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Mode of action of pyrazinamide: disruption of *Mycobacterium tuberculosis* membrane transport and energetics by pyrazinoic acid

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Pyrazinamide is an important sterilizing drug that shortens tuberculosis (TB) therapy. However, the mechanism of action of pyrazinamide is poorly understood because of its unusual properties. Here we show that pyrazinoic acid, the active moiety of pyrazinamide, disrupted membrane energetics and inhibited membrane transport function in *Mycobacterium tuberculosis*. The preferential activity of pyrazinamide against old non-replicating bacilli correlated with their low membrane potential and the disruption of membrane potential by pyrazinoic acid and acid pH. Inhibitors of membrane energetics increased the antituberculous activity of pyrazinamide. These findings shed new light on the mode of action of pyrazinamide and may help in the design of new drugs that shorten therapy.

Keywords: tuberculosis, mechanism of action, membrane potential, *M. tuberculosis*

Introduction

Pyrazinamide is an important front-line drug for the treatment of tuberculosis (TB). Pyrazinamide, along with isoniazid and rifampicin, forms the cornerstone of modern TB therapy. Pyrazinamide plays a unique role in shortening the therapy from previously 9–12 months to 6 months,[1–4] because it kills a population of semi-dormant tubercle bacilli in acidic pH environments that are not killed by other TB drugs.[5] Pyrazinamide is a paradoxical and unconventional drug.[6] Despite its remarkable sterilizing activity *in vivo*,[7,8] pyrazinamide is not active against *Mycobacterium tuberculosis* under 'normal' culture conditions near neutral pH.[9] Pyrazinamide is only active against *M. tuberculosis* at acid pH,[10] an environment that is produced during active inflammation, and its activity is closely related to the acidity of the medium.[11,12] Even at acid pH (e.g. pH 5.6), pyrazinamide kills *M. tuberculosis* slowly and incompletely with no more than 76% of the bacterial population being killed by 1000 mg/L pyrazinamide,[13] which is 10–20 times higher than the already high minimum inhibitory concentration (MIC) of 50–100 mg/L.[6] We have shown that the acid pH requirement of pyrazinamide action is the result of increased accumulation of pyrazinoic acid (POA), the active form of pyrazinamide, in the tubercle bacilli at acid pH but not at neutral pH.[14] Unlike conventional antibiotics, which are more active against the actively growing bacteria than non-growing bacteria, pyrazinamide is exactly

the opposite, that is, it is less active against young growing tubercle bacilli but is more active against old non-growing bacilli.[12]

Despite many studies since its discovery over 50 years ago,[15] the mechanism of action of pyrazinamide is the least understood of all TB drugs because of its unusual properties. Pyrazinamide is a prodrug that is converted into the active form pyrazinoic acid (POA) by bacterial nicotinamidase/pyrazinamidase (PZase),[16,17] and pyrazinamide-resistant *M. tuberculosis* clinical isolates lose PZase activity[16] owing to mutations in the *pncA* gene encoding PZase.[17] *M. tuberculosis* is uniquely susceptible to pyrazinamide, and this unique pyrazinamide susceptibility correlates with a deficient pyrazinoic acid efflux mechanism in this organism, whereas the naturally pyrazinamide-resistant *Mycobacterium smegmatis* has a highly active pyrazinoic acid efflux mechanism that quickly extrudes pyrazinoic acid out of the cell.[14] Although the fatty acid synthase-I (FAS-I) has been proposed as a target of pyrazinamide in a study using *M. smegmatis* and 5-Cl-pyrazinamide, this proposition has been questioned in a recent study, which showed that FAS-I is the target of 5-Cl-pyrazinamide[18] but not the target of pyrazinamide.[19] In fact, various lines of evidence suggested that pyrazinoic acid does not appear to have a specific cellular target.[6,20] The action of pyrazinamide has been proposed to be the result of a weak acid effect of pyrazinoic acid on the tubercle bacilli.[6,20] In this study we present evidence that pyrazinoic acid and pyrazinamide could de-energize the membrane by collapsing the membrane potential and affect the membrane transport function at acid

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pH as a mechanism of action. In support of this proposition, we have shown that inhibitors of membrane potential production enhanced the antituberculosis activity of pyrazinamide.

