Steady-state Kinetics and Inhibitory Action of Antitubercular Phenothiazines on Mycobacterium tuberculosis Type-II NADH-Menaquinone Oxidoreductase (NDH-2)*

Takahiro Yano†§, Lin-Sheng, Li†, Edward Weinstein§, Jiah-Shin Teh†, and Harvey Rubin‡§

From the Departments of†§ Medicine and ‡§ Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Type-II NADH-menaquinone oxidoreductase (NDH-2) is an essential respiratory enzyme of the pathogenic bacterium Mycobacterium tuberculosis (Mtb) that plays a pivotal role in its growth. In the present study, we expressed and purified highly active Mtb NDH-2 using a Mycobacterium smegmatis expression system, and the steady-state kinetics and inhibitory actions of phenothiazines were characterized. Purified NDH-2 contains a non-covalently bound flavin adenine dinucleotide cofactor and oxidizes NADH with quinones but does not react with either NADPH or oxygen. Ubiquinone-2 (Q2) and decylubiquinone showed high electron-accepting activity, and the steady-state kinetics and the NDH-2 oxidoreductase reaction were found to operate by a ping-pong reaction mechanism. Phenothiazine analogues, trifluoperazine, Compound 1, and Compound 2 inhibit the NDH-Q2 reductase activity with IC_{50} = 12, 11, and 13 μM, respectively. Trifluoperazine inhibition is non-competitive for NADH, whereas the inhibition kinetics is found to be uncompetitive in terms of Q2.

The Gram-positive bacterium Mycobacterium tuberculosis (Mtb) causes tuberculosis, one of the leading causes of morbidity and mortality in the world. Each year nine million active cases of the disease are diagnosed, accounting for three million deaths. Multidrug-resistant tuberculosis and the existence of “persistent” organisms that are tolerant to antibiotics exacerbate the problem, for which more effective and efficient treatments need to be urgently developed. Mtb is traditionally considered an obligate aerobe, yet during the normal course of events in the infective cycle, the bacillus is able to survive in conditions of low oxygen and nutrient concentrations, such as those postulated to exist within granulomas. Mtb adapts its metabolic activity, cellular transcription, and protein expression accordingly (1). It is therefore of great importance to understand how Mtb generates ATP under a variety of environmental conditions.

Type-II NADH-dehydrogenase (NDH-2) is a critical enzyme in the life cycle of Mtb. The enzyme has been purified from Saccharomyces cerevisiae (2), Escherichia coli (3, 4), Bacillus subtilis (5), Methylococcus capsulatus (6), Corynebacterium glutamicum (7, 8), Acidimans ambivalens (9, 10), and Sulfolobus metallicus (11) and is, in general, composed of a single polypeptide chain, which contains a flavin as a sole cofactor. It is noteworthy that this enzyme is not found in mitochondria. The essential role of NDH-2 in Mtb is supported by extensive evidence from biochemical (12) and transcriptional studies (13), gene deletion analysis, investigation of bacterial growth in various media and under various culture conditions, and animal experiments (12). Mtb contains two copies of ndh genes (ndh and ndhA). The Mtb NDH-2 and NDH-2A share 67% sequence identity, and the genes are separated by 17 kb. Mtb NDH-2 is highly homologous to those of Mycobacterium leprae and Mycobacterium smegmatis with 91 and 81% amino acid sequence identity, respectively. A strain of Mtb in which ndh has been disrupted by transposon mutagenesis is nonviable (14); however, a ndhA deletion mutant of Mtb can be easily isolated (15). We previously demonstrated that purified NDH-2A is a competent oxidoreductase (12). Therefore, we suggest that ndhA, although present in Mtb, is probably not expressed and cannot rescue mutations in Mtb ndh. NDH-2 is likely to be the sole NADH-dehydrogenase enzyme in the Mtb respiratory chain utilized for growth in an aerobic environment.

Isoniazid (INH) and ethambutol are two of the standard anti-tuberculosis medications used throughout the world. Increasing resistance to these medications is recognized as a serious global public health threat. The discovery that a mechanism of INH drug resistance in Mtb is linked to mutations in Mtb NDH-2 that decrease its activity is profoundly important (16–18). Although measured indirectly, INH-resistant mutant NADH oxidase activity is decreased 10–50% compared with the wild-type level. It has been hypothesized that reduced NDH-2 activity in the mutants leads to an increase in the intracellular NADH/NAD+ balance and accounts for the mechanism of INH resistance (16, 18). The present understanding of this effect is that increased concentrations of NDH decrease binding of activated INH adduct to InhA, which is an NADH-dependent enoyl-ACP reductase necessary for mycolic acid synthesis in Mtb.

