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

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Introduction

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.

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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 [3H]tetraphenylphosphonium bromide (TPP+) and [3H]tetraphenylphosphonium chloride (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 pyrazinamide 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 × 10⁶ cells/mL. [35S]Methionine, [3H]uracil and L-[14C]serine, [14C]benzoic acid and [3H]tetraphenylphosphonium bromide (TPP+) were obtained from Amersham.

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 [35S]methionine as a precursor for protein synthesis and [3H]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, pyrazinamide 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, pyrazinamide 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, pyrazinamide 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 synthesis, respectively, rather than by inhibition of a specific component of the RNA or protein synthesis machinery.

Because conversion of the produg 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-[14C]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...
Bacillus subtilis\textsuperscript{22} and supports the notion that POA inhibits the membrane transport function of \textit{M. tuberculosis}.

Disruption of membrane potential in \textit{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 \textit{M. tuberculosis}, we measured the membrane potential of \textit{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 \textit{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 \textit{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.

\textit{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.\textsuperscript{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 \textit{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).
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\(_{1}\)F\(_{0}\) 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 \( M. \) \( \text{tuberculosis} \) (Figure 4). DCCD at 1 mM produced a higher synergic effect with pyrazinamide (over 100-fold decrease in cfu) than rotenone at 4 \( \mu \)M and 1 mM azide (over 10-fold decrease in cfu) (Figure 4). This indicates that the F\(_{1}\)F\(_{0}\) 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
bacteria, 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, 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. 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 and a relatively poor ability to maintain membrane energetics 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 $F_0F_1$ ATPase, 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 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. Our demonstration that pyrazinamide disrupts the membrane energetics in $M. tuberculosi$s 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.

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References

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