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Pyrazinoic Acid Efflux Rate in *Mycobacterium Tuberculosis* is a better proxy of Pyrazinamide Resistance

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Abstract

Pyrazinamide is one of the most important drugs in the treatment of latent *Mycobacterium tuberculosis* infection. The emergence of strains resistant to pyrazinamide represents an important public health problem, as both first- and second-line treatment regimens include pyrazinamide. The accepted mechanism of action states that after the conversion of pyrazinamide into pyrazinoic acid by the bacterial pyrazinamidase enzyme, the drug is expelled from the bacteria by an efflux pump. The pyrazinoic acid is protonated in the extracellular environment and then re-enters the mycobacterium, releasing the proton and causing a lethal disruption of the membrane. Although it has been shown that mutations causing significant loss of pyrazinamidase activity significantly contribute to pyrazinamide resistance, the mechanism of resistance is not completely understood.

The pyrazinoic acid efflux rate may depend on multiple factors, including pyrazinamidase activity, intracellular pyrazinamidase concentration, and the efficiency of the efflux pump. Whilst the importance of the pyrazinoic acid efflux rate to the susceptibility to pyrazinamide is recognized, its quantitative effect remains unknown.

Thirty-four *M. tuberculosis* clinical isolates and a *Mycobacterium smegmatis* strain (naturally resistant to PZA) were selected based on their susceptibility to pyrazinamide, as measured by Bactec 460TB and the Wayne method. For each isolate, the initial velocity at which pyrazinoic acid is released from the bacteria and the initial velocity at which pyrazinamide enters the bacteria were estimated.

The data indicated that pyrazinoic acid efflux rates for pyrazinamide-susceptible *M. tuberculosis* strains fell within a specific range, and *M. tuberculosis* strains with a pyrazinoic acid efflux rate below this range appeared to be resistant. This finding contrasts with the high pyrazinoic acid efflux rate for *Mycobacterium smegmatis*, which is innately resistant to pyrazinamide: its pyrazinoic acid efflux rate was found to be 900 fold higher than the average efflux rate for *M. tuberculosis* strains.

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No significant variability was observed in the pyrazinamide flux rate. The pyrazinoic acid efflux rate explained 61% of the variability in Bactec pyrazinamide susceptibility, 24% of Wayne activity, and 51% of the Bactec 460TB growth index. In contrast, pyrazinamidase activity accounted for only 27% of the Bactec pyrazinamide susceptibility. This finding suggests that mechanisms other than *pncA* mutations (reduction of pyrazinamidase activity) are also implicated in pyrazinamide resistance, and that pyrazinoic acid efflux rate acts as a better proxy for pyrazinamide resistance than the presence of *pncA* mutations. This is relevant to the design of molecular diagnostics for pyrazinamide susceptibility, which currently rely on *pncA* gene mutation detection.

Keywords

Mycobacterium tuberculosis; POA efflux rate; PZA flux rate; PZA resistance

Introduction

Tuberculosis (TB) remains one of the major causes of sickness and death worldwide.¹ The resurgence of the disease in industrialized countries and the emergence of multidrug resistant (MDR) strains have renewed scientific interest in the possible mechanisms that contribute to drug resistance in this bacterium.²

Pyrazinamide (PZA) is one of the most important drugs in the treatment of TB. It is the most effective drug against latent *M. tuberculosis* infection,^{3–5} and in addition, its inclusion in isoniazid- and rifampin-containing regimens has enabled the shortening of the treatment period from 9 months to 6.⁵ The emergence of strains resistant to PZA represents an important public health problem, as both first- and second-line treatment regimens include PZA. Yet, it has received remarkably little attention from the TB research community to date.

In TB-endemic developing countries, PZA resistance rates are high. For example in Peru, PZA resistance is around 30–50% in MDR and retreated patients⁶.

Microbiological tests for PZA resistance are not reliable because the need for an acidic pH, which inhibits normal growth of the organism^{7, 8}. Alternative molecular tests based on the detection of mutations in the *pncA* gene are in development^{9–11}.

Unlike other antibacterials, PZA has no defined target of action. PZA is a pro-drug that enters bacteria by passive diffusion. In the cytoplasm it is converted to its active form, pyrazinoic acid (POA), by bacterial nicotinamidase, which has pyrazinamidase (PZAse) activity. The mechanisms of action and resistance to PZA in *M. tuberculosis* are not entirely understood. In PZA-susceptible *M. tuberculosis*, the pyrazinamidase, which is constitutively expressed,^{12, 13} converts PZA to POA.^{3, 14, 15} POA is expelled from the mycobacterium by an efflux pump, is protonated (to POAH) extracellularly under acidic conditions, and is reabsorbed into the bacilli, releasing the proton. This cycle is repeated resulting in POA accumulation within the mycobacterium, reducing intracellular pH, and provoking lethal disruption of membrane permeability and cellular damage.^{12, 13, 15}

