The Antioxidant Mimetic, MnTE-2-PyP, Reduces Intracellular Growth of *Mycobacterium abscessus*

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Mycobacterium abscessus is a rapidly growing environmental mycobacterium that can cause severe skin, soft tissue, and lung infections. *M. abscessus* grows inside macrophages, and these cells release a vast number of proinflammatory cytokines in response to infections. The metalloporphyrin, MnTE-2-PyP, is a broad antioxidant that reduces inflammatory cell signaling. Macrophage-like THP-1 cells were infected with *M. abscessus* in the presence or absence of MnTE-2-PyP. MnTE-2-PyP significantly decreased, in a dose-dependent manner, the number of *M. abscessus* organisms recovered from infected THP-1 cells 4 and 8 days after infection. Furthermore, when combined with clarithromycin, MnTE-2-PyP additively reduced the number of cells associated with *M. abscessus*. A mechanism of bacterial growth inhibition by MnTE-2-PyP was then elucidated. It was found that MnTE-2-PyP promoted the survival of infected THP-1 cells and increased fusion of *M. abscessus*-containing phagosomes with lysosomes.

Keywords: antioxidant mimetic; MnTE-2-PyP; *Mycobacterium abscessus*; THP-1 cells

Mycobacterium abscessus is a rapidly growing mycobacterium that is ubiquitously present in the soil and in both natural and potable water (1). Although most individuals are able to resist infection with *M. abscessus*, this organism can cause skin, soft tissue, and chronic lung infections that are often difficult to treat (1, 2). Patients with cystic fibrosis may be chronically infected with *M. abscessus*, and the presence of this bacterium has been associated with a decline in lung function (3). Immunocompromised individuals are also more susceptible to *M. abscessus* infection. These infections are typically difficult to eradicate because the organism has an inherently high level of drug resistance (1). Indeed, the only class of oral drugs that has *in vitro* activity against *M. abscessus* is the second generation macrolides, clarithromycin and azithromycin (1). However, resistance to these antibiotics has been observed clinically, which leaves few options for physicians treating this infection.

Very little research has been conducted on this particular mycobacterium, partly because *M. abscessus* has only recently been described as an independent species; for years it had been categorized under the *Mycobacterium chelonae* complex (1). Little is known about how the host controls *M. abscessus* infection. A recent study implies that having a healthy adaptive immune system, adequate T cell immunity in particular, is required for controlling *M. abscessus* infection (4). The adequate

production of IFN- γ and TNF- α also appears to be crucial in containing *M. abscessus* infections (4, 5).

It is known that *M. abscessus* can infect and multiply within human lung macrophages and fibroblasts (6). The bacteria have also been shown to grow in the lung cells of immune-compromised SCID mice (6). Unlike nonpathogenic bacteria, which succumb to death via oxidative burst and the acidic environment inside the lysosome, the *M. abscessus*-containing phagosome fails to fuse with lysosomes (7). Thus, the mycobacteria can grow and replicate intracellularly (6). Interestingly, *M. tuberculosis* can survive hostile oxidative environments of the macrophage by secreting superoxide dismutase, presumably to reduce the oxidative stress around the bacteria (8).

While a certain level of inflammatory response is necessary for host defense against an invading pathogen, excessive amounts may be deleterious to the host. For example, oxidative stress triggered by infections can result in damage to host tissues (9, 10). Recent *in vivo* studies show that reducing the oxidative stress associated with infections also reduces the accompanying inflammation. Mita and coworkers showed that metallothionein, a reactive oxygen species (ROS) scavenger, can protect against *Helicobacter pylori*-induced gastric lesions by reducing inflammatory cell activity (11). The ROS scavenger did not reduce bacterial load, but protected against gastric injury (11). Another study showed that influenza-infected mice treated with the metalloporphyrin antioxidant, MnTE-2-PyP, had reduced immunopathology and increased macrophage numbers in their airways (12). The investigators proposed that airways protected from oxidative stress had decreased cellular apoptosis, which led to the survival of the airway macrophages (12). Thus, in certain infections associated with an exuberant inflammatory response, attenuation of the inflammation may protect the host tissue from damage and is overall beneficial to the host.

The metalloporphyrin-based catalytic antioxidant, MnTE-2-PyP (chemical name: Manganese (III) *Meso*-Tetrakis-(*N*-Methylpyridinium-2-yl) porphyrin) has been shown to have broad antioxidant properties. Metalloporphyrins can scavenge superoxide, lipid peroxides, ONOO $\cdot$, and H $_2$ O $_2$ (13–17). MnTE-2-PyP inhibits inflammation and injury induced by radiation, bleomycin, or lipopolysaccharide exposure (18–20). MnTE-2-PyP also reduces NF- κ B signaling by changing the redox environment around this transcription factor (19). It is believed that MnTE-2-PyP is able to affect such a wide variety of different disease states through its ability to alter cell signaling (21).

To determine how altering the redox state may affect intracellular survival of *M. abscessus*, we examined the *in vitro* effects of MnTE-2-PyP on the infection of human macrophages with *M. abscessus*. We found that the antioxidant mimetic, MnTE-2-PyP, reduced the number of *M. abscessus* from infected macrophages. In arriving at a possible mechanism for the reduction in bacterial numbers, we found that MnTE-2-PyP promoted macrophage survival while enhancing bacterial-contained phagosomes to fuse with lysosomes.

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MATERIALS AND METHODS

THP-1 Cells

THP-1 cells, a human monocyte-derived cell line, were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cells were grown in suspension in 100-mm plates in RPMI medium supplemented with 10% (vol/vol) fetal bovine serum (FBS) and 1% (vol/vol) penicillin/streptomycin (Mediatech, Inc., Herndon, VA). Antibiotics were used in the growth medium of THP-1 cells to reduce contamination of the propagating cells. The cells were stimulated with 15 ng/ml of phorbol 12-myristate 13-acetate (PMA; Sigma, St. Louis, MO) for 24 hours to differentiate the cells into a macrophage-like phenotype. Before infection of *M. abscessus*, the cells were placed in RPMI medium with 10% FBS and no antibiotics.

Primary Human Monocytes

The study design and consent procedure were approved by the National Jewish Health Institutional Review Board. After informed consent was obtained, venous blood was collected in BD Vacutainer CPT tubes (cell preparation tubes with sodium citrate; Becton Dickinson, Franklin Lakes, NJ) and centrifuged at $1,800 \times g$ for 30 minutes at 25°C. The cloudy monolayer containing cells was removed and centrifuged at $300 \times g$ for 5 minutes at 25°C to pellet the cells. The cells were washed twice in PBS, counted, and plated in a 24-well plate ($\sim 3 \times 10^6$ cells/well). Cells were plated in RPMI medium supplemented with 10% FBS and colony-stimulating factor-1 (CSF-1, 10 ng/ml). Monocytes were allowed to adhere to the plate for approximately 6 hours. The cells that did not adhere were removed and the remaining cells ($\sim 0.5 \times 10^6$) were allowed to differentiate for 24 hours before use in an experiment.

M. abscessus Preparation

M. abscessus was prepared as previously described (22). *M. abscessus* was obtained from ATCC. The mycobacteria were grown to log phase at 37°C under agitation in 7H9 medium containing Middlebrook 7H9 broth (Fisher, Pittsburgh, PA) supplemented with 10% (vol/vol) ADC Enrichment Broth. The *M. abscessus* stock cultures were stored at a concentration of 1.0 McFarland turbidity standard (10^8 bacilli/ml) at -80°C .

Cell Infection with *M. abscessus* and *M. abscessus* Growth Assays

THP-1 cells (0.5×10^6 /well) were treated with PMA (15 ng/ml) containing medium. After 24 hours of treatment, the cells were adhered to the plate and the medium on the cells was replaced with fresh 10% FBS RPMI medium without antibiotics $\pm$ MnTE-2-PyP (0–60 μM). Primary human monocytes were pre-incubated with 10% FBS RPMI medium with CSF-1 $\pm$ MnTE-2-PyP (30 μM). After 1 hour of incubation, the cells were infected with 5×10^6 bacilli/well (for a multiplicity of infection [MOI] equal to 10) for 1 hour. All infectious medium was removed and replaced with fresh medium $\pm$ MnTE-2-PyP. In some cases, clarithromycin (0.5 $\mu\text{g/ml}$) was also added to the cells. In other cases, cells were not pre-incubated or infected in the presence of MnTE-2-PyP, but instead MnTE-2-PyP was added after infection for the remainder of the experiment. For the Day 0 time point, the cells were washed twice with a 1:1 solution of RPMI:PBS. Adherent cells were lysed with 250 μl of 0.25% SDS per well for approximately 5 minutes at 37°C. 7H9 plating broth (250 μl /well) was then added to neutralize the SDS. Serial dilutions of cell lysates were then plated in duplicate on Middlebrook 7H10 agar and bacteria were allowed to grow for 4 days at 37°C. The numbers of *M. abscessus* colony-forming units (CFU) on the plates were counted and the mean CFU calculated. Cells at Days 2, 4, and 8 after infection were harvested in the same manner as those at the Day 0 time point.

M. abscessus Growth on Agar in the Presence of MnTE-2-PyP

M. abscessus, 15 μl of 10^8 bacilli/ml, was spread evenly on 7H10 agar plates and allowed to dry. Sterilized filter wafers were soaked in RPMI medium containing 0–60 μM of MnTE-2-PyP and placed on the agar plates. The bacteria were allowed to grow for 4 days at 37°C and the plates were inspected for growth inhibition around the filter wafers.

Phagocytosis Assay

THP-1 cells (0.5×10^6) were stimulated with PMA for 24 hours. One hour before infection, the PMA medium was removed and fresh RPMI medium without antibiotics was added in the presence or absence of MnTE-2-PyP (0 or 30 μM) for 1 hour. Bacteria were fluorescently labeled by incubation in 1M Na_2CO_3 (pH 9.5) and 200 μM of fluorescein isothiocyanate (FITC) for 1 hour at 37°C, washed in PBS, and resuspended in RPMI medium. Cells were then incubated for 1 hour at 37°C with FITC-labeled *M. abscessus* (2.5×10^7) at a ratio of 50 bacteria to one THP-1 cell (MOI = 50). Because the majority of cells remained stuck to the plate, 250 μl of trypsin-versene with EDTA (Lonza, Basel, Switzerland) was added to each well to remove the cells. The cells were collected, cold $1 \times$ PBS (500 μl) was added to stop the phagocytosis, and the cells centrifuged at $300 \times g$ for 10 minutes at 4°C. The pelleted cells were resuspended in a trypan blue solution (200 $\mu\text{g/ml}$ in PBS) to quench the fluorescence of bacteria that were attached to the surface of the THP-1 cells but not phagocytosed. The cells were then fixed in 4% paraformaldehyde, resuspended in $1 \times$ PBS, and subjected to analysis in a fluorescence-activated cell sorter (FACS) (FACScalibur; Becton Dickinson Immunometry Systems, San Jose, CA). FACS was used to measure intracellular fluorescence in the THP-1 cells. Light transmission data from cells passing through a laser beam generated by a single air-cooled, argon ion laser (488 nm excitation) were collected by a forward scatter (FSC) detector and side scatter (SSC) detector and a fluorescence detector. FSC indicates cell size and SSC indicates the granularity and viability of each cell. Fluorescence data, which indicated internalized bacteria, were collected on a log scale, with green fluorescence measured at 530 nm. Data from 10,000 events (cells) per condition were collected and analyzed with CellQuest software (Becton Dickinson Immunometry Systems). To eliminate background fluorescence of the THP-1 cells, uninfected PMA stimulated THP-1 cells were run as a control and made equal to 1.0 for each experiment conducted.

