Original Contribution

*Mycobacterium tuberculosis* has diminished capacity to counteract redox stress induced by elevated levels of endogenous superoxide

Priyanka Tyagi\textsuperscript{a,b,1}, Allimuthu T. Dharmaraja\textsuperscript{c,1}, Ashima Bhaskar\textsuperscript{a}, Harinath Chakrapani\textsuperscript{c,*}, Amit Singh\textsuperscript{a,*}

\textsuperscript{a} Department of Microbiology and Cell Biology, Centre for Infectious Disease Research (CIDR), Indian Institute of Science, Bangalore-12, India
\textsuperscript{b} International Centre for Genetic Engineering and Biotechnology, New Delhi 67, India
\textsuperscript{c} Department of Chemistry, Indian Institute of Science Education and Research, Pune 08, India

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**A B S T R A C T**

*Mycobacterium tuberculosis* (Mt) has evolved protective and detoxification mechanisms to maintain cytoplasmic redox balance in response to exogenous oxidative stress encountered inside host phagocytes. In contrast, little is known about the dynamic response of this pathogen to endogenous oxidative stress generated within Mt. Using a noninvasive and specific biosensor of cytoplasmic redox state of Mt, we for the first time discovered a surprisingly high sensitivity of this pathogen to perturbation in redox homeostasis induced by elevated endogenous reactive oxygen species (ROS). We synthesized a series of hydroquinone-based small molecule ROS generators and found that ATD-3169 permeated mycobacteria to reliably enhance endogenous ROS including superoxide radicals. When Mt strains including multidrug-resistant (MDR) and extensively drug-resistant (XDR) patient isolates were exposed to this compound, a dose-dependent, long-lasting, and irreversible oxidative shift in intramycobacterial redox potential was detected. Dynamic redox potential measurements revealed that Mt had diminished capacity to restore cytoplasmic redox balance in comparison with *Mycobacterium smegmatis* (Msm), a fast growing nonpathogenic mycobacterial species. Accordingly, Mt strains were extremely susceptible to inhibition by ATD-3169 but not Msm, suggesting a functional linkage between dynamic redox changes and survival. Microarray analysis showed major realignment of pathways involved in redox homeostasis, central metabolism, DNA repair, and cell wall lipid biosynthesis in response to ATD-3169, all consistent with enhanced endogenous ROS contributing to lethality induced by this compound. This work provides empirical evidence that the cytoplasmic redox poise of Mt is uniquely sensitive to manipulation in steady-state endogenous ROS levels, thus revealing the importance of targeting intramycobacterial redox metabolism for controlling TB infection.

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**Introduction**

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) produced by the host enzymes are critical for controlling *Mycobacterium tuberculosis*, the causative agent of tuberculosis [1–3]. Moreover, both clinical observations in patients suffering from chronic granulomatous disease and recent experimental studies in zebra fish indicate a major role of superoxide (O\textsubscript{2} \textsuperscript{•})

**Abbreviations**: CFZ, clofazimine; DHQ, dihydroethidium; MABA, microplate Alamar blue assay; MDR, multidrug-resistant; Msm, *Mycobacterium smegmatis*; Mt, *Mycobacterium tuberculosis*; XDR, extensively drug-resistant
* Corresponding authors.
\textsuperscript{1} These authors have contributed equally to this work.

E-mail addresses: harinath@iiserpune.ac.in (H. Chakrapani), asingh@mcbl.iisc.ernet.in (A. Singh).

Generated by NOX2 in neutrophil-mediated containment of mycobacterial infection in vivo [4,5]. Collectively, these studies suggest a beneficial role of host-generated ROS and RNS in limiting survival of Mt during infection. Despite the toxic effects of ROS and RNS, Mt survives and persists within macrophages, indicating that Mt has defense mechanisms to effectively counter host-generated exogenous oxidative stress. In line with this, several studies have reported the essential role of cell wall-associated lipids (cyclopropanated mycolic acids, PDIM, etc.) [6,7], secretory antioxidant enzymes (superoxide dismutase [SodA, SodC], catalase [KatG], etc.), and secretory redox buffer ergothionine (ERG) in providing an excellent anatomical barrier to and detoxification of exogenous oxidants [8]. Additionally, Mt produces cytosolic reducing buffers such as mycothiol (MSH) and thioredoxins (Trxs) to protect the cytoplasmic redox environment from oxidative insult. However, while lack of antioxidant secretion and cell wall lipid components adversely

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affect survival of Mtb in vivo [9,10], disruption of the cytoplasmic redox systems MSH or Trx does not attenuate Mtb replication in vivo [11]. These findings indicate that cell surface-associated mechanisms (e.g., SodA, KatG, mycolic acids) are necessary and sufficient to alleviate toxicity mediated by exogenous ROS and these mycobacterial adaptive mechanisms seem to be important for maintaining intrabacterial redox balance and persistence of Mtb during infection. A modest increase in the endogenous $O_2^\bullet$ levels is known to substantially damage DNA, proteins, and lipids in diverse bacteria [12,13], indicating that in the case of Mtb infection, host-generated $O_2^\bullet$ are effectively countered by mycobacterial protective systems to prevent overwhelming amplification of endogenous oxidants in Mtb which exceeds the organism’s detoxification and repair capabilities. To gain a direct insight into this mechanism, we have recently developed an elegant tool (Mrx1 repair capabilities. To gain a direct insight into this mechanism, we generated O2 bacteria [12,13], indicating that in the case of Mtb in vivo – a sensitive superoxide radical generator provided evidence for the sensitivity of Mtb toward oxidative stress, how Mtb responds to specific elevation in endogenous ROS levels has never been studied. Intriguingly, recent studies indirectly indicate that Mtb might be sensitive to increases in endogenous ROS levels. For example, rifampicin, a known RNA polymerase inhibitor, was found to enhance ROS including hydroxyl radicals in Mtb [15,16]. However, the mechanism by which rifampicin generate ROS is poorly understood and thus its lethality cannot be attributed to ROS alone. Similarly, clofazimine has been shown to act through induction of oxidative stress due to elevated ROS [17]. Due to its ability to intercalate DNA [18], this drug is not a reliable prototype to study the specific effects of elevated ROS. Although the antimalarial drug artemisinin that acts through induction of oxidative stress is itself not a Mtb inhibitor, it has recently been reported that when covalently linked with mycobacin, a siderophore which enhances cell permeability, the resulting conjugate is a potent antmycobacterial agent [15]. However, the requirement for iron as a trigger for oxidative stress induction by artemisinin as well as the presence of the iron-chelating mycobacin complicates mechanistic interpretations regarding the mycobacterial role of ROS. Together, while these studies partly provide evidence for the sensitivity of Mtb toward oxidative stress, a clear understanding of how pathogens respond to endogenous ROS generated within Mtb cells remained uncharacterized.