Any alteration to the POA cycle that prevents its intracellular accumulation and cytoplasm acidification could change the mycobacterium's resistance profile. The loss of PZAse activity had been described as the major mechanism of PZA resistance in *M. tuberculosis*.¹³ Moreover, *M. kansasii*, which is naturally resistant to PZA, has approximately 5-fold lower PZAse activity and approximately 25-fold lower nicotinamidase activity than *M.*

tuberculosis.^{16, 17} Several studies have demonstrated an association between PZA resistance and mutations in the PZase coding gene (*pncA*).^{12–14} In a previous study, we analyzed the relationship between the PZase activity of the recombinant PZase and the PZA susceptibility level of clinical strains. The level of PZase activity was able to explain only 27% of the variability in the level of PZA resistance, as determined by the Bactec growth index percentage.¹⁸ This suggests that, in addition to mutations in the *pncA* gene, ancillary mechanisms related to PZA resistance in *M. tuberculosis* must also exist. The Wayne test detects POA released by bacilli into the culture media; negative Wayne activity is usually associated with PZA resistance^{7, 19}. An inability of the bacteria to release POA to the extracellular environment may be caused by low expression of PZase or enzymatic malfunction due to *pncA* mutations^{13, 14, 20–22}. However, some PZA-resistant isolates with *pncA* mutations retain PZase activity^{9, 21, 23, 24}, suggesting either inappropriate gene expression or an alternative mechanism such as an altered POA efflux pump.

Previous studies have demonstrated that POA efflux is also implicated in mycobacterial resistance to PZA.^{16, 17} The rate at which POA is expelled from bacteria may depend on multiple factors. The most relevant factors include the pyrazinamidase kinetic parameters (k_{cat} , K_M), the intracellular pyrazinamidase concentration, and the efficiency of the POA efflux pump. The individual contribution of each of these factors to the POA efflux rate is unknown. Non-tuberculous *M. smegmatis* and *M. avium* are innately resistant to PZA, but this resistance is not linked to a mutated *pncA* gene. Resistance in these two mycobacteria has been attributed to a highly active efflux mechanism that extrudes the active POA at a high rate from the bacterial cell, preventing lethal intracellular POA accumulation^{16, 17}. The POA efflux mechanism of *M. smegmatis* has been shown experimentally to be significantly more potent than that of *M. tuberculosis*¹⁷.

Early studies of transport in bacteria focused on identifying how certain substances enter the cells. Levy et al.²⁵ and Nikaido et al.^{26, 27} studied active efflux to explain decreased drug accumulation as a resistance mechanism. Today, it is accepted that resistant phenotypes are often produced through the activity of drug efflux pumps. More recently, Zhang et al. measured the accumulation of [¹⁴C]POA in *M. tuberculosis* H37Rv by adding [¹⁴C]PZA to the culture media, with which it was possible to estimate the POA extrusion rate.¹⁷

In this study, the rate at which POA is expelled from the bacteria (efflux rate), and the rate at which PZA enters the cell (flux rate), were measured in various *M. tuberculosis* clinical isolates and their quantitative association with the level of PZA resistance was evaluated.

Materials and Methods

Mycobacterium strains

Thirty-four *M. tuberculosis* clinical isolates, including the PZA-susceptible reference strain H37Rv, and an *M. smegmatis* strain, were analyzed in this study. Eleven of these strains were analyzed in a previous study, from which data concerning PZase activity and PZA susceptibility based on Bactec 460TB and Wayne activity were available.¹⁸ The *pncA* gene and its promoter were sequenced, and mutations in both regions were detected.

These strains were selected based upon their Wayne activity; strains displaying a range of negative, weak, and positive Wayne activities were selected. Sixteen strains were PZase wild-type (*pncA* sequence identical to H37Rv reference strain). Ten of them were randomly selected from a larger sample resistant to PZA. Sixteen strains showed PZase mutations. Mutated PZases comprised mutations in or close to the metal binding site (D49N, H51R, K48T, H57R), mutations close to the active site (F94L, T135P, D136G), and mutations

relatively distant from both of these regions (L116P, V139A, C14G, G78C, D12A, D12G, G24D, Y34D, and F58L).

PZA susceptibility

PZA susceptibility of each strain was determined by Bactec 460TB and the Wayne method, as previously reported.^{18, 14, 17, 19, 28, 29} Briefly, Bactec 460TB was performed at pH 6.0 using 100 µg/ml PZA. The Bactec PZA resistance level was estimated using the ratio of the radioactive growth index of the culture media containing bacteria and PZA to the radioactive growth index of the control (i.e. bacteria in culture media without PZA), expressed as a percentage (percentage of growth). Strains with a Bactec PZA resistance level greater than 20% were considered resistant to PZA. The Wayne test detected POA released by the bacilli into the culture media. Based on the color intensity, the strains were classified as having positive, weak, or negative Wayne activity.