The anti-mycobacterial activity of phenothiazines has been reported for a number of years (19–24). Trifluoperazine (TPZ) (Fig. 1), for example, reduced in vitro ATP synthesis in M. leprae, suggesting that one of the target sites might be the electron transport pathway itself (25). TPZ is effective against a virulent Mtb strain H37Rv in a macrophage model of infection, and it is synergistic with both INH and rifampicin (26, 27). In our previous investigation (12), we have shown that phenothiazines block NDH-dependent oxygen consumption by Mtb membranes. Furthermore, we have demonstrated that phenothiazines inhibit purified recombinant ndh and ndhA and hinder growth of Mtb both in culture and in a mouse model of tuberculosis.

Although NDH-2 is essential for growth in Mtb and plays an important role in drug resistance, little is known about the catalytic reaction
mechanism of NDH-2 nor is the mode of action of inhibitors of the enzyme well characterized. Therefore, in-depth understanding of the reaction catalyzed by NDH-2 is crucial to predicting and controlling the behavior of the organism. In the present study, the kinetic properties of the enzyme and the mode of action of phenothiazines were investigated in detail using highly active NDH-2 enzyme purified in an *M. smegmatis* expression system.

**EXPERIMENTAL PROCEDURES**

**Bacterial Culture and Conditions**—*E. coli* strains and their plasmid-harboring derivatives were grown in Luria broth medium containing appropriate antibiotics (100 μg/ml ampicillin, 50 μg/ml hygromycin) at 37 °C. *M. smegmatis* and its transconjugants were aerobically grown in 7H9 medium containing 50 μg/ml hygromycin at 37 °C.

**Molecular Cloning of Mtb ndh and ndhA Genes and Construction of Expression Plasmids**—All DNA manipulations were carried out according to standard protocols as described in Ref. 28. Mtb *ndh* was amplified by PCR with genomic DNA from *M. tuberculosis* as a template using the following PCR primers: *ndh* forward primer, 5'-AGCTATGGATCCATGAGTCCCCAGCAAGAACCCACA-3'; *ndh* reverse primer, 5'-AGCTATTCTAGACTAATGATGATGATGATGGCTGGCCACCTTAGCGCTTGC-3'. PCR was carried out by 30 cycles of amplification step gradient (98 °C for 30 s, 54 °C for 30 s, and 72 °C for 2 min). The PCR product of the *ndh* gene was digested by BamHI and XbaI and was inserted under an acetamidase upstream promoter of the pOLYG plasmid (a kind gift from Dr. V. Mizrahi, National Health Laboratory Service). The final construct (*Ndh/polyace*) was verified by restriction enzyme digestion and DNA sequencing.

**Expression of Recombinant Mtb Ndh in M. smegmatis**—The expression plasmid Ndh/polyace was transferred into *M. smegmatis* cells by electroporation. A well isolated colony was picked and inoculated in 2 ml of 7H9 medium containing 50 μg/ml hygromycin. To induce protein expression, 2% (w/v) acetamide was added and cells were grown to stationary phase at 37 °C for 2–3 days. Harvested cells were suspended in Hepes/phosphate buffer (50 mM Hepes/100 mM potassium phosphate, pH 6.8, containing 1 mM phenylmethylsulfonyl fluoride) and stored at −80 °C.

**Purification of Mtb Ndh from M. smegmatis**—The cells were broken by sonication on ice with a Brown Sonifier in a pulse mode with 30% output for 5 min × 3 times. The suspension was centrifuged at 6,000 revolutions/min for 15 min to precipitate unbroken cells and debris. The supernatant was further ultracentrifuged at 45,000 revolutions/min for 30 min. The pellet was resuspended in Hepes/phosphate buffer and ultracentrifuged again. Potassium cholate and potassium deoxycholate was added to the membrane suspensions (~5 mg/ml) at a final concentration of 2% (w/v) for each detergent, and the suspension was sonicated briefly and incubated on ice for 1 h with gentle stirring. The detergent
extract was obtained by ultracentrifugation at 45,000 revolutions/min for 30 min. TALON resin (1 ml bed volume) was added to the detergent extract, and the suspension was incubated at 4 °C for 1 h. The resin was transferred to a small column (1 × 10 cm) and washed with 20 ml of Hepes/phosphate buffer containing 1% (w/v) cholate followed by 40 ml of Hepes/phosphate buffer containing 1% (w/v) cholate and 10 mM imidazole. The bound proteins were eluted with Hepes/phosphate buffer containing 1% (w/v) cholate and 150 mM imidazole, and active fractions were pooled. Glycerol was added to be 40% (w/v), and the enzyme was quick-frozen in liquid nitrogen and stored at −80 °C until use.

