Pyrazinoic acid decreases the proton motive force, respiratory ATP synthesis activity and cellular ATP levels

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Abstract

Pyrazinoic acid, the active form of the first-line anti-tuberculosis drug pyrazinamide, decreased the proton motive force and respiratory ATP synthesis rates in sub-cellular mycobacterial membrane assays. Pyrazinoic acid also significantly lowered cellular ATP levels in *Mycobacterium bovis* BCG. These results indicate that the predominant mechanism of killing by this drug may be due to the depletion of cellular ATP reserves.

Shortening tuberculosis treatment duration is a key objective in order to reduce non-compliance and to combat recently emerging multi-drug resistant strains of *Mycobacterium tuberculosis* [6, 19, 36]. Pyrazinamide (PZA), an important first-line drug employed in tuberculosis chemotherapy, played a key role in shortening of tuberculosis treatment from 9 months to 6 months [22]. PZA is a sterilizing drug that efficiently kills populations of *Mycobacterium tuberculosis* residing in acidic environments, as found during active inflammation [1, 7, 10, 11, 20, 21, 29, 33]. Despite the importance of PZA, no cellular target proteins have clearly been identified [4, 23, 31, 41], and its mechanism of action probably is the least understood of all first- and second line anti-tuberculosis drugs. A better understanding of PZA action may help in development of new drugs to further shorten tuberculosis treatment.

PZA constitutes a pro-drug that in the mycobacterial cell is hydrolyzed by pyrazinamidase to yield the active entity, pyrazinoic acid (POA) [15, 16]. According to the hypothesis put forward by Zhang and colleagues, POA, a weak acid (pKₐ 2.9), acts as an uncoupling agent by breaking down the bacterial membrane potential [39, 40]. POA in its unprotonated form can leave the cell by an unknown efflux system.
take up a proton in the acidic environment and enter the mycobacterial cell again in its protonated, less polar form [39]. The thereby caused decrease in proton motive force then blocks, amongst other processes, uptake of metabolites required for growth [40]. However, it is not known if the decreased membrane potential observed for PZA in whole mycobacterial cells [40] is due to the postulated uncoupling effect or indirectly caused by interference of PZA/POA with other cellular targets. Moreover, the impact of POA on respiratory ATP synthesis and on cellular ATP levels has not been investigated. In this report we use sub-cellular and cellular assays to address these open questions. We use *M. bovis* BCG as model system, which is resistant to PZA due to mutations in pyrazinamidase [16, 30, 31], but is fully susceptible to POA [15, 32, 37].

POA directly interferes with the proton motive force. We isolated membrane vesicles from *M. bovis* BCG as reported previously [9]. In this sub-cellular system, the cytosolic fraction is removed, allowing for a more specific investigation of drug action on membrane components [8,9]. First we determined if pyrazinoic acid (POA) directly interferes with the proton motive force. The proton motive force was monitored with the ACMA quenching method as described previously [9]. Addition of succinate caused fluorescence quenching, which was eased upon addition of an uncoupler (SF6847), proving that the quenching was caused by a proton motive force across the membrane (Figure 1). In the presence of POA the proton motive force decreased in a dose-dependent manner (Figure 1). This effect was more pronounced at pH 5.5 as compared to pH 6.5 (Figure 1A and B). The POA concentrations needed to significantly decrease the proton motive force were comparable to values reported earlier for *M. tuberculosis* at whole-cell level [40]. This result shows that POA directly interferes with membrane energetics.
POA decreases rates of respiratory ATP synthesis. Next, we investigated to which extend POA, by decreasing the proton motive force, interferes with respiratory ATP production. ATP synthesis rates by the mycobacterial membranes were determined as described [8, 9]. As depicted in Figure 2, POA inhibited ATP synthesis in a dose-dependent manner. The affinity for POA significantly decreased at higher pH, with IC$_{50}$ values of 200 µg/ml at pH 5.5 compared with 850 µg/ml at pH 6.5. (Figure 2A and B, closed bars). This result is consistent with the enhanced killing previously observed at acidic pH for PZA in vivo and for POA in vitro [38, 40]. As a control, for PZA only a minor effect was observed, with IC$_{50}$ values exceeding 2000 µg/ml at both pH values tested (Figure 2A and B, open bars). These results strongly suggest that POA is the active entity, which diminishes rates of ATP synthesis. By interfering with both uptake of metabolites as reported earlier [40] and with respiratory ATP synthesis as shown here, POA thus exerts at least dual action, which potentially renders it an exceptionally powerful drug. This unusual property of POA may hold in particular for bacteria under low energy supply [12, 34] and may in part explain why pyrazinamide appears to constitute an essential component of anti-tuberculosis drug regimens.

POA decreases cellular ATP levels. We investigated the impact of ATP synthesis inhibition by POA on cellular ATP levels. Addition of POA to M. bovis BCG grown in liquid culture significantly decreased cellular ATP levels in both time-dependent and dose-dependent manner (Figure 3). The concentrations of POA needed to reduce cellular ATP levels correlate well with those required for ATP synthesis inhibition, e.g. 800 µg /ml POA (IC$_{50}$ in ATP synthesis assay) decreased cellular ATP by ~ 40 % after 1 day and > 60% after 6 days. As control, PZA did not significantly change ATP levels. These results suggest that POA interference with respiratory ATP
synthesis has a significant impact on cellular ATP levels, which may be the cause of bacterial killing.

PZA/POA share several interesting characteristics with a new series of ATP synthesis inhibitors, the diarylquinolines [2,5], as follows: both drugs show a delayed action in vitro and in vivo, with time-dependent killing observed only from day 3-4 on [28,40]. Moreover, both drugs display a particularly strong effect on mycobacteria under (semi-) dormant conditions [10,18,25,27,38]. Finally, similar to our data reported here for POA, TMC207 reduces cellular ATP levels [17,18,27]. Based on this similarity in signature we suggest that both drugs share the same predominant mechanism of killing by depleting the cellular ATP pool. It needs to be determined to which extend alternative targets are involved and contribute to the pronounced bactericidal action of pyrazinamide.

The chain of events leading from (too) low ATP levels to bacterial killing presumably is complex [14,24] and factors involved need to be elucidated. Drugs interfering with cellular energy pools appear to be very powerful, in particular against dormant bacteria. Membrane function and respiratory ATP synthesis may constitute a new generation of antibiotic targets for treatment of persistent infections [3,13,19,40]. The sub-cellular membrane assay described here for characterization of pyrazinoic acid can be applied for screening and characterization of this new generation of compounds targeting respiratory ATP production.

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ATP synthase in slow- and fast-growing mycobacteria is active in ATP


Figure Legends

**Figure 1: Pyrazinoic acid decreases the proton motive force in a sub-cellular assay.** The proton motive force in membrane vesicles from *M. bovis* BCG was monitored in the presence of pyrazinoic acid at the indicated concentrations (µg/ml) by quenching of ACMA as fluorescence indicator at pH 6.5 (A) and pH 5.5 (B). Succinate and the uncoupler SF6847 were added to establish and collapse the proton motive force at the time point indicated by the arrows. Each experiment was carried out in three-fold, representative results are shown.

**Figure 2: Pyrazinoic acid inhibits ATP synthesis.** ATP synthesis activity by membrane vesicles was determined by quantifying produced ATP with the glucose-6-phosphate method [9]. The reaction was carried out in the presence of the indicated concentrations of pyrazinoic acid (closed bars) or pyrazinamide (open bars) at pH 6.5 (A) or pH 5.5 (B). Each graph shows mean values of three independent experiments with standard deviation.

**Figure 3: Pyrazinoic acid decreases cellular ATP levels.** *M. bovis* BCG was grown in liquid culture (pH = 6.5) as in [9]. Cellular ATP levels in the presence of pyrazinoic acid or pyrazinamide were determined at the indicated time points using the luciferase bioluminescent method [17], based on cell volumes described in [26]. Each bar shows mean values of three independent experiments with standard deviation.
Figure 1

A

B

Fluorescence Intensity

Time (min)

A

B

Succinate
Uncoupler

1000
500
0

120
110
100
90
80
70
60
50
40
30

15 20 25 30 35 40 45

15 20 25 30 35 40 45

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Figure 2

A

B