In order to better understand the effects of elevated ROS in Mtb, we decided to test the consequences of exposure of Mtb to a set of novel, cell-permeable, reliable, and efficient superoxide radical generators (ATD series). Using multiple experimental approaches involving sensitive chemical, enzymatic, genetic, and expression assays, we investigated the potential of ATD compounds in (i) enhancing endogenous mycobacterial ROS production and (ii) impairing intramyobacterial redox homeostasis. The exceptional growth inhibitory activity shown by ATD compounds against drug-susceptible and drug-resistant strains of Mtb unambiguously demonstrates that intramyobacterial redox state is exceptionally sensitive to endogenous oxidative stress. Our mechanistic findings open up fresh avenues of redox research pertaining to the development of ROS generating anti-TB drugs.

**Materials and methods**

**Dihydroethidium assay for superoxide detection**

Dihydroethidium (DHE) assay was used for superoxide detection [19–21]. A stock solution of dihydroethidium (0.93 mg, 2.95 μmol) was prepared in DMSO (295 μl) and stored in the dark at $-20^\circ$ C until its use. A stock solution of the compound (5 μl, 10 mM) and DHE (5 μl of 10 mM) were reacted in acetonitrile:phosphate buffer of pH 7.4 (1:1, v/v, 100 mM, final volume 100 μl) for 3 h at 37° C. The reaction mixture was diluted to 50 μM using acetonitrile:phosphate buffer of pH 7.4 (1:1, v/v, to a final volume of 1 ml). The reaction mixture was filtered (0.22 μm) and injected (25 μl) in an Agilent high performance liquid chromatograph (HPLC) attached with a fluorescence detector (excitation at 356 nm; emission at 590 nm). The column used was a Zorbax SB C-18 reversed-phase column (250 x 4.6 mm, 5 μm), the mobile phase was water:acetonitrile containing 0.1% trifluoroacetic acid and a gradient starting with 90% → 0 min, 90: 10 to 70: 30 → 45 min, 30:70 to 0: 45–50 min, 0: 50–55 min, 10: 90 → 55–60 min was used with a flow rate of 0.5 ml/min. Under these conditions literature reports indicate that if superoxide is produced, a peak for 2-hydroxyethidium (2-OH-E$^\bullet$), which elutes at 286 min, would be observed [19–21]. When other oxidative species are generated ethidium (E$^\bullet$) would be formed. Again, according to literature reports E$^\bullet$ elutes at 29.5 min [19–21]. We independently confirmed the formation of 2-OH-E$^\bullet$ and E$^\bullet$ by mass spectrometry.

**Intracellular superoxide detection**

For intracellular superoxide detection [19–21], Mycobacterium smegmatis (MC$^\bullet$155) was cultured in 5 ml of middle brook M7H9 medium (M7H9, with 10% albumin–dextrose–saline (ADS) supplement) at 37° C for 24 h. The cultured bacteria were centrifuged to aspirate out the medium and resuspended to an OD $600_{nm}$ of 0.5 with fresh M7H9 medium. This bacterial solution was incubated with 50 μM of each ATD compound and 50 μM dihydroethidium independently for 30 min in the dark by covering the falcon tube in an aluminum foil. The suspension was centrifuged to aspirate out any excess of the compound and/or DHE in the medium. The collected bacterial pellet was resuspended with acetonitrile and the cells were lysed using a probe sonicator for 3 min on ice. The cell lysate was then removed by centrifugation and the supernatant acetonitrile was separated and stored at $-20^\circ$ C before injecting in HPLC. The HPLC method used was as described previously [19–21]. A similar protocol was followed for the detection of intracellular superoxide generation by ATD-3169 in Mycobacterium bovis BCG.

**Preparation of mycobacterial cells for $E_{MSH}$ measurements by flow cytometry**

Mycobacterial strains were grown in Middlebrook 7H9 medium (Difco) supplemented with 10% ADS, 0.2% glycerol, 0.05% Tween 80 treated with 10 mM NEM for 5 min at room temperature (RT) followed by fixation with 4% PFA for 15 min at RT. After washing thrice with 1X phosphate buffer saline (PBS), bacilli were analyzed using a BD FACS Verse Flow cytometer (BD Biosciences). The biosensor response was measured by analyzing the ratio at a fixed emission (510 nm) after excitation at 405 and 488 nm. Data were analyzed using the FACSuite software.

**MIC assays**

MIC was determined using a microplate Alamar blue assay (MABA). The Alamar blue assay was performed in 96-well flat bottom plates. Mtb strains were cultured in 7H9-ADS medium and grown till exponential phase (OD $600_{nm}$ ~0.4). Approximately $1 \times 10^5$ bacteria were taken per well in a total volume of 200 μl of 7H9-ADS medium. Wells containing no Mtb were the autofluorescence control. Additional controls consisted of wells containing cells and medium only. After 5 days of incubation at 37°C, 20 μl of 10X Alamar blue was added and plates were reincubated for 24 h. The fluorescence readings were then recorded. Fluorescence intensity.
was measured in a SpectraMax M3 plate reader (Molecular Device) in top-reading mode with excitation at 530 nm and emission at 590 nm. Percentage inhibition was calculated based on the relative fluorescence units and the minimum concentration that resulted in at least 90% inhibition was identified as MIC.

**Checkerboard synergy assay**

Checkerboard synergy assay was performed using Alamar blue dye in a 96-well plate as described [22]. Synergy was defined by fractional inhibitory concentration index (FICI) values of 0.5, antagonism by FICI values of >4.0, and no interaction by FICI values from 0.5 to 4.0. The FICIs were calculated as follows: $\Sigma FICI= FICI A + FICI B$, where FIC A is the MIC of drug A in the combination/MIC of drug A alone, and FIC B is the MIC of drug B in the combination/MIC of drug B alone.

Other assay protocols, synthetic procedures, and characterization data are available in the Supplemental Information.

**Results**

**2,3-Dihydro-1,4-naphthoquinone-based small molecules generate ROS inside mycobacteria**

To examine the response of *Mtb* toward endogenous oxidative stress, our first challenge was to synthesize cell-permeable redox-oriented compounds that specifically enhance ROS levels within *Mtb* cells. Our preliminary analysis recently demonstrated growth inhibitory activity of ROS generators based on a 2,3-dihydro-1,4-naphthoquinone scaffold against mycobacteria [23]. While these molecules are believed to generate intrabacterial ROS [23], a direct proof for this and the exact mechanism by which they perturb mycobacterial redox physiology to exert antimycobacterial activity remain uncharacterized. To investigate this, we first synthesized a new series of ROS-generating compounds (ATD series) in 80–90% yield (Fig. 1A and Table S1) and characterized by NMR (see SI Experimental procedures). These compounds undergo enolization in buffer to produce a diolate, which spontaneously produces superoxide by reacting with oxygen to give a quinone which in turn is known to undergo bireduction to generate superoxide.

To examine both possibilities, we first monitored the ability of ATD compounds to generate $O_2^•−$ under ambient aerobic conditions in buffer adjusted to pH 8.0 using a widely reported luminal-based chemiluminescence assay [25]. Xanthine oxidase (XO), an enzyme which oxidizes hypoxanthine (X) to produce $O_2^•−$ and other oxidants, served as a positive control in our assays. As a negative control, we have synthesized a closely related derivative of ATD compounds (ATD-4110), which is a naphthoquinone and is therefore incapable of spontaneously producing $O_2^•−$ in buffer. The compounds ATD-3169, ATD-4064, and ATD-4054 produced significantly higher levels of $O_2^•−$ as revealed by ~2- to 15-fold increased chemiluminescence as compared to positive control at 20 min postincubation (Fig. 2A; also see Supplemental Information Fig. S1). A time-dependent analysis demonstrated an initial increase in $O_2^•−$ production for 20 min followed by a gradual decline (Fig. 2A). Since the main aim of this work is to induce intramycobacterial $O_2^•−$ generation by ATD compounds, we determined $O_2^•−$ production using a well-established and freely cell-permeable $O_2^•−$ indicator, dihydroethidium [26]. It has been shown that DHE specifically reacts with $O_2^•−$ to release fluorescent product 2-hydroxyethidium (2-OH-E•), which can be conveniently detected by analytical techniques such as HPLC, fluorescence microscopy, etc. [26]. The reaction of DHE with other oxidants produces ethidium (E•). We first confirmed $O_2^•−$ generation in the buffer at pH 8.0 using DHE. An HPLC spectrum of $O_2^•−$ reaction products clearly showed varying levels of 2-OH-E• and E• generated by ROS generators (Fig. 2C). As expected, peaks correspond to 2-OH-E• and E• were not detected in buffer alone or in the case of compound ATD-4110 (Fig. 2C).

To evaluate if our ROS generators are capable of promoting $O_2^•−$ inside mycobacterial cells, we exposed *Mycobacterium smegmatis* to ATD compounds and DHE and detected fluorescent peaks by HPLC. Since cell death induced by ATD compounds might also elevate intracellular ROS levels, $O_2^•−$ generation was monitored at an early time point (60 min). Further, to minimize the contribution of extracellular $O_2^•−$ present in the culture medium, we thoroughly washed samples of 7H9 growth medium and performed measurements on cells suspended in 1X PBS. As shown in Fig. 2D, intramycobacterial $O_2^•−$ was generated by all of the ATD compounds. Interestingly, ATD-4110 generated comparable levels of intramycobacterial $O_2^•−$, thus validating the redox-cycling-based mechanism of ROS production within *Msm* cells by these compounds. Exogenous $O_2^•−$ cannot penetrate the cell membrane [27], suggesting that ATD compounds are permeating into the cell interior and promoting endogenous $O_2^•−$ production possibly by catalyzing electron cycling with flavin, Fe-S clusters, and oxygen

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**Fig. 1.** (A) Structures of ATD series of compounds prepared in this study. (B) Mechanism of $O_2^•−$ radical generation from 2,3-dihydro-1,4-naphthoquinones involves enolization in buffer to a diolate, which spontaneously produces superoxide by reacting with oxygen to give a quinone which in turn is known to undergo bireduction to generate superoxide.
[28]. In a separate experiment, Msm cells pretreated with DHE were then exposed to ATD-3169 and we similarly found an increase in 2-OH-E^+ , supporting the capability of this compound to permeate mycobacteria to enhance intracellular ROS levels (see Supplemental Information Fig. S2).

Since O2^- rapidly undergoes dismutation to H2O2 and molecular oxygen (O2) either spontaneously or by superoxide dismutase (SOD) [28], we next analyzed the activity of ATD compounds to generate H2O2. We incubated Msm with 50 μM of compounds for 60 min and H2O2 released in the extracellular medium was measured using a well-established fluorescence-based Amplex Red assay [29]. A calibration curve was generated with varying concentrations of H2O2 and the levels of extracellular H2O2 can be quantified by this assay (Fig. S1). We observed that while all of the compounds produce H2O2, ATD-3169 showed exceedingly greater levels of H2O2 in 2 h (Fig. 2B). Furthermore, consistent with the O2^- data, time–kinetic analysis demonstrated an initial increase in H2O2 production reaching a maximum value at 2 h posttreatment, followed by a steady decrease (Fig. 2B). The eventual decrease in O2^- and H2O2 levels is in line with our recent study demonstrating the presence of an efficient antioxidant buffer system (mycothiol; MSH) to rapidly counterbalance oxidative stress in Msm [14]. Taken together, we have successfully designed novel small molecules capable of enhancing intracellular ROS in mycobacteria.

**ROS generators modulate mycothiol redox balance in Msm**

We have shown the generation of O2^- in buffer and inside Msm by ATD compounds using redox-active fluorogenic dyes and HPLC. Although important for detecting ROS in vitro, intracellular detection of ROS by fluorogenic dye-based disruptive technologies can introduce oxidation artifacts during cell lysis, which prohibits dynamic measurements in real time [30,31]. Therefore, to validate our in vitro findings and to find out if ATD compounds are efficient in overwhelming mycobacterial antioxidant capacity, we next exploited a recently reported noninvasive tool to measure dynamic changes in intramycobacterial oxidation–reduction state [14]. Actinomycetes including mycobacteria produce millimolar concentrations of an antioxidant, mycothiol, which serves as a major cytoplasmic buffer that protects bacteria from oxidative stress [32]. Since oxidative stress rapidly oxidizes reduced mycothiol (MSH) to oxidized mycothiol (MSSM), the ratio of mycothiol redox couple (MSH/MSSM) or mycothiol redox potential (EMSH) provides a direct indicator of mycobacterial redox state [14]. To examine if the ROS generators perturb mycobacterial redox balance, we first performed dynamic measurement E_MSH of Msm in response to ATD compounds using a highly sensitive and specific biosensor of intramyobacterial mycothiol redox potential (E_MSH; Mrx1-roGFP2) [14]. In Mrx1–roGFP2, oxidation–reduction-sensitive GFP (roGFP2) is covalently fused to Mtb mycothiol-specific oxidoreductase mycoredoxin-1 (Mrx-1), which reversibly transfers electrons between the mycothiol redox buffer and the thiol groups of roGFP2 [14]. Therefore, continuous formation and release of the roGFP2 disulfide bridge are coupled with the actual redox potential of the mycothiol buffer inside mycobacteria [14]. The oxidation and reduction state of Mrx1–roGFP2 thiols can be easily detected by measuring fluorescence intensity at 405 and 488 nm excitation wavelengths with a fixed emission wavelength of 510 nm [14]. Increase or decrease in Mrx1–roGFP2 ratio (405/488) indicates oxidative or reductive shift in E_MSH of a mycobacterial cell, respectively [14].

Msm expressing Mrx1–roGFP2 biosensor was independently exposed to various concentrations of ATD compounds (1.5 to 25 μM) and two known ROS generators (H2O2 and cumene hydroperoxide [CHP]). The ratemic response was measured by flow cytometry at 24 h posttreatment. Surprisingly, despite showing increased generation of ROS inside Msm, most of the ATD compounds were ineffective in increasing the Mrx1–roGFP2 ratio at each concentration tested, indicating an effective induction of mycothiol antioxidant system in response to ROS generators (Fig. 3A). Similar results were obtained with H2O2 and CHP treatment (Fig. 3A). Only treatment with 25 μM of ATD 3169 and ATD 4064 induced significant oxidative shift in E_MSH of Msm (Fig. 3A). To examine if enhanced oxidative E_MSH induced by ATD-3169 and ATD 4064 correlated with growth inhibition, we determined minimal inhibitory concentrations (MICs) of these compounds against Msm using MABA. Alamar blue (AB) is an oxidation–reduction indicator dye that has been widely used to measure the sensitivity of mycobacteria to anti-TB drugs [33]. A change in nonfluorescent blue to fluorescent pink indicates reduction of AB due to growth, whereas inhibition of growth by antmycobacterial compounds interferes with AB reduction and color development. Consistent with the Mrx1–roGFP2 findings, most of the ROS generators were inefficient in inhibiting growth of Msm even at concentrations higher than 200 μM (Table 1). Importantly, ATD-3169 completely prevented Msm growth at a minimum inhibitory concentration (MIC) of 50 μM, while lower oxidative stress induced by ATD-4064 correlated with the higher MIC value against Msm (200 μM, Table 1). It can be argued that the use of
redox-cycling compounds can influence AB oxidation–reduction to compromise the MABA assay. To address this issue, we validated the inhibitory effect of a few ATD compounds (ATD-3169, ATD-4064, and ATD-4110) on Msms by analyzing colony forming units (CFUs). As shown in Fig. 3B, Msms treated with ATD compounds at MIC concentrations displayed ~2.5 log reduction in CFUs as compared to untreated cells at 24 h posttreatment. These results confirm that both MABA and CFU analyses are in reasonable agreement with each other and that ATD compounds exert bactericidal effects on mycobacterial cells.

A direct linkage between oxidative $E_{OX}$ and growth inhibition suggests that disruption of mycolith redox homeostasis is the underlying mechanism by which ATD-3169 exerts an antimycobacterial effect. However, ATD-3169 was ineffective in reducing Msms growth at concentrations lower than 25 μM. We reasoned that this could be due to efficient recruitment of the mycolith antioxidant system to dissipate ROS generated by lower concentrations of ATD-3169. To show this, we exposed Msms expressing Mrx1–roGFP2 to nontoxic concentrations of ATD-3169 (3.1, 6.25, 12.5 μM) and the ratiometric response was measured at early time points to capture dynamic changes in $E_{OX}$. Addition of ATD-3169 resulted in a rapid increase in Mrx1–roGFP2 ratio in a concentration-dependent manner (Fig. 3C). As indicated in Fig. 3C, maximum oxidation induced by various concentrations of ATD-3169 was achieved within 15 min postexposure. Interestingly, the observed increase in Mrx1–roGFP2 ratio was not long lasting and cells recovered from the oxidative insult as indicated by a decrease in 405/480 ratio to basal levels within 180 min posttreatment (Fig. 3C), indicating activation of an MSH-dependent antioxidative mechanism. As a control, we have similarly tracked $E_{OX}$ on treatment of Msms with a toxic concentration (i.e., 50 μM) of ATD-3169. In this case, Msms displayed a sustained and irreversible increase in 405/488 ratio, confirming the role of overwhelming mycolith oxidation caused by higher concentrations of ATD-3169 (Fig. 3C). Lastly, to confirm the role of Msh in tolerating oxidative stress generated by ATD-3169, we measured the MIC of ATD-3169 against MSH-negative strains of Msms (MsmsmshA and MsmsmshD). As shown in Tables 1, a 2-fold lower concentration of ATD-3169 was sufficient to inhibit growth of MSH-negative strains as compared to wt Msms. Consistent with these findings, Amplicex Red assay showed that MsmsmshA consistently displayed higher levels of extracellular H$_2$O$_2$ as compared to wt Msms (Fig. 3D). These results confirmed that Msms responds to ATD compounds by inducing dynamic changes in MSH redox signaling and homeostasis, and suggest that efficient generation of intrabacterial ROS may induce killing by compromising reductive capabilities of mycobacteria.

ATD-3169 perturbs mycolith redox homeostasis in Mtb

Several studies have indicated that slow growing mycobacteria, including Msms, lack protective oxidative stress responses due to the absence of a functional copy of oxidative stress regulators such as OxyR and SoxR [34]. Moreover, pathogenic and drug-resistant Msms strains displayed relatively oxidized steady-state intrabacterial

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**Table 1** Minimal inhibitory concentrations (MICs) of ROS generators against diverse bacterial species.

