EFFECTS OF (1-DECYL)TRIPHENYLPHOSPHONIUM ON *MYCOBACTERIUM TUBERCULOSIS* INFECTIONS *IN VITRO* AND *IN VIVO*

by

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(Under the Direction of Kaori Sakamoto)

ABSTRACT

Mycobacterium tuberculosis is the greatest cause of infectious disease by a single agent, and expresses multiple virulence factors to avoid destruction and maintain residence inside the host cell to survive, replicate, and avoid immune system detection. *M. tuberculosis* is difficult to target with antibiotics, and many strains are emerging as multidrug-resistant. (1-Decyl) triphenylphosphonium (dTPP) has been used in many previous studies, and the purpose of this study was to investigate antimycobacterial efficacy and cytotoxic effects of dTPP in murine bone marrow-derived macrophages. Bacterial load was significantly reduced by dTPP treatment, but the compound also exerted significant cytotoxic effects *in vitro.* Efficacy and safety of dTPP usage *in vivo* also were tested using a murine model to determine synergistic effects when combined with a known antimycobacterial agent, Rifampin. Within 24-hours of treatment with dTPP, all mice were dead or presented with neurologic signs upon physical examination. This is the first study reporting adverse effects of dTPP.

INDEX WORDS: *Mycobacterium tuberculosis*, (1-Decyl)triphenylphosphonium (dTPP), Tuberculosis

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Tuberculosis is a serious, life-threatening disease with a complex pathogenesis and long history in much of the world and the United States. The World Health Organization reports an estimated 9.6 million people developed tuberculosis in the year 2014, with 1.5 million people dying from the disease.[17] Due to the disease prevalence and mortality, *Mycobacterium tuberculosis*, the primary causative agent of tuberculosis in humans, is now the greatest cause of infectious disease by a single agent.[15,17] Furthermore, multidrug-resistance of *M. tuberculosis* is a major concern, with 3.3% of new cases and 20% of previously-treated cases demonstrating resistance to multiple antimycobacterial agents, and 9.7% of cases were reported to be extensively drug-resistant, which is a recorded 0.1% increase from the report from 2014.[17] The greatest risk factor for contracting tuberculosis or reactivating latent tuberculosis is immune system status as affected by poverty/homelessness, overcrowding, alcoholism/drug abuse, smoking, HIV co-infection, diabetes, and use of immunosuppressive agents.[13]

Mycobacteria are acid-fast, Gram-positive bacilli, which appear as beaded, branching rods approximately 1 to 4 μm in length.[41] These organisms are slow-growing, non-motile, and non-spore-forming bacteria with a cell wall composed of mycolic acids, which makes them hydrophobic, resistant to environmental stressors, and difficult to target with antibiotics.[9,41] The *M. tuberculosis* complex is composed of several *Mycobacterium spp.* reported to cause tuberculosis in different hosts with different phenotypic profiles, and is thought to have evolved

approximately three million years ago from an ancient progenitor named *Mycobacterium prototuberculosis.*[21] *M. tuberculosis* bacilli are inhaled via aerosolized droplets expelled by an infected person; one droplet can contain up to 400 bacilli, and the infectious dose of *M. tuberculosis* is reported to be as low as 1 to 4 bacilli.[4]

Bacilli interact with alveolar macrophage surface receptors to induce phagocytosis, and while phagocytosis by macrophages usually proceeds to destruction of the invading microbe, *M. tuberculosis* expresses multiple virulence factors to maintain residence inside the host cell to survive, replicate, and avoid immune system detection. Endocytic phagosomes in macrophages normally undergo a maturation process, fuse with lysosomes to acquire enzymatic contents, and acidify to destroy internalized microbes. *M. tuberculosis* circumvents this process by closely associating with the membrane of the phagosome and delaying maturation.[10,41] de Chastallier *et al*. reported that attaching beads 0.1 μm in diameter to the outer membrane of *Mycobacterium avium* physically blocked association with the phagosomal inner membrane, and the phagosomes matured and fused with lysosomes as evident by the co-presence of lysosomal contents.[10] Thi *et al*. cited PE-PGRS62 as the protein responsible for arrest of phagosomal maturation, as their study showed that *Mycobacterium smegmatis* can prevent the vesicle from progressing to the late-endosomal stage when induced to express PE-PGRS62.[44] The researchers cited that phagosomes containing PE-PGRS62-expressing *Mycobacterium smegmatis* significantly lacked two proteins associated with the late-endosome and lysosome: Rab7, a small G-protein that regulates trafficking for endosomal-lysosomal fusion, and LAMP-1 (lysosomal associated membrane protein 1), which is a protein whose presence indicates fusion of endosomal-lysosomal membranes.[24,26,45] Thi *et al*. demonstrated that PE-PGRS62 localizes to the cell wall of *M. tuberculosis* and hypothesized that the protein interacts with receptors located on the

phagocytic membrane to prevent acquisition of Rab7 and LAMP-1, thereby preventing phagolysosome maturation and prolonging the intracellular persistence of *M. tuberculosis*.[44] In addition, the study found that PGRS62-expression by *Mycobacterium smegmatis* reduced post-transcriptional expression of nitric oxide synthase (iNOS) in infected macrophages.[44] Further studies reported that phagosomes containing *M. tuberculosis* retain major histocompatability complex II (MHC II), thereby interrupting antigen processing and export to the plasma membrane for presentation.[5,6] By inhibiting phagolysosomal fusion and suppressing iNOS expression, *M. tuberculosis* prolongs intracellular replication time without the threat of degradation or antigen processing.

Macrophages are activated by phagocytosis of the bacilli, and then secrete cytokines and chemokines to ignite the host immune response and recruit other immune cells. The trademark of *M. tuberculosis* pathology is the development of a highly-organized grouping of host immune cells called a granuloma, which serves to restrain spread and proliferation of the *M. tuberculosis* bacilli.[36] The center of the granuloma consists of infected macrophages, which are "walled-off" by rings of epithelioid macrophages, foamy macrophages, and peripheral lymphocytes.[41] The waxy, impenetrable nature of the *M. tuberculosis* cell wall resists killing by host cells, and extended cell recruitment leads to granuloma formation in an attempt to prevent the spread of infection. Occasionally throughout the granuloma, macrophages will fuse to form multinucleated giant cells, which function to destroy bacilli previously ingested by individual macrophages prior to giant cell formation.[32] The hypoxic center of the granuloma has a high protein and lipid content due to the dead, infected macrophages, and a caseous gross appearance develops once the granuloma becomes necrotic.[41] In this hypoxic state, the normally aerobic, carbohydrate metabolism of *M. tuberculosis* can shift upon changes in bacterial gene expression

to become dormant and lipid-metabolizing.[28] The collagenous, fibrous capsule surrounding the granuloma can also make the bacteria difficult to access by antibiotics.

Many strains of *M. tuberculosis* are emerging as multidrug-resistant (MDR) or extremely drug-resistant (XDR), and the "W" strain of *M. tuberculosis* demonstrates resistance to most first and second-line antimycobacterial agents.[1] Current treatment regimens for drug-resistant tuberculosis cases can last from six months to two years of cocktails consisting of multiple drugs, making the treatment expensive with concerns of toxicity.[19,34,43] Lalloo and Ambaram call attention to "a need for drugs that are safe and effective against resistant strains, able to shorten the course of treatment, effective for latent TB infection, and that have minimal interactions with retroviral drugs."[31] The mounting trend of resistance with limited effective treatment options calls for the development of new antimycobacterial agents and investigating repurposing of known drugs not currently part of the tuberculosis treatment regimen.[18] Furