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Mechanisms of Pyrazinamide Action and Resistance

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Abstract

PZA is a unique anti-tuberculosis drug that plays a key role in shortening the TB therapy. PZA kills non-replicating persisters that other TB drugs fail to kill, and thus making it an essential drug for inclusion in any drug combinations for treating drug susceptible and drug-resistant TB such as MDR-TB. PZA acts differently from common antibiotics by inhibiting multiple targets such as energy production, trans-translation and perhaps pantothenate /coenzyme A required for persister survival. Resistance to PZA is mostly caused by mutations in the *pncA* gene encoding pyrazinamidase involved in conversion of the prodrug PZA to the active form POA. Mutations in the drug target RpsA are also found in some PZA-resistant strains. The recent finding that *panD* mutations are found in some PZA-resistant strains without *pncA* or *rpsA* mutations may suggest a third PZA resistance gene and a potential new target of PZA. Current phenotype based PZA susceptibility testing is not reliable due to false resistance, and sequencing of the *pncA* gene represents a more rapid, cost-effective and more reliable molecular test for PZA susceptibility testing and should be used for guiding improved treatment of MDR/XDR-TB. Finally, the story of PZA has important implications for not only TB therapy but also chemotherapy in general. PZA serves as a model prototype persister drug and hopefully a 'tipping point' that inspires new efforts at developing a new type of antibiotics or drugs that target non-replicating persisters for improved treatment of not only TB but also other persistent bacterial infections.

The History: The Unusual Discovery and the Roller Coaster of PZA

Pyrazinamide (PZA), a nicotinamide analogue (Fig. 1), was first chemically synthesized in 1936 (1) but its antituberculosis was not recognized till 1952 (2). Its discovery as a TB drug was based on a serendipitous observation that nicotinamide had certain activity against mycobacteria in animal models (3). Subsequent synthesis of nicotinamide analogs and direct testing in the mouse model of tuberculosis (TB) infection without in vitro testing led to the identification of PZA as a most active agent (4, 5). Before 1970s, PZA was mainly used as a second-line TB drug for the treatment of drug resistant TB or in treatment of relapsed TB because of the hepatic toxicity caused by higher PZA dosage (3.0 g) and longer treatment

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used in earlier clinical studies. However, largely encouraged by the impressive mouse studies by McDermott and colleagues that demonstrated high sterilizing activity of PZA in combination with isoniazid (INH) (6), the British MRC conducted clinical trials in East Africa with lower PZA doses (1.5 – 2.0 g daily), which is not significantly hepatotoxic. PZA was found almost as effective as rifampin (RIF) as a sterilizing drug as judged by more frequent sputum conversion at 2 months and by the relapse rates. Subsequent clinical studies showed that the effects of RIF and PZA were synergistic. These studies showed that treatment could be shortened from 12 months or more to 9 months if either RIF or PZA was added to the regimen but to 6 months if both were included (7). PZA has since been used as a first-line agent for treatment of drug susceptible TB with RIF and INH and ethambutol, which is currently the best TB therapy. PZA is also an integral component of treatment regimens for MDR-TB (8) and also of any new regimens in conjunction with new TB drug candidates in clinical trials (9).

Importance of PZA in Shortening TB Therapy

PZA is a critical frontline TB drug that plays a unique role in shortening the treatment period from 9–12 months to 6 months (7, 10, 11). The inclusion of PZA with isoniazid (INH) and rifampin (RIF) forms the basis for our current short course chemotherapy based on the work by McDermott and colleagues in a mouse model of TB infection (6, 12). This powerful sterilizing activity is due to PZA killing a population of *M. tuberculosis* persisters that are not killed by other drugs (13). PZA is used during the first 2-month intensive phase of the 6 month therapy as giving PZA longer than 2 months does not appear to add additional benefit (7). This is presumably because inflammation leading to an acid environment in the lesions had decreased after 2 months. More recent efforts to find optimal drug combinations with new drug candidates for shortening TB treatment in the mouse model suggest that PZA is the only drug that cannot be replaced without compromising treatment efficacy (14–16). In view of its unique and indispensable sterilizing activity among all TB drugs including new drug candidates in clinical trials, there is recent unprecedented interest in PZA as seen by three workshops on PZA in about a year (http://www.cdc.gov/tb/publications/newsletters/notes/TBN_4_12/labbranch_update.htm).

Mechanisms of PZA Action

PZA is a mysterious, unconventional and paradoxical drug. The mode of action of PZA is unusual and has puzzled investigators ever since its clinical use in 1952. The main reason is that PZA is very different from common antibiotics that are primarily active against growing bacteria and no or little activity for non-growing persisters. However, PZA is exactly the opposite to common antibiotics as it has no or little activity against growing tubercle bacilli and is primarily active against non-growing persisters (17, 18).

Despite its powerful in vivo sterilizing activity demonstrated both in the animal model (6, 12) and in humans in shortening TB chemotherapy (13), PZA has no activity in vitro under normal culture conditions at neutral pH (19), but is active only at an acid pH (e.g. pH 5.5) in vitro (20). Furthermore, unlike other TB drugs, the activity of PZA increases with decreasing metabolic activity. PZA only kills *M. tuberculosis* slowly in vitro at acid pH (21).

In vivo, PZA has high sterilizing activity against persisters in an acidic environment that is present during inflammation (22, 23), which is responsible for its ability to shorten TB therapy. Despite its use for the past 70 years, and despite its importance as an irreplaceable frontline drug in shortening TB therapy, the mode of action of PZA is the least understood of all TB drugs (24). However, new progress has been made in our understanding of PZA in recent years. Much of the historical and clinical aspects of PZA was covered in a previous review article published in 2003 (24) and will not be discussed here. We will mainly focus on new developments in PZA since then while retaining the basic important information.

PZA is a prodrug that is converted to the active form pyrazinoic acid (POA) by PZase/nicotinamidase encoded by the *pncA* gene in *M. tuberculosis* (25) (Fig. 1). The purified recombinant *M. tuberculosis* PncA is a Mn^{2+} and Fe^{2+} containing enzyme that is a monomer (26). The mechanism of PZA conversion to POA may be similar to that of the nitrilase superfamily in which nucleophilic attack by active site cysteine generates a tetrahedral intermediate that collapses with the loss of ammonia and subsequent hydrolysis of the thioester bond by water (27, 28).

Based on various studies (17, 25, 29–31), the following model for the mode of action of PZA was proposed (Fig. 2)(24, 30). PZA enters bacilli through passive diffusion and is converted into POA (a moderately strong acid with pKa of 2.9) by the cytoplasmic PZase encoded by *pncA*. POA then gets out of the cell through passive diffusion and a deficient efflux mechanism in *M. tuberculosis* (29). Once POA is outside the cell, if the extracellular pH is acidic (e.g. pH 5.5), a small proportion of POA will become uncharged protonated acid HPOA, which readily permeates through the membrane. The acid-facilitated POA influx can overcome the weak deficient POA efflux, which causes accumulation of POA in *M. tuberculosis* cells at acid pH over time (29). The HPOA brings protons into the cell and this could eventually cause cytoplasmic acidification such that vital enzymes could be inhibited. In addition, POA could de-energize the membrane by collapsing proton motive force and affect membrane transport inhibiting protein and RNA synthesis (30). At neutral or alkaline pH, there is little POA found in *M. tuberculosis* (29), because over 99.9% of POA is in charged anion form (17) and does not get into cells easily and remains outside the cells (29). This observation explains why PZA is active at acidic pH but not at neutral pH (20) and also explains the correlation between the MIC of PZA and acidic pH values, which can be expressed by the Henderson-Hasselbalch equation (17). It is worth noting that acid pH not only allows POA to re-enter and accumulate in the bacilli (29) but also decreases the membrane potential and inhibits growth and metabolism required for the drug action. The unique activity of PZA against *M. tuberculosis* appears to be due to a deficient POA efflux mechanism (29) that is unable to counteract the acid-facilitated POA influx, which could cause increased accumulation of POA and eventual acidification of the cytoplasm, de-energized membrane (30), inhibition of various targets (see below on mechanisms of action) and cell death, especially in non-growing persisters with low metabolism at acid pH.

Various conclusions can be derived from these hypotheses concerning the activity of PZA. Activity of PZA is strongly related to the pH of the micro-environment of the bacilli, with activity increasing with acidity (20) (17). As diffusion of POA into the cell occurs passively but its removal requires energy to run the efflux pump (29, 30), bactericidal activity is

greatest when bacterial energy sources are at their lowest. This conclusion was first demonstrated by the finding that old cultures were more susceptible to PZA than young actively growing cultures (17). In addition, the above model predicted that energy inhibitors would enhance PZA activity which was subsequently shown to be case (30)(see below). The specificity of this PZA action on persisters was then demonstrated using the Hu/Coates models of persisters (18) and with work that showed increased bactericidal activity when low metabolic activity was produced by energy inhibitors (30, 32), by anaerobic conditions (33) and when incubation temperatures were reduced from 37 °C to 15–25 °C (34).

PZA activity in persister models

The Hu/Coates models explore the action of drugs on persisters and therefore substantiate the hypothesis that PZA has unusual activity against persister sub-population. The results obtained with them are therefore crucial to the overall thesis concerning the bacillary populations against which it is most effective. In model 1, cultures of *M. tuberculosis* incubated without shaking for up to 100 days were sampled, PZA added and bactericidal activity measured. As the duration of incubation and starvation increased from 4 days (log phase) to 30 days and then 100 days, the bactericidal activity of PZA increased. In model 2, the cultures were sampled immediately after selection of the RIF-tolerant population when re-growth was occurring upon subculture into fresh RIF-free medium and when bacterial metabolism was high, and PZA had little bactericidal activity against these actively growing bacilli. In model 3, the cultures were sampled at 3 days after inoculation in RIF-containing liquid medium when growth and metabolism of the sub-population would be expected to be minimal, and PZA was highly bactericidal against this population. The action of PZA in these three models demonstrates clearly that PZA is most bactericidal when cultures of *M. tuberculosis* are the most static.

We found that starvation decreased membrane potential in old bacilli and enhanced PZA activity (35). In addition, it is worth noting that starved *M. tuberculosis* had increased expression of *pncA* (36), which could increase PncA enzyme levels needed for enhanced conversion of PZA to active form POA, and may thus contribute to increased killing of tubercle bacilli by PZA under starvation conditions.