| Strain                          | ATD-3167 | ATD-3169 | ATD-4053 | ATD-4054 | ATD-4064 | ATD-4110 |
|---------------------------------|----------|----------|----------|----------|----------|----------|
| Staphylococcus aureus           | >100 μM  | >100 μM  | >100 μM  | >100 μM  | >100 μM  | >100 μM  |
| E. coli                         | >100 μM  | >100 μM  | >100 μM  | >100 μM  | >100 μM  | >100 μM  |
| Salmonella typhimurium           | >100 μM  | >100 μM  | >100 μM  | >100 μM  | >100 μM  | >100 μM  |
| Klebsiella pneumoniae           | >100 μM  | >100 μM  | >100 μM  | >100 μM  | >100 μM  | >100 μM  |
| Pseudomonas aeruginosa          | >100 μM  | >100 μM  | >100 μM  | >100 μM  | >100 μM  | >100 μM  |
| Msms mc155                      | 200 μM   | 50 μM    | 200 μM   | 200 μM   | 200 μM   | 200 μM   |
| H37Rv                           | 100 μM   | 3.125 μM | 100 μM   | 50 μM    | 12.5 μM  | 12.5 μM  |
| MDR (JL 2287)                   | 50 μM    | 6.25 μM  | 50 μM    | 50 μM    | 6.25 μM  | 6.25 μM  |
| XDR (Myc-431)                   | 300 μM   | ND       | ND       | ND       | ND       | ND       |
| MDR (JL 2261)                   | ND       | 3.125 μM | ND       | ND       | ND       | ND       |
| MDR (JL 1934)                   | ND       | 3.125 μM | ND       | ND       | ND       | ND       |
| MDR (BND320)                    | ND       | 3.125 μM | ND       | ND       | ND       | ND       |
| M. bovis RCC                    | ND       | 3.125 μM | ND       | ND       | ND       | ND       |
| H37Rv ΔmshA                     | ND       | 0.3125 μM| ND       | ND       | ND       | ND       |
| H37Rv ΔmshA comp                 | ND       | 3.125 μM | ND       | ND       | ND       | ND       |
| MsmsmshA                       | ND       | 25 μM    | ND       | ND       | ND       | ND       |
| MsmsmshD                        | ND       | 25 μM    | ND       | ND       | ND       | ND       |

MICs of ATD compounds against a range of Gram-positive and Gram-negative bacteria were measured using the microbroth dilution technique as recommended by National Committee for Clinical Laboratory Standards. MIC, minimum inhibitory concentration; ND, not determined.
oxidative shift in strains Jal 2287 and MYC 431 was comparable to that of stressed throughout the course of the experiment (Fig. 4A). The Mtb In orchestrating an efficient and dynamic MSH-speciﬁc antioxidant response by generating overwhelming endogenous oxidative stress.

**Table 2**

Data showing synergy between ATD-3169 and clofazimine (CFZ).

| Strain   | Drug combination | MIC (µM for 3169, µg/ml for clofazimine) | FIC | FICI |
|----------|------------------|-----------------------------------------|-----|------|
| H37Rv    | 3169             | 3.125/0.7                                | 0.25 | 0.47 |
| Clofazimine | 3169           | 0.25/0.0125                             | 0.25 | 0.49 |
| Jal2287 (MDR) | 3169         | 3.125/0.7                                | 0.25 | 0.49 |
| Clofazimine | 3169           | 0.125/0.0125                             | 0.25 | 0.49 |
| Myc 431 (XDR) | 3169          | 6.25/1.5                                 | 0.25 | 0.49 |
| Clofazimine |                | 0.5/0.125                                | 0.25 | 0.49 |

Influence of ATD-3169 and CFZ combination against drug-sensitive (H37Rv), multidrug resistant (MDR: Jal 2287), and extensively drug-resistant (XDR: Myc 431) strains of Mtb was examined using a checkerboard assay.

**E<sub>EMSH</sub>** as compared to nonpathogenic Msm, and exhibited exceptional sensitivity to endogenous increase in oxidant levels [14,34,35]. On this basis, we hypothesized that Mtb strains might show differential sensitivity toward ATD-3169 as compared to Msm. To examine this possibility, we expressed Mrx1-roGFP2 in the virulent laboratory strain (Mtb H37Rv), and two Indian patient isolates of Mtb, i.e., a multidrug-resistant strain (Jal-2287) and an extensively drug-resistant strain (MYC 431) [14].

Mtb strains expressing Mrx1-roGFP2 were exposed to lower concentrations of ATD-3169 (3.1, 6.25, 12.5 µM) at which Msm robustly maintained intrabacterial **E<sub>EMSH</sub>** and survival. As a positive control, we treated Mtb H37Rv with 1 mM H<sub>2</sub>O<sub>2</sub>. To minimize the contribution of cell death in generating oxidative stress, we measured the Mrx1-roGFP2 ratio at early time points. Addition of ATD-3169 rapidly increased the Mrx1-roGFP2 ratio in a concentration-dependent manner among the Mtb strains (Fig. 4A, B, and C). Similar oxidation of Mrx1-roGFP2 was detected on exogenous exposure to H<sub>2</sub>O<sub>2</sub> (Fig. 4D). However, while the Mrx1-roGFP2 ratio gradually decreased to baseline values in the case of H<sub>2</sub>O<sub>2</sub>-challenged Mtb (Fig. 4D), ATD-3169-treated Mtb remained oxidatively stressed throughout the course of the experiment (Fig. 4A).

The ratiometric ﬂuorescence pattern displayed by the drug-resistant strains Jal 2287 and MYC 431 was comparable to that of Mtb H37Rv (Fig. 4B and C). Since we have shown that ATD-3169 induces an oxidative shift in **E<sub>EMSH</sub>** of Mtb by generating endogenous ROS such as O<sub>2</sub>• via redox cycling, we asked if a similar mechanism exists for Mtb. However, due to technical and biosafety challenges associated with handling and processing a BSL3 class pathogen such as Mtb for HPLC analysis, we measured O<sub>2</sub>• levels inside a related but nonpathogenic slow growing vaccine strain of mycobacteria, Mycobacterium bovis BCG. It has been previously reported that the intrabacterial **E<sub>EMSH</sub>** of BCG is similar to that of Mtb, indicating a comparable cytoplasmic redox state of these two species under normal growing conditions [14]. Moreover, BCG was found to be equally sensitive to ATD-3169 as compared to Mtb strains (Table 1). Using a DHE assay, we found that BCG cells treated with ATD-3169 generate peaks corresponding to O<sub>2</sub>•• (2-OH•E•) and other ROS (E<sup>+</sup>•) (Fig. 4E). As a positive control, we used a well-known O<sub>2</sub>•• generator (menadione) in our assay and similarly detected a 2-OH•E• peak, conﬁrming the generation and detection of O<sub>2</sub>•• by menadione and ATD-3169 inside BCG (Fig. 4E). These results indicate that ATD-3169 severely impaired the ability of Mtb to orchestrate an efﬁcient and dynamic MSH-speciﬁc antioxidant response by generating overwhelming endogenous oxidative stress.

Low concentrations of ATD-3169 efﬁciently inhibit growth of drug-resistant Mtb strains

Having shown that ATD-3169 is efﬁcient in perturbing mycothiol redox balance in Mtb strains, we next determined the MIC of ATD-3169 against several virulent and drug-resistant strains of Mtb using MABA assay. These include a laboratory strain (Mtb H37Rv), and several clinical drug-resistant strains of Mtb, i.e., single drug-resistant (BND 320), multidrug-resistant (MDR: Jal 2261, 1934, Jal 2287), and extensively drug-resistant (XDR: MYC 431) [14]. In complete agreement with the Mrx1-roGFP2 ﬁndings, virulent and drug-resistant clinical strains showed exceptional sensitivity toward ATD-3169. A concentration range of 3.125–6.25 µM was sufﬁcient to uniformly inhibit the growth of Mtb strains (Table 1). The killing potential of ATD-3169 was also conﬁrmed by enumerating colony-forming units (CFUs) of Mtb at various time points posttreatment (Fig. 4F). Lastly, when tested against human embryonic kidney cells HEK293, a cell viability assay revealed no signiﬁcant inhibition at 25 µM [24]. Thus, at concentrations where complete inhibition of Mtb was observed, the compounds were well tolerated by mammalian cells. These results suggest that the endogenous production of O<sub>2</sub>•• and H<sub>2</sub>O<sub>2</sub> by ATD-3169 effectively perturbed mycothiol redox balance to speciﬁcally compromise growth of virulent and drug-resistant Mtb strains.

**Targeting mycothiol pathway increases sensitivity of Mtb to ATD-3169**

Earlier studies have shown that MSH-deﬁcient mycobacteria are susceptible to broad classes of antibiotics [36,37]. Recently we have shown that while anti-TB drugs do not alter **E<sub>EMSH</sub>** of Mtb during growth in culture medium, they speciﬁcally induce intramycobacterial oxidative **E<sub>EMSH</sub>** to exert efﬁcient mycobacterial activity during infection [14]. Collectively, these ﬁndings underscore the importance of MSH-redox homoeostatic mechanism(s) in protecting mycobacteria against antibiotics. Contrary to known anti-TB drugs, treatment with ATD-3169 produces ROS inside mycobacteria and induces an intrabacterial oxidative shift in **E<sub>EMSH</sub>** of Mtb in culture medium in vitro. To decisively show that ATD-3169 functions by disturbing mycothiol redox balance, we assessed the effect of ATD-3169 on an Mtb H37Rv strain deﬁcient in mycothiol biosynthesis (MtbΔmshA). The MtbΔmshA strain does not produce mycothiol and showed sensitivity to oxidants [38,39]. We found that the MtbΔmshA strain was 10 times more sensitive to ATD-3169 as compared to wt Mtb H37Rv (MIC 0.3125 µM) (Table 1). Complementation of MtbΔmshA with an integrated copy of wt mshA restored the sensitivity to Mtb H37Rv levels. These results suggest that the mycothiol system is one of the main intrinsic mechanisms for protection against ATD-3169 in Mtb.

Similar to ATD-3169, another redox-cycling drug, clofazimine (CFZ), has been shown to induce mycobacterial killing by generating ROS and inducing an oxidative shift in **E<sub>EMSH</sub>** during growth in culture medium [14]. Therefore, we checked if the CFZ and ATD-3169 combination acts synergistically on Mtb. First the MICs of CFZ and ATD-3169 were determined independently using an AB assay and found to be consistent with previously reported MIC values for CFZ (Table 2). Second, using a checkerboard assay (see Experimental procedures), compound interactions were determined by growing Mtb strains in sub-MIC concentrations of CFZ and sub-MIC fractions of ATD-3169. The **ΣFIC** (fractional inhibitory concentration) for each combination was calculated and given in Table 2. The combination of CFZ and ATD-3169 gave a **ΣFIC** of ≤0.5 against the drug-sensitive laboratory strain (H37Rv) and drug-resistant clinical strains (Jal 2287 and MYC 431).
together, our data for the first time revealed that Mtb is uniquely sensitive to elevation in endogenous ROS levels, and suggest selective targeting of mycobacterial redox homeostasis by producing excessive oxidative stress within Mtb cells as a potential new intervention strategy against TB.

ATD-3169 modulates expression of genes associated with the antioxidant system, DNA repair, central metabolism, and cell wall lipid biosynthesis

To further understand the mechanism of ATD-3169 action, we performed microarray analysis of Mtb H37Rv exposed to 30 μM ATD-3169 for 4 h (see SI Experimental procedures). A transcriptional response showed differential regulation of ~500 genes in response to ATD-3169 (2-fold cutoff, \( P \leq 0.05 \), Table S2). Since ROS generally damage DNA, proteins, and lipids, a transcriptional response to ATD-3169 is largely composed of genes involved in DNA repair, cell wall lipid biosynthesis, iron homeostasis, sulfur metabolism, redox-active proteins, citric acid cycle proteins, and transcriptional regulators (Fig. 5A and Table S2). Given that ATD-3169 induced substantial oxidative stress, expression of katG was highly upregulated (~35-fold) on treatment. Additionally, several genes involved in the oxidation–reduction reactions such as hpx (heme haloperoxidase), Rv1786 (ferredoxin), oxidoreductases (Rv0068, Rv0149, Rv0183), and monoxygenases (Rv0892, Rv3083, Rv1393c) were influenced by ATD-3169 (Fig. 5B). Because DNA is one of the sensitive targets of ROS, we expected to see many genes involved in DNA metabolism to be differentially expressed in response to ATD-3169. Consistent with this, among the highly upregulated genes were those which encode for helicases (uvrC, uvrD), DNA repair and recombination (recA, rada, dinP, alkB, nei, etc.), DNA supercoiling (gyrA, gyrB), SOS response (lexA), etc. (Fig. 5C). The upregulation of several genes involved in cell wall lipid/polyketide biosynthesis (mma3, mma2, pks2, pks7, papa1, papa3, etc.) and fatty acid metabolism (fas, fadD9, fadD22, fadD31, etc.) indicates damage of the cell wall lipid architecture by ATD-3169 (Fig. 5D). Since ATD-3169 inhibits growth of Mtb by perturbing
mycothiol redox homeostasis, we reasoned that pathways involved in the biosynthesis of intracellular thiols might be affected by this compound. In agreement with this, genes involved in the biosynthesis of reduced sulfur metabolites such as cysteine and methionine \[ \text{metH, cysN, cysD, sahH, etc.} \] and the prominent sulfur-containing cell wall glycolipid, sulfolipid-1 \((\text{pks2, papA1})\) were induced on treatment (Fig. 5E).