PZase activity

The *pncA* genes were cloned, expressed, and purified, and recombinant PZases were analyzed to estimate their enzymatic activity, as previously described.¹⁸ Briefly, PZase activity was calculated using a PZA hydrolysis reaction with some modifications.³⁰ PZA (1mM) was incubated with 1 µM PZase in 50 mM sodium phosphate pH 6.5. 10 µL of 20% FeNH₄(SO₄)₂ was added, followed immediately by 890 µL of 100 mM glycine-HCl (pH 3.4) to terminate the reaction. Optical density (OD) was measured spectrophotometrically at 450 nm. The quantity of POA produced was estimated by interpolation in a standard curve of known concentrations. The enzymatic activity was estimated as the amount of POA produced in a 1-min reaction divided by the total amount of enzyme. Each recombinant PZase was tested 3 times.

POA efflux and PZA flux rates

Suspension of *M. tuberculosis* strains—All 34 *M. tuberculosis* strains were cultured on 7H10 medium, retrieved, and weighed using a digital scale. An average of 2 mg of bacterial dry weight was obtained for each strain. The bacterial cells were washed with 10 mM citrate buffer pH 6.2 by centrifugation at 2 800 X g for 20 min at 4°C and suspended in 30 ml of 10 mM citrate buffer pH 6.2, which was then divided into twelve 2.25 ml aliquots per strain.

To measure PZA uptake, the bacteria were incubated at 37°C for 12 h, 24 h, 36 h, and 48 h. For each incubation period, 1mM PZA was added to two samples, and one sample without PZA was included as a negative control (blank in the spectrophotometer). The twelve samples were incubated together and after the indicated time intervals, the three samples were removed and centrifuged at 2 800 X g for 20 min at 4°C.

Extracellular fraction—The supernatants were recovered and heated at 100°C for 20 min to kill any viable bacteria, and stored at 4°C until needed.

Intracellular fraction—The sediment was suspended in 10 mM citrate buffer pH 6.2 to give a volume of 2.5 ml, and heated at 100°C for 20 min to kill and lyse the bacteria. After centrifugation at 2 800 X g for 20 min at 4°C, the supernatants were transferred to new tubes and the protein concentration measured using the Bradford method, then stored at 4°C.

Quantification of POA and PZA in the extracellular and intracellular fraction

POA measurement—20 μ l of $\text{FeNH}_4(\text{SO}_4)_2$ was added to 1 ml of supernatant from the extracellular and intracellular fractions and centrifuged at 10 000 X g for 10 min. The absorbance of the color intensity was read at 450 nm to give the initial POA concentration.

PZA measurement—5 μ l of PZAse was added to 1 ml of supernatant from the extracellular and intracellular fractions to obtain a final concentration of 1 μ M. The suspension was incubated at 37°C for 1 h, which was considered sufficient time to hydrolyze all of the PZA present.

Subsequently, 20 μ l of $\text{FeNH}_4(\text{SO}_4)_2$ was added and the mixture was centrifuged at 10 000 X g for 10 min. The absorbance of the color intensity was read at 450 nm to give the final POA concentration. The difference between these values represents the PZA concentration ($\text{PZA} = \text{POA}_{\text{final}} - \text{POA}_{\text{initial}}$).

Estimation of POA efflux and PZA flux rates

The initial velocity at which PZA enters the bacteria ($V_{\text{PZA}} = \text{PZA flux rate}$) and the initial velocity at which POA is released from the bacteria ($V_{\text{POA}} = \text{POA efflux rate}$), were estimated with 95% confidence intervals. The concentrations of PZA and POA measured in the intra- and extracellular environments at the different time points after initial incubation with PZA were fitted to a second order linear regression in time. The initial velocity was estimated by the linear regression coefficient.

Although the measurement of the POA efflux rate is based on the Wayne reaction, the assay has been modified for this study. Whereas Wayne activity is measured at 0.8 mM PZA in agar media after 7 days of growth, in this study POA efflux rate was determined in Wayne-based reactions using liquid media with 1 mM PZA after 48 hours.

Statistical analysis

To compare the POA efflux rate and PZA flux rate between sensitive and resistant *M. tuberculosis* strains based on Bactec and Wayne tests, a t-test was performed. The variability of the Bactec growth index was modeled in a linear regression by the POA efflux rate and the PZA flux rate. The Wayne activity was dichotomized: negative versus 'weak or positive'. The variability of Wayne activity and Bactec susceptibility were modeled in a logistic regression. The correlation between the PZAse activity and the POA efflux rate was estimated by the Pearson correlation test and by a linear regression. The statistical analysis was conducted with a 5% significance level using the statistical software Stata 10 (StataCorp, College Station, TX).