Enzyme Assay—The standard reaction buffer contained 50 mM sodium phosphate buffer (pH 7.0) and 150 mM NaCl. For NAD(P)H-Q oxidoreductase assay, 100 μM NAD(P)H and 40 μM Q were used. The reaction was initiated by adding Q and was monitored spectrophotometrically by following the absorbance changes at 340–400 nm (Δε₃₄₀−₄₀₀ nm = 6000 M⁻¹ cm⁻¹) at 30 °C. The specific activities mentioned above were calculated using the respective extinction coefficients. For inhibitor titration, the enzyme was preincubated with various concentrations of inhibitors in the reaction buffer at room temperature for 2–3 min.

Flavin Extraction Analysis—Extraction of flavin was attempted from the purified protein in the presence of 12% (w/v) trichloroacetic acid on ice for 10 min followed by centrifugation for 15 min. The purified enzyme was also subjected to proteinase K digestion in the presence of 1% (w/v) SDS at 65 °C overnight. Trichloroacetic acid was added to be 6% (w/v), and the mixture was incubated on ice for 10 min followed by centrifugation. Fluorescence of the supernatant obtained above was measured with an excitation wavelength at 446 nm and an emission...
The purified NDH-2 had very low specific activities (0.1 unit mg\(^{-1}\)) and were not stable. In our efforts to obtain highly active and stable enzymes, we have expressed Mtb NDH-2 in E. coli and have obtained enzymatically active preparations. The enzymes oxidized NADH with quinone and were inhibited by the ndh and ndhA genes) in a highly homogenous state (Fig. 2A). The purified Mtb NDH-2 was ~50 kDa, as expected from the DNA sequence, and specifically recognized by the anti-His tag antibody (Fig. 2B). The purified NDH-2 exhibited specific activity as high as 20–25 units mg\(^{-1}\), which is >200-fold higher than that of the previous preparations. Approximately 2 mg of a highly pure NDH-2 enzyme was obtained from 15 g (wet weight) of cells.

**Spectroscopic and Enzymatic Properties of the Purified Mtb NDH-2**—The Mtb NDH-2 exhibited several noteworthy features. The absorption spectra of the light-yellow Mtb NDH-2 is shown in Fig. 3A. The enzyme, as purified, exhibited broad absorption peaks at 365 and 450 nm, characteristic of flavin. A slight contamination of heme-like impurities was recognized at 420 nm. This peak height varied among preparations. The protein was weakly fluorescent. With an excitation at 446 nm, a fluorescence emission was detected at 523 nm (data not shown). Upon the addition of 2.2 \(\mu\)M guanidine-HCl, the emission band increased 2.8-fold higher, indicating that the protein was unfolded and the flavin was exposed to solvent. Fig. 3B shows emission and excitation spectra of the purified Mtb NDH-2 in the presence of 2.2 \(\mu\)M guanidine-HCl. The excitation spectrum of the flavin exhibited two peaks at 378 and 448 nm, whereas only one emission band at 530 nm was observed. The bound flavin could be extracted with 12% (w/v) trichloroacetic acid. By fluorescence spectroscopy, we identified the bound flavin to be flavin adenine dinucleotide (FAD). Mtb NDH-2 expressed in *M. smegmatis* rapidly lost its catalytic activity at pH 8.0 and higher. This alkaline-sensitive nature was also observed for the purified Mtb NDH-2 (see next paragraph). The NDH-2 was also highly sensitive to detergents. Among a number of different types of detergents tested, only potassium cholate was able to solubilize Mtb NDH-2 from the membranes without losing enzymatic activity. Mtb NDH-2 lost activity immediately after the addition of Triton X-100 and dodecyl-\(\beta\)-d-maltoside, which have been successfully used for purification of NDH-2 from other organisms, such as *E. coli* (3, 4) and *C. glutamicum* (7, 35). Incubation of Mtb cytoplasmic membranes with these detergents or in alkaline buffer resulted in immediate loss of the enzymatic activity of the native NDH-2. The purified NDH-2 retained its high catalytic activity in the presence of 40% (w/v) glycerol and could be stored at \(-80^\circ\)C for up to one month without any noticeable loss of the enzymatic activity.