Materials and methods

Chemicals

Pyrazinamide and pyrazinoic acid were obtained from Sigma–Aldrich Co. Pyrazinamide was dissolved in sterile deionized water at 10 mg/mL and pyrazinoic acid was dissolved in dimethyl sulphoxide (DMSO) at 50 mg/mL. N,N'-dicyclohexyl carbodiimide (DCCD) was dissolved in 95% ethanol at a stock solution of 200 mM, and sodium azide was dissolved in sterile deionized water at 10 mM. Radiochemicals such as [^{35}S]methionine, [^{3}H]uracil, L-[^{14}C]serine, [^{14}C]benzoic acid and [^{3}H]tetraphenylphosphonium bromide (TPP$^+$) were obtained from Amersham.

Growth of mycobacteria

M. tuberculosis strain H37Ra was grown in 7H9 liquid medium (Difco) supplemented with 0.05% Tween 80 and 10% bovine serum albumin-dextrose-catalase (ADC) enrichment (Difco) at 37°C for various times ranging from a few weeks to a few months with occasional agitation.

Effect of pyrazinoic acid on protein and RNA synthesis in *M. tuberculosis*

A 2-week-old *M. tuberculosis* H37Ra culture was centrifuged and the cell pellet was resuspended in Sauton's medium adjusted to pH 3.0, 5.0 and 7.0, respectively, at a concentration of about 2.5×10^8 cells/mL. [^{35}S]Methionine, [^{3}H]uracil and L-[^{14}C]serine were added to the cell suspensions at 10 µCi/mL. Pyrazinoic acid or pyrazinamide at a concentration of 0.4 (about 50 mg/L) or 4 mM (500 mg/L) and dinitrophenol (DNP) at 4 mM as a control were added to the cell suspensions and the mixtures were incubated at 37°C for various time points. At 15, 45, 240 and 960 min, aliquots of cell suspensions were removed and washed with Sauton's medium by filtration on 0.45 µm nitrocellulose filters under a vacuum. For the serine uptake study, H37Ra cells were resuspended in 7H9 medium at pH 5.0 or 7.0 and then treated with POA at 100 or 400 mg/L for 3 h before the addition of L-[^{14}C]serine at a final concentration of 1 µCi/mL. At different time points, aliquots of cell suspensions were removed and filtered through 0.45 µm filters as above. The amount of radioactivity associated with the bacterial cells was determined by scintillation counting of the radioactive filters.

Measurement of membrane potential

Membrane potential was measured with [^{3}H]tetraphenylphosphonium bromide (TPP$^+$) using the method described previously.[21] Briefly, 20-day-old or 130-day-old *M. tuberculosis* H37Ra cells were resuspended in Sauton's medium at pH 3–8. [^{3}H]TPP$^+$ (380 µCi/mmol) at 10 µM final concentration was then added to the cell suspension and the mixture was fully mixed before silicone oil was added and the mixture was incubated for another 10 min. The mixture was spun at 12 000 rpm for 3 min, and 100 µL of supernatant was taken for scintillation counting. The cell pellets were then snap-frozen in an alcohol dry ice bath. The bottoms of the tubes containing cell pellet were cut off for scintillation counting.

Effect of rotenone, DCCD and azide on pyrazinamide activity

M. tuberculosis H37Ra culture was cultivated as described above, harvested by centrifugation, washed in one volume of PBS (pH 7.0) and resuspended in citrate buffer (pH 5.5). Cells were treated with rotenone (4 µM), DCCD (1 mM), sodium azide (1 mM) or pyrazinamide (50 mg/L), or rotenone, DCCD or sodium azide in combination with pyrazinamide for 0 and 5 days, when the cells were washed with PBS buffer and colony forming units for each sample were determined by serial dilution and plating on 7H11 agar supplemented with ADC enrichment.

Results

Effect of pyrazinoic acid and pyrazinamide on the inhibition of membrane transport in *M. tuberculosis*