Measurement of Superoxide and Nitric Oxide Production

Dihydroethidium (DHE) fluorescent probe (Invitrogen/Molecular Probes, Eugene, OR) was used to detect the presence of superoxide. Briefly, THP-1 cells were washed three times with Hanks' buffered saline solution (HBSS). The cells were infected with *M. abscessus*, at an MOI = 10 for a range of time (5–90 min). The cells were then treated for 15 minutes with DHE (10 μM), placed on ice, and then analyzed by FACS using an excitation of 488 nm and wavelength emission of 585 nm.

Nitric oxide concentrations were measured by using a Sievers Model 280 Nitric Oxide Analyzer (Boulder, CO) with the help of Dr. Garry Buettner at the University of Iowa, as previously described (23). Briefly, PMA-treated THP-1 cells were pre-treated in RPMI medium $\pm$ MnTE-2-PyP (30 μM) for 1 hour. THP-1 cells were then infected for 1 to 24 hours. The cell supernatants were collected at 0, 1, and 24 hours after infection and nitrite levels were analyzed (23).

NF- κ B Electromobility Shift Assay

PMA-treated THP-1 cells were pre-incubated in RPMI medium $\pm$ MnTE-2-PyP (30 μM). Cells were infected with *M. abscessus* for 0, 0.5, 1, 3, or 6 hours and in some cases for 1 and 2 days. The nuclei were isolated from the cells using the NXTRACT CellLytic NUCLEAR Extraction Kit (Sigma). Briefly, cells were washed in cold $1 \times$ PBS and scraped off the plate in 1 ml of cold $1 \times$ PBS. The cells were centrifuged at $300 \times g$ for 5 min at 4°C and the supernatant discarded. $1 \times$ lysis buffer (100 μl) was added to each cell pellet, mixed up and down, and incubated on ice for 10 minutes. The cell lysate was then passed through a 26-g needle 10 times. The samples were centrifuged at $3,000 \times g$ for 10 minutes at 4°C. The supernatant was discarded and the pellet resuspended with extraction buffer and incubated on ice for 10 minutes. The samples were spun at $18,000 \times g$ for 10 minutes and the supernatant (nuclear extract) was collected and stored at -80°C . The protein concentrations of the nuclear extracts were determined by using a bicinchoninic acid (BCA) kit (Pierce, Rockford, IL). The NF- κ B probe was labeled with ^{32}P -ATP according to manufacturer's directions (Gel Shift Assay Core System; Promega, Madison, WI). The nuclear extracts were then incubated with the radioactive NF- κ B probe for 20 minutes at room temperature before the proteins were separated on a 5% TBE gel; in some cases cold NF- κ B

oligo was incubated with samples for a negative control. The gel was dried and exposed to radiosensitive film. Densitometric analysis was performed on the films to semi-quantify NF- κ B binding (Quantity One; Bio-Rad, Hercules, CA).

Measurement of Cytokines

PMA-stimulated THP-1 cells were pretreated for 1 hour in RPMI medium $\pm$ MnTE-2-PyP (30 μ M) and then infected with *M. abscessus* (MOI = 10). Supernatants were collected at 0, 6, 12, 24, and 48 hours after infection. TNF- α was measured from the collected supernatants using a Quantikine human TNF- α ELISA kit (R&D Systems, Minneapolis, MN) according to manufacturer's instructions. The collected supernatants were also assayed for IL-8 concentration by electrochemiluminescence (ECL) as previously described (24).

Cell Viability Assay

PMA-stimulated THP-1 cells were pre-treated for 1 hour in RPMI medium $\pm$ MnTE-2-PyP (30 μ M) and then infected with *M. abscessus* for 1 hour. The infectious medium was replaced and the appropriate fresh medium was then added. The cells were incubated for 2 days and then harvested. Some cells were incubated with trypan blue, and cells that excluded the dye and dyed cells were separately counted. Other cells were washed in 1 $\times$ PBS and fixed in 4% paraformaldehyde for 30 minutes. The THP-1 cells were then TUNEL stained using the In Situ Cell Death Detection Kit, Fluorescein (Roche, Mannheim, Germany). Briefly, the cells were permeabilized on ice for 2 minutes, washed with 1 $\times$ PBS, and then incubated in label/enzyme solution for 1 hour at 37°C. The cells were washed twice with 1 $\times$ PBS and then analyzed by FACS as described above.