**Steady-state Electron Transfer Kinetics of the Purified Mtb NDH-2**—To characterize the enzymatic properties of the purified Mtb NDH-2, we first examined the pH dependence and substrate specificity. Mtb NDH-2 catalyzes NADH-Q oxidoreductase reaction with a pH optimum of 7.0 (Fig. 4). The enzymatic activity sharply dropped at pH 8.0.

**TABLE 1** Apparent \(K_m\) and \(V_{\text{max(app)}}\) values of the NADH-acceptor reductase activities of the purified Mtb NDH-2 and % inhibition by TPZ

| Substrate               | \(K_m(\text{app})\) | \(V_{\text{max(app)}}^a\) | \(V_{\text{max(app)}}/K_m(\text{app})\) | Inhibition by 50 \(\mu\)M TPZ* |
|------------------------|---------------------|-----------------------------|-----------------------------------------|---------------------------------|
| Ubiquinone-2\(^e\)     | 6.4                 | 11.0                        | 2.75                                    | 85                             |
| DBQ\(^e\)              | 4.8                 | 0.95                        | 0.12                                    | 81                             |
| Phylloquinone\(^e\)    | 7.8                 | 12.2                        | ND                                      | ND                             |
| Menaquinone-4\(^a\)    | 12.2                | 3.4                         | ND                                      | 90                             |
| 1,4-Naphthoquinone\(^a\) | ND                  | 5.9                         | ND                                      | 29                             |

\(^{a}\) One unit is defined as 1 \(\mu\)mol of NADH oxidized min\(^{-1}\).

\(^{b}\) Percentage inhibition of the NADH-acceptor reductase activities by 50 \(\mu\)M TPZ was determined using 100 \(\mu\)M NADH and 40 \(\mu\)M quinone analogues.

\(^{c}\) ND, not determined.

\(^{d}\) Specific activities were given for these electron acceptors. Activities were measured using 100 \(\mu\)M NADH and 40 \(\mu\)M quinone analogues.
and higher due to irreversible inactivation of the enzyme. We also examined NADPH as an electron donor; however, Mtb NDH-2 did not show any detectable NADPH-Q oxidoreductase activity within a pH range of 4.0–9.0. In addition, we did not detect electron transfer activity to oxygen (NAD(P)H oxidase activity), unlike NDH-2 from S. cerevisiae and C. glutamicum, as reported earlier (35).

We measured the electron transfer activities using various quinone analogues. Among the ubiquinone analogues tested, ubiquinone-2 was found to show the highest activity (Table 1). Reduction of Q2 and n-decylubiquinone was inhibited by 50 μM TPZ to 85 and 76%, respectively. NADH-Q2 reductase activity was inhibited to 95% by 100 μM TPZ.

Because the NADH oxidase activity of the NDH-2-dominant Mtb respiratory chain is completely blocked by TPZ, as demonstrated earlier (12), the TPZ-sensitive electron transfer activities of the purified NDH-2 with Q2 and n-decylubiquinone seem to reflect the physiological electron transfer reactions. On the other hand, the NADH-Q1 reductase activity was only partially sensitive to TPZ and could not be fully inhibited by high concentrations of TPZ. Menaquinone analogues, phylloquinone (vitamin K1) and menaquinone-4 (vitamin K2), showed lower electron transfer activities compared with those for ubiquinone analogues. The \( K_m \) value for phylloquinone is 7.8 μM. The electron transfer activities with these menaquinone analogues are also sensitive to TPZ. Water-soluble naphthoquinone analogues, 1,4-naphthoquinone (α-naphthoquinone) and 2-methyl-1,4-naphthoquinone (menadione), were also reduced by Mtb NDH-2 with moderate specific activities 3.4 units/mg and 5.9 units/mg, respectively. These electron transfer activities were not fully sensitive to TPZ, indicating that reduction of these compounds may not reflect the physiological electron transfer reactions.

