findings of Shi et al. (108), several groups evaluated whether targeted sequencing of *rpsA* could be used to predict PZA resistance in *M. tuberculosis* clinical isolates bearing a wild-type *pncA* locus. Mutations within *rpsA* were identified in a limited number of strains, although no clear association with PZA resistance has been established (23, 108, 120–126). Alexander et al. (120) speculated that the RpsA C-terminal region is tolerant to amino acid substitutions and may be innocuous with respect to PZA action. In response to a comment posted by Simons and colleagues (121), Alexander et al. (127) cautioned that research attempting to attribute drug resistance to novel mutations must be tempered with experiments demonstrating linkage between phenotype and genotype. Indeed, two independent groups reconstructed the *rpsA* ΔA438 allele in *M. tuberculosis*, and both showed a <2-fold change in PZA susceptibility (128). Importantly, mutations in *rpsA*, including Δ438, have been identified in PZA-susceptible clinical isolates of *M. tuberculosis* (129, 130). Moreover, 10-fold overexpression of *rpsA* had no measurable impact on PZA susceptibility (128). Lack of an association between *rpsA* and PZA resistance is consistent with previous reports by Spiers et al. and Klemens et al., which demonstrated that *M. tuberculosis* strain DHMH444 is fully susceptible to POA *in vitro* (118) and to PZA in a murine model

of infection (131), respectively. Collectively, these observations demonstrate that *rpsA* is not associated with PZA or POA susceptibility, and the low-level PZA resistance of *M. tuberculosis* strain DHMH444 is most likely due to its documented reduced level of PncA activity (118).

To reevaluate the possible interaction between POA and *M. tuberculosis* RpsA, Dillon et al. (128) repeated ITC ligand interaction studies described by Shi et al. (108). Studies with *E. coli* (132) and *Pseudomonas* (133) have shown that RpsA binds to single-stranded RNA and is important for translation initiation. When RpsA was titrated with poly(C) RNA, a robust bimodal interaction signal was observed, indicative of two high-affinity single-stranded RNA binding sites of RpsA. However, when 100 μM RpsA (pH 7.4) was titrated with a saturated solution of POA (pH 7.4), no change in free energy was detected, indicating that these solutes do not show a measurable interaction. In contrast, when the pH of the saturated solution of POA (pH 2.3) was not adjusted to match the diluent buffer and was titrated into near neutral (pH 7.4) phosphate buffer, a robust exothermic signal was observed (128), similar to that reported by Shi et al. (108). This signal was abolished when the pH of the saturated POA solution was adjusted to that of the diluent buffer (128). Thus, it is most probable that the signal reported by Shi et al. was a result of pH-dependent proton dissociation and not reflective of interaction between RpsA and POA.

Despite the ability of RpsA to interact with tmRNA (134), RpsA has been shown to be entirely dispensable for *trans*-translation in species in which its role has been evaluated, such as *E. coli* (135) and *Thermus thermophilus* (136, 137). To further examine the connection between POA action and mycobacterial *trans*-translation, Dillon et al. conducted cell-free *in vitro trans*-translation assays in reaction mixtures containing *M. tuberculosis* ribosomes supplemented with a charged tRNA mixture, appropriately processed and charged tmRNA, and *M. tuberculosis* SmpB (128). Rather than relying on translational stalling and mRNA cleavage to trigger *trans*-translation, a non-stop DHFR transcript was used (128). In these assays, *trans*-translational tagging of the non-stop DHFR was confirmed and tagging could be inhibited by an antisense oligonucleotide directed against tmRNA (115). Importantly, no inhibitory effect on *trans*-translation was observed with as much as 1 mM POA (128). Thus, the antitubercular activity of PZA is independent of *trans*-translation and RpsA.

MODEL 4: PYRAZINOIC ACID BLOCKS COENZYME A SYNTHESIS THROUGH INHIBITION OF L-ASPARTATE DECARBOXYLASE

In order to discover novel mechanisms for PZA resistance in *M. tuberculosis*, Zhang et al. isolated and characterized a large number of spontaneous PZA-resistant isolates (138). Of 174 strains that were analyzed, 169 had mutations in *pncA*, while 5 carried wild-type *pncA* and were subjected to full-genome resequencing. These *pncA* wild-type strains were found to harbor missense mutations within the *panD* (*Rv3601c*) gene, which encodes L-aspartate decarboxylase (138), a rate-limiting step in the CoA biosynthetic pathway (139, 140). In this pathway, β-alanine and L-pantoate are ligated by PanC (141) to form pantothenate, which is processed in five additional steps to afford CoA (Fig. 1D) (142). Further work revealed that PZA resistance phenotypes could be recapitulated in H37Ra overexpression of wild-type or mutant *panD* as well as *panD* from *E. coli* and *M. smegmatis* (143). Consistent with a role for POA in disruption of CoA biosynthesis, multiple recent studies have demonstrated that supplementation of culture medium with pathway intermediates, such as β-alanine, pantothenate, and pantetheine, can potently antagonize PZA- and POA-mediated growth inhibition of *M. tuberculosis* (143–145). In addition, Dillon et al. (144) demonstrated that other β-alanine-containing metabolites as well as the β-alanine structural analogs 3-aminopropanol and propanoic acid could antagonize PZA action. However, the β-alanine precursor, aspartate, and cosubstrate in pantothenate synthesis, pantoate, were not sufficient to induce an antagonistic effect (144). These data suggest that POA interacts in some way with the CoA biosynthetic pathway.