Because iron homeostasis is intrinsically coupled to ROS-mediated lethality \([12,40]\), genes involved in iron storage \((\text{bfrB, Rv2455c})\) and iron regulation \((\text{idcR, furA})\) were highly induced in our expression data (Fig. 5F). The induction of iron-repressor and iron-storage genes indicates the increase in free iron content on treatment with ATD-3169, suggesting a possible leaching of iron by endogenous \(O_2^{*}\) from iron-binding proteins such as Fe–S

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Fig. 5. Global changes in the expression of \(\text{Mtb H37Rv}\) genes in response to ATD-3169. \(\text{Mtb H37Rv}\) was grown to an O.D_{600 nm} of 0.4 and treated with 30 \(\mu\text{M}\) ATD-3169 for 4 h at 37 °C. Total RNA was isolated and subjected to microarray analysis as noted under Materials and methods. (A) Pie chart corresponding to differentially expressed genes in response to ATD-3169, functionally classified in 13 classes based on the annotation given in the tuberculist database. Differentially regulated genes were shown in the heat maps. (B) Detoxification, miscellaneous oxidoreductases, and oxygenases. (C) DNA replication, repair, recombination, and restriction/modification. (D) fatty acid catabolism and lipid anabolism. (E) sulfur metabolism. (F) transcriptional regulators. (G) TCA cycle and glyoxylate cycle genes, and (H) drug efflux pumps. (I) qRT-PCR validation of a select number of genes differentially regulated in the microarray data. Data shown are the representative of at least two difference experiments performed in triplicate. Error bars represents standard deviations from the mean.
cluster proteins. In line with this, we observed overexpression of cysD, which encodes for a cysteine desulphurase involved in the repair of oxidatively damaged Fe–S clusters [41]. One of the unified mechanisms by which ROS inhibit growth of prokaryotic and eukaryotic organisms is by releasing iron from the citric acid cycle enzyme like [Fe–S]-dependent dehydratase, aconitase [28]. Interestingly, we found that ATD-3169 induced expression of multiple genes which encode for citric acid cycle enzymes including Fe–S containing aconitase (acon), 2-oxoglutarate dehydrogenase (kgd), and dihydrolipoamide dehydrogenase (Rv0794c), indicating a direct role of endogenous ROS generated by ATD-3169 in inactivating these mycobacterial enzymes (Fig. 5G). Additionally, genes involved in glyoxylate cycle (isocitrate lyase; icl) and gluconeogenesis (phosphoenolpyruvate carboxykinase; pck), which counterbalance oxidative stress by increasing the yield of reducing power, were found to be upregulated by ATD-3169 (Fig. 5G). These results indicate a functional linkage between energy metabolism and intracellular redox balance, and suggest a major role of central metabolism in increasing the efficiency of Mtb to tolerate oxidative stress.

Increased expression of multiple drug efflux pumps provides a critical defense against redox-cycling drugs in Escherichia coli [44]. Similarly, induction of genes encoding efflux pumps, including Rv2688c, Rv1410c, Rv2326c, phoY2, etc., indicates that Mtb tries to reduce endogenous ROS levels by limiting intracellular accumulation of ATD-3169 (Fig. 5H). Mtb’s adaptive response to oxidative stress is dependent on multiple transcriptional regulators and stress-dependent sigma factors [8]. A large number of gene regulators including Rv3160c, Rv1474c, Rv3055, Rv0067c, Rv3050c, idER, farA, etc. were induced by ATD-3169 (Fig. 5F). Lastly, using quantitative reverse-transcriptase PCR (qRT-PCR), we verified the microarray results by monitoring the differential expression of a select number of Mtb genes in response to ATD-3169 (Fig. S1; Table S2, S4). In sum, data generated from multiple techniques clearly show that the efficient antimycobacterial activity shown by the ATD-3169 is a consequence of increased intrabacterial oxidative stress and associated damage to cellular pathways involved in ROS detoxification, and repair of essential metabolites such as DNA, proteins, and lipid in Mtb.

Discussion

Increased emergence of drug-resistant Mtb strains coupled with a meager number of new antibiotics in the pipeline has necessitated an urgent need to enhance our portfolio of anti-TB drugs. In this context, a paradigmatic approach would be to induce long-lasting and irreversible changes in cellular homeostatic mechanisms of Mtb. Several studies in humans, animal models, and macrophages clearly indicate that exogenous oxidents such as O$_2^\bullet$ produced by a phagocytic respiratory burst are critical components of host defense against bacterial infection [3-5]. Furthermore, owing to exceptionally higher rate constants (10$^{8}$ M$^{-1}$ s$^{-1}$) for O$_2^\bullet$-mediated inactivation of essential metabolic enzymes such as dehydratase and mononuclear enzymes, a modest increase in endogenous O$_2^\bullet$ levels elicits substantial enzymatic and growth defects in bacteria [45]. Despite the remarkable antimicrobial potency of exogenous oxidants, Mtb seems to express several mechanisms specifically directed to dissipate host-generated oxidative stress for persistence in vivo. While Mtb’s ability to resist host-generated redox stress is widely studied, to the best of our knowledge how mycobacteria respond to a specific increase in endogenous O$_2^\bullet$ level has not been characterized to date. Consequently, we have developed a series of cell-permeable redox-cycling drugs which generate ROS, specifically O$_2^\bullet$ via undergoing bioactive activation inside mycobacteria. Because O$_2^\bullet$ is a charged species at physiological pH (pK$_a$=4.8), it cannot penetrate cellular membranes [27]. Keeping this in mind, ATD compounds were specifically designed to generate O$_2^\bullet$ inside a mycobacterial cell. The specific detection of O$_2^\bullet$ in the cytoplasm of mycobacteria validates our chemical synthesis approach and indicates a mechanism involving abstraction of electrons from low-potential metal centers (Fe–S clusters), respiratory quinones, and flavins followed by univalent reduction of O$_2$ to generate O$_2^\bullet$ by ROS generators. Our results demonstrating exceptional sensitivity of Mtb (including MDR/XDR strains) to compounds that generate endogenous ROS, along with the reported resistance of Mtb toward exogenous oxidants, revealed crucial differences in defense mechanisms exploited by Mtb to tolerate ROS produced inside or outside the bacterial cell. Such disparate responses to ROS were not observed in other bacterial systems. For example, while under normal respiratory conditions basal scavenging enzymes (e.g., Sod, Kat) are sufficient to protect E. coli from endogenous O$_2^\bullet$ and H$_2$O$_2$, any abnormal increase in endogenous ROS levels by exogenous oxidative stress agents or redox-cycling drugs is rapidly sensed by OxyR and SoxRS systems, respectively, to mount an effective antioxidant response [46,47]. Interestingly, major antioxidant enzymatic systems in Mtb are either secretory (SodA, KatG) or located in the periplasm (SodC), indicating that the pathogen is evolutionarily equipped to neutralize extracellular O$_2^\bullet$ derived from phagocytes during infection. The above findings, along with the lack of a functional cytosolic redox-sensing system such as OxyR or SoxR, indicate that Mtb is genetically programmed to divert the majority of cellular antioxidant machinery to mitigate exogenous ROS and to maintain cytoplasmic redox balance. While important in the natural context of infection, these studies indicate that Mtb might be vulnerable to redox-cycling drugs that permeate inside bacteria to specifically elevate endogenous ROS levels and perturb redox homeostasis. Consistent with this, virulent H37Rv and MDR/XDR strains have shown exceptional sensitivity to an increase in endogenous ROS/RNS by redox-cycling drugs such as nitroimidazopyrans, CFZ, and vitamin C [12,27,44]. In contrast, other bacterial species (including Msm) known to contain nonsecretory forms of antioxidant enzymes and capable of inducing an OxyR-type protective oxidative stress response were comparatively more tolerant to killing by nitroimidazopyrans, vitamin C, and ATD compounds (Table 1) [35,48].

The production of millimolar concentrations of cytoplasmic redox buffer, MSH, is the main mechanism by which mycobacteria maintain a reduced cytosolic state [49]. We reasoned that if ATD-3169 specifically elevates endogenous ROS levels, then one would expect to observe perturbation of mycothiol redox homeostasis in Mtb. Using a novel mycothiol biosensor [14], we showed that ATD-3169 induces a rapid and long-lasting oxidative stress in $E_{Msm}$ of Mtb, indicating impairment of MSH-controlled reductive capabilities of Mtb by ATD-3169. A similar oxidative stress in $E_{Msm}$ was induced in MDR/XDR strains, raising an interesting possibility of targeting mycothiol redox pathway to impact survival of both drug-sensitive and drug-tolerant Mtb strains. The importance of the mycothiol pathway in tolerating endogenous ROS is further revealed by our findings showing an exceptionally high sensitivity of $Mtb\Delta mshA$ toward ATD-3169. The reported higher antioxidant capacity of $Msm$ ($E_{Msm}=−300$ mV) as compared to Mtb ($E_{Msm}=−270$ mV) may have partly contributed to differential susceptibility of these strains toward ATD-3169 in our study [14]. Finally, our microarray data validate our estimates that ATD-3169 induces endogenous oxidative stress and perturbs mycobacterial redox metabolism. Similar to other redox-cycling drugs, ATD-3169 appears to function by disrupting iron homeostasis. Induction of genes encoding the Fe–S cluster containing TCA cycle enzymes, iron-storage proteins, Fe–S cluster repair proteins, and iron repressors clearly indicates elevated iron levels due to damage of Fe–S clusters by ATD-3169. Because iron is the coactivant in the Fenton reaction, which increased the chances of DNA damage, a large number of DNA repair genes were upregulated. Interestingly, we also observed activation of the gluconeogenesis pathway and drug-efflux pumps in response to ATD-3169. Since NADPH depletion
is a most likely a consequence of redox cycling, it is possible that induction of genes involved in gluconeogenesis may be a mechanism to restore cellular NADPH levels. Similarly, upregulation of drug-efflux pumps is one of the predominant strategies exploited by bacteria to excrete redox-cycling drugs. Of note, the drug-efflux pumps induced by ATD-3169 were similarly regulated in response to a macrophage environment, anti-TB drugs, and redox agents, indicating their broader role in detoxification and maintaining cellular homeostasis [50,51]. While induction of many oxidative stress responsive genes was clearly evident, expression of sodA, ErG, and MSH biosynthetic genes was not affected on ATD-3169 treatment. Although unexpected, other expression studies have shown that many antioxidant systems including SOD are constitutively overexpressed in Mtb and that their expression remained uninfluenced in response to an oxidative or nitrosative insult [34,52]. Alternatively, it is likely that some posttranscriptional mechanisms are involved in regulating the levels of these antioxidants in Mtb. For example, levels of SodA are directly modulated by the activity of SecA2 secretion system in Mtb [53]. Similarly levels of ERG can be regulated by the transport system(s) involved in its secretion [54]. Lastly, changes in metabolite levels can alter levels of MSH. Since cysteine and methionine biosynthetic genes are differentially expressed in response to ATD-3169, MSH levels can be regulated by changes in the flux of cysteine or methionine on oxidative stress. Interestingly, homologues of many ATD-3169-inducible genes were similarly regulated by O2− and H2O2 in a SoxR- and/or OxyR-dependent manner in other bacteria, indicating the existence of novel redox-sensitive transcription factors in Mtb. In this context, some of the members of the Mtb whiB family (Fe-S cluster containing redox-sensitive transcription factors) appear to modulate the oxidative stress response in Mtb [55,56].

In summary, our biochemical, genetic, and expression experiments indicate that ATD-3169 enhances intramycobacterial ROS levels that trigger bactericidal activity by weakening redox homeostasis in Mtb. Due to the pressing need for developing novel interventional methodologies to counter Mtb, the effects of directly increasing endogenous ROS, in real time, on the intracellular redox potential of Mtb and its effects on mycobacterial physiology and survival were studied. We find that Mtb is uniquely sensitive to perturbation in redox homeostasis when it encounters elevated ROS within cells. Mtb secretes large amounts of major antioxidant enzymes such as catalase and superoxide dismutase to mitigate ROS encountered inside host phagocytes. Impaired survival of Mtb strains lacking either secretory antioxidants or major cell wall lipids in animal models of experimental tuberculosis further emphasizes the importance of mechanisms to alleviate toxicity associated with exogenous oxidants generated during infection. The evolutionary role of effectively countering exogenous ROS appears to have compromised Mtb’s ability to overcome endogenous ROS. We anticipate that our findings will play an important role in high content screening of small-molecule inhibitors of intrabacterial redox homeostasis and in delineating novel redox pathways involved in persistence, drug resistance, and pathogenesis of Mtb. Increased sensitivity displayed by Mtb toward elevated endogenous ROS may be relevant to other intracellular pathogens (e.g., Salmonella and Meningococcus) which mainly exploit secretory antioxidant enzymes/systems to effectively divert exogenous superoxide during infection. Thus, our findings may have relevance to several intracellular pathogens causing chronic and relapsing infections where persistence and drug tolerance pose challenges for treatment.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2015.03.008.

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