Electron Transfer by Mtb NDH-2 Follows a Ping-Pong Tetra Uni Reaction Mechanism—Next, we investigated the kinetic mode of the electron transfer reactions of Mtb NDH-2 with Q2 as an electron acceptor. Fig. 5 shows a Hanes-Woolf plot of the NADH-Q2 oxidoreductase activity of the purified Mtb NDH-2. Q2 reductase activities at various Q2 concentrations were plotted in a Hanes-Woolf plot ([Q2] versus \( [Q2]/v \)) and a Lineweaver-Burk plot (1/[Q2] versus 1/v, inset). NADH concentrations were varied: 10 μM (closed square), 20 μM (open triangle), 30 μM (closed triangle), 50 μM (open circle), and 100 μM (closed circle).

![FIGURE 5. Hanes plots of the steady-state kinetics of the NADH-Q2 oxidoreductase activity of the purified Mtb NDH-2. Q2 reductase activities at various Q2 concentrations were plotted in a Hanes-Woolf plot ([Q2] versus [Q2]/v) and a Lineweaver-Burk plot (1/[Q2] versus 1/v, inset). NADH concentrations were varied: 10 μM (closed square), 20 μM (open triangle), 30 μM (closed triangle), 50 μM (open circle), and 100 μM (closed circle).](image-url)
Inhibition Kinetics of Phenothiazine, TPZ on Mtb NDH-2—Previously (12), we have demonstrated that phenothiazines effectively suppress the growth of Mtb in vitro as well as in a mouse model and have provided biochemical evidence that the drugs inhibit the electron transfer activity of the Mtb NDH-2 in membranes as well as the purified recombinant enzymes. To better understand the mode of action of phenothiazines on Mtb NDH-2, we examined the effects of phenothiazines on the steady-state kinetics of the highly active purified enzyme. Fig. 6 shows the titration of the NADH-Q2 oxidoreductase activity with several phenothiazine analogues. TPZ, Compound 1, and Compound 2 effectively inhibit NADH-Q2 oxidoreductase activity with IC$_{50}$ values of 12, 11, and 13 μM, respectively, indicating nearly equal potency under the conditions used. The compounds inhibited the oxidation of NADH with Q2 almost completely (>95%) at 80 μM and higher, suggesting that reduction of Q2 occurs at the physiological Q binding site as observed in situ. An inhibitor, flavone, on the other hand, poorly inhibited the NADH-Q2 oxidoreductase activity of Mtb NDH-2 with IC$_{50}$ = 170 μM, and the activity could not be completely inhibited by higher concentrations of flavone (data not shown). Fig. 7A shows a double reciprocal plot of TPZ inhibition kinetics of the NADH-Q2 oxidoreductase activity in terms of NADH. The lines diverge and intersect on the 1/[NADH] axis, suggesting that the inhibition kinetics is non-competitive in terms of NADH. The inhibition kinetics with respect to Q2 revealed a different inhibition pattern. In a double reciprocal plot shown in Fig. 7B, the slopes of the lines are nearly constant at different concentrations of TPZ, suggesting that the inhibition kinetics follows an uncompetitive mode.

DISCUSSION

In this study, we successfully obtained highly active and stable recombinant enzyme by using M. smegmatis as a host. Characterization of the Mtb NDH-2 revealed several intriguing properties. One of the findings is that the Mtb NDH-2 contains a non-covalently bound FAD as a cofactor. NDH-2 has been classified into three distinct groups (groups A, B, and C) depending on characteristic structural signatures in the primary sequence (36). Members of group A have two nucleotide binding motifs where NAD(P)H and flavin are bound. Group A NDH-2 enzymes, such as those from E. coli and C. glutamicum, contain a non-covalently bound FAD. The primary sequence of Mtb NDH-2 indicates that this enzyme is categorized as group A. The biochemical properties of the Mtb NDH-2 revealed in the present study are in good agreement with those predicted for group A.