Co-Localization of *M. abscessus* and Lysosomes

PMA-stimulated THP-1 cells were grown on a glass chamber slide and were pretreated for 1 hour in RPMI medium $\pm$ MnTE-2-PyP (30 μ M) and then infected with Syto 62-labeled *M. abscessus*. The bacteria were labeled with Syto 62 according to manufacturer's instruction (Invitrogen/Molecular Probes). Cells were infected for 4 or 6 hours. Two hours before the infection was complete, LysoTracker Red DND-99 (50 nM) was added to the cells to label the lysosomes (Invitrogen/Molecular Probes). After infection was complete, the medium was removed and fresh medium was added back to the cells. The cells were then viewed at $\times 40$ under oil immersion with an inverted Zeiss 200M microscope with a 175 watt xenon lamp in a DG4 Sutter instruments lamp housing (Carl Zeiss, Thornwood, CA). The Cy3 filter was used to detect the lysosomal staining and the Cy5 filter was used to detect the bacteria. Fifteen random pictures were taken per well for each experiment. For each picture taken, the number of bacteria containing cells was counted as well as the number of cells containing bacteria that overlapped with a lysosome. This analysis was conducted for each random picture and was counted by two individuals. A sum of all the numbers tallied for the 15 pictures was made and the percentage of cells containing co-localized bacteria from cell-containing bacteria alone was then calculated.

Statistics

All data were derived from at least three independent experiments. We calculated all fluorescence data as the total number of fluorescent cells and normalized the data by making the control condition equal to 1. The data were analyzed by a one-way ANOVA followed by a Student-Newman-Keuls test or a paired *t* test.

RESULTS

MnTE-2-PyP Reduces the Number of Intracellular *M. abscessus* Recovered from Macrophages

To determine the effect of the antioxidant mimetic, MnTE-2-PyP, on *M. abscessus* growth, THP-1 cells were infected in the presence of increasing amounts of MnTE-2-PyP (0–60 μ M). As shown in Figure 1, on Day 0 (1 h after infection) there was no difference in the amount of bacteria harvested from the THP-1 cells with or without MnTE-2-PyP. However, MnTE-2-PyP (15,

Figure 1. MnTE-2-PyP reduces the number of intracellular *Mycobacterium abscessus*. THP-1 cells were pre-incubated for 1 hour in RPMI medium or medium containing 15, 30, or 60 μ M MnTE-2-PyP before infection of *M. abscessus*. After 1 hour of infection, the cells were lysed and *M. abscessus* was cultured on solid agar (Day 0). For Days 4 and 8 conditions, the cells were incubated in their respective medium for an additional 4 or 8 days followed by bacterial culturing. Cells incubated in MnTE-2-PyP (15, 30, or 60 μ M) all showed significantly reduced bacterial numbers on Days 4 and 8 after infection as compared with the control condition (0 μ M MnTE-2-PyP). All data represent mean $\pm$ SE, *n* = 4; *significant change from control (*P* < 0.05).

30 and 60 μ M) significantly inhibited the number of *M. abscessus* colonies recovered at both 4 and 8 days after infection as compared with the infected alone control (0 μ M MnTE-2-PyP; Figure 1). In the presence of MnTE-2-PyP, the average reduction in growth was 74% and 72% on Days 4 and 8, respectively. We also investigated if MnTE-2-PyP given after infection provided any therapeutic beneficial effect. We found no significant reduction of *M. abscessus* 4 days after infection ($\sim 10.0\%$ reduction $\pm 11.4\%$). However, at 8 days after infection, there was significant reduction in the number of cell-associated bacteria recovered ($\sim 60.1\%$ reduction $\pm 8.9\%$, *P* < 0.05).

Since all of these experiments were conducted in a monocyte cell line, we wanted to determine the effects of MnTE-2-PyP on the growth of *M. abscessus* in primary human monocytes. Therefore, we conducted *M. abscessus* growth assays in primary monocyte cells and found that MnTE-2-PyP significantly inhibited the number of bacteria recovered from these primary cells at 8 days after infection as well (55.9% reduction $\pm 5\%$, *P* < 0.05).

Because clarithromycin is one of the most effective drugs used to treat *M. abscessus* infections, we next determined whether MnTE-2-PyP could enhance the *in vitro* activity of clarithromycin. THP-1 cells were infected with *M. abscessus* in the presence or absence of MnTE-2-PyP (30 μ M) for 1 hour. The infectious medium was removed and in some cases clarithromycin (0.5 μ g/ml) was added to the replacement medium. As shown in Figure 2, clarithromycin alone significantly reduced growth of *M. abscessus* on Day 2 (85% reduction $\pm 5.6\%$, *P* < 0.05) and Day 4 after infection (78% reduction $\pm 6.4\%$, *P* < 0.05) as compared with controls. In the presence of both clarithromycin and MnTE-2-PyP, intracellular bacterial numbers were further reduced as compared with the clarithromycin alone condition (Day 2: 96% reduction $\pm 1.3\%$, *P* < 0.05; Day 4: 93% reduction $\pm 1.4\%$, *P* < 0.05; Figure 2). Thus, MnTE-2-PyP acted additively with clarithromycin to inhibit growth of *M. abscessus* intracellularly.

Figure 2. MnTE-2-PyP and clarithromycin (CAM) additively reduce the number of intracellular *M. abscessus*. THP-1 cells were pre-incubated for 1 hour in RPMI medium $\pm$ 30 μ M MnTE-2-PyP before infection of *M. abscessus*. After 1 hour of infection, the cells were lysed and *M. abscessus* was cultured on solid agar (Day 0). In some cases, clarithromycin (0.5 μ g/ml) was then added. For Days 2 and 4 conditions, the cells were incubated in their respective medium for an additional 2 or 4 days, followed by bacterial culturing. As expected, MnTE-2-PyP alone and CAM alone significantly reduced bacterial growth on Days 2 and 4 after infection when compared with control. The combination of MnTE-2-PyP and CAM further significantly reduced bacterial growth when compared with MnTE-2-PyP alone or CAM alone conditions. All data represent mean $\pm$ SE, $n = 7$; *significant change from control ($P < 0.05$), #significant change from CAM alone.