We previously hypothesized that pyrazinoic acid as a weak acid could potentially inhibit membrane transport function as a possible mechanism of action.[20] To test this hypothesis, we assessed the effect of POA on the incorporation of [^{35}S]methionine as a precursor for protein synthesis and [^{3}H]uracil as a precursor for RNA synthesis in *M. tuberculosis*. As shown in Figure 1 (a and b), whereas acid pH alone (pH 3.0 and 5.0) had a non-specific inhibitory effect, owing to lowered membrane potential required for the uptake of methionine and uracil by acid pH (see below), the presence of the weak acid POA caused further inhibition of protein (Figure 1a) and RNA synthesis (Figure 1b). In contrast, at pH 7.0, pyrazinoic acid had little effect on protein or RNA synthesis. Dinitrophenol (DNP) as a positive control significantly inhibited the RNA and protein synthesis at both acid and neutral pH. At 4 h of incubation, pyrazinoic acid at 50 mg/L significantly inhibited RNA synthesis at pH 3, but had less effect at pH 5.0 and 7.0 (data not shown); however, pyrazinoic acid greatly inhibited RNA synthesis at a higher concentration of 500 mg/L at acidic pH 5.0 (Figure 1a). The degree of inhibition of protein and RNA synthesis is a function of pyrazinoic acid concentration, acidic pH and time of incubation. That pyrazinoic acid simultaneously inhibited both RNA and protein synthesis indicates that the inhibition is caused by reduced transport of uracil and methionine needed for RNA and protein synthesis, respectively, rather than by inhibition of a specific component of the RNA or protein synthesis machinery.

Because conversion of the prodrug pyrazinamide into pyrazinoic acid by PZase is slow in *M. tuberculosis,*[14] unlike pyrazinoic acid, pyrazinamide at early time points such as a few hours had little effect on protein or RNA synthesis (data not shown). Therefore we examined the effect of pyrazinamide on the protein and RNA synthesis after exposure of *M. tuberculosis* cells to pyrazinamide for 2 days to ensure its complete conversion into pyrazinoic acid. Indeed, under this condition, pyrazinamide, like pyrazinoic acid, also inhibited the protein synthesis (Figure 1c) and RNA synthesis (Figure 1d) at both 50 and 500 mg/L at acid pH 5.0, but not at neutral pH. There was little difference in the degree of inhibition between 50 and 500 mg/L pyrazinamide at acid pH 5.0 (Figure 1c and d). That pyrazinamide itself had little effect on protein or RNA synthesis at early time points because of slow conversion into pyrazinoic acid provides a possible explanation as to why previous attempts at identifying the mechanism of pyrazinamide action using short time points commonly used for the study of the mode of action of antibiotics were unsuccessful.