As CoA is an essential acyl carrier for hundreds of reactions in central metabolism

(142), CoA depletion would provide an enticing explanation for the action of POA that unifies previous observations, such as the previously discussed impairments of energy metabolism and fatty acid synthesis. Notably, several groups have shown stress conditions that decrease cellular CoA pools, such as nutrient starvation and hypoxia, augment PZA susceptibility in *M. tuberculosis* (88–90). In fact, PZA treatment in anaerobic cultures of *M. tuberculosis* resulted in a 2-log reduction of bacterial CFU after 5 days (88). Recent work by Gopal et al. (145) has shown that wild-type *M. bovis* BCG displayed a significant decrease of cellular CoA after 12 and 24 h of POA treatment. Importantly, mutations in *panD* abrogated POA-mediated CoA depletion, resulting in CoA levels that were comparable to those of the no-drug control (145). Further, this study demonstrated that CoA depletion is specific for POA, as structural analogs, nicotinic acid and benzoic acid, did not significantly alter cellular CoA levels. In a separate study, Gopal et al. (146) conducted a metabolomic analysis on *M. bovis* BCG treated with POA to assess the effect of POA on intermediates of the CoA biosynthetic pathway. POA treatment resulted in a $>$10-fold reduction in β-alanine levels and depletion of numerous downstream intermediates in this pathway within 4 h. Additionally, depletion of CoA by POA resulted in the concomitant increase in the concentrations of medium-chain, dicarboxylate, and long-chain fatty acids within 24 h of treatment. Accumulation of fatty acids may contribute to bacterial cell death through impairment of oxidative phosphorylation and eventual collapse of membrane potential (147). Consistent with these findings, Rosen et al. showed that loss-of-function mutations in *fadD2*, an acyl-CoA ligase responsible for the detoxification of fatty acids, leads to hypersusceptibility of POA (148).

Based on genetic analysis of *panD* and the ability of POA to broadly disrupt CoA homeostasis, it is possible that POA interferes with activity of *M. tuberculosis* L-aspartate decarboxylase (Fig. 1D). In *M. tuberculosis*, PanD functions as a tetramer and shares sequence and structural similarity with other members of the PanD family (139). Yet *M. tuberculosis* PanD has a 13-amino-acid C-terminal extension which serves as the central contact point for tetramer formation (139). It is interesting that the majority of spontaneous *panD* mutations identified by Zhang et al. occurred within the portion corresponding to the last 13 amino acids of the C terminus (138). Similar *panD* missense mutations corresponding to the C terminus were subsequently described by Gopal et al. (145). In support of L-aspartate decarboxylase as a molecular target of POA, Gopal et al. (146) demonstrated interaction between PanD and POA (K_D [equilibrium dissociation constant] $= 6.1\ \mu M \pm 0.88\ \mu M$). Mutations within the N terminus and C terminus of PanD abrogated this interaction, which suggests that PanD-related PZA resistance is likely due to a loss of binding. Collectively, these studies support a model in which POA binds to PanD and inhibits synthesis of β-alanine, which ultimately leads to CoA insufficiency and broadly impaired central metabolism. However, it is important to note that an *M. tuberculosis* pantothenate auxotrophic strain (mc^{2}7000) containing a *panD* deletion remained susceptible to PZA when cultured in medium containing a subantagonistic concentration of panthetheine (144). Similar to the case with the parental strain, PZA susceptibility of *M. tuberculosis* mc^{2}7000 could be antagonized by exogenous pantothenate. However, unlike for the parental strain, PZA susceptibility of strain mc^{2}7000 was not antagonized by supplementation with β-alanine. These data demonstrate that if PanD is indeed a target of POA, additional targets likely exist within the CoA biosynthetic pathway. Future studies are necessary to further clarify the mechanism behind disruption of CoA biosynthesis and PZA activity.

Despite the isolation of POA-resistant *panD* missense mutants using laboratory strains of *M. tuberculosis*, analogous mutations have yet to be described for clinical isolates (123, 149). However, it is worth noting that the naturally PZA-resistant *Mycobacterium canetti* harbors a PanD M117T amino acid substitution (138). Importantly, recent work described by Gopal and colleagues (150) showed enrichment of POA-resistant *M. tuberculosis* strains from infected BALB/c mice that had been treated for 8 weeks with POA. Approximately 80% of *M. tuberculosis* POA-resistant isolates derived from infected mice contained mutations in *panD*, with the majority of these mutations

corresponding to the C terminus. These recovered *panD* mutant strains were not reevaluated for PZA resistance *in vivo*. Yet infectivity of a previously characterized *M. tuberculosis* POA-resistant *panD* mutant (POA^R 1) (145) was assessed using a low-dose aerosol infection in BALB/c mice. After 6 weeks of infection, this strain was found to have *in vivo* growth comparable to that of a matched wild-type control, suggesting that *panD* mutant strains remain infective (150). Furthermore, a recent study conducted by Ramirez-Busby et al. (125) analyzed 224 extensively drug-resistant (XDR) *M. tuberculosis* clinical isolates that showed PZA resistance, of which one *pncA* wild-type isolate contained a heterogeneous mutation (-G291) in *panD*. While the collective data demonstrate an incontrovertible association between POA action and CoA metabolism, the clinical relevance of *panD* to PZA resistance demands further analysis.