MnTE-2-PyP Is Not Directly Antimicrobial

We next investigated the mechanism by which MnTE-2-PyP inhibited the intracellular growth of *M. abscessus*. To determine if MnTE-2-PyP has direct antimicrobial or toxic effects against *M. abscessus*, bacteria were plated on agar and filter papers soaked in different concentrations of the mimetic (0–60 μ M) were placed on the bacterial-seeded agar plate. After 4 days, the bacterial growth pattern was observed. Growth inhibition rings were not observed around the filter papers soaked in MnTE-2-PyP, indicating that MnTE-2-PyP is not directly toxic to *M. abscessus* (data not shown).

MnTE-2-PyP Does Not Affect Phagocytosis of *M. abscessus*

Since MnTE-2-PyP did not have any direct effects on the growth of *M. abscessus*, we hypothesized that the mimetic was acting on the THP-1 cell to inhibit the number of intracellular bacteria. Although Figure 1 (Day 0 time points) would suggest that MnTE-2-PyP is not inhibiting the phagocytosis of *M. abscessus*, we corroborated this with additional experiments using fluorescein-labeled bacteria. PMA-stimulated THP-1 cells were infected with FITC-labeled *M. abscessus* for 1 hour in the presence or absence of MnTE-2-PyP (30 μ M). As shown in Figure 3, there was approximately 8-fold induction in intracellular fluorescence detected when cells were incubated with FITC-labeled bacteria as compared with uninfected cells, indicating that *M. abscessus* are readily phagocytosed by THP-1 cells. In the presence of MnTE-2-PyP, THP-1 cells phagocytosed similar amount of fluorescent bacteria (Figure 3). We conclude from these studies that MnTE-2-PyP did not inhibit phagocytosis of *M. abscessus* by macrophages.

Figure 3. MnTE-2-PyP does not inhibit phagocytosis of *M. abscessus* by THP-1 cells. THP-1 cells were pre-incubated for 1 hour in RPMI medium or medium containing MnTE-2-PyP (30 μ M) before infection of FITC-labeled *M. abscessus*. After 1 hour of infection, the cells were fixed and intracellular fluorescence was analyzed via fluorescence-activated cell sorter (FACS). THP-1 cells readily phagocytose *M. abscessus* as indicated by the increased intracellular fluorescence. In the presence of MnTE-2-PyP, there was no significant difference in the level of phagocytosis of *M. abscessus*. The control mean fluorescence was normalized to 1.0 for each experiment. All data represent mean $\pm$ SE, $n = 3$; *significant change from control ($P < 0.05$).

MnTE-2-PyP Does Not Reduce Intracellular Growth of *M. abscessus* by Altering Superoxide or Nitric Oxide Cellular Levels

MnTE-2-PyP has broad antioxidant properties and scavenges a variety of free radicals. It has been shown that another mycobacterium species, *M. tuberculosis*, can withstand oxidative environments inside macrophages by up-regulating redox-sensing proteins (8). *M. tuberculosis* is somewhat susceptible to nitric oxide production; when nitric oxide production is blocked, certain strains of *M. tuberculosis* displayed enhanced intracellular growth (25). Little is known about the ability of *M. abscessus* to withstand the oxidative environment of a stimulated macrophage. Therefore, we measured superoxide and nitric oxide production by THP-1 cells infected with *M. abscessus* in the presence or absence of MnTE-2-PyP (Figure 4). *M. abscessus* infection alone produced a modest induction of superoxide, peaking 15 minutes after infection (Figure 4A). In the presence of MnTE-2-PyP, superoxide production was reduced when compared with infected alone cells (Figure 4A). THP-1 cells infected with *M. abscessus* produced increased concentrations of nitrite, a metabolite of nitric oxide, as the infection progressed (Figure 4B). The addition of MnTE-2-PyP had no effect on nitrite production (Figure 4B). We conclude from these experiments that it is unlikely that MnTE-2-PyP reduces the number of intracellular *M. abscessus* by altering overall superoxide or nitric oxide production.

MnTE-2-PyP Only Reduces NF- κ B Binding at Early Times of Infection

MnTE-2-PyP has previously been shown to inhibit NF- κ B signaling and, as a result, reduce inflammation (19). NF- κ B activity is increased early after *M. tuberculosis* infection (0.5–6 h after infection), and this increased level correlates with higher numbers of intracellular *M. tuberculosis* (26). Therefore, we hypothesized that MnTE-2-PyP may reduce *M. abscessus* growth by inhibiting NF- κ B activity. PMA-stimulated cells were pre-treated for 1 hour with or without MnTE-2-PyP (30 μ M) and then infected with *M. abscessus* for 0.5 to 6 hours or for 1 to 2 days.

Figure 4. The effects of MnTE-2-PyP on superoxide and nitric oxide production by *M. abscessus*-infected THP-1 cells. (A) Cells were stained with DHE to detect superoxide production. THP-1 cells were infected for various times $\pm$ MnTE-2-PyP and then incubated with DHE. The cellular fluorescence was then analyzed via FACS. MnTE-2-PyP reduced the overall production of superoxide in the infected THP-1 cell. These data are representative of two other independent experiments. (B) PMA-treated cells were incubated in RPMI or medium containing 30 μ M of MnTE-2-PyP for 1 hour. The cells were infected with *M. abscessus* for 0 to 24 hours. The supernatant from these different infection periods were analyzed for nitrite levels. MnTE-2-PyP did not affect the overall production of nitrite by infected THP-1 cells. All data represent mean $\pm$ SE, $n = 3$.

