Inhibitors of type II NADH:menaquinone oxidoreductase represent a class of antitubercular drugs

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Mycobacterium tuberculosis **(Mtb) is an obligate aerobe that is capable of long-term persistence under conditions of low oxygen tension. Analysis of the Mtb genome predicts the existence of a branched aerobic respiratory chain terminating in a cytochrome *bd* system and a cytochrome aa_3 system. Both chains can be initiated with type II NADH:menaquinone oxidoreductase. We present a detailed biochemical characterization of the aerobic respiratory chains from Mtb and show that phenothiazine analogs specifically inhibit NADH:menaquinone oxidoreductase activity. The emergence of drug-resistant strains of Mtb has prompted a search for antimycobacterial agents. Several phenothiazines analogs are highly tuberculocidal *in vitro*, suppress Mtb growth in a mouse model of acute infection, and represent lead compounds that may give rise to a class of selective antibiotics.**

Mycobacterium tuberculosis | respiratory chain

The World Health Organization estimates that two billion people are infected with *Mycobacterium tuberculosis* (Mtb), and two million people die of the disease each year (1). Most individuals infected with the organism are latent carriers who have a 2–23% lifetime risk of developing reactivation tuberculosis (TB). The risk dramatically increases if the carrier's immune system is suppressed. Also, drug resistance is a serious concern; the isoniazid (INH)-resistance rate is ≈10%, and the rifampicin (RIF) resistance rate is ≈1%, with lower numbers in countries with effective TB programs and higher numbers in countries with deficient TB programs. The World Health Organization declared TB infections to be a global public health emergency (1), and the need to identify targets for antimicrobial therapy remains urgent.

Mtb is capable of establishing persistent infection in the host by using a complex interplay between the host immune system and bacterial survival mechanisms. In the persistent infection, Mtb adapt to depletion of available oxygen and nutrients and enter a stage of nonreplicating persistence (NRP) in granulomatous or necrotic lesions. NRP Mtb are resistant to INH, ethambutol, and RIF, but they become sensitive to metronidazole *in vitro* (2). Given the critical role of oxygen in the generation of cellular energy and bacterial long-term survival, there is surprisingly little information on oxidative phosphorylation in Mtb. Clearly, oxidative phosphorylation is a central component in the production of ATP and the subsequent growth and pathogenesis of Mtb. Here, we characterize the aerobic respiratory pathway and show that NADH:menaquinone oxidoreductase (Ndh) is a key target for TB agents.

Materials and Methods

Media and Strains. Mtb $H_{37}R_v$ was a gift from C. Imperatrice (Clinical Infectious Diseases, Hospital of the University of Pennsylvania) and *Mycobacterium smegmatis* Mc^2155 was obtained from V. Mizrahi (National Health Laboratory Service, Johannesburg). Bacteria were cultured in 7H9 broth supplemented with 10% oleic acid-albumin-dextrose catalase/0.5% glycerol/0.05% Tween 80. Solid agar (15 g/liter) was added to liquid media to create solid media as required.

Spectroscopy. Aerobically grown, gamma-irradiated Mtb $H_{37}R_v$ whole cells were obtained from John Belisle (Colorado State University, Fort Collins) under National Institute of Allergy and Infectious Diseases Contract NO1-A1-75320. Cells (12 g of wet weight) were washed with 10 ml of phosphate–digitonin buffer (50 mM sodium phosphate, pH 7.4, containing 0.1% digitonin) and resuspended in the same buffer. The bacteria were lysed by passage through a French press at 14,000 kPa, and the suspension was centrifuged at 12,000 × *g* for 15 min to remove cellular debris. The supernatant was further clarified by centrifugation at 50,000 × *g* for 50 min. This supernatant was used as the membrane-containing fraction (or cell-free extract) for spectral studies. To isolate cell membranes, the 50,000 × *g* supernatant was centrifuged at 150,000 × *g* for 60 min. The resulting pellet was washed with phosphate–digitonin buffer, resuspended, and then centrifuged at 150,000 × *g* for 60 min a second time. Protein concentrations were determined by bicinchoninic assay (BCA, Pierce) by using BSA as the standard. UV-visible spectra were recorded at room temperature on a Cary 4E dual-beam spectrophotometer (Varian) by using a scan speed of 120 nm/min and a slit width of 1 nm. Reduction of 21.3 mg/ml membrane protein was accomplished by a 3-min incubation with 5 mM NADH. To illustrate the spectral perturbation due to CO binding of terminal oxidases, CO gas was gently bubbled into the sample for 5 min before NADH reduction. For the samples containing trifluoperazine (TPZ), drug was added to the sample and incubated for 5 min before the addition of NADH.

Amperometric Assay. Oxygen consumption by respiration of Mtb cytoplasmic membranes was measured by a polarographic method. Mtb membranes (50 mg/ml) were suspended in 0.1 M potassium phosphate buffer (pH 7.4), and 10 mM NADH was added to initiate the respiration. Subsequently, 1 mM TPZ or vehicle and then 10 mM sodium ascorbate plus 1 mM 3,3,5,5-tetramethylphenylenediamine (TMPD) were added into the reaction mixture, as shown in Fig. 2*B*.

NADH-Quinone Oxidoreductase Activity Assay. For the NADH-quinone oxidoreductase activity assay of Mtb membrane, the membrane was dialyzed against 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA at 4°C overnight to remove endogenous reducing substrates. The Mtb membrane (3

Abbreviations: cfu, colony-forming units; INH, isoniazid; RIF, rifampin; TPZ, trifluoroperazine; CPZ, chlorpromazine; Mtb, *Mycobacterium tuberculosis*; Ndh, NADH:menaquinone oxidoreductase; MIC, minimum inhibitory concentration; RC, respiratory chain; TMPD, 3,3,5,5-tetramethylphenylenediamine; TB, tuberculosis; GI, growth index.

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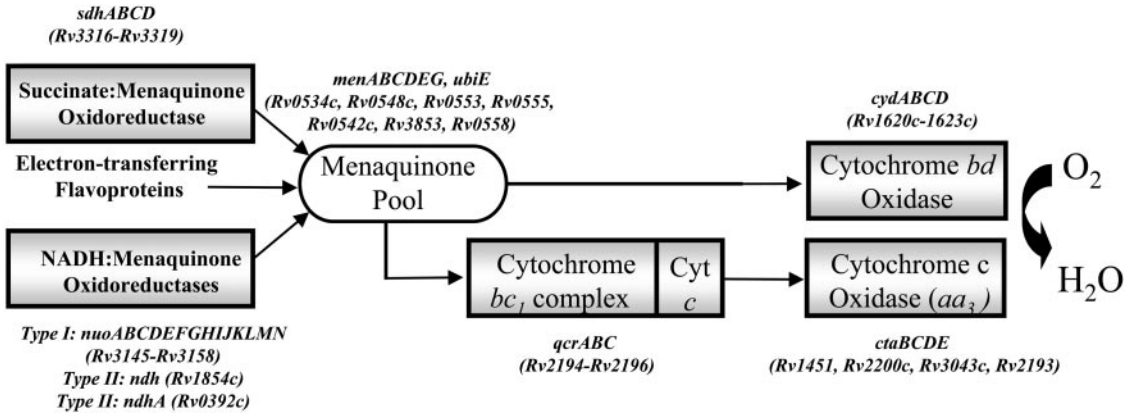

Fig. 1. Proposed pathway of aerobic electron flow in mycobacteria. Complexes are shown in boxes, with corresponding gene names and GenBank accession nos. given outside of the boxes.

mg/ml) was suspended in the phosphate buffer containing 10 mM KCN and 100 μM menaquinone 1 (MK_1) or Q_1. Drug or vehicle was added as desired, and the reaction mixture was incubated for 5 min at 30°C. The reaction was initiated by adding 100 μM NADH, and the absorbance change at 340 nm was monitored by using a custom-built 1098 spectrophotometer (Hitachi, Tokyo). NADH oxidation did not occur in the absence of membrane protein or quinone. For the purified Ndh or NdhA, NADH-quinone oxidoreductase activity was measured in 50 mM sodium phosphate buffer (pH 7.0) containing 150 mM NaCl and 2% (wt/vol) potassium cholate. The reaction mixture contained 50 μM Q_2/1 μg of purified enzyme, and it was initiated by adding 100 μM NADH and monitored by following the absorbance change at 340–400 nm with a PerkinElmer 557 double-beam dual-wavelength spectrophotometer at 37°C. For chlorpromazine (CPZ)-inhibition assays, ≈1 μg of purified enzyme and 50 μM Q_2 were incubated in the reaction mixture with various amounts of CPZ for 2–3 min at room temperature before the addition of NADH.

Expression and Purification of Mtb *ndh* and *ndhA* in *Escherichia coli*. The Mtb *ndh* and *ndhA* genes were amplified by PCR using the Mtb genomic DNA and template, and the products were cloned in pET16b expression plasmid, which contains an N-terminal His-6-tag. Mtb Ndh and NdhA were reproducibly expressed in *E. coli* strain BL21(DE3), and the products were localized to the cytoplasmic membrane. The *E. coli* cell suspension in buffer A (50 mM Hepes, pH 7.0/100 mM KCl/1 mM PMSF) was passed through a French press twice at 14,000 kPa. Next, the suspension was centrifuged at 12,000 × *g* for 15 min to remove unbroken cells and debris. The resulting supernatant was ultracentrifuged at 130,000 × *g* for 30 min. The membrane pellet was resuspended in buffer A and ultracentrifuged at 130,000 × *g* again for 15 min. The cytoplasmic membranes thus obtained were resuspended in buffer A, 2% (wt/vol) sodium cholate (pH 7.2) was added, and the mixture was solubilized by incubation at 4°C for 1 h. The mixture was next ultracentrifuged at 130,000 × *g* for 30 min, and solubilized proteins in the supernatant were collected. We added 1 ml of buffer A-equilibrated Talon (BD Biosciences) resin, and the mixture was incubated at 4°C for 1 h. The resin was transferred into a column was washed with 30 ml of buffer A with 1% cholate, followed by 50 ml of buffer A with 1% cholate and 10 mM imidazole. Last, the bound proteins were eluted with buffer A with 1% cholate and 100 mM imidazole. Purified recombinant Mtb Ndh and NdhA were used immediately for activity assays.

Determination of the Minimum Inhibitory Concentration (MIC) by the Bactec MGIT 960. The MIC for each compound was determined by the Bactec MGIT (mycobacteria growth indicator tube) 960 system. Mtb $H_{37}R_v$ was grown in Middlebrook 7H9 broth until the growth index (GI) reached 75 (GI is a scale in the Bactec system (Becton Dickinson), which reflects the amount of growth) and was then diluted 2,500-fold and used as the inocula. The vials were dispensed with different dilutions of drug to reach final concentrations ranging 0.2–26 μg/ml. All of the drug-containing vials were inoculated with 0.5 ml of the bacterial suspensions prepared as described above. Six drug-free controls were included with each test; three were inoculated with 0.5 ml of the suspension, and the remaining three were inoculated with 0.5 ml of a 1:100 dilution of the suspension. The vials were incubated at 37°C and read in a Bactec 960 reader every day until the GI in the control diluted 1:100 reached 75, with an increase in the GI of at least 10 for 3 consecutive days. The time to positive was 10 days for the undiluted control. MIC was defined as the lowest concentration of the drug that caused an increase in the GI equal to or less than the increase in the GI of the control diluted 1:100.

Animal Studies: Test of Phenothiazine Efficacy in a BALB/c Mouse Model of Acute Mtb Infection. Each treatment group consisted of five female mice intranasally infected with 10^2 colony-forming units (cfu) of H37Rv Mtb on day 0. INH, RIF, or compound 1 was given orally on days 1–11. After 11 days of the indicated treatment, mice were killed, and the lungs and spleens were aseptically collected. Serial 10-fold dilutions were prepared of tissue homogenates in 7H9 media and were plated on 7H11 agar at 37°C for cfu enumeration after 4 weeks of incubation.

Results

Genetic Characterization of Aerobic Respiration in Mtb. By analyzing the Mtb genome (3), we found the presence of a respiratory chain (RC) with a quinol oxidase branch and a cytochrome oxidase branch (Fig. 1). Cytochrome *bd* oxidase is a quinol oxidase encoded by *cydABCD*, and cytochrome aa_3 is a cytochrome *c* oxidase encoded by *ctaBCDE*. We recently demonstrated (4) that the *d*-type oxidase is induced in mycobacteria under low-oxygen conditions and is important for microaerobic growth.

By homology, the aa_3-type oxidase belongs to the heme-copper oxidase superfamily of respiratory oxidases critical for exponential growth under oxygen-rich conditions (5). Mtb membrane preparations readily oxidize reduced bovine and yeast cytochromes *c*, yet extraction of Mtb membranes under high salt conditions failed to release free cytochrome *c* (data not shown).

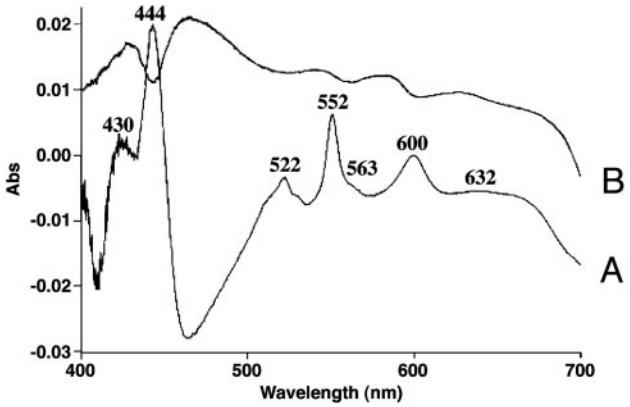

Fig. 2. The reduced and the CO-binding pigments of H37Rv cell extracts. (*A*) NADH-reduced minus air-oxidized difference spectrum. Reduction was accomplished 5 min after the addition of 10 mM NADH. (*B*) (NADH-reduced plus CO) minus (NADH-reduced) difference spectrum. To show the spectral perturbation due to CO binding, CO was bubbled through the sample before reduction.

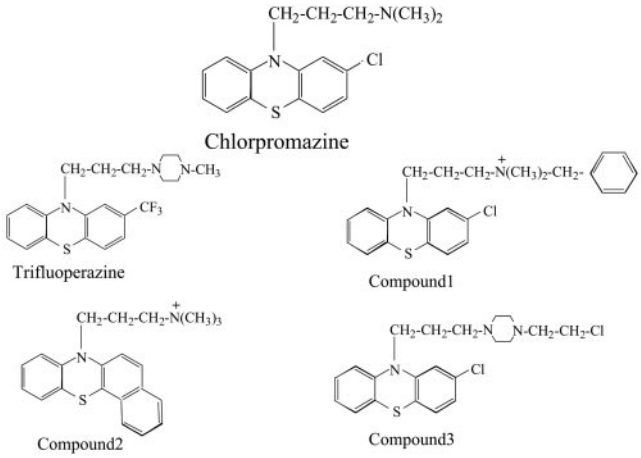

Fig. 3. Structures of analogs of CPZ.

Inspection of the genome indicates that cytochrome *c* is fused to the third subunit (QcrQ) of the bc_1 complex. Therefore, Mtb is a second member of a clade of G+C rich Gram-positive organisms with a diheme cytochrome c_1 subunit in the bc_1 complex (6). The physiologic electron donor to the bc_1 complex is demethylmenaquinone 8 (DMK-8), which is presumably synthesized by the enzymes encoded by *menABCDEG*.

The quinone pool in Mtb is reduced by succinate:menaquinone oxidoreductase, which is comprised of four subunits, encoded by *sdhABCD* (complex II) and Ndh. We identified two types of Ndh in the Mtb genome (Fig. 1). NDH-1 is a 14-subunit (*nuoABCDEFGHIJKLMN*) complex that is generally coupled to proton translocation. NDH-2, is an alternative, nonproton pumping, single-subunit oxidoreductase present in two copies (*ndh* and *ndhA*). The type II NADH:menaquinone oxidoreducatases, Ndh and NdhA, share 67% sequence identity, and their genes are separated by 17 kb. Other mycobacterial species, such as *Mycobacterium leprae* and *M. smegmatis* contain single copies of the *ndh* gene with 91% and 83% identical residues, respectively, compared with Mtb *ndh*. Furthermore, *M. leprae* depends solely on Ndh activity, as evidenced by the loss of all type I genes during the course of evolution except for a *nuoN* pseudogene. Inactivating mutations in *M. smegmatis ndh* lead to a thermosensitive lethal and auxotrophic phenotype (7). In *E. coli*, type II NADH dehydrogenase is dominant under conditions of aerobic growth, whereas type I NADH dehydrogenase is induced under anaerobic conditions (8–11). Therefore, type II NADH dehydrogenase is an important respiratory enzyme for growth of Mtb in an aerobic environment.

Spectroscopic Characterization of Cytochrome Components. To characterize the cytochromes corresponding to the bc_1 complex and terminal oxidases of Mtb, we collected reduced minus oxidized difference spectra (Fig. 2*A*) of Mtb membrane particles. Peaks were observed at 563, 532, and 430 nm, which are characteristic of the *b*-type cytochromes (12). Peaks at 552 and 522 nm, which are typical of cytochrome *c* of the bc_1 complex, were also visible. A broad peak at 600 nm with a resonance peak at 444 nm presumably corresponded to cytochrome aa_3. The bacilli were grown under fully aerobic conditions, making the discrete *bd* oxidase peak at 632 nm difficult to discern (4). CO binds to terminal oxidases and causes a shift in heme absorbance. To confirm that the peak at 600 nm represented a terminal oxidase, [NADH-reduced plus CO] minus [NADH-reduced] spectra were obtained (Fig. 2*B*). CO binding produced a trough at 444 nm and a peak at 429 nm. The cytochrome *c* peak of the bc_1 complex at 552 nm was not visible in the [NADH-reduced plus CO] minus [NADH-reduced] difference spectrum, consistent with a cytochrome that does not bind oxygen.

Determination of the Mechanism of Action of Phenothiazines. The steady-state rate of respiration of Mtb membrane particles was determined in a cell-free amperometric assay. Addition of NADH to Mtb membrane particles resulted in an immediate linear consumption of oxygen, but consumption was fully inhibited by the addition of the phenothiazine TPZ (1 mM) (Fig. 3). Respiration in the drug-arrested membranes was restored by the addition of 10 mM ascorbate and 1 mM TMPD, which donate electrons at the level of cytochrome *c* (Fig. 4*A*). Thus, the phenothiazine affected both respiratory branches in Mtb. The recovery of oxygen consumption implied that the site of inhibition by TPZ is upstream of cytochrome *c*. To confirm this site of action, we tested the ability of TPZ to inhibit the reduction of bovine cytochrome *c*. By using the 550- to 540-nm wavelength pair, we determined that TPZ (100 μM) inhibited the rate of reduction of cytochrome *c* by 75%.

To determine whether this block affected *bd* oxidase or the bc_1 complex, we collected [NADH-reduced] minus [air-oxidized] difference spectra in the presence of TPZ (Fig. 4*B*). After incubation with 100 μM TPZ, the peaks corresponding to cytochromes *b*, *c*, *a*, and *d* not only failed to increase but were oxidized below baseline. Thus, phenothiazines inhibit electron transport in Mtb at a point before the reduction of cytochrome *c*.

To test whether phenothiazines inhibited Ndh activity, the electron-transport chain was blocked with 10 mM KCN, and the rate of oxidation of NADH in the presence of menaquinone 1 (MK_1) was measured. The addition of compound 1 resulted in a concentration-dependent decrease in Ndh activity with an IC_{50} of 158 μM. Flavone, a weak inhibitor of type II NADH dehydrogenase, suppressed NADH oxidation with an IC_{50} of 750 μM. Classic inhibitors of type I NADH dehydrogenase, such as rotenone (10 μM), piericidin A (10 μM), and pyridaben (10 μM) did not inhibit Mtb Ndh activity. Also, deamino-NADH, a type I NADH dehydrogenase specific substrate, was not oxidized by Mtb membranes. Therefore, type II NADH dehydrogenase is a specific target site of phenothiazines.

Next, we tested whether phenothiazines inhibited succinate dehydrogenase activity (complex II). In this assay, the dye dichlorophenolindophenol was used as an electron acceptor and

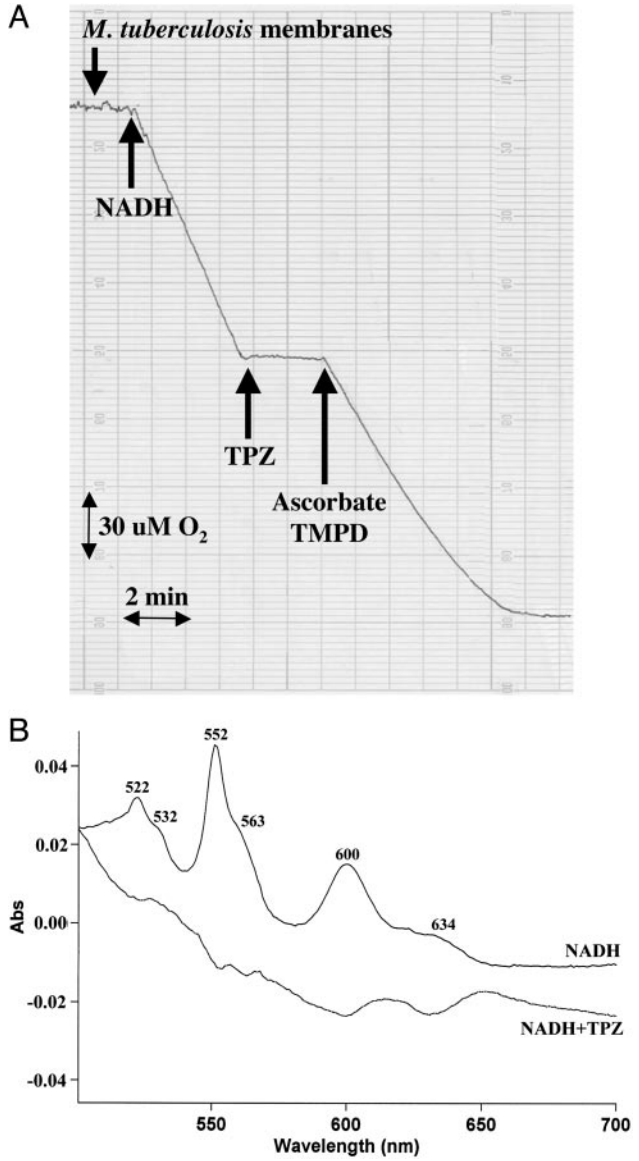

Fig. 4. Phenothiazine inhibition of Mtb respiration. (*A*) TPZ inhibition of NADH-dependent oxygen consumption by Mtb membranes. Mtb membrane protein was added to phosphate buffer in a vessel equipped with a Clark-type oxygen electrode. Respiration was initiated by the addition of 10 mM NADH and arrested upon the addition of 1 mM TPZ. Addition of 10 mM ascorbate and 1 mM TMPD produced an immediate resumption of respiration. (*B*) [NADH-reduced] minus [air-oxidized Mtb] membranes in the absence (solid line) or presence (dotted and hashed lines) of 100 μM TPZ. For each trace, 10 mM NADH was added to a 1-ml quartz semimicrocuvette. For the samples containing TPZ, drug was added to the sample and incubated for 5 min before the addition of NADH. Samples were suspended in 50 mM Hepes buffer (pH 7.4) at a protein concentration of 21.3 mg/ml.

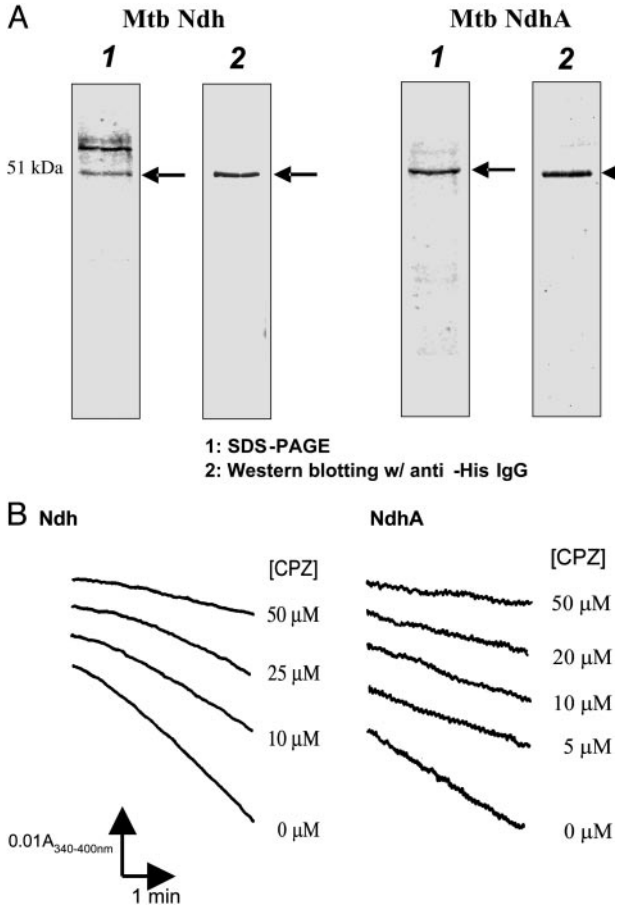

Fig. 5. Phenothiazines inhibit recombinant Ndh and NdhA. (*A*) The Mtb Ndh (*Left*) and NdhA (*Right*) were expressed in *E. coli* with an N-terminal His-6-tag. Lane 1 shows a Coomassie brilliant blue-stained polyacrylamide gel, and lane 2 shows Western blotting with anti-His tag IgG. The Ndh and NdhA products are indicated by arrows. Their apparent molecular mass is 51 kDa. (*B*) CPZ titration of NADH-Q_2 oxidoreductase activity of the purified recombinant Mtb Ndh (*Left*) and NdhA (*Right*). The IC_{50} is 10 μm for both Ndh and NdhA. Specific activity is $\approx$0.12 μmol of oxidized NADH per min/mg protein.

succinate as the electron donor. Phenothiazine concentrations as high as 400 μM did not inhibit the reduction of dye. These results were confirmed by using the amperometric assay with succinate as the electron donor. The rate of oxygen consumption in this assay was not altered by the phenothiazines. This result further confirms that the inhibitory activity of phenothiazines is specific for type II NADH dehydrogenase.

Phenothiazines Inhibit Recombinant Ndh and NdhA. As described above, Mtb contains two copies of type II NADH dehydrogenase genes, *ndh* and *ndhA*. To determine whether Ndh or NdhA are specific targets for phenothiazine action, we cloned and independently expressed each of the genes in *E. coli* with N-terminal His-6-tags. The recombinant proteins were purified by affinity chromatography, and anti-His-6 Western blotting indicated single bands of 51 kDa (Fig. 5*A*), consistent with the expression of Ndh and NdhA gene products. The recombinant proteins were capable of oxidizing NADH in the presence of ubiquinone 2. Next, a titration of the NADH:unquinone 2 oxidoreductase activity with CPZ demonstrated an IC_{50} of $\approx$10 μM for both Ndh and NdhA enzymes (Fig. 5*B*). Therefore, both Ndh and NdhA are inhibited by the action of phenothiazines.

Screening Phenothiazine Analogs. To investigate this class of drugs further, a series of phenothiazines were tested in an Mtb growth-inhibition assay. We screened 50 phenothiazine analogs (GlaxoSmithKline, Collegeville, PA) for inhibition of Mtb growth. The MIC for each compound was determined by using the Bactec MGIT 960 system. The MICs of compound 1 (1.11 μg/ml), compound 2 (3.44 μg/ml), compound 3 (3.96 μg/ml), TPZ (19.2 μg/ml), CPZ (9.23 μg/ml), RIF (0.5 μg/ml), and INH (0.15 μg/ml) were determined by using the Bactec 960 system. The bacteriocidal effect of these drugs was demonstrated by the failure to recover colonies on plates from cultures at 35 days.

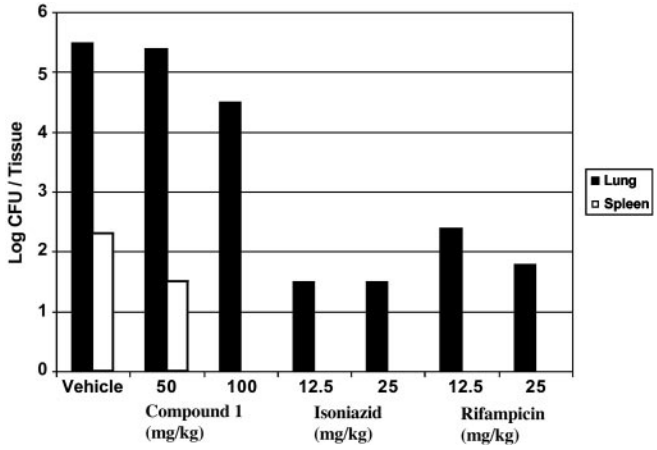

Fig. 6. Test of phenothiazine efficacy in a BALB/c mouse model of acute Mtb infection. Each treatment group consists of five female mice intranasally infected with 100 cfu of $H_{37}R_v$ Mtb. After 11 days of the indicated treatment, mice were killed, and the levels of Mtb in the lung and spleen were determined.

Antimycobacterial Activity of the Phenothiazines in an Animal Model. The convincing antimycobacterial activity of phenothiazine compounds in our earlier studies led us to test whether the compounds were effective in a more clinically relevant mouse model of acute infection. We tested compound 1 for *in vivo* activity against H37Rv Mtb in female BALB/c mice (Fig. 6). Mice were initially intranasally infected with Mtb on day 0 and then given INH, RIF, or compound 1 orally on days 1–11. Mice were then killed, and the levels of Mtb in the lung and spleen were determined. Animals receiving 100 mg/kg compound 1 showed a statistically significant 90% decrease in cfu in the lung, compared with animals receiving vehicle. Animals treated with compound 1 showed no cfu in the spleen, whereas three of five animals receiving vehicle alone had recoverable organisms.

