Inhibition of *Mycobacterium tuberculosis* AhpD, an Element of the Peroxiredoxin Defense against Oxidative Stress

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The resistance of *Mycobacterium tuberculosis* to isoniazid (INH) is largely linked to suppression of a catalase-peroxidase enzyme (KatG) that activates INH. In the absence of KatG, antioxidant protection is provided by enhanced expression of the peroxiredoxin AhpC, which is itself reduced by AhpD, a protein with low alkylhydroperoxidase activity of its own. Inhibition of AhpD might therefore impair the antioxidant protection afforded by AhpC and make KatG-negative strains more sensitive to oxidative stress. We report here that the 3(E),17-dioxime of testosterone is a potent competitive AhpD inhibitor, with a K_i of 50 ± 2 nM. The inhibitor is stereospecific, in that the 3(E) but not 3(Z) isomer is active. Computational studies provide support for a proposed AhpD substrate binding site. However, the inhibitor does not completely suppress the in vitro activity of AhpC/AhpD, because a low titer of AhpD suffices to maintain AhpC activity. This finding, and the low solubility of the inhibitor, explains its inability to suppress the growth of INH-resistant *M. tuberculosis* in infected mouse lungs.

It is estimated that approximately 2 billion people are infected with *Mycobacterium tuberculosis* (14). The symbiosis between human immunodeficiency virus and tuberculosis, together with the appearance of multidrug-resistant strains, has contributed greatly to the resurgence of the disease (18). The renewed threat of tuberculosis and determination of the *M. tuberculosis* genome (5) have led to renewed efforts to find agents that are active against drug-resistant strains, completely sterilize the infection, and/or shorten the duration of drug therapy.

The resistance to isoniazid (INH) largely results from mutations in the *M. tuberculosis* catalase-peroxidase KatG that diminish its ability to convert INH to its active form (13, 21, 24, 26, 28, 29). A decrease in the antioxidant protection provided by KatG is potentially deleterious, as the organism resides in the highly oxidative environment of host macrophages. AhpC, a peroxiredoxin that is elevated in KatG-negative strains (4, 6, 22, 23, 25, 27, 28), reduces peroxides and peroxynitrite and may help to compensate for the decreased KatG activity in INH-resistant strains (1, 16). The peroxiredoxins employ cysteine sulfhydryl groups to remove peroxides from the environment. The AhpC from *M. tuberculosis* has three cysteines, Cys61, Cys174, and Cys176 (11). One of these cysteines reacts with the peroxide to give a sulfenic acid (-SOH) (12, 20), which is then converted to a disulfide by intramolecular or intermolecular reaction with a second sulfhydryl group. In the final step, the disulfide bond is reduced by an external thiol.

The AhpC disulfide bond is reduced by different mechanisms in different organisms. In yeast, the reduction is medi-

ated by thioredoxin and thioredoxin reductase (3), in *Salmonella enterica* serovar Typhimurium it is by the flavoprotein AphF (7), and in *M. tuberculosis* it is apparently by a system consisting of AhpD, dihydrolipoamide succinyltransferase (SucB), and dihydrolipoamide dehydrogenase (2). SucB can be replaced in vitro by dihydrolipoamide. The expression of AhpD, which has no sequence homology to AhpC, AhpF, or other previously characterized proteins, is controlled by the same promoter as AhpC (22). AhpD has limited alkylhydroperoxidase activity of its own in the presence of surrogate electron donors such as the *S. enterica* serovar Typhimurium AhpF (11). The alkylhydroperoxidase activity of AhpC can also be supported by a surrogate electron donor, in which case AhpD is not required for activity (11).

AhpD is a homotrimer of a 177-amino-acid protein ($M_r = 18,781$ Da) containing two cysteines, Cys130 and Cys133, in a Cys-His-Ser-Cys motif within a novel protein fold (2, 17). Both cysteines are required for enzymatic activity (2, 11, 15). A shallow hydrophobic groove in each subunit in the crystal structure that extends from the outer solvent-exposed edge to the Cys residues may be the substrate-binding site of the protein, whether it be AhpC or a peroxide (17). Among the best substrates for AhpD in the reaction supported by AhpF are cholesterol 7-hydroperoxide ($K_m = 132$ μ M) and cumene hydroperoxide ($K_m = 50$ μ M) (11).

To inhibit AhpD, and therefore the AhpC/AhpD system, a search has been carried out for potential inhibitors in which the hydroperoxide (—OOH) group in structures related to the known substrates was replaced by the isosteric oxime (=NOH) moiety. These studies have yielded a potent in vitro inhibitor of AhpD that has been used to explore the potential of AhpD as a target for antituberculosis drug development.

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MATERIALS AND METHODS

Materials. Lipoamide (*D,L*-6,8-thioctic acid amide), INH, cyclodextran, and bovine intestinal mucosal lipoamide dehydrogenase (EC 1.8.1.4; 100 U/mg of protein) were purchased from Sigma (St. Louis, Mo.). *Escherichia coli* strain BL21(DE3) was from Novagen, and strain DH5 α was from Life Technologies (Gaithersburg, Md.). Q-Sepharose Fast Flow was purchased from Amersham Biosciences (Peapack, N.J.), and polyethyleneimine (10% solution) was from Research Biotechnologies, Inc. (Natick, Mass.). Luria-Bertani medium was obtained from Life Technologies. Sterol ketones and compound 10 were from Steraloids (Newport, R.I.). Purified proteins were concentrated using Millipore YM10-regenerated cellulose ultrafiltration membranes. Isopropyl β -D-thiogalactopyranoside was from Promega (Madison, Wis.). Trityl chloride resin was purchased from Novabiochem Company. *S. enterica* serovar Typhimurium AhpF was provided by Patrick Hillas (11). AhpC and AhpD and their mutants were expressed and purified as previously reported (11). A Hewlett-Packard HP-8452 UV-visible spectrophotometer was used for all electronic absorption measurements.

AhpF-dependent activity assays. The rates of hydroperoxide reduction were determined anaerobically in a coupled assay with AhpF by monitoring the decrease in absorbance at 340 nm due to NADH oxidation (11). The assay mixtures typically contained 2 mM hydroperoxide substrate in 100 mM potassium phosphate buffer (K_Pi), pH 7.0, with 1 mM EDTA, 0.25 mM NADH, 20 μ M AhpD or mutant, and 10 μ M AhpF. The background NADH oxidation caused by AhpF alone was monitored before the hydroperoxide substrate was added, and the rate was measured.

AhpD-dependent AhpC activity assays. The rates of NADH oxidation catalyzed by AhpC in the presence AhpD, lipoamide, and bovine lipoamide dehydrogenase were measured by monitoring the change in absorbance at 340 nm (2). Typical conditions for the assays were 50 mM K_Pi (pH 7.0), 1 mM EDTA, 200 μ M NADH, 2.5 μ M AhpC, 2.5 μ M AhpD, 0.2 U of bovine dihydrolipoamide dehydrogenase, and 50 μ M lipoamide. For steady-state kinetics assays, the substrate concentration was varied and data were fit to the equation $v = V_{\max} [S] / (K_m + [S])$.

Bacteria. *M. tuberculosis* H37Rv (ATCC 27294) was used as the wild-type strain. Bacteria were grown in vitro in Middlebrook 7H9 broth (Difco) containing albumin (bovine, fraction V)-dextrose-catalase (ADC) and Tween 80 (0.05%, vol/vol) using a rotary shaker (150 rpm) at 37°C. The bacteria were harvested at the mid-logarithmic growth phase (optical density at 650 nm, 0.5 to 1.0) and frozen at -70°C before being used in experimental infections of mice.

Mice. C57BL/6 mice were used as the infection model. Five- to 8-week-old female mice were purchased from Taconic (Germantown, N.Y.). Experimental mice were housed in rigid isolator filter system racks both before and after their infection with *M. tuberculosis* strains. Mice were used according to guidelines set forth by National Institutes of Health protocol LIG-2E as approved by the National Institute of Allergy and Infectious Diseases Animal Care and Usage committee.