To determine whether pyrazinoic acid inhibits the membrane transport of amino acids, we examined the effect of pyrazinoic acid on the uptake of L-[^{14}C]serine at acid and neutral pH conditions. Pyrazinoic acid at 100 and 400 mg/L inhibited the uptake of serine at acid pH 5.0 (Figure 2a), but had little effect at pH 7.0 (Figure 2b). The inhibitory effect of pyrazinoic acid on serine transport was more pronounced at 400 mg/L than at 100 mg/L at acid pH (Figure 2a). The above data are consistent with the finding that weak acids inhibit transport of various nutrients such as amino acids as shown in
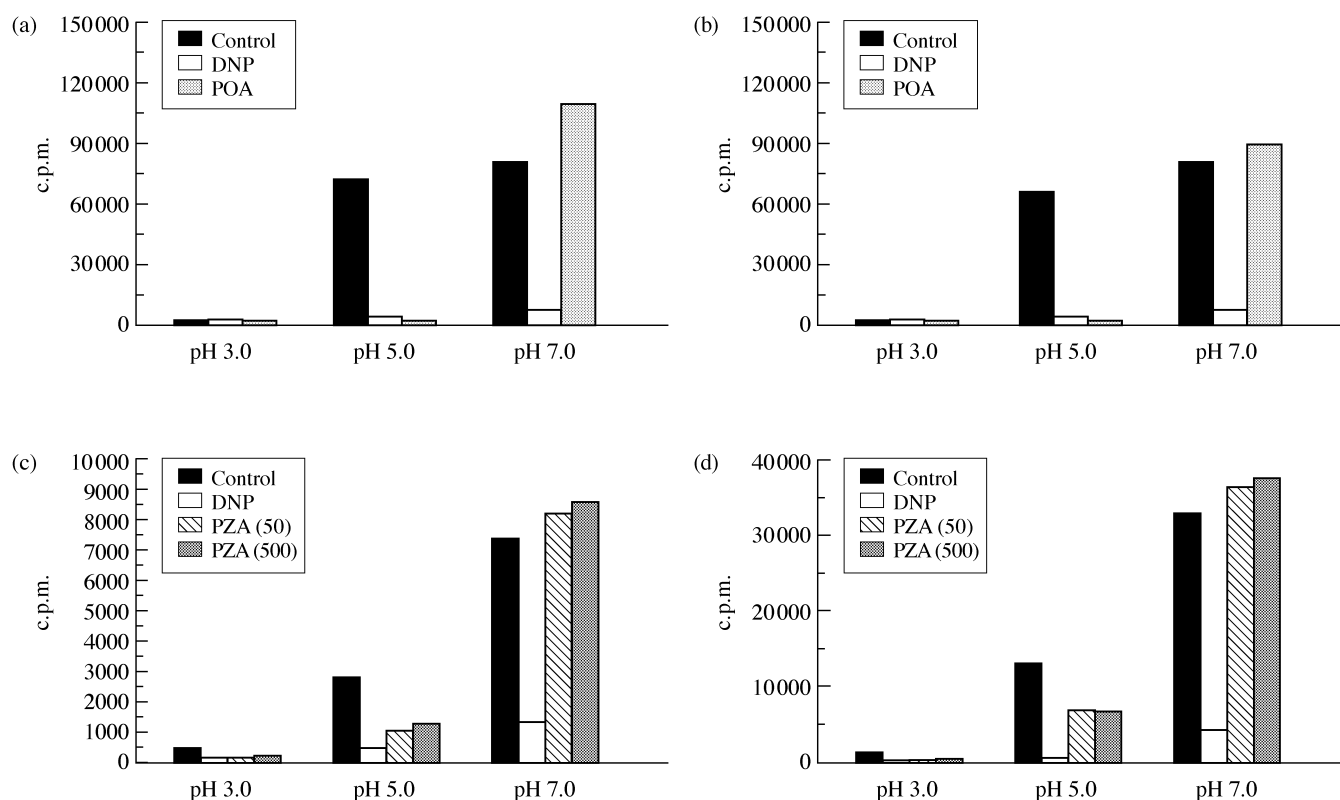
Figure 1. Effect of pyrazinoic acid and pyrazinamide on protein and RNA synthesis in *M. tuberculosis*. The effect of pyrazinoic acid (POA) (500 mg/L, 4 mM) on protein (a) and RNA synthesis (b) as measured by the amount of radioactive [^{35}S]methionine or [^{3}H]uracil associated with the tubercle bacilli (counts per minute, c.p.m.) was monitored after exposure for 4 h at pH 3.0, 5.0 and 7.0. The effect of pyrazinamide (PZA) (50 and 500 mg/L) on protein (c) and RNA synthesis (d) was similarly determined after exposure of H37Ra to pyrazinamide for 2 days. Dinitrophenol (DNP) (4 mM) was included as a positive control in the experiment.

Bacillus subtilis[22] and supports the notion that POA inhibits the membrane transport function of *M. tuberculosis*.

Disruption of membrane potential in M. tuberculosis by pyrazinoic acid

Weak acids are proton carriers at acid pH that could potentially decrease the membrane potential required for the transport of various nutrients. To determine whether the weak acid pyrazinoic acid could disrupt membrane potential in *M. tuberculosis,* we measured the membrane potential of *M. tuberculosis* exposed to pyrazinoic acid, with benzoic acid as a weak acid control, at pH 5.5. Indeed, pyrazinoic acid, like benzoic acid, disrupted the membrane potential in *M. tuberculosis* at acid pH (pH 5.5) (Figure 3a). The simultaneous inhibition of transport of methionine and uracil required for protein and RNA synthesis and of the serine uptake by pyrazinoic acid (Figures 1 and 2) is best explained by the weak acid effect of pyrazinoic acid on decreasing the membrane potential, which is required for transport of many nutrients. These data indicate that pyrazinoic acid targets the membrane and interferes with the membrane energetics required for the transport function of the membrane.

### Effect of acid pH on the decrease in membrane potential

We next examined the effect of external pH on the membrane potential in *M. tuberculosis*. The membrane potential was lower at acid pH than at neutral pH (Figure 3b). The decreased membrane potential by acid pH is most likely the cause for the reduced uptake of uracil, methionine and serine at acid pH as seen in Figures 1 and 2. The decreased membrane potential by acid pH could potentiate the effect of pyrazinoic acid, which further reduces the membrane potential.

Old non-replicating bacilli have a lower membrane potential than young replicating bacilli

Pyrazinamide is more active against old non-growing bacilli than against young growing bacilli.[12] Since pyrazinoic acid disrupted the membrane potential (Figure 3a), we suspected that old bacilli might have a lower membrane potential with less active metabolism than young replicating bacilli, which may underlie the differential activity of pyrazinamide against the old bacilli. To test this, we first compared the membrane potential of a 20-day-old fresh *M. tuberculosis* H37Ra culture and that of a 130-day-old culture. For both young and old bacilli, the membrane potential values were lower at acid pH but higher at neutral or alkaline pH (Figure 3b). The membrane potential for the old culture was generally lower than that of the young culture at external pH of 4–8.5, except at very acidic pH 3 (Figure 3b). At pH 5.0, the membrane potential of the old bacilli (–62.26 ± 6.44 mV) was about 59 units lower than that of the young bacilli (–121.1 ± 3.85 mV). We then compared the membrane potential of old and young bacilli in the presence of POA. As shown in Figure 3(a), POA (at 4 mM equivalent to about 500 mg/L POA) caused a significant decrease in membrane potential in both young and old cells. Benzoic acid (4 mM) as a weak acid control similarly decreased the membrane potential. In contrast, rifampicin, which inhibits RNA synthesis, did not have any significant effect on the membrane potential (data not shown).
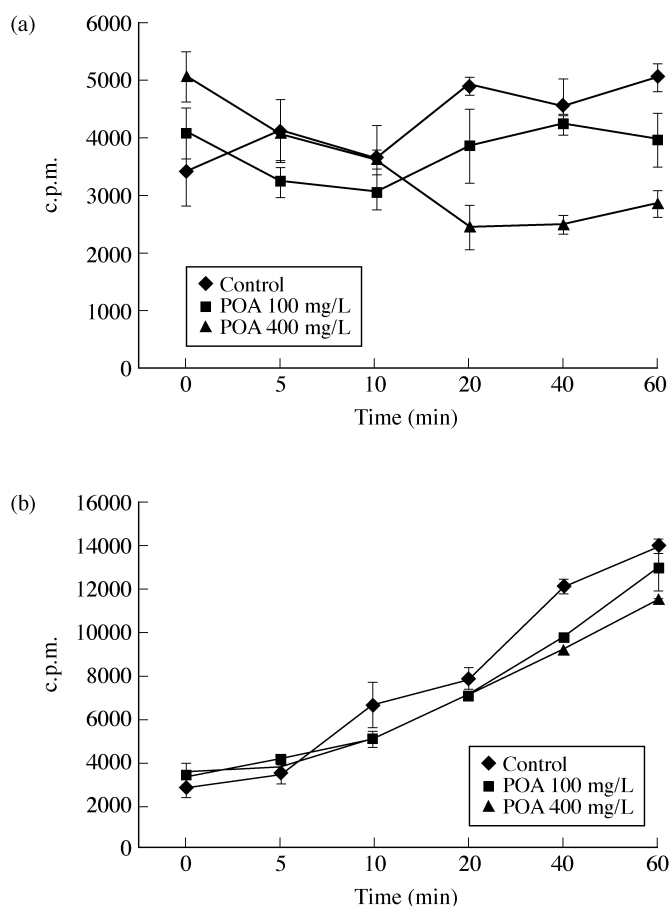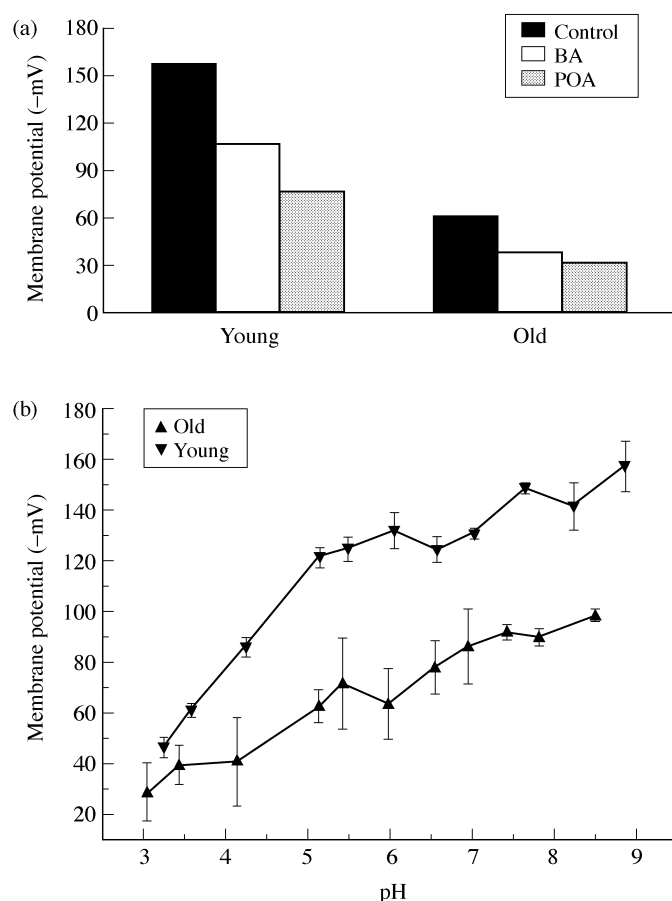
Figure 2. Effect of pyrazinoic acid on L-[¹⁴C]serine uptake. The uptake of L-[¹⁴C]serine in *M. tuberculosis* H37Ra cells treated with pyrazinoic acid (POA) (100 or 400 mg/L) at pH 5.0 (a) and pH 7.0 (b) is presented. At different times, the amount of radioactivity (c.p.m.) associated with the cells was determined.