Energy inhibitors enhance PZA activity

Since PZA depletes membrane potential in *M. tuberculosis* (30), we reasoned that energy inhibitors could enhance PZA activity. Indeed, we found that the activity of PZA was significantly enhanced by energy inhibitors such as DCCD (F1F0-ATP synthase inhibitor), rotenone (NADH dehydrogenase I-Complex I inhibitor), and azide (cytochrome c oxidase inhibitor) (30), and also by CCCP, dinitrophenol (DNP), valinomycin and cyanide (32). The subsequent observation in 2005 that bedaquiline (a diarylquinoline), an inhibitor of F1F0 ATP synthase, could synergize with PZA activity (37) – a finding just like DCCD synergy with PZA shown earlier in 2003 (30), provides further support for the model of PZA. These energy inhibitors deplete membrane energy, which renders tubercle bacilli more susceptible to the energy depleting action of POA. This effect of energy inhibitors on enhancing PZA activity is specific to PZA, as they did not enhance the activity of other control drugs like INH or RIF (32).

Mutations in energy production and NAD pathways and ion homeostasis cause increased PZA susceptibility

Based on the model of PZA (30), we predicted that energy production defects either due to either chemical energy inhibitors or genetic mutations will lead to increased susceptibility to PZA. Indeed, this has proven to be true in the case of the bedaquiline and PZA synergy reported subsequently (37, 38). Furthermore, we found that *M. tuberculosis* mutants defective in energy production (made available through TARGET, JHU), had higher PZA susceptibility (MIC=10 µg/ml) than the parent strain (MIC=50 µg/ml) (Table 1) (Ying Zhang, unpublished data). These mutants include mutations in NADH dehydrogenase subunits H and N (*nuoH*, *nuoN*), nitrate reductase *narH*, and formate dehydrogenase *fdhF*, and *kdpA* (potassium transport ATPase) and *yjcE* (Na⁺/H⁺ exchanger) involved in potassium and sodium ion transport, and *pncB1* (Rv1330c) involved in NAD recycling. NADH dehydrogenase *nuoH* and *nuoN* mutants, nitrate reductase *narH* mutant, and formate dehydrogenase *fdhF* mutant, involved in energy production under anaerobic conditions, were also highly susceptible to PZA with a 5-fold reduction in MIC from 50 µg/ml in the parent strain to 10 µg/ml in the mutants. It is of interest to note that mutation in *pncB1*, involved in NAD recycling and energy metabolism, is also more susceptible to PZA. The observation that mutations in *kdpA* and *yjcE* involved in potassium and sodium ion transport, respectively, caused higher susceptibility to PZA, suggests that potassium and sodium ion homeostasis involved in pH regulation may also be important for PZA action. However, mutations in transcription regulator *marR*, MT3006 (ATP binding protein) and MT3981 (putative ATPase) did not have significant effect on PZA susceptibility. The above findings are consistent with our current model of PZA (24, 30) and confirm that energy production pathways and pH homeostasis are important for PZA action. It is unlikely that all the above diverse energy production enzymes whose mutations cause increased PZA susceptibility represent targets of POA. It is more likely as we predicted in the model of PZA that since POA disrupts membrane energy (30), any defect in energy production pathways or ion homeostasis could potentiate PZA activity as shown previously (30, 32). These findings may have implications for developing new drugs that synergize with PZA for improved treatment of TB.

Anaerobic and hypoxic conditions potentiate PZA activity

PZA activity is significantly enhanced under hypoxic or anaerobic conditions compared with atmospheric conditions with ambient oxygen (33). Under microaerophilic or anaerobic conditions, bacteria produce less energy (ATP and membrane potential) due to less efficient nitrate or fumarate electron acceptor usage, compared with respiration with oxygen in aerobic conditions, which produces more energy using oxygen as an electron acceptor. The preferential activity of PZA against tubercle bacilli under hypoxic or anaerobic conditions is presumably due to low energy production under such conditions so that the bacilli are more prone to the energy depleting effect of PZA. By contrast, supplementation of alternative electron donor nitrate to supply energy under anaerobic conditions antagonized PZA activity (33). While energy inhibitors rotenone and azide still enhanced PZA activity under anaerobic conditions (33), DCCD, which enhanced PZA activity under aerobic ambient oxygen condition (30) failed to do so under anaerobic conditions (33). It is worth noting that

PZA acts beyond a general weak acid effect, since although energy inhibitors such as DCCD, rotenone, azide could increase the activity of both PZA and other weak acids, weak acids have no activity under anaerobic conditions where PZA had increased activity for *M. tuberculosis* (33, 39).

PZA activity at different incubation temperatures

When the incubation temperature is below 28°C, cultures of *M. tuberculosis* survive but do not multiply (34). They would be expected to have low energy requirements decreasing as the temperature dropped towards 8°C when active metabolism is minimal. When incubation temperatures were reduced from 37°C to 25°C or 22°C, there was a considerable increase in the bactericidal activity of PZA (34).

Certain weak acids enhance PZA activity

Also predicted from our model of PZA, weak acids, which disrupt membrane energy, could enhance the activity of PZA. We have shown that weak acids including benzoic acid (2 mM), propyl hydroxybenzoic acid (1 mM), and sorbic acid (1 mM) could indeed enhance PZA activity in vitro (35, 39). However, the weak acid enhancement of PZA activity is mainly seen for old bacilli and is not as effective as energy inhibitors, which indicates an additive effect between weak acids and PZA rather than a synergistic effect between energy inhibitors and PZA. However, some weak acids such as lactic acid and fatty acid C10 had no effect on enhancing PZA activity (35). Common clinically used weak acids aspirin and ibuprofen enhanced the activity of PZA in the mouse model of TB infection (40).