Infection of mice. Frozen stocks of mycobacterial strains were thawed and diluted into sterile phosphate-buffered saline containing 0.05% Tween 80 to obtain a final concentration of 10⁷ CFU ml⁻¹. This inoculation mixture was delivered to mice using a nose-only aerosol generator (CH Technologies) that is capable of infecting 24 mice simultaneously. Briefly, a bioaerosol-nebulizing aerosol generator apparatus generated a controlled mycobacterial aerosol that delivered 50 to 200 infectious particles to the mice over a 10-min exposure time. Four mice from the infection group were sacrificed 1 day after aerosol delivery to ensure equivalent inocula. Each time point consisted of four mice.

CFU assay. Bacterial loads in infected lung tissue as well as in vitro cultures were measured by performing serial, 10-fold dilutions of either organ homogenates or broth cultures and plating these in duplicate onto Middlebrook 7H11 agar (Difco) plates supplemented with oleic acid and ADC. Plates were incubated at 37°C for 4 weeks before CFU were determined. Plates were rechecked after 6 weeks of incubation to ensure the accuracy of CFU counts.

Inhibitor delivery. After the infection was allowed to proceed for 13 weeks, mice were randomly divided into four groups and administered the study drug(s) for 14 days. Group 1 received no inhibitor. Group 2 received AhpD inhibitor 4 at a once-daily dose of 50 mg/kg of body weight, dissolved in 100 μ l of cyclodextran. Group 3 received INH at a once-daily dose of 25 mg/kg by gavage in 100 μ l of 0.05% agar solution. Group 4 received AhpD inhibitor 4 subcutaneously at a once-daily dose of 50 mg/kg combined with INH (25 mg/kg). All mice under study were sacrificed 15 weeks after infection (2 weeks of therapy), and the CFU were determined.

MIC determinations. INH, AhpD inhibitor 4, or the two in combination were added to 96-well plates and serially diluted in Middlebrook 7H9 broth (Difco)

containing ADC and Tween 80 (0.05%, vol/vol). H37Rv, isolate 202 (an INH-resistant clinical isolate), and a *katG* mutant strain were passed twice from frozen stock to early log phase and then added to the wells at a final concentration of 10⁴ CFU/ml. The plates were incubated for 3 weeks and then examined for growth. The MIC was defined as the lowest inhibitor concentration at which no growth was observed.

Computations. The AMBER and DOCK 4.0 programs were used to explore the interaction between the sterol ligand and AhpD (8, 19). The AhpD crystal structure was first minimized in vacuum using the SANDER module of the AMBER program. The minimization was done using 250 steps of steepest descent followed by 250 steps of Newton-Raphson cycles. Rigid docking was then pursued, using DOCK 4.0 to find the possible binding site of the ligand to AhpD. The ligand structure was constructed by replacing the two carbonyl groups with oxime (C=N—OH) moieties in the crystal structure of androstenedione. There are several conformations of the ligand due to rotation around the C-N bond. Both rigid and flexible conformations of the ligand were docked to the protein. The DOCK program scored each orientation of the ligand using an interaction force field, which included electrostatic and Van der Waal (VDW) terms.

Rigid docking, in which the internal geometry of the ligand remains fixed, did not give a structure with significant interaction between the protein and the ligand. A flexible docking calculation was therefore performed in which different rotamers of the ligand were considered in the docking efforts. Two different VDW repulsion parameters were used in the calculation, one with a 1/*r*¹² term and the other with a softer 1/*r*⁹ term, to permit slightly closer contacts to be explored. In both the cases the attractive part of the VDW term was -1/*r*⁶.

Procedure for the preparation of sterol oximes. The ketone or aldehyde, hydroxylamine hydrochloride (1.2 eq) and NaO-acetate (1.5 eq) in 95% ethanol was stirred at 80°C for 20 h or at reflux overnight. The reaction was monitored by thin-layer chromatography, using *p*-anisaldehyde for visualization. The mixture was then cooled to 25°C, saturated aqueous Na₂CO₃ was added, the resulting solution was extracted with CH₂Cl₂, and the extract was concentrated. The residue was purified by column chromatography (silica gel; ethyl acetate-hexane at 1:2 or CH₂Cl₂-methanol at 20:1). A white solid was generally obtained with a ~95% yield. The structures of all the compounds were consistent with their ¹H-nuclear magnetic resonance (NMR), ¹³C-NMR, and high-resolution mass spectra.