Figure 3. Effect of pyrazinoic acid and pH on membrane potential in young and old tubercle bacilli. Young (20 day) and old (130 day) bacilli were incubated with pyrazinoic acid (POA, 4 mM) and benzoic acid (BA, 4 mM) at acid pH 5.5 for 1 h before the membrane potential was measured (a). The effect of varying external pH on membrane potential in young and old bacilli is shown in (b). MP, membrane potential, is in negative units (mV) on *y*-axis.

Energy inhibitors synergize with the antituberculous activity of pyrazinamide

Because pyrazinoic acid decreased membrane potential (Figure 3a), we reasoned that energy inhibitors that interfere with membrane potential production might enhance the activity of pyrazinamide. Indeed, *N,N′*-dicyclohexylcarbodiimide (DCCD), which inhibits membrane-bound F_1F_o proton-ATPase and reduces the generation of membrane potential,[24] and rotenone, a specific inhibitor of NADH dehydrogenase—Complex I,[25] and azide, which inhibits membrane-bound cytochrome *c* oxidase and reduces generation of membrane potential by decreased proton pumping,[26] all increased the activity of pyrazinamide against *M. tuberculosis* (Figure 4). DCCD at 1 mM produced a higher synergic effect with pyrazinamide (over 100-fold decrease in cfu) than rotenone at 4 μM and 1 mM azide (over 10-fold decrease in cfu) (Figure 4). This indicates that the F_1F_o proton-ATPase is likely to play a more important role than NADH dehydrogenase and cytochrome *c* oxidase in maintaining the membrane energetics in non-growing cells at acid pH.

Discussion

The recent interest in developing new tuberculosis drugs that can shorten the lengthy 6 month therapy has highlighted the importance of understanding the mode of action of pyrazinamide.[6] In this study, we have shown that pyrazinoic acid inhibited the protein and RNA synthesis and serine uptake as well as disruption of membrane potential at acid pH. The observation that uptake of uracil and methionine was significantly reduced in the presence of pyrazinoic acid at acid pH implies that both RNA and protein synthesis were inhibited by pyrazinoic acid. In various bacterial systems, transport of many nutrients into the cell requires a proton motive force (membrane potential or delta pH).[27] The simultaneous inhibition of synthesis of different macromolecules (protein, RNA) and serine uptake by pyrazinoic acid is best explained by its effect on decreasing membrane potential (Figure 3), which is required for membrane transport. These data indicate that pyrazinoic acid or pyrazinamide targets the membrane and interferes with the energetics and function of the membrane.