Effects of iron, oxidative stress and DNA damage on PZA activity

The separation of PZA activity in vitro and in vivo prompted us to examine the effect of iron, which could potentially be elevated in local inflammatory lesions, on PZA activity in vitro. We found that iron enhanced PZA and POA activity against *M. tuberculosis* (41). Other metal ions such as Mg^{2+} , Ca^{2+} and Zn^{2+} did not enhance the activity of PZA or POA (41). Iron is known to enhance oxidative stress by producing reactive oxygen, which can damage DNA and membranes causing inhibition of cell division, a condition that allows PZA to act more effectively. Alternatively, iron has been shown to enhance the enzyme activity of PncA (26), which in turn results in increased PZA conversion to the active form POA, causing more effective killing. Sodium nitroprusside (1 mM), a reactive nitrogen nitric oxide (NO) producer, enhanced PZA activity (100 µg/ml) against old *M. tuberculosis* cultures but not young cultures (32). However, other oxidative stress agents such as menadione and hydrogen peroxide did not significantly enhance PZA activity.

UV, which is known to damage DNA and membrane causing inhibition of cell division and depleting membrane energy, respectively, has been shown to enhance the activity of PZA against *M. tuberculosis* (32, 39). Since iron, UV and acid stress all cause oxidative damage to DNA, it is quite likely that oxidative damage may be related to PZA action. It is possible DNA damage leads to growth inhibition or cell stasis, which could then potentiate PZA activity. But the detailed mechanisms remain to be determined.

Overall, PZA is a peculiar TB drug whose activity is influenced not only by concentration but is also increased by local acid pH (20, 29), by a low metabolic state of the bacilli and by other factors such as hypoxia and iron concentration. Of particular importance, factors that reduce the metabolic activity of bacilli tend to enhance the activity of PZA, i.e., PZA activity is increased when bacterial metabolic activity is decreased.

Targets of PZA

The target of PZA was suggested to be fatty acid synthase-I (Fas-I) in a study using *M. smegmatis* and 5-Cl-PZA (42). However, no mutations in Fas-I have been found in PZA-resistant *M. tuberculosis* strains. A subsequent study showed that Fas-I is the target of 5-Cl-PZA but not the target of PZA (43). In fact, recent studies have shown that 5-Cl-PZA and PZA act very differently as 5-Cl-PZA is converted by PzaA (a second PZase enzyme not related to PncA not present in *M. tuberculosis*) to less active 5-Cl-POA (44) and is not converted by PncA, the enzyme involved in PZA conversion to POA (45). Despite some in vitro activity of 5-Cl-PZA, it has no activity against *M. tuberculosis* or *M. bovis* in the mouse model (46). Overexpression of Fas-I (target of 5-Cl-PZA) and PzaA (involved in inactivating 5-Cl-PZA) caused 5-Cl-PZA resistance in *M. smegmatis*. However, overexpression of Fas-I in *M. tuberculosis* was toxic. The studies on Fas-I as a possible target of PZA in cell-free assays or in whole cells are questionable as extremely high concentrations of PZA or POA above the physiological concentrations were used (47). Attempts to isolate POA-resistant mutants have failed (48), and clinical isolates of *M. tuberculosis* resistant to PZA with *pncA* mutations are still susceptible to POA (48). Our current model (Fig. 2) can best explain the various unusual features of PZA, including the requirement of acid pH for drug activity (29), relationship between pH and PZA MIC (17), preferential activity against old non-growing bacilli over growing bacilli (17), unique susceptibility of PZA against *M. tuberculosis* (29), higher activity of PZA at hypoxic and anaerobic conditions than at normoxia (33), enhancement of PZA activity by energy inhibitors (30, 32), UV (32) and iron (41) and weak acid enhancement of PZA activity (32).

A new target of PZA, RpsA (ribosomal protein S1) involved in the process of trans-translation, was recently identified (31). Overexpression of RpsA caused resistance to PZA in *M. tuberculosis* as seen by a 5-fold increase in the MIC of PZA (MIC=500 µg/ml) compared with the vector control (MIC=100 µg/ml, at pH 5.5). In addition, we found that a low level PZA-resistant clinical strain DHM444 without *pncA* mutations (48) contained a deletion of amino acid alanine at the 438th residue (438 A) due to a 3-bp GCC near the C-terminus of the RpsA (31). Importantly, POA was found to bind to the wild type RpsA but not the mutant RpsA A438 from the PZA-resistant strain DHM444 or only weakly with the RpsA from naturally PZA-resistant *M. smegmatis* or *E. coli*. POA specifically inhibited the trans-translation of *M. tuberculosis* but not the canonical translation of *M. tuberculosis* or the trans-translation of *M. smegmatis* or *E. coli*. Trans-translation is a process that removes toxic protein products formed under stress conditions by adding a tmRNA tag which is the protease recognition sequence and then sent for degradation by proteases (49). Trans-translation is dispensable during active growth but becomes important for bacteria in managing stalled ribosomes or damaged mRNA and proteins under stress conditions (50, 51). It is required for stress survival and pathogenesis in some bacteria (49). More recently,

we identified a new gene, *panD* encoding aspartate decarboxylase that is involved in PZA resistance (52) (see below). PanD is involved in synthesis of β -alanine that is a precursor for pantothenate and co-enzyme A biosynthesis. It is likely that PanD is a target of PZA and that POA binding to PanD could inhibit synthesis of pantothenate and co-enzyme A which may be critical for persister TB bacteria. The findings that POA inhibits the trans-translation and possibly pantothenate and CoA synthesis in *M. tuberculosis* help to explain why diverse stress conditions such as starvation, acid pH, hypoxia, and energy inhibitors and other drugs could all potentiate PZA activity (17, 30). Based on our current and previous studies, we propose a revised model of mechanisms of action of PZA that can better explain the peculiar features of this unique and paradoxical drug (Fig. 2).