RESULTS

Oximes as AhpD inhibitors. Ten steroid oximes (Fig. 1) and 8 terpene-, phenyl-, or naphthyl-oximes were either purchased or synthesized from the corresponding ketones or aldehydes by condensation with hydroxylamine. The compounds were selected because of their similarities to cholesterol 7-hydroperoxide, cumene hydroperoxide, or monoterpene hydroperoxides, all of which are directly reduced by AhpD (11). In each potential inhibitor, the hydroperoxide of the substrate was replaced by the isosteric oxime (=NOH). The nonsteroidal compounds, all of which were inactive, were the oximes of camphor, acetophenone, benzophenone, 2-phenylpropional, 4,6,6-trimethylbicyclo(3.1.1)hept-3-en-2-one, 6,6-dimethylbicyclo(3.1.1)-hepta-2-carboxaldehyde, and 6,6-dimethylbicyclo(3.1.1)hept-2-ene-2-carboxaldehyde.

The effects of the compounds on the reduction of H₂O₂ were evaluated in two systems: (i) the reconstituted system, consisting of AhpC, AhpD, lipoamide, bovine dihydrolipoamide dehydrogenase, and NADH (Fig. 2) (2, 15), and (ii) a system consisting of AhpD and the *S. enterica* serovar Typhimurium AhpF, which measures the ability of AhpD to directly reduce peroxide substrates (Fig. 2) (11). Compound 4, with a *K_i* of 50 $\pm$ 2 nM (mean $\pm$ standard deviation) (see below), was the best inhibitor. However, even at high nominal concentrations, compound 4 did not fully suppress the catalytic activity in the reconstituted AhpC-dependent system. In contrast, compound 4 completely blocked the direct alkylhydroperoxidase activity of AhpD in the AhpF-dependent assay (Fig. 2). These agents

FIG. 1. Structures of steroid oximes evaluated as AhpD inhibitors.

acted through AhpD, as they did not directly inhibit either AhpC or AhpF.

Inhibition by compound 4. In both activity assays, inhibition of AhpD by compound 4 was dose dependent and exhibited a K_i of ~ 70 nM. However, synthetic 4 was a 72:28 mixture of the 3(*E*) and 3(*Z*) stereoisomers that could be separated by column chromatography and whose stereochemistry was assigned by NMR (10). Only the 3(*E*) isomer inhibited AhpD, whereas the 3(*Z*) isomer was completely inactive (Fig. 3). As the K_i was

determined with the synthetic isomer mixture, the true K_i value for the 3(*E*) isomer is therefore 50 ± 2 nM. As shown by Lineweaver-Burk plots, compound 4 is a competitive inhibitor (Fig. 4).

To test the reversibility of the inhibition of AhpD by compound 4, charcoal was used to extract the inhibitor from the solution. Treatment of a solution of AhpD and compound 4 (isomeric mixture) with charcoal restored the activity of the system to the level obtained when AhpD alone was treated

FIG. 2. Effects of steroidal oximes on the AhpC peroxidase activity supported by AhpD (filled column) and by AhpF (open column); 100% activity corresponds to the activity in the absence of steroidal oximes. Each bar represents the average of three independent determinations. For activity supported by AhpD, incubation mixtures contained 2 mM inhibitor, 0.5 mM H_2O_2 , 200 μ M NADH, 1 mM EDTA, 2.5 μ M AhpD, 2.5 μ M AhpC, 0.2 U of bovine dihydrolipoamide dehydrogenase, and 50 μ M lipoamide in 50 mM KP_i (pH 7.0), and reactions were carried out at 4°C to facilitate activity measurements. For activity supported by AhpF, incubation mixtures contained 2 mM inhibitor, 2 mM cumene hydroperoxide, 250 μ M NADH, 1 mM EDTA, 20 μ M AhpD, and 10 μ M AhpF in 100 mM KP_i (pH 7.0), and reactions were carried out at 25°C.

with charcoal. Addition of compound 4 again reduced the activity (data not shown). The conclusion that inhibition of AhpD by compound 4 is reversible was confirmed by the finding that the mass spectrum of AhpD after incubation with 4

exhibited the same molecular ion as the untreated protein. No peaks corresponding to adducts of AhpD and 4 were observed.