Results

PZA-susceptibility and PZAse activity in *M. tuberculosis* strains

Twenty-four strains were PZA-resistant (mean % Growth index= 68.41 (SD=24.83)), and 10 strains PZA-susceptible (mean % Growth index= 9.30 (SD=7.47)), according to Bactec 460TB. Twenty strains were Wayne assay negative, of which 19 were PZA-resistant according to Bactec 460TB. 14 strains were Wayne positive and among these, 9 were Bactec PZA-susceptible (Table I). Among the 16 *pncA* wild type (PZAse sequence identical to H37Rv) strains, 10 were resistant and 6 were susceptible according to Bactec 460TB. Among the 16 PZAse mutated strains, 13 were resistant according to Bactec 460TB. Testing of the recombinant enzyme showed that the *pncA* wild type strains WT14, WT15, and WT16, had high PZAse activity, similar to that of the H37Rv strain. We considered all other *pncA* wild type strains to have the same PZAse activity. The selection of the *pncA* wild type

strains included 7 Wayne negative and 10 were PZA resistant according to Bactec 460TB (Table I). With the exception of L116P, all PZAse mutated strains showed low PZAse activity. Despite its high PZAse activity, L116P was Wayne negative and PZA resistant. Mutations affecting the metal binding site (D49N, H51R) or a mutation close to the active site (T135P) resulted in very low PZAse activity. Two *pncA* wild type strains (WT9 and WT16) showed a mutation in the *pncA* promoter. WT16 strain showed a low POA efflux rate, possibly due to a lack of PZAse expression caused by the mutation in the promoter. Although the mutation in the promoter, WT9 showed an intermediate POA efflux rate (Table I). Two strains with the same mutation (C14G) were present. We measured the PZAse activity on the recombinant PZAse of one and considered the other had the same activity. Both had close values for the percentage of growth, however one resulted sensitive and the other resistant due to the cutoff selection (Table I).

Comparison of the POA efflux and PZA flux rates between *M. tuberculosis* and *M. smegmatis*

The estimated POA efflux rate and PZA flux rate for the *M. tuberculosis* and *M. smegmatis* strains are shown in Table 2. The POA efflux rate was estimated as the rate at which POA appeared in the extracellular media. The PZA flux rate was estimated as the rate at which PZA disappeared from the extracellular media.

The average POA efflux rate for the *M. tuberculosis* strains was 5.13 (SD = 5.80) nmol POA/(mg protein)/min. The mean POA efflux rate for *M. smegmatis* was 5222 nmol POA/(mg protein)/min, 900 fold higher than the average POA efflux rate for *M. tuberculosis* ($P<0.001$). The average PZA flux rate for the *M. tuberculosis* strains was 10.73 (SD = 12.72) nmol PZA/(mg protein)/min. The mean PZA flux rate for *M. smegmatis* (9055 nmol PZA/(mg protein)/min) was 840 fold higher than the corresponding rate for *M. tuberculosis* ($P<0.001$).

Relationship between POA efflux/PZA flux rates, PZA-susceptibility, PZAse activity, and mutations

The distribution of the POA efflux rates for the PZA-susceptible and the PZA-resistant *M. tuberculosis* strains as determined by Bactec 460TB, as well as for *M. smegmatis*, are shown in Figure 1. In this schematic representation, PZA-resistant *M. tuberculosis* strains can be seen to be clustered in a group with a lower POA efflux rate than the PZA-susceptible strains.

This evidence suggests that the POA efflux rate for PZA-susceptible strains is likely to fall within a critical range. Strains with a POA efflux rate below this range appear to be resistant. The highest POA efflux rate corresponded to the PZA-resistant *M. smegmatis*, being 900 fold higher than for *M. tuberculosis*. One single *M. tuberculosis* strain (V139A) that was PZA-resistant exhibited the highest POA efflux rate amongst the *M. tuberculosis* strains tested. This rate was approximately 3-fold higher than the average rate for the PZA-susceptible strains. After excluding this outlier isolate, the mean POA efflux rate of the PZA-resistant strains (2.48 (SD=1.89) nmol POA/(mg protein)/min) was significantly lower than the corresponding rate for the PZA-susceptible strains (8.83 (SD=4.14) nmol POA/(mg protein)/min, $P<0.0001$). Similarly, the mean POA efflux rate for the Wayne-negative strains (2.01 (SD=1.93) nmol POA/(mg protein)/min) was significantly lower than that for the Wayne-positive strains (5.77 (SD=4.27) nmol POA/(mg protein)/min, $P=0.0037$); (Figure 2A). POA efflux rate was highly and significantly associated with both Wayne activity and PZA-susceptibility. About 23% of the variability of POA efflux rate was explained by the Wayne activity. The mean POA efflux rate in the Wayne positive/weak group (1.51 (SD=0.19) nmol POA/(mg protein)/min) was significantly higher than that of

the Wayne negative (0.40(SD=0.32) nmol POA/(mg protein)/min) group ($P=0.002$, two-sample t-test) (Figure 2B). For each increase of 1 nmol POA/(mg protein)/min in POA efflux rate there was a 64% increase in the odds of being Wayne positive, and a 67% decrease in the odds of being PZA-resistant according to Bactec 460TB. Under the same conditions, for each increase of 1 nmol POA/(mg protein)/min in POA efflux rate there was a reduction in the Bactec percentage of growth index of 6.07 units. POA efflux rate was able to explain 61% of the variability of Bactec PZA-susceptibility, 51% of the percentage of growth index, and 24% of Wayne activity.