Nuclear fractions were isolated and EMSAs were performed on the nuclear fractions using a 32 P-labeled oligonucleotide that corresponds to the consensus binding site for activated NF- κ B. THP-1 cells infected with *M. abscessus* alone caused a significant increase in NF- κ B binding, with the highest binding occurring approximately 3 hours after infection (Figures 5A and 5B). Cells infected with *M. abscessus* in the presence of MnTE-2-PyP displayed reduced NF- κ B binding throughout the 6 h period as compared with infected alone cells (Figures 5A and 5B). At 6 hours after infection, MnTE-2-PyP significantly reduced the amount of NF- κ B binding as compared with the infected alone groups (Figure 5B). However, when cells were analyzed 1 and 2 days after infection, no difference in NF- κ B binding was observed between infected alone cells and cells infected in the presence of MnTE-2-PyP (Figure 5C).

MnTE-2-PyP Does Not Alter the Production of TNF- α and IL-8 during Infection

NF- κ B regulates the release of several cytokines, including TNF- α and IL-8. Therefore, PMA-stimulated THP-1 cells were pre-treated for 1 hour with or without MnTE-2-PyP (30 μ M) and then infected with *M. abscessus* for 0 to 48 hours. TNF- α and IL-8 levels were measured in the collected supernatant (Figure 6). Infection with *M. abscessus* caused an increase in TNF- α production, peaking at approximately 12 to 24 hours after infection (Figure 6A). MnTE-2-PyP had negligible effect on TNF- α pro-

Figure 5. The effects of MnTE-2-PyP on NF- κ B activity in *M. abscessus* infected THP-1 cells. PMA-treated cells were incubated in RPMI or medium containing 30 μ M of MnTE-2-PyP for 1 hour. The cells were infected with *M. abscessus* for 0 to 6 hours or 1 to 2 days. Nuclear extracts were made and electrophoretic mobility shift assays (EMSA) were performed for NF- κ B. (A) Representative NF- κ B EMSA. *M. abscessus* infection caused an increase in NF- κ B activation at 0.5 to 6 hours after infection and MnTE-2-PyP reduced the NF- κ B activation. Controls for the NF- κ B EMSA assay are also shown. The positive control contained stimulated HeLa cells obtained from Promega and the negative control contained cold NF- κ B oligo added to the EMSA reaction. (B) Densitometric analysis of NF- κ B EMSA blots (p50-p65 band) from 0.5 to 6 hours after infection. EMSA blots are from four independent experiments. The mean OD of the reactive bands in the control group was normalized to 1.0. *Significant difference from the control, 0 time point ($P < 0.05$); #significant difference from cells treated with MnTE-2-PyP compared with the control condition ($P < 0.05$). (C) EMSA blots from 1 or 2 days after infection. NF- κ B activity was not reduced with the addition of MnTE-2-PyP at Days 1 or 2 after infection. EMSA blots are representative of three independent experiments.

Figure 6. MnTE-2-PyP does not affect the production of TNF- α and IL-8 from infected THP-1 cells. PMA-treated cells were incubated in RPMI or medium containing 30 μ M of MnTE-2-PyP for 1 hour. The cells were infected with *M. abscessus* for 0 to 48 hours and cell supernatants were collected. TNF- α or IL-8 concentrations were then measured. (A) TNF- α . *M. abscessus* infection caused an increase in TNF- α production, peaking around 12 hours after infection. MnTE-2-PyP had little effect on the release of TNF- α . (B) IL-8. THP-1 cells infected with *M. abscessus* produced increasing amounts of IL-8. In the presence of MnTE-2-PyP, IL-8 production was slightly reduced; however, this effect was not significant. All data represent mean $\pm$ SE, $n = 4$.

duction (Figure 6A). *M. abscessus* infection also caused an increase in IL-8 production from THP-1 cells that was observed throughout the course of the experiment (Figure 6B). MnTE-2-PyP caused a slight decrease in IL-8 release from THP-1 cells at 12 to 48 hours after infection as compared with infected alone cells (Figure 6B). However, this observed trend was not statistically significant. Thus, MnTE-2-PyP had little effect on TNF- α or IL-8 production from *M. abscessus*-infected THP-1 cells.

MnTE-2-PyP Increases the Survival of *M. abscessus*-Infected THP-1 Cells

Throughout the course of an *M. abscessus* growth assay (0–8 d after infection), it was observed via microscopy that cells infected in the presence of MnTE-2-PyP appeared more viable than cells infected alone. Therefore, THP-1 cell viability was further examined during infection. PMA-stimulated THP-1 cells were pre-incubated for 1 hour with or without MnTE-2-PyP (30 μ M) and then infected for 1 hour. A trypan blue assay was conducted on cells 2 days after infection. Cells infected with *M. abscessus* alone were $28.9\% \pm 2.4\%$ viable. Cells infected with *M. abscessus* in the presence of MnTE-2-PyP showed a significant increase in viability at $46.8\% \pm 2.4\%$ as compared with infected alone cells. A fluorescent TUNEL stain assay was used to further confirm the results of the trypan blue staining. Cells infected with or without MnTE-2-PyP for 2 days were TUNEL-stained and analyzed via FACS. Cells infected with *M. abscessus* alone displayed approximately 20-fold induction in positively TUNEL-stained cells when compared with control cells (Figure 7). This indicates that by 2 days of infection, there is a relatively large increase in cell death. However, in the presence of MnTE-2-

Figure 7. *M. abscessus* promotes THP-1 cell death at 2 days after infection, and MnTE-2-PyP reduces the cell death triggered by the bacteria. THP-1 cells were pre-incubated for 1 hour in RPMI medium or medium containing 30 μ M MnTE-2-PyP before infection with *M. abscessus*. The cells were cultured for 2 days, TUNEL stained and analyzed by flow cytometry. A significant increase in cell death was observed in THP-1 cells infected with *M. abscessus* alone. MnTE-2-PyP significantly reduced this increased cell death. The control mean fluorescence was normalized to 1.0 for each experiment. All data are mean $\pm$ SE, $n = 3$; *significant change from control, #significant change from *M. abscessus* alone condition ($P < 0.05$).