Discussion

The type II NADH dehydrogenase is a unique and important antimicrobial target. Human mitochondria use only type I NADH dehydrogenase, whereas Mtb contains both type I and type II NADH dehydrogenases. It is not known whether the Mtb NDH-1 genes are expressed in any growth conditions. DNA-array analysis revealed that the level of transcription of Mtb NDH-1 is down-regulated upon starvation *in vitro* (14), but transposon insertions apparently are tolerated in the *nuoA*~*N* gene cluster, suggesting that NDH-1 is dispensable for growth *in vitro* (15). In the case of *E. coli*, type II NADH dehydrogenase is dominantly expressed under aerobic growth conditions, whereas type I NADH dehydrogenase is induced under semi-aerobic conditions (8, 10, 11). Recent studies (16) showed that the knockout of *ndhA* is possible in Mtb, and the lack of a viable strain containing an insertionally inactivated *ndh* suggests that it is a lethal phenotype. Furthermore, *M. leprae* depends solely on Ndh activity as evidenced by the loss of all type I genes except a *nuoN* pseudogene during the course of evolution (3). Inactivating mutations in *M. smegmatis ndh* lead to a thermosensitive lethal and auxotrophic phenotype (7). Therefore, it is reasonable to suggest that type II NADH dehydrogenase is the sole NADH dehydrogenase enzyme in the Mtb RC for growth in an aerobic environment.

The antimycobacterial activity of these phenothiazines has been sporadically reported over the past 40 years (17–19). The efficacy of phenothiazines as antitubercular drugs has not been investigated thoroughly, and the target sites of phenothiazines have remained unknown. Previous investigations suggested that phenothazines affect multiple sites of action in Mtb, including synthesis of lipids, proteins, and DNA. Of particular interest is an observation that TPZ exhibited a significant effect on *in vitro* ATP synthesis by *M. leprae* (20). Cellular ATP level of *M. leprae* was dramatically reduced in the presence of 5 μg/ml TPZ in the growth media, suggesting that one of the target sites is the electron transport pathway. This prompted us to test whether these compounds were able to suppress Mtb growth *in vivo* and to identify type II NADH dehydrogenase as a target site of phenothiazines in the Mtb oxidative phosphorylation system.

In agreement with the literature, our data indicate that phenothiazines are potent inhibitors of Mtb growth *in vitro*. Phenothiazines exert significant tuberculocidal activity against Mtb strains resistant to INH, rifampin, streptomycin, pyrazinamide, and ethambutol combined (21, 22). TPZ is effective against virulent Mtb strain $H_{37}R_v$ in a macrophage model of infection, and it is reported as synergistic with both INH and RIF (23, 24). Because the *in vitro* concentration required for bacteriocidal activity is >100-fold greater than the clinical concentration of drug *in vivo* (24), we endeavored to find analogs with greater potency. Our data indicate that compound 1 is 12-fold more potent than TPZ in the Bactec 960 growth assay. Phenothiazines are reported to be concentrated within macrophages, which may reduce the required dose for treatment significantly (21). In contrast to TPZ, compound 1 is water soluble at pH 7.4, and initial cellular-toxicity studies of this compound in A-537 fibroblasts indicate an LD_{50} of >28 μg/ml (data not shown). In our mouse model of acute infection, a 100 mg/kg dose of compound 1 produced a 1-log decrease in cfu within the lung. Therefore, compound 1 is an important lead compound for the development of antitubercular drugs.

It is reasonable to speculate that the modulation of electron transport in mycobacteria by phenothiazines may have a profound effect on the entrance and maintenance of dormancy. Recently, the DosS/DosT, Dos R (Rv3132c/Rv2027c Rv3133c) system has been described, which is an oxygen- and NO-regulated, two-component system in Mtb, composed of two histidine kinases (DosS and DosT) and the response-regulator DosR (25, 26). The Dos system has been implicated in the ability of mycobacteria to adapt to hypoxic environmental conditions (27, 28). During a hypoxic exposure, the quinone pool shifts to a reduced state because of the absence of oxygen as an electron acceptor. Voskuil *et al*. (29) observed that CN reverses the NO induction of the Dos regulon and suggested that the Dos response could be mediated by cytochrome aa_3. This suggestion is consistent with the recent results of Boshoff *et al*., who demonstrated that CN does not up-regulate the Dos regulon (30). A drug such as phenothiazine, which is capable of blocking electron flow to menaquinone, would presumably result in an increase in the oxidized state of the entire RC, and thereby, it would prevent the organism from entering a Dos-mediated state. As a result, when the RC is clamped in the oxidized state, the bacteria behave in the short term as if they were under aerobic conditions, even if oxygen is limiting. In fact, CPZ treated bacteria show more robust short-term growth than bacteria not treated with CPZ under low-oxygen conditions (H.R. and E.A.W., unpublished data). Phenothiazines may accelerate the course of TB treatment by interfering with bacterial persistence, thus making other anti-TB agents more effective.

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1. Maher, D. & Raviglionem M. C. (1999) in *Tuberculosis and Nontuberculous Mycobacterial Infections*, ed. Schlossberg, D. (Saunders, Philadelphia), 4th Ed., pp. 104–115.
2. Wayne, L. G. (1994) *Eur. J. Clin. Microbiol. Infect. Dis.* **13,** 908–914.
3. Cole, S. T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S. V., Eiglmeier, K., Gas, S., Barry, C. E., III, *et al.* (1998) *Nature* **393,** 537–544.
4. Kana, B. D., Weinstein, E. A., Avarbock, D., Dawes, S. S., Rubin, H. & Mizrahi, V. (2001) *J. Bacteriol.* **183,** 7076–7086.
5. Ferguson-Miller, S. & Babcock, G. T. (1996) *Chem. Rev.* **96,** 2889–2907.
6. Sone, N., Nagata, K., Kojima, H., Tajima, J., Kodera, Y., Kanamaru, T., Noguchi, S. & Sakamoto, J. (2001) *Biochim. Biophys. Acta* **1503,** 279–290.
7. Miesel, L., Weisbrod, T. R., Marcinkeviciene, J. A., Bittman, R. & Jacobs, W. R. (1998) *J. Bacteriol.* **180,** 2459–2467.
8. Wackwitz, B., Bongaerts, J., Goodman, S.D. & Unden, G. (1999) *Mol. Gen. Genet.* **262,** 876–883.
9. Calhoun, M. W. & Gennis, R.B. (1993) *J. Bacteriol.* **175,** 3013–3019.
10. Unden, G. & Bongaerts, J. (1997) *Biochim. Biophys. Acta* **1320,** 217–234.
11. Green, J. & Guest, J. R. (1994) *Mol. Microbiol.* **12,** 433–444.
12. Jones, C. W. & Poole R. K. (1985) in *Methods in Microbiology*, ed. Gottschalk, G. (Academic, London), pp. 285–328.
13. Young, I. G., Jaworowski, A. & Poulis, M. I. (1978) *Gene* **4,** 25–36.
14. Betts, J. C., Lukey, P. T., Robb, L. C., McAdam, R. A. & Duncan, K. (2002) *Mol. Microbiol.* **43,** 717–731.
15. Sassetti, C. M., Boyd, D. H. & Rubin, E. J. (2003) *Mol. Microbiol.* **48,** 77–84.
16. McAdam, R. A., Quan, S., Smith, D. A., Bardarov, S., Betts, J. C., Cook, F. C., Hooker, E. U., Lewis, A. P., Woollard, P., Everett, M. J., *et al.* (2002) *Microbiology* **148,** 2975–2986.
17. Ratnakar, P. & Murthy, P. S. (1992) *FEMS Microbiol. Lett.* **76,** 73–76.
18. Molnar, J., Beladi, I. & Földes, I. (1977) *Zentralbl. Bakteriol. Mikrobiol. Hyg. Ser. A* **239,** 521–526.
19. Kristiansen, J. E. & Vergmann, B. (1986) *Acta Pathol. Microbiol. Scand. Sec. B.* **94,** 393–398.
20. Katoch, V. M., Saxena, N., Shivannavar, C. T., Sharma, V. D., Katoch, K., Sharma, R. K. & Murthy, P. S. (1998) *FEMS Immunol. Med. Microbiol.* **20,** 99–102.
21. Amaral, L., Kristiansen, J.E., Abebe, L. S. & Millett, W. (2001) *J. Antimicrob. Chemother.* **47,** 505–511.
22. Gadre, D. V., Talwar, V., Gupta, H. C. & Murthy, P. S. (1998) *Int. Clin. Psychopharm.* **13,** 129–131.
23. Crowle, A. J., Douvas, G. S. & May, M. H. (1992) *Chemotherapy* **38,** 410–419.
24. Reddy, M. V., Nadadhur, G. & Gangadharam, P. R. J. (1996) *J. Antimicrob. Chemother.* **37,** 196.
25. Voskuil, M. I., Schnappinger, D., Visconti, K. C., Harrell, M. I., Dolganov, G. M., Sherman, D. R. & Schoolnik, G. K. (2003) *J. Exp. Med.* **198,** 705–713.
26. Roberts, D. M., Liao, R. P., Wisedchaisri, G., Hol, W. G. J. & Sherman, D. (2004) *J. Biol. Chem.*, **279,** 23082–23087.
27. O'Toole, R., Smeulders, M. J., Blokpoel, M. C., Kay, E. J., Lougheed, K. & Williams H. D. (2003) *J. Bacteriol.* **185,** 1543–1554.
28. Georgellis, D., Kwon, O. & Lin, E. C. (2001) *Science* **292,** 2314–2316.
29. Voskuil, M. I., Visconti, K. C. & Schoolnik, G. K. (2004) *Tuberculosis* **84,** 218–227.
30. Boshoff, H. I., Myers, T. G., Copp, B. R., McNeil, M. R., Wilson, M. A. & Barry, C. E., III, (2004) *J. Biol. Chem.***279,** 40174–40184.