In contrast, PZA flux rate was not significantly associated with Wayne activity or PZA susceptibility (Table 3). No significant difference was observed in the PZA flux rate between the PZA susceptible and the PZA resistant strains ($P=0.75$). Similarly, no significant difference was observed in the PZA flux rate between the Wayne-positive and the Wayne-negative strains ($P=0.45$); (Figure 2B).

POA efflux rate was positively and significantly correlated with PZase activity (Pearson rho-correlation parameter = 0.41, $P=0.03$) after a logarithmic transformation to achieve normality. In a linear regression, 27% of the variability of POA efflux rate was explained by the PZase activity after excluding the *pncA* wild type strains that were PZA resistant because they were selected to search evidence of alternate mechanisms of PZA resistance.

PZase activity was significantly associated with the presence of a mutation in the PZase. The presence of a mutation reduced the PZase activity by 28.46 $\mu\text{mol POA}/\text{min}/\text{mg PZase}$ on average ($P<0.001$). The existence of a PZase mutation explained about 73% of the variability of PZase activity. It was not possible to assess the association between resistance and mutations, because some of the *pncA* wild type strains were selected among isolates resistant to PZA.

Discussion

This study presents a novel experimental approach to quantitatively estimate POA efflux rate and PZA flux rate in clinical isolates of *M. tuberculosis* and *M. smegmatis*. The relationship between these rates and the level of PZA-resistance of the strains was analyzed. The data demonstrate that POA efflux rate is variable within *M. tuberculosis* strains, and is significantly associated with both Bactec 460TB level of PZA susceptibility and Wayne activity. For the first time, a quantitative relationship between the POA efflux rate and the level of PZA susceptibility has been demonstrated in *M. tuberculosis* strains. Variation in the POA efflux rate was able to explain PZA resistance in certain strains in which PZase activity was not affected, supporting the presence of an additional mechanism of resistance other than a reduction in PZase activity. Mutations that alter the efficiency of the POA efflux pump might be responsible for PZA resistance in these cases.

A previous study suggested that *M. tuberculosis* has a low POA efflux rate that permits the lethal accumulation of POA. In contrast, *M. smegmatis*, which is naturally resistant to PZA, was shown to have a more active POA efflux mechanism that results in a fast extrusion of POA and does not permit its accumulation, even at an acidic pH.¹⁵ The authors proposed that a weak POA efflux mechanism underlies the unique susceptibility of *M. tuberculosis* to PZA, and that the natural PZA resistance of *M. smegmatis* may be due to a highly active POA efflux mechanism.

This study, however, demonstrates that PZA resistance in *M. tuberculosis*, in contrast with *M. smegmatis*, is associated with a low, rather than a high, POA efflux rate, in strains with PZase activity as well as in strains with *pncA* mutations and impaired PZase. *M. tuberculosis* strains exhibited different levels of POA efflux rates, varying in a range within

120 fold, and resulting in different levels of PZA susceptibility. POA efflux rates from susceptible *M. tuberculosis* strains were found to fall within a critical range, and strains with a POA efflux rate below this range appeared to be resistant to PZA. This variability in POA efflux rate was able to explain 51–61% of the PZA resistance as determined by Bactec 460TB.

The association of PZA resistance with a low, rather than a high, POA efflux rate suggests that the accumulation of POA within the cell may not in itself be of importance. The expulsion of POA from the bacterium may in fact be the rate-limiting step in the cycle of cell destruction. Following its expulsion, as described above, the POA is protonated in the extracellular environment to POAH and re-enters the mycobacterium, probably through a passive mechanism, where it dissociates to POA and H. This introduction of a proton into the cell is responsible for intracellular acidification and, ultimately, membrane disruption and cell death. The POA, meanwhile, can be subsequently expelled again by the same efflux pump, re-protonated, and re-enter the cell, thus causing further acidification. Therefore, a reduction in the rate of expulsion of the POA would lead to a reduction in acidification; this may explain why a low POA efflux rate, even though in strains with an optimal PZase activity, would cause PZA resistance, in contrast to what was previously thought. The high POA efflux rate found in *M. smegmatis* suggests that other mechanisms are of importance in this mycobacterium; these factors remain unclear.