PyP, the THP-1 infected cells displayed only about 5-fold increase in TUNEL-stained cells as compared with controls (Figure 7). Thus, MnTE-2-PyP protected infected THP-1 cells from death.

MnTE-2-PyP Enhances Phagosome-Lysosome Fusion

We hypothesized that THP-1 cells infected in the presence of MnTE-2-PyP were surviving longer, allowing the macrophages longer time to kill the phagocytosed bacteria. One mechanism in which mycobacteria contained within phagosomes are killed is by promoting phagosome-lysosome fusion. The lysosome destroys phagocytosed bacteria in part because of its highly acidic environment. In instances in which *M. abscessus* infection becomes established, the bacteria are able to survive within macrophages and fibroblast cells by residing in phagosomes that never fuse with lysosomes. Therefore, we determined whether MnTE-2-PyP could enhance phagosome-lysosome fusion. PMA-stimulated THP-1 cells were pre-incubated for 1 hour with or without MnTE-2-PyP (30 μ M) and then infected for 4 or 6 hours with Syto 62-labeled *M. abscessus*. Two hours before the completion of infection, LysoTracker was added to the cells to label the cells' lysosomes. The cells were then viewed under a microscope and the amount of co-localization of bacteria with lysosomes was calculated (Figure 8). At 4 hours after infection there was more co-localization observed in the presence of MnTE-2-PyP; however, this difference was not significant. By 6 hours after infection, there is a significant increase (~ 2 -fold) in the amount of bacteria co-localizing with lysosomes in cells incubated in MnTE-2-PyP as compared with control medium (Figure 8C). We conclude from these experiments that MnTE-2-PyP reduces intracellular *M. abscessus* numbers, at least in part, by enhancing phagosome-lysosome fusion.

DISCUSSION

In the present study, we investigated the effects of the antioxidant mimetic, MnTE-2-PyP, on the growth of *M. abscessus* in human macrophages. We found that MnTE-2-PyP significantly inhibited the number of intracellular *M. abscessus* in both THP-1 cells and primary human monocytes. Intracellular bacterial numbers were reduced by the presence of MnTE-2-PyP whether

Figure 8. MnTE-2-PyP causes increased co-localization of *M. abscessus* with the lysosome. THP-1 cells were pre-incubated for 1 hour in RPMI medium or medium containing 30 μ M MnTE-2-PyP before infection of Syto 62-labeled *M. abscessus*. The cells were infected for 4 or 6 hours and then stained with LysoTracker to detect the lysosomes. (A) Representative control micrograph of macrophages infected for 6 hours without MnTE-2-PyP; very little co-localization of bacteria with lysosomes was observed. *Cells containing bacteria that are not co-localized to the lysosome. (B) Representative micrograph of cells infected for 6 hours in the presence of MnTE-2-PyP; co-localization of bacteria with lysosomes was enhanced. *Cells containing bacteria that are not co-localized to the lysosome. The arrows indicate cells containing bacteria that are co-localized to lysosomes. (C) Quantitative analysis of all micrograph images at 4 and 6 hours after infection. At 6 hours after infection, there was a significant increase in bacteria co-localization with the lysosome in the presence of MnTE-2-PyP as compared with control conditions. All data are mean $\pm$ SE, $n = 3$; [#]significant change from the control ($P < 0.05$).

it was given before or after infection; however, a greater reduction was observed when MnTE-2-PyP was administered before the infection. Since *M. abscessus* infections are very difficult to treat, with only a few effective antibiotics available, we next determined if MnTE-2-PyP could be used in combination with an effective antibiotic. We chose clarithromycin because it is one of the most effective agents against *M. abscessus* infections (1). MnTE-2-PyP reduced the recovery of cell-

associated *M. abscessus* and had an additive effect with clarithromycin. This novel finding is promising because there are very limited options for patients whose *M. abscessus* develops multi-drug resistance.

A series of experiments were then conducted to determine the mechanism by which the antioxidant mimetic reduced the number of intracellular *M. abscessus*. We first examined if MnTE-2-PyP was directly toxic to the bacteria and found that MnTE-2-PyP did not have direct antimicrobial activity. From this finding, we decided to examine the effects of MnTE-2-PyP on the infected macrophage.

One way in which MnTE-2-PyP may reduce bacterial numbers inside THP-1 cells is by inhibiting the phagocytosis of the *M. abscessus*. A large increase in intracellular fluorescence was observed with cells incubated with fluorescent *M. abscessus* both with and without MnTE-2-PyP. There did appear to be slightly less bacteria phagocytosed in the presence of MnTE-2-PyP when compared with the *M. abscessus* infected alone condition; however, this difference was not statistically significant. Because there was such a large amount of bacteria phagocytosed in the presence of MnTE-2-PyP, we concluded that phagocytosis inhibition was not the mechanism used by the antioxidant mimetic to reduce intracellular numbers of *M. abscessus*.