Acid pH is known to be essential for pyrazinamide activity.[10] We have shown in a previous study that the role of acid pH is to facilitate formation of protonated pyrazinoic acid (HPOA), which readily permeates through the membrane, and to cause increased accumulation of pyrazinoic acid anions and protons in the cell.[14] In this study, we demonstrated yet another role of acid pH in potentiating pyrazinamide action as its ability to decrease the membrane potential. Whereas acid pH is known to lower membrane potential in other
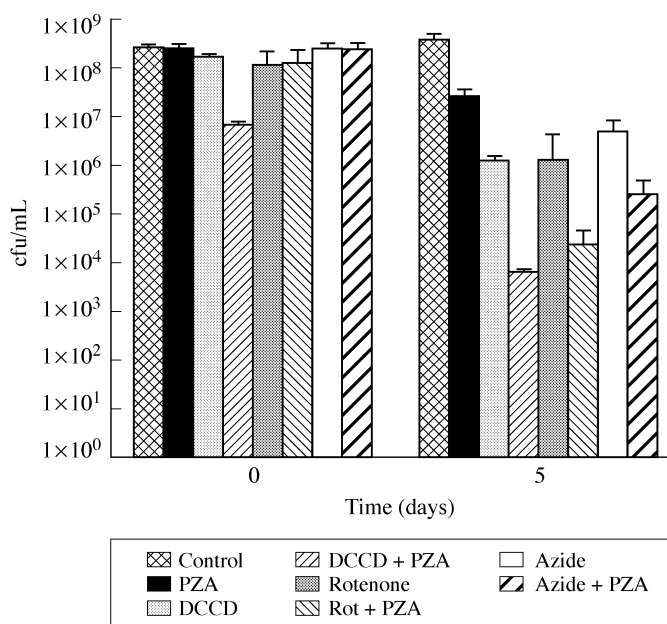
Figure 4. Effect of proton motive force inhibitors on pyrazinamide activity. *M. tuberculosis* H37Ra was exposed to pyrazinamide (50 mg/L) in a citrate buffer (pH 5.5) in combination with 1 mM DCCD, 1 mM sodium azide or 4 μM rotenone. Pyrazinamide (PZA) alone and energy inhibitors alone were included as controls. The number of surviving bacilli at day 0 and day 5 was determined. The *y*-axis is cfu/mL in log scale. Rot, rotenone.

bacteria,[23] this point was not appreciated previously in the context of pyrazinamide action until we have shown here that pyrazinoic acid also disrupts the membrane potential.

Pyrazinamide is more active against old bacilli than against fresh young bacilli,[12] which is consistent with the fact that pyrazinamide is involved in shortening the tuberculosis therapy by killing non-replicating 'semi-dormant' bacilli in an acidic environment.[5] This study provides a plausible explanation for this observation. Old or 'semi-dormant' bacilli have a less active metabolism and less energy reserves as they have a lower membrane potential (Figure 3a). The low membrane potential in old and non-replicating tubercle bacilli in the context of a deficient POA efflux mechanism[14] and a relatively poor ability to maintain membrane energetics[21] provides yet another weak point (Achilles heel) for attack by weak acid POA at acid pH, which further decreases the membrane potential to even lower levels. Since membrane potential, but not pH gradient, is essential for the synthesis of ATP by the F_1F_o ATPase,[28] the decreased membrane potential caused by POA will in turn inhibit ATP production in old or dormant bacilli with less energy reserves and deplete energy in the cell, leading to reduced viability. The observation that inhibition of membrane potential generation enzymes proton-ATPase, NADH dehydrogenase and cytochrome *c* oxidase by DCCD, rotenone and azide, respectively synergizes with pyrazinamide activity, also lends further support to the notion that pyrazinamide or pyrazinoic acid targets membrane energetics as a mechanism of action. Such a mechanism of action provides the best explanation for the unusual properties of pyrazinamide, such as the acid pH requirement, the slow killing and relatively high MIC for young growing bacilli with more energy reserves, and the preferential activity against old non-replicating bacilli with less energy reserves.

Antibiotics are generally active against multiplying bacteria, but are much less effective against non-replicating bacteria as in stationary phase or in biofilm. Pyrazinamide is exactly the opposite[6] and represents the prototype of a class of new antibiotics that kills non-growing persisters more effectively than growing bacilli. The paradoxical features of pyrazinamide challenge the conventional wisdom of developing antibiotics against growing bacteria as an effective means to control non-growing bacteria in persistent infections and call for re-evaluation of the reliance on low MICs as the sole criteria for identifying antibiotics. The ineffectiveness of current antibiotics to kill non-growing or dormant bacteria is believed to be underlying the need for prolonged therapy.[29–31] Our demonstration that pyrazinamide disrupts the membrane energetics in *M. tuberculosis* may have implications for developing new drugs that target energy metabolism in dormant or non-replicating organisms and shorten the treatment of TB and perhaps also other persistent bacterial infections.

Acknowledgements

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Mode of action of pyrazinamide

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