The POA efflux rate appears to depend on at least five factors: (1) the capacity of the pyrazinamide to enter the mycobacterium, which is likely to depend on a passive diffusion system; (2) the enzymatic activity of the PZase, which is affected by mutations in the *pncA* gene; (3) the concentration of PZase in the bacteria, which determines the total capacity to hydrolyze PZA into POA; (4) the efficiency of the efflux pump, which is likely to be affected by mutations in the gene encoding the pump; and (5) the concentration of efflux pumps in the bacterial membrane, which determines the total capacity to expel POA. The individual contribution of each of these factors to the POA efflux rate is unknown. With the exception of the PZase activity, the individual contribution of these factors to the PZA-susceptibility level is also unknown. In a previous study, the PZase activity was found to explain 27.3% of the PZA susceptibility.¹⁸ Further studies are required to explore the effects of the remaining factors on the POA efflux rate and on PZA susceptibility.

A PZA susceptible *M. tuberculosis* strain would be expected to contain a sufficiently high concentration of active PZase to convert PZA into POA. PZA must enter the bacteria at an appropriate rate; and POA efflux pumps must be present at an appropriate concentration and with an adequate efficiency in order to expel POA at the required rate. Finally, protonated POA (POAH) must return to the mycobacterium at an appropriate rate. Any deviation from these conditions would alter the equilibrium of the cycle, resulting in a change in the level of PZA susceptibility. The experimental design employed in this study did not discriminate between these individual factors. In this study the total combined effect of all existing factors was measured, and translated into the net rate at which POA is expelled from the bacteria. The variability that was identified in the POA efflux rate could be caused by an alteration of any one or more of the factors described above.

The PZase activity explained about 27% of the variability in POA efflux rate. Therefore, other factors must be present and should account for the remaining unexplained variability. This study confirmed the existence of PZA resistant strains with *pncA* wild type and with a similar PZase activity to the H37Rv strain that showed a low POA efflux rate and did not show a mutation in the *pncA* promoter (strains WT11 and WT12). Similarly, strains with mutated PZases showing competent PZase activity were also PZA resistant and associated with a low POA efflux rate (strains Y34D, K48T). A low POA efflux rate might be expected

to be a reflection of impaired PZase activity (caused by *pncA* mutations). However, the normal levels of PZase activity amongst these particular strains suggest that a different mechanism of resistance must occur in these particular cases. It is possible that mutations in the POA efflux pump could affect its efficiency and alter the rate at which POA is expelled. The identity of the gene encoding the POA efflux pump is still unknown and further studies are required.

Our data also showed that some PZA resistant strains showed a POA efflux rate higher than the corresponding to some susceptible strains. In particular, the V139A strain is of particular interest because it was a PZA resistant strain that showed a higher POA efflux rate than the average rate of the PZA susceptible strains, making it an outlier in the data set; the reasons for this are unclear. These strains had an active PZase and showed a Wayne activity. The mechanism of resistance in these particular strains is not likely to be associated with impaired PZase activity or an altered efficiency of the POA efflux pump. A recent study³¹ identified an alternate mechanism of PZA resistance in *pncA* wild-type strains. It has been shown that POA binds to RpsA, a ribosomal protein, inhibiting the trans-translation process, which is lethal to the mycobacteria. Mutations in the C-terminus of the RpsA prevents the binding of POA and the inhibition of trans-translation, generating resistance to PZA.

Under experimental conditions, this study found that the POA efflux rate for *M. smegmatis* was 900 fold higher than the average rate for *M. tuberculosis*. This is consistent with previous studies.¹⁷ The more efficient POA efflux mechanism in *M. smegmatis* could be explained by any of the following: a higher PZase expression; the presence of an efflux pump isoform with a higher activity; or a higher concentration of efflux pumps within the bacterial cell membrane. Further studies are required to clarify this.

In a previous study, the rate of POA extrusion in the susceptible *M. tuberculosis* H37Rv strain (approx. 0.3 pmol/mg/min) was found to be more than 2 orders of magnitude (1:233) lower than that for *M. smegmatis* (approx. 70 pmol/mg/min).¹⁵ In the present study, the POA efflux rate for H37Rv *M. tuberculosis* was 15.98 nmol POA/(mg protein)/min, approximately two orders of magnitude (1:567) lower than that for *M. smegmatis* (9055 nmol POA/(mg protein)/min). This difference in values could be due to variations in the experimental conditions; the ratio of the POA efflux rate between H37Rv *M. tuberculosis* and *M. smegmatis* was similar in the two studies. The POA efflux rate was significantly associated with Bactec PZA susceptibility (OR=0.33; for a one unit decrease in POA efflux rate, there was approximately a 3-fold increase in the risk of being a resistant strain) and with Wayne activity (OR=1.64). Considering that the maximum variation in the POA efflux rate among the analyzed strains was 29 units, it is apparent that even small variations in the POA efflux rate result in notable variations in the level of PZA susceptibility, to the extent where at low POA efflux rates there is a PZA-resistant phenotype.

The results of this study showed that the PZA flux rate was, on average, 10–30 fold higher than the POA efflux rate. This difference is likely to be necessary for the accumulation of a lethal concentration of POA in the bacteria. The PZA flux rates of the analyzed strains were less variable than the corresponding POA efflux rates, and were not significantly associated with PZA susceptibility. This supports previous findings that entry of PZA into the bacteria occurs by passive diffusion, and thus is not likely to vary significantly across strains.¹⁷

Even though the entry of PZA into the bacteria is likely to occur by passive diffusion, this study found that the PZA flux rate for *M. smegmatis* was 840 fold higher than that for *M. tuberculosis*. This difference can be explained by the fact that POA is released 900 times faster in *M. smegmatis*, thus drawing the reaction towards the hydrolysis of PZA. This

increases the external/internal PZA gradient and favors its passage into the bacteria, resulting in a higher PZA flux rate.

Although the mean POA efflux rate for the PZA-resistant strains was significantly lower than that for the PZA-susceptible strains, there were some PZA-resistant strains with POA efflux rates similar to those for susceptible strains. For these strains, not only were the POA efflux rates similar, but the PZA flux rates also. This suggests that in these strains, an alternative mechanism other than an alteration in the POA efflux rate may be causing PZA resistance. For example, it is possible that in these strains the protonated POA (POAH) may not be returning to the mycobacterium at an appropriate rate. These mechanisms require further study.

This study demonstrated that in some of the analyzed strains both the entry of PZA and the expulsion of POA from the mycobacteria decelerated significantly (data not shown). The deceleration in flow of PZA can be attributed to changes over time in the concentration gradient: as PZA enters the bacteria its initially high extracellular concentration gradually decreases, whilst it tends to accumulate in equilibrium with POA within the bacteria, causing a reduction in the concentration gradient driving its entry. Similarly, the expulsion of POA from the bacteria leads to a reduction in intracellular POA concentrations, reducing the rate at which the efflux pumps are able to expel further POA. Neither the PZA flux deceleration rate nor the POA efflux deceleration rate was significantly associated with the level of PZA resistance.

Molecular methods to determine PZA resistance have to date relied on the detection of *pncA* mutations based on the assumption that this was the main mechanism of resistance. This study suggests that a better proxy for PZA resistance is the POA efflux rate, which is the result of multiple factors, amongst which PZAse activity is just one. This could be of importance to the development of novel molecular, biochemical, or electrochemical methods to detect PZA resistance more accurately.

Conclusion: PZA-susceptible isolates of *M. tuberculosis* are characterized by demonstrating a POA efflux rate falling within an optimal range, and strains that have a POA efflux rate below this range appear to be resistant. The POA efflux rate was able to explain approximately 61% of the variability in PZA resistance as determined by Bactec 460TB. Mechanisms other than *pncA* mutations and PZAse activity that affect the POA efflux rate, and factors affecting entry of POAH into the cell, must explain the remaining variability in PZA resistance and should be further studied. The POA efflux rate could be used as a proximate predictor of PZA resistance in *M. tuberculosis*.

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Figure 1. Schematic representation of POA efflux rate for different *M. tuberculosis* strains and its relationship with PZA Bactec susceptibility. Arrow direction/scale color indicates higher efflux rate.

Figure 2. Comparison between POA efflux/PZA flux rates and PZA-susceptibility. a) POA efflux, b) PZA flux. Bar plot compares mean of POA efflux and PZA flux rates between PZA-susceptibility tests (Bactec and Wayne). S, PZA-susceptible; R, PZA-resistant. P, Wayne-positive; N, Wayne-negative.

Table I
Pyrazinamide susceptibility of selected *Mycobacterium tuberculosis* strains. POA efflux and PZA flux rates

<i>pncA</i> mutation	Mutation in promoter	PZAse activity (μmol POA/ min/mg PZAse)		Wayne activity	Bactec 460TB		POA efflux-rate [95% CI] nmol POA/(mg protein)/ min	PZA flux-r; [95% CI] nmol PZA/(mg protein)/ min
		Median	IQR		% Growth index ¹	Susceptibility ²		
H57R*	N	0.204	0.02	Neg	100	R	4.32[3.10, 5.55]	16.54[6.41, 26.68]
WT8	N	iden.	iden.	Neg	53	R	1.758[1.00, 2.51]	11.32[2.93, 19.71]
WT9	Y	iden.	iden.	Neg	53	R	5.92[4.66, 7.19]	62.46[33.30, 91.63]
WT11	N	iden.	iden.	Neg	91	R	0.52[0.31, 0.72]	1.59[1.23, 1.95]
WT12	N	iden.	iden.	Neg	97	R	0.67[0.70, 0.91]	0.71[0.05, 1.48]
WT14	N	38.40	18.36	Neg	69	R	1.45[0.88, 2.01]	1.67[0.26, 3.08]
WT15	N	38.40	18.36	Neg	63	R	3.07[2.56, 3.59]	3.83[1.99, 5.68]
WT16	Y	38.40	18.36	Neg	88	R	0.25[0.16, 0.34]	0.94[0.06, 1.95]
D49N*	N	0.045	0.014	Neg	74	R	0.65[0.31, 0.99]	1.39[0.83, 1.85]
H51R*	N	0.006	0.001	Neg	90	R	0.45[0.36, 0.54]	0.60[0.04, 1.24]
L116P*	N	50.15	5.68	Neg	46	R	4.39[2.16, 6.62]	20.88[5.90, 35.88]
T135P*	N	0.02	0.02	Neg	98	R	0.67[0.53, 0.81]	1.35[0.17, 2.52]
C14G [†]	N	0.90	0.47	Neg	8	S	12.59[9.98, 15.20]	17.14[9.39, 24.88]
V139A	N	3.46	1.96	Weak	100	R	29.22[18.06, 40.37]	37.90[34.30, 41.52]
G78C*	N	6.96	0.83	Weak	27	R	6.39[5.17, 7.61]	28.19[7.92, 64.30]
F94L*	N	21.19	8.89	Weak	70	R	4.18[3.95, 4.41]	7.63[3.76, 11.50]
D12A*	N	9.24	3.19	Weak	77	R	3.61[0.81, 6.41]	10.96[5.40, 16.53]
D12G*	N	14.00	2.52	Weak	70	R	1.53[0.84, 2.22]	1.84[0.25, 3.44]
G24D*	N	4.28	3.25	Weak	68	R	0.72[0.49, 0.96]	2.96[0.54, 5.39]
Y34D*	n.a.	20.58	8.35	Weak	78	R	1.42[1.10, 1.74]	2.36[2.35, 2.38]
H37Rv	N	38.40	18.36	Pos	1	S	15.98[10.04, 21.91]	22.18[12.64, 31.72]
C14G [†]	N	0.90	0.47	Pos	13	S	7.24[5.00, 9.49]	8.16[5.48, 10.84]
F58L	N	14.13	10.81	Pos	88	R	5.17[4.29, 6.05]	5.64[0.17, 11.44]
WT4	N	iden.	iden.	Pos	1	S	6.91[5.32, 8.50]	9.92[4.35, 15.50]

<i>pncA</i> mutation	Mutation in promoter	PZAse activity (μmol POA/ min/mg PZAse)		Wayne activity	Bactec 460TB		POA efflux-rate [95% CI] nmol POA/(mg protein)/ min	PZA flux-r; [95% CI] nmol PZA/(mg protein)/ min
		Median	IQR		% Growth index ¹	Susceptibility ²		
WT5	N	iden.	iden.	Pos	1	S	14.42[7.84, 21.00]	18.19[15.43, 20.95]
WT6	N	iden.	iden.	Pos	68	R	2.04[1.54, 2.54]	2.15[0.49, 3.82]
WT7	N	iden.	iden.	Pos	32	R	2.58[1.97, 3.19]	2.70[1.64, 3.75]
WT10	N	iden.	iden.	Pos	22	R	3.89[1.93, 5.81]	13.70[10.21, 17.18]
WT13	N	iden.	iden.	Pos	16	S	3.97[3.52, 4.43]	3.99[3.17, 4.81]
WT17	n.a.	iden.	iden.	Pos	19	S	9.19[5.71, 12.68]	17.62[11.20, 24.04]
WT18	N	iden.	iden.	Pos	12	S	5.94[4.85, 7.04]	6.20[6.19, 6.21]
WT19	N	iden.	iden.	Pos	19	S	4.72[3.29, 6.16]	6.11[3.66, 8.56]
D136G	N	12.34	6.84	Pos	3	S	7.29[5.55, 9.04]	10.95[8.67, 13.23]
K48T*	N	10.45	1.06	Pos	20	R	1.33[0.97, 1.70]	5.07[0.01, 10.14]
<i>M. smegmatis</i>	n.a.	n.a.	n.a.	Pos	n.a.	R	5222[3150, 7293]	9055[8394, 9715]

WT, *pncA* wild type strain. ND, non-determined.

* Previously reported strain 18

[†] Different clinical isolates sharing the same PZAse mutation.

¹ % Growth index, percentage of growth on 100 μg/ml PZA-broth compared to a PZA-free broth (% growth index larger than 20% is considered PZA-resistant).

² Susceptibility. S, PZA-susceptible; R, PZA-resistant. 95% confidence interval is shown between brackets. WT, wild type. NS, not significant. *iden*, PZAse activity is identical to the corresponding of H37Rv n.a., not available

Table II

Single regression models for determination of PZA susceptibility variability.

	Wayne activity		Bactec 460TB	
	Logistic regression Odds ratio, (P-value)	% Growth index Linear regression Coefficient, (P-value)	Susceptibility Logistic regression Odds ratio, (P-value)	
V _{POA}	1.64 (0.017)	−6.07 (0.000)	0.33 (0.012)	
V _{PZA}	1.00 (0.892)	0.88 (0.079)	1.02 (0.504)	