Another mechanism used by macrophages to kill ingested bacteria is to create an oxidative burst. Although free radical production has been shown to have minimal effects on the survival of *M. tuberculosis*, there are little data on the effects of superoxide and nitric oxide production on *M. abscessus* viability (8, 25). We found that THP-1 cells produced little superoxide in response to the phagocytosed *M. abscessus*. In the presence of MnTE-2-PyP, the overall production of superoxide was reduced. These findings are not surprising, since MnTE-2-PyP is a superoxide scavenger. The overall reduced superoxide production may contribute to the enhanced macrophage survival, but not to the increased bacterial killing. However, it must be taken into consideration that the data are based on superoxide production from the entire macrophage. Therefore, there could be localized oxidative stress differences in different compartments inside the macrophage that our assay was not sensitive enough to detect. These localized differences in redox state could alter cellular activity. We also showed that nitrite levels were increased in macrophages infected with *M. abscessus* in the presence or absence of MnTE-2-PyP. Thus, nitric oxide production does not appear to affect *M. abscessus* viability inside THP-1 cells, and MnTE-2-PyP is not a nitric oxide scavenger. From these data, we conclude that free radical production has very little effect on bacterial viability in THP-1 cells and MnTE-2-PyP is not reducing growth of the bacteria by altering superoxide or nitric oxide production directly.

MnTE-2-PyP has been shown to alter cell signaling and reduce inflammation by reducing NF- κ B activity (19). Piganelli and coworkers showed that MnTE-2-PyP inhibits T cell proliferation and that LPS-stimulated macrophages treated with MnTE-2-PyP inhibit TNF- α and NADPH release of superoxide (21). This group later showed that MnTE-2-PyP was mediating the aforementioned anti-inflammatory properties by inhibiting NF- κ B activation (19). In particular, they showed that MnTE-2-PyP oxidizes the p50 subunit of NF- κ B, which results in a conformational change that renders the subunit unable to bind to DNA (19). Although little is known about the effects of *M. abscessus* infection on host cell signaling, much is known about the host's response to *M. tuberculosis*. For example, *M. tuberculosis* induces NF- κ B activation in macrophage cells (26). This increased NF- κ B activity is thought to prevent the host cell from undergoing apoptosis and allowing the infected cell to harbor the growing bacteria longer (26). Therefore, we investigated whether MnTE-

2-PyP was reducing NF- κ B activity, which could account for the reduced bacterial growth observed in THP-1 cells. We found that NF- κ B activity is increased early (~ 3 h after infection) with *M. abscessus* infection of THP-1 cells. We also found that MnTE-2-PyP significantly reduced the activation of NF- κ B in infected THP-1 cells during this time. However, later during infection, specifically Days 1 and 2 after infection, MnTE-2-PyP no longer inhibited NF- κ B activity. To confirm these findings, we measured cytokine levels from infected THP-1 cells with and without MnTE-2-PyP. We found that TNF- α and IL-8 production were increased with *M. abscessus* infection (0–2 d after infection). MnTE-2-PyP did not affect cytokine production from infected macrophages. These data illustrate that overall cytokine release was not altered by MnTE-2-PyP and, thus, indicated to us that MnTE-2-PyP does not reduce bacterial growth by altering NF- κ B cell signaling.

We next investigated THP-1 cell viability after *M. abscessus* infection. While conducting the bacterial growth assays, it was noticed that there were more dying or dead cells in cells infected with *M. abscessus* alone as compared with cells infected in the presence of MnTE-2-PyP. To quantify this observation, a TUNEL assay was performed. We found a large increase in TUNEL-positively stained cells when THP-1 cells were infected for 2 days. In the presence of MnTE-2-PyP, the number of TUNEL-stained cells was significantly lower when compared with bacteria alone infected cells. These data indicate that in the presence of MnTE-2-PyP, the infected THP-1 cells live longer and are healthier as compared with cells infected alone. TUNEL staining is supposed to specifically label DNA strand breaks associated with apoptosis; however, extensive DNA fragmentation may occur in certain forms of necrosis (manufacturer's notes). Therefore, we cannot distinguish between apoptosis and necrosis in our study.

We hypothesized that one reason MnTE-2-PyP may keep the cells alive longer is that the phagocytosed bacteria are being killed in the macrophage. It is known that *M. abscessus*, like other mycobacteria, enter the host cell and never fuse with the lysosome (6, 7). We found that MnTE-2-PyP causes the bacteria to colocalize to the lysosome. Therefore, it appears that MnTE-2-PyP is reducing the number of phagocytosed bacteria by enhancing phagosome-lysosome fusion. How MnTE-2-PyP induces phagosome-lysosome fusion is not known. It is possible that MnTE-2-PyP is able to change the redox environment or cause a cell-signaling event inside the macrophage that triggers the fusion of the phagosome and lysosome. It is known that calcium release and actin polymerization are needed for phagosome-lysosome fusion (27). Both of these events can be controlled by changes in redox environment (28, 29). However, at this time more research needs to be conducted to fully understand how MnTE-2-PyP is causing *M. abscessus* to be localized to the lysosome.

In the present study, we showed that the antioxidant mimetic, MnTE-2-PyP, reduced the intracellular number of *M. abscessus* by approximately 70% when compared with control. We also demonstrated that the growth-inhibiting effect of MnTE-2-PyP on intracellular *M. abscessus* was additive with the antibiotic clarithromycin. We showed that macrophages infected in the presence of MnTE-2-PyP survived longer and had a healthier morphology as compared with cells infected alone. We also determined that cells infected in the presence of MnTE-2-PyP most likely killed the internalized bacteria by promoting lysosome-phagosome fusion. Directing phagocytosed *M. abscessus* to lysosomes allows for clearance of the bacteria while maintaining host cell integrity.

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