The specific activity of the Mtb NDH-2 expressed in M. smegmatis is 200-fold greater than the previous preparations that were expressed in E. coli. The expression and purification of the highly active enzyme permitted us to characterize the enzymatic properties of Mtb NDH-2, observing a number of unique properties of the enzyme. First, Mtb NDH-2 exclusively catalyzes the oxidation of NADH with quinones and does not use either NADPH or oxygen as substrates under any conditions examined in this study. NDH-2 homologues from E. coli (37),
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C. glutamicum (35), and Trypanosoma brucei (38) have been reported to reduce oxygen by a single electron to produce harmful reactive oxygen species. Although it remains unknown whether reactive oxygen species produced by other NDH-2s play any physiological role, electron transfer without electron leak to oxygen, as seen in Mtb NDH-2, ensures efficient respiratory activity and reduces exogenous oxidative stress. Second, the purified Mtb NDH-2 is able to reduce various ubiquinone analogues, and thus far, Q2 shows the highest catalytic activity. The electron transfer activity is fully sensitive to TPZ inhibition. On the other hand, the reductase activity with less hydrophobic Q1 is only partially inhibited by TPZ. These results suggest that the Mtb NDH-2 interacts with these quinones differently. It is implied that the hydrophobic alkyl chain at the 3 position plays an important role in the interactions of quinone with the physiological binding site of the NDH-2. The Mtb NDH-2 can reduce menaquinone analogues, phyloquinone and menaquinone-4, in a TPZ-sensitive manner; however, the specific activities are much lower than those with ubiquinones. Because Mtb contains menaquinine-9 in the Q pool (39), the low catalytic activities of the Mtb NDH-2 with menaquinones are rather unexpected. A low midpoint redox potential of menaquinone (Em = −100 mV versus Em for ubiquinone = +90 mV) partially contributes to the slower electron transfer rates. It should be noted that Mtb NDH-2 in Mtb or M. smegmatis membranes also show much lower electron transfer activities with menaquinone than ubiquinone. Thus, it is possible that the low catalytic activity with menaquinone is intrinsic to the nature of Mtb NDH-2.

The present steady-state kinetics study revealed that the NADH-Q2 oxidoreductase reaction of the purified NDH-2 follows a ping-pong reaction mechanism, where NDH-2 reacts with NADH and Q in two separate reaction steps without forming a ternary complex. The mechanism predicts that following reduction of FAD with NADH to FADH2 and release of NAD+, Q binds to its binding site and accepts electrons from an isoalloxazine ring of flavin. The ping-pong reaction mechanism has been proposed for NDH-2 homologues from fungal mitochondria (40, 41). Without structural support and further analysis, the current kinetic data cannot provide any information about the mechanism of the interaction of Mtb NDH-2 with the two substrates separately. It is suggested that NADH in the cytoplasm and menaquinone-9 in the membrane approach the isoalloxazine ring of FAD through distinct pathways to carry out the respective electron transfer reactions. NADH binds to the NDH binding site containing the GXXGXXG motif (Mtb numbering). Although secondary structure analytical programs could not find any transmembrane α-helices in the Mtb NDH-2, several programs (DAS, HMMTOP, SOSUI, and THM) predict one or possibly two α-helices near the C terminus (between Ala185 and Ile410) that may form an amphipathic α-helix rich in positively charged amino acid residues (His, Arg, and Lys) and hydrophobic amino acid residues (Tyr, Trp, etc.). The present study suggests that the hydrophobic isoprenoid group plays an important role in preferential binding to the site mentioned above. Therefore, it is tempting to speculate that the membrane interface domain provides an amphipathic structure that guides a hydrophilic menaquinone ring part of menaquinone-9 to the isoalloxazine ring of flavin from the lipid layer. Structural information describing the catalytic sites is essential for better understanding of the reaction mechanism of the NDH-2. The successful expression and purification of the highly active NDH-2 demonstrated in this study opens a way to characterize structural details of Mtb NDH-2 by utilizing site-directed mutagenesis and several biochemical approaches. Such attempts are currently being made in our laboratory to understand the kinetic and structural mechanism of Mtb NDH-2.

All three phenothiazine compounds examined in the present study inhibit the Mtb NDH-2 activity with the same potency of IC50 = 10 μM, despite the fact that chemical structures of these compounds vary as shown in Fig. 1. Phenothiazines are composed of two major structural units in general, a large heterocyclic 3-ring and a side chain attached to the N terminus of the phenothiazine ring. These unique structures do not resemble either NADH or quinone. Therefore, it is highly unlikely that phenothiazines bind to the NDH or Q binding site and competitively inhibit the enzymatic reactions. TPZ appears to combine with and inhibit an intermediate species formed during catalysis, such as NDH-(FADH2)-NAD+ complex or NDH(FADH2). It is implied that the binding of TPZ analogues interfere with release of products or binding of substrate. The present study suggests that a structure common among these three compounds, indicated by the broken line in Fig. 1, is essential for their inhibitory activities. In conjunction with the in vivo studies, our inhibition study with Mtb NDH-2 provides valuable information on the structure-activity relationships of phenothiazines. Such information may be useful for development of more potent anti-tubercular drugs in the future.

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