Mechanisms of PZA Resistance

Although PZA resistance in *M. tuberculosis* was shown by McDermott's group to be related to loss of nicotinamidase and pyrazinamidase in 1967 (53), the mechanism of PZA resistance was not known until 1996 when mutation in the *pncA* gene encoding nicotinamidase and pyrazinamidase was demonstrated to cause PZA resistance (25).

Mutations in *pncA*

In vitro studies suggest that mutations leading to PZA resistance seem to occur frequently at a frequency of 10^{-5} (54). PZA-resistant *M. tuberculosis* strains typically lose PZase/nicotinamidase activity (53); a monomeric enzyme that appears to contain manganese and ferrous iron at a 1:1 ratio (26). There is a good correlation between loss of PZase activity and PZA resistance in *M. tuberculosis* (53, 55, 56). Mutations in *pncA* gene encoding nicotinamidase/pyrazinamidase (PZase) are the major mechanism of PZA resistance (25, 48). The nature of the identified *pncA* mutations is largely missense mutations causing amino acid substitutions, and in some cases nucleotide insertions or deletions, nonsense mutations in the *pncA* structural gene, or in the putative promoter region of *pncA* (e.g. at -11 position) (48, 57). The *pncA* mutations are highly diverse and scattered along the gene (48) (Fig. 3). The diverse nature of *pncA* mutations is unique to PZA resistance and is not understood. The role of various *pncA* mutations in affecting PncA enzyme activity and contributing to PZA resistance was evaluated recently by expressing the mutant PncA enzymes and assessing their enzyme activity (58). It was found that mutations causing varying PncA enzyme activities in general correlated with the level of PZA resistance, but were not sufficient to explain a high variability of PZA resistance levels (58). The authors suggested that complementary mechanisms for PZA resistance with mutations in *pncA* might play a role (58). However, inaccurate PZA susceptibility testing may underlie some of the discrepancies between PZase activity and levels of PZA resistance. It would be of interest to validate the proposal of complementary mechanisms of PZA resistance with *pncA* mutations by transformation studies with the mutant *pncA* encoding varying enzyme activity in the background of a null PZase *M. tuberculosis* strain.

Despite the highly diverse and scattered distribution of *pncA* mutations, there is some degree of clustering at three regions of PncA, 3–17, 61–85, 132–142 (48) (Fig. 3). These regions happen to contain catalytic sites and metal-binding sites of the PZase enzyme (27, 59). The crystal structure of *P. horikoshii* PncA (37% identity with *M. tuberculosis* PncA) has

provided some insight into how *pncA* mutations in *M. tuberculosis* might cause PZA resistance (59). The three regions where *pncA* mutations appear to cluster correspond to three of the four loops that contribute to the scaffold of the active site. Mutations at C138, D8, K96, D49, H51, H71, modify the active site triad and metal binding site. Residues F13, L19, H57 (position of characteristic mutation of H57D in *M. bovis*), W68, G97, Y103, I113, A134, H137, line up the active site and mutations at these positions are also predicted to cause loss of enzyme activity. Mutations at Q10, D12, S104 and T142 are predicted to disrupt hydrogen-bonding interactions between the side chain and main chain atoms. Loss of PZase activity due to mutations at other sites can be attributed to potential perturbation of the active site or disruption of the protein core. Recently, the crystal structure of *M. tuberculosis* PncA was solved and provided some insight into how very diverse mutations could contribute to PZA resistance (60). The wild type *M. tuberculosis* PncA structure was found to be a monomer that contains manganous and ferrous ions, confirming earlier studies (26). It would be of interest to determine mutant PncA structures and see how mutant PncAs affect PZA activation and cause PZA resistance.

Most PZA-resistant *M. tuberculosis* strains (up to 99.9%) have mutations in *pncA* (24, 61, 62). However, some PZA-resistant strains without *pncA* mutations have been reported (48, 63–65). These could be due to false resistance as well as a small number of genuine PZA-resistant strains without *pncA* mutations. The average of PZA-resistant strains having *pncA* mutations from all published studies including those that reported low percentage of PZA-resistant strains without *pncA* mutations is about 85%. The real percentage of PZA-resistant strains with *pncA* mutations could be higher after excluding false resistance. A few genuine PZA-resistant strains that do not have *pncA* mutations have the following phenotypes; one is PZase-negative with high levels of resistance (63, 66, 67), indicating mutations in an undefined *pncA* regulatory gene may be involved in PZA resistance. This is very rare. Another type of such strains has low level PZA resistance and positive PZase activity, and is presumably due to alternative mechanisms of resistance, such as mutations in drug target *rpsA* gene (31) or other unknown genes. The clinical significance of the rare low level PZA-resistant PZase positive strains is unclear as they may still respond to PZA treatment in vivo. The virulence and fitness of PZA-resistant strains with *pncA* mutations seem to be unaltered and such strains appear to not only be capable of causing active transmission of disease (63) but also seem to be more virulent as shown in a more recent study (68).