The aqueous solubility of compound 4 is low, as its spectroscopically determined concentration in the buffer decreases

FIG. 3. Differential inhibitory activities of the *E* and *Z* isomers of compound 4 on the peroxidase activity of AhpC supported by AhpD. The incubation mixtures contained 0.5 mM H_2O_2 , 200 μ M NADH, 1 mM EDTA, 2.5 μ M AhpD, 2.5 μ M AhpC, 0.2 U of bovine dihydrolipoamide dehydrogenase, and 50 μ M lipoamide in 50 mM KP_i (pH 7.0). Incubations were carried out at 4°C.

FIG. 4. (A) Lineweaver-Burk double-reciprocal plot of the inhibition by compound 4 of the AhpD-dependent peroxidase activity (V) of AhpC. The cumene peroxide concentration ranged from 50 to 150 μM ($[S]$). The concentrations of compound 4 were 0.015, 0.035, 0.07, and 0.125 μM . (B) Lineweaver-Burk double-reciprocal plot for the inhibitory effect of compound 4 on the AhpF-dependent peroxidase activity (V) of AhpD at concentrations of cumene peroxide ranging from 40 to 125 μM ($[S]$). The concentrations of compound 4 were 0, 0.05, 0.14, 0.28, 0.5, and 0.7 μM . The data in both plots are representative of at least three similar experiments.

with time. Consequently, the inhibition by 4 in both activity assays decreased with time, and after 1 h the compound was inactive. The use of polyethylene glycol 300-4000 as a surfactant did not detectably improve the inhibitory properties of compound 4.

A very low ratio of AhpD was required to support the catalytic activity of AhpC, as 20% of the maximal activity of AhpC was preserved by 0.004 eq of AhpD (Fig. 5). This explains the residual activity of AhpC/AhpD at high concentrations of compound 4. Clearly, the ability of AhpD to provide the reducing equivalents required for turnover is not the rate-limiting step in the reaction.

Docking of compound 4 to AhpD. Rigid docking of 4 to the proposed AhpD substrate-binding groove was examined after computational relaxation of the AhpD crystal structure (15, 17). The ligand for these studies was constructed by replacing the carbonyl groups in the crystal structure of androstenedione with oximes. Rigid docking of this structure to AhpD gave complexes in which only one oxime group interacted with the protein, while the other pointed away from the protein surface. Similar results were obtained when one allowed a flexible conformation of the sterol but applied a standard but fairly rigid repulsion parameter. On the other hand, when flexible conformations of the sterol oxime were docked employing a softer

FIG. 5. AhpD-dependent peroxidase activity of AhpC. (a) $V_{\max}$ values at various ratios of AhpD to AhpC; (b) semilogarithmic dependence of $V_{\max}$ on the ratio of AhpD to AhpC. Conditions were as follows: 2 mM cumene peroxide, 200 μM NADH, 1 mM EDTA, 2.5 μM AhpC, 0.2 U of bovine dihydrolipoamide dehydrogenase, and 50 μM lipoamide in 50 mM K_2P_i (pH 7.0) at 4°C.

TABLE 1. MICs for cultures of *M. tuberculosis* strain R37Rv, clinical isolate 202, and a mutant *katG* strain treated with INH alone, compound 4 alone, or the two in combination

Compound	Isolate	MIC (mg/ml)	Synergy with inhibitor 4
INH	R37Rv	0.05–0.1	No change in MIC
	Strain 202	12.5	No change in MIC
	<i>katG</i> mutant	No inhibition	No inhibition
Inhibitor 4	H37Rv	No inhibition	
	Strain 202	No inhibition	
	<i>katG</i> mutant	No inhibition	

VDW repulsion term, orientations were observed in which both oxime functions interacted with the protein. The interactions between the ligand and the protein are anchored by two hydrogen bonds: in one, the distance between an oxygen of Glu118 and the proximal N-OH hydrogen is 2.64 Å; in the other, the distance between the sulfur of Cys133 and the other N-OH hydrogen is 2.24 Å. The use of a softer potential allowed the ligand to come closer to AhpD and to form two hydrogen bonds.