OTHER GENES ASSOCIATED WITH PZA RESISTANCE

Several other genes associated with PZA and POA resistance have recently been reported. Two independent laboratories have demonstrated a connection between mutations in *clpC1* and POA resistance (150–153). ClpC1 (154, 155) is a class II AAA+ ATPase that provides chaperone activity for the essential cytoplasmic Clp protease (156–158). It is unclear whether *clpC1*-related POA resistance is due to a direct or indirect mechanism and how this relates to previous findings involving the CoA biosynthetic pathway.

Other research groups have expanded the list of potential *M. tuberculosis* targets responsible for PZA resistance. Njire et al. (159) have associated an Asp67Asn substitution in Rv2783 with PZA resistance. Rv2783 is a bifunctional enzyme that catalyzes the metabolism of RNA, single-stranded DNA, and ppGpp and was identified in a POA affinity chromatography assay by Shi et al. (108). Additional studies have associated PZA and POA resistance with mutations in numerous genes of unknown function (153). The roles of the corresponding functions of these various genes in resistance to PZA have yet to be elucidated but indicate that susceptibility and resistance of *M. tuberculosis* to PZA are quite complex.

FUTURE DIRECTIONS

Despite the identification of *M. tuberculosis* POA-resistant isolates *in vitro*, the *in vivo* relevance of the corresponding mutations to PZA resistance remains unclear. Correlation between *in vitro* findings and clinical efficacy are not yet straightforward and will require additional studies to resolve. As a first step, resistant strains identified *in vitro* should undergo extensive confirmation in animal models of TB infection in order to bridge the gap between *in vitro* and *in vivo* findings. Further, studies involving animals with defined impairments in cell-mediated immunity can help to elucidate the relevance of specific host responses that are critical for PZA efficacy. These animal experiments should utilize PZA and POA concentrations similar to those used in TB patients in order to represent standard treatment.

In addition to detailed characterization of novel PZA resistance mechanisms, future research should focus on other compounds that synergize with PZA. Niu and colleagues (160) screened a clinical drug library containing 1,524 substances for compounds that showed synergy with PZA. One hundred thirty hits were found to enhance PZA activity against stationary-phase cultures of *M. tuberculosis* strain H37Ra. Eighty-three of these hits were compounds that have FDA approval for other medical indications and should be evaluated for their potential in repurposing for enhancing PZA action. The identification and study of synergistic compounds will provide insight on the mode of action of PZA and could lead to shorter, more effective treatment regimens.

Recent elegant studies of PZA pharmacokinetics in TB patients (161) and animal models (28, 29, 162–164) have highlighted the importance of drug distribution and penetration into various lesion types as well as the reliance of the intracellular environment for PZA activity. In addition to measuring pH and tissue penetration, future studies should seek to characterize PZA metabolites in the caseum throughout the TB

disease spectrum. Recently, Marakalala and colleagues have characterized the host proteomes of multiple lesion types and regions (caseous granuloma, caseous granuloma caseum, cavitary granuloma, cavitary granuloma caseum, and solid granuloma) (165). Interestingly, this study showed that greater differences occurred within regions of the same granuloma than among different lesion types. The centers of the granuloma were found to contain multiple proinflammatory signals, antimicrobial peptides, reactive oxygen species, and proinflammatory eicosanoids. In contrast, the tissue surrounding the caseum displayed an anti-inflammatory profile. Mapping of various granuloma landscapes should be expanded to include the characterization of resident *M. tuberculosis* subpopulations by single-cell analysis. Proteomic evaluation of heterologous granulomas and specific regions and *M. tuberculosis* subpopulations paired with targeted PZA pharmacokinetic data will provide researchers with a robust model of drug efficacy, potentiation by the host, and responsive or nonsusceptible bacterial cells. This model may be utilized to design antibiotic adjuvants and adjunctive therapeutics to enhance the host response and circumvent PZA resistance (166).

CONCLUDING REMARKS

While questions regarding PZA action that were posed by Feldman over 60 years ago have not been fully resolved, significant steps have been undertaken to understand this crucial drug. Recent advances have cleared some of the prevailing dogma that has surrounded PZA and indicated a correlation between metabolic activity and the drug's activity. Future studies will expand upon these findings through examining the activity of PZA in the context of its associated host microenvironment. We are fortunate to be in a period of scientific research with unprecedented productivity bolstered by advances in genomics, high-throughput drug screens, and pharmacokinetics, all of which will be crucial to finally solve Feldmann's 1954 questions concerning the basis for PZA activity against *M. tuberculosis*.

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