Mutations in *rpsA*

Recently, it was shown that some PZA-resistant clinical isolates such as DHM444 without *pncA* mutations (48) and *M. canettii* had mutations in the drug target RpsA (31, 69). Although it was initially thought that the C-terminus of RpsA, which harbors the alanine deletion in strain DHM444, might be the drug binding site, more recent studies suggest that mutations in the middle or near the N-terminal part of the RpsA (69) may also be involved in drug binding as such strains have been found. For example, *M. canettii*, a *M. tuberculosis* complex organism that is naturally resistant to PZA without meaningful *pncA* mutations (62), has multiple *rpsA* mutations including Thr5Ala, Pro9Pro, Thr210Ala, Glu457Glu (69). It is worth noting that RpsA target mutations are usually associated with low level of PZA

resistance (MIC=200–300 µg/ml PZA). Future studies are needed to assess if the mutations identified in *rpsA* are responsible for low level PZA resistance.

Mutations in *panD*

Although mutations in the *pncA* and the *rpsA* account for most PZA-resistant strains, some other PZA-resistant strains lack mutations in either *pncA* or *rpsA*. To identify potential new mechanisms of PZA resistance, we recently analyzed a large panel of 174 PZA-resistant mutants generated in vitro and found 5 of them harbored a new gene, *panD* encoding aspartate decarboxylase that is involved in PZA resistance (52). *panD* mutations were identified in naturally PZA-resistant *M. canettii* strains and a PZA-resistant MDR-TB clinical isolate. PanD is involved in synthesis of β-alanine that is a precursor for pantothenate and co-enzyme A biosynthesis, which is known to be important for survival and pathogenesis in vivo (70). It is likely that PanD is a target of PZA and that POA binding to PanD could inhibit pantothenate and co-enzyme A biosynthesis, which is critical for central metabolism required for energy production and fatty acid metabolism in *M. tuberculosis*. Future studies are underway to address the role of *panD* mutations in PZA resistance and confirm PanD as a new target of PZA. However, there may still be a small number of PZA-resistant strains that do not have mutations in *pncA*, *rpsA*, or *panD*. The mechanism of PZA resistance in such strains remains to be determined.

The observation that most PZA-resistant *M. tuberculosis* strains have mutations in *pncA* gene has implications for rapid detection of PZA resistance. The current phenotype based PZA susceptibility testing is not routinely performed as it is not reliable due to false resistance (24)(71), and sequencing of the *pncA* gene represents a more rapid, cost-effective and perhaps more reliable molecular test for PZA susceptibility testing and avoids the problems of phenotype based susceptibility testing, which is slow and subject to false resistance. Rapid detection of PZA susceptibility by *pncA* sequencing should be used for guiding improved treatment of MDR/XDR-TB(72).

Implications of PZA for Developing a New Generation of Persister Drugs

PZA is an important frontline drug that plays a unique role in our fight against TB. The increasing emergence and outbreaks of MDR/XDR-TB calls for urgent development of new drugs (73, 74). It is increasingly recognized that the new drugs should not only be active for drug-resistant TB but also more importantly shorten the current 6 month therapy (75–78). Developing new drugs that have activity for persister TB bacteria is critical for further shortening the current TB therapy. Although there are currently several drug candidates in clinical development (78, 79), none can replace PZA and all the drug candidates, including the highly potent bedaquiline and PA-824 or Delamanid (OPC-67683), will have to be used together with PZA since any drug combination without PZA is invariably inferior in animal studies (14, 15, 37, 80). Since demonstration of PZA depleting membrane energy maintenance in 2003 (30) and the synergy between PZA and bedaquiline which itself inhibits ATP synthesis in 2005 (37), there is currently significant interest to develop new drugs targeting energy production pathways in *M. tuberculosis* for improved treatment of TB (81). It is interesting to note that PZA has recently been shown to enhance autophagy of host cells to facilitate clearance or killing of intracellular *M. tuberculosis* (82), a finding that

may partly explain its high sterilizing activity in vivo. This finding has implications for design of enhancers of autophagy as a novel approach to increasing PZA activity in vivo for improved treatment of TB.

PZA validates a principle that drugs active against non-replicating persisters are important for improved treatment of persistent infections. PZA is a prototype model persister drug that plays an indispensable role in any new drug combination. Indeed, in a recent study on the sterilizing activities of new drug regimens in the mouse model, all regimens contained PZA (83). Improved understanding of how PZA works is important for design of new drugs that further shorten the therapy. From the prototype persister drug PZA, one sees the future of antibiotic and cancer drug development. Drugs like PZA should be developed to target persisters and cancer stem cells (equivalent to persisters in cancer) for improved treatment of not only tuberculosis but also other persistent bacterial infections and cancers.