In vivo assay of inhibitor 4. The MIC evaluation revealed no synergy (Table 1) when compound 4 was combined with INH. The MIC of INH for the H37Rv strain of *M. tuberculosis* was 0.05 to 0.1 µg/ml, with a significantly higher MIC for INH-resistant isolate 202. There was no change in the sensitivity to INH of a *katG* mutant when the AhpD inhibitor was added to the MIC plates.

In vivo, administration of compound 4 with INH did not enhance the eradication of tuberculosis in a murine model. After 14 days of daily INH therapy in chronically infected mice, the log₁₀ CFU per ml of lung tissue was 4.83 (± 0.18), compared with 5.63 (± 0.10) in the untreated group and a statistically indistinguishable value for treatment with inhibitor 4 alone. There was no statistically significant enhancement of INH activity with the additional provision of subcutaneous compound 4, which resulted in a log₁₀ CFU/ml of 4.64 in the fourth group. Similarly, reduction of the bacillary burden in the spleen was not significantly enhanced by the addition of compound 4 (data not shown).

DISCUSSION

Replacement of the hydroperoxide by an oxime in structures similar to those of AhpD substrates led to identification of compound 4 as a potent inhibitor of AhpD (Fig. 2). The specific structure of compound 4 is important for its inhibitory activity, as closely related sterol oximes, including monooximes related to inhibitor 4, exhibited lower activities. Most impressively, the 3(*E*) isomer of 4 inhibited AhpD (*K_i* = 50 nM) in both activity assays, whereas the 3(*Z*) isomer was inactive (Fig. 3).

Compound 4 completely inhibited the alkylhydroperoxidase activity of AhpD supported by the surrogate electron donor AhpF, but a residual 20% of the activity was observed even at high nominal concentrations of compound 4 in the reaction supported by AhpC, AhpD, lipoamide, and lipoamide dehydrogenase. This limitation was partially due to the low aqueous solubility of 4 and its propensity to aggregate, as evidenced by

the finding that the degree of inhibition decreased to zero 1 h after compound 4 was added to the buffer. However, a major factor in preventing complete inhibition is the very low ratio of AhpD to AhpC required to maintain substantial AhpC activity. Thus, although compound 4 completely inhibits the direct alkylhydroperoxidase activity of AhpD supported by AhpF, it does not completely block the AhpD-dependent AhpC activity. Both activities appear to involve binding of compound 4 to the same site, as both have similar *K_i* values and exhibit a similar dependence on inhibitor structure.

Compound 4 binds to AhpD reversibly with competitive kinetics (Fig. 4) rather than through the formation of a covalent bond. Computational docking experiments using a soft VDW repulsion potential suggest that compound 4 binds to the proposed substrate binding groove of AhpD in an orientation that allows hydrogen bonds to be formed between both oxime groups and the protein surface (15, 17). This involvement of both oximes is consistent with the inhibitor data, which show that elimination of either oxime weakens the interaction. These studies provide evidence that the substrate for binding both alkylhydroperoxides and oxidized AhpC has been correctly identified.

Standard short-course chemotherapy in adults for drug-sensitive pulmonary tuberculosis typically requires a four-drug regimen for the first 2 months (e.g., INH, rifampin, pyrazinamide, and ethambutol) followed by two-drug therapy (e.g., INH and rifampin) for 4 months (9). One reason for these 6 months of therapy is that a metabolically distinct population of bacilli exists that, by their very persistent nature, makes them difficult to eradicate. Recent work has demonstrated that a chronic murine model of tuberculosis mirrors this posit, exhibiting decreasing INH sensitivity over time. We have demonstrated here that the muted efficacy of INH in a chronic aerogenic murine tuberculosis model is not reversed after 14 days of therapy with the AhpD inhibitor compound 4.

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