Conclusions

PZA is a unique anti-tuberculosis drug that plays a key role in shortening TB therapy. PZA is particularly effective in killing non-replicating persisters that other TB drugs fail to kill, making it an essential drug for inclusion in any current or new drug combinations for treating both drug susceptible and drug-resistant TB such as MDR-TB. PZA acts quite differently from common antibiotics by inhibiting multiple targets such as energy production, trans-translation and perhaps pantothenate /coenzyme A required for persister survival. Resistance to PZA is mostly caused by mutations in the *pncA* gene encoding pyrazinamidase involved in conversion of the prodrug PZA to the active form POA. Mutations in the drug target RpsA are also found in some PZA-resistant strains. The recent finding that *panD* mutations are found in some PZA-resistant strains without *pncA* or *rpsA* mutations may suggest a third PZA resistance gene and a potential new target of PZA. Current phenotype based PZA susceptibility testing is not reliable due to false resistance, and sequencing of the *pncA* gene represents a more rapid, cost-effective and more reliable molecular test for PZA susceptibility testing and avoids the problem of phenotype based susceptibility testing and should be used for guiding improved treatment of MDR/XDR-TB. Finally, the story of PZA has important implications for not only TB therapy but also chemotherapy in general: that is, persister drugs by killing the non-growing bacterial persisters are critical for shortening the therapy and reducing relapse. PZA serves as a model prototype persister drug and hopefully a ‘tipping point’ that inspires new efforts at developing a new type of antibiotics or drugs that target non-replicating persisters, i.e., bacterial persisters or cancer stem cells (the equivalent of cancer persisters) for improved treatment of not only TB but also other persistent bacterial infections such as persistent Lyme disease and urinary tract infections, biofilm infections and even cancer.

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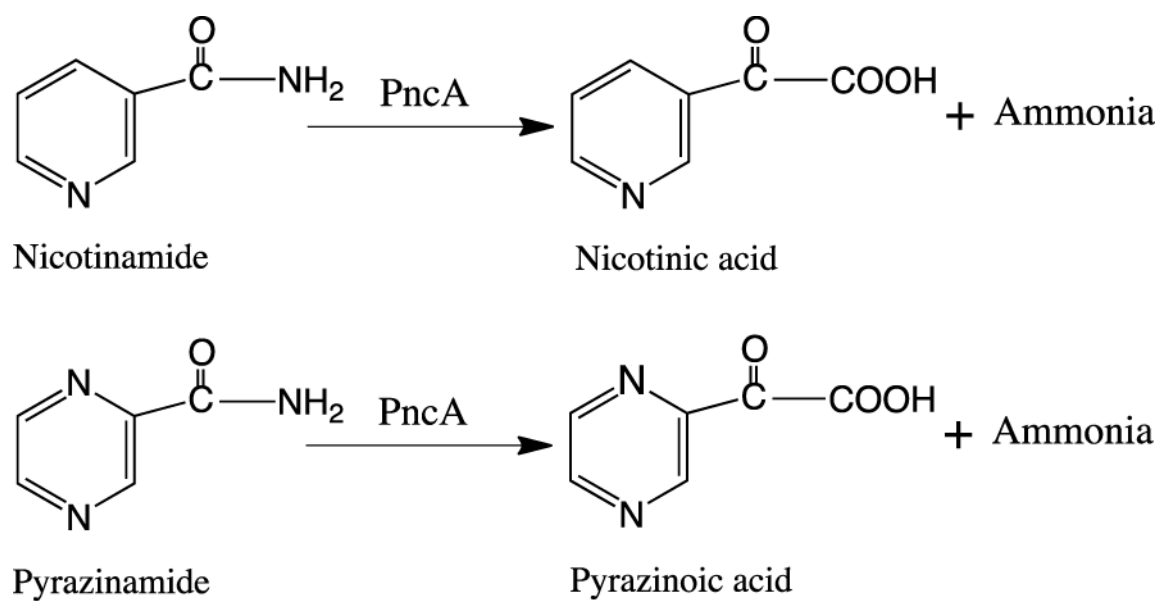


Figure 1.

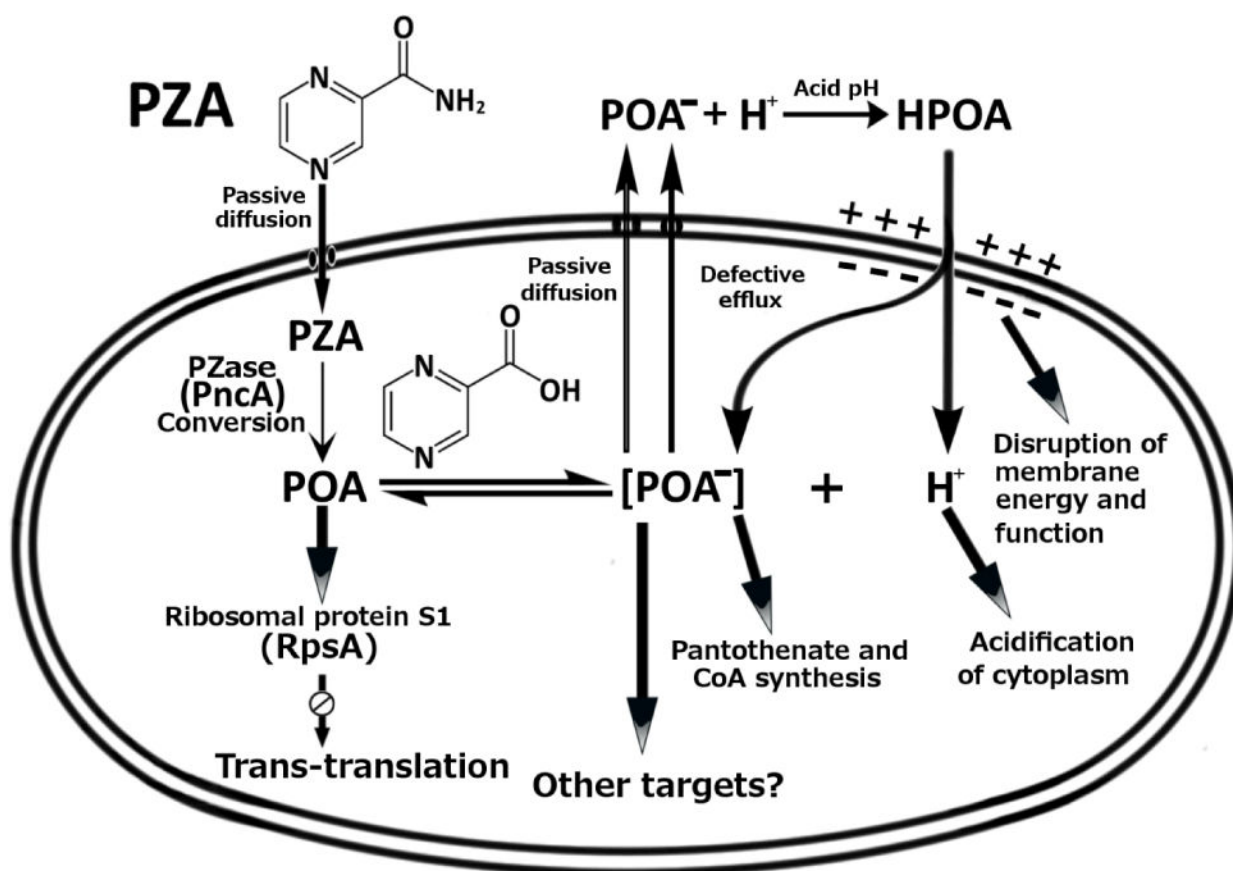


Figure 2.

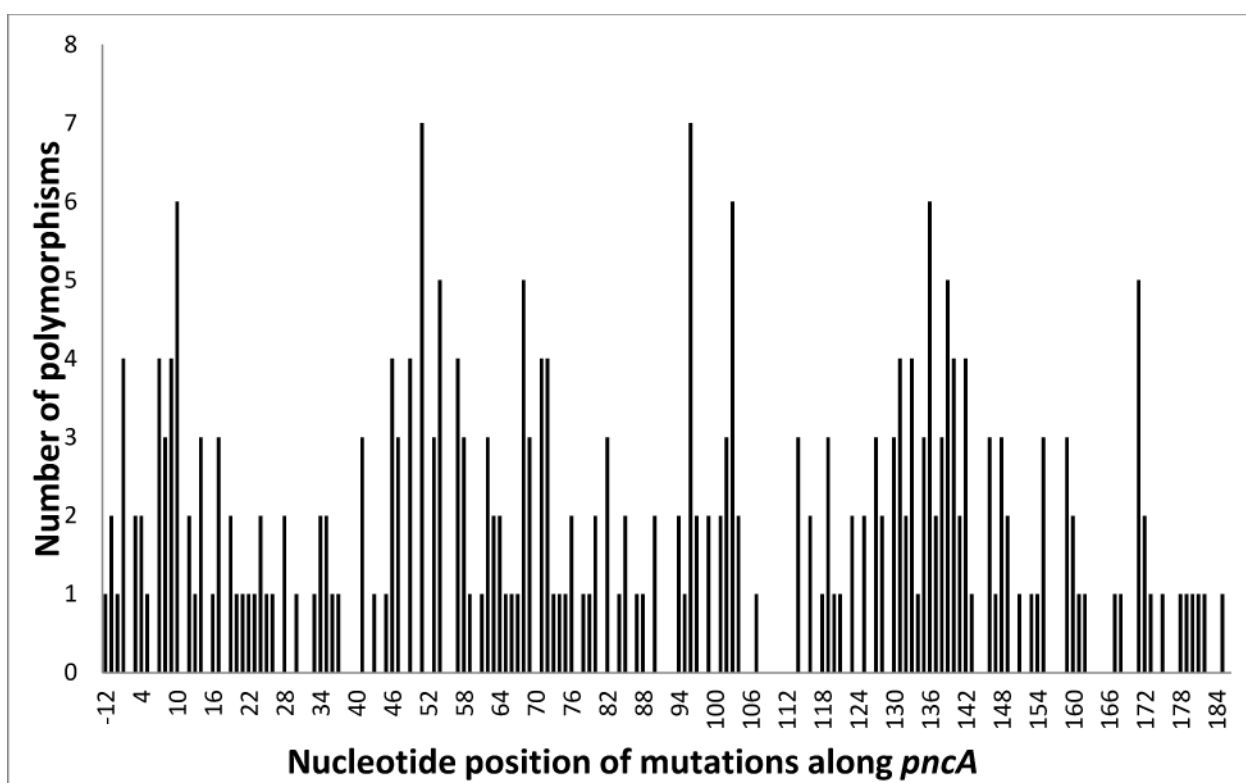


Figure 3.

Table 1Increased PZA susceptibility of *M. tuberculosis* mutants with defect in energy metabolism

Strain	Gene/Function	MIC (µg/ml) (pH 5.5)
CDC1551	Wild type	25–50
MT3240 mutant	<i>nuoH</i> , NADH dehydrogenase	10
MT3246 mutant	<i>nuoN</i> , NADH dehydrogenase	10
MT2968 mutant	<i>fdhF</i> , formate dehydrogenase α-subunit	10
MT1199 mutant	<i>narH</i> , nitrate reductase β-subunit	10
MT1372 mutant	<i>pncB1</i> , nicotinatephosphoribosyltransferase	10
MT2345 mutant	<i>yjcE</i> , Na/H exchanger	10
MT1058 mutant	<i>kdpA</i> , potassium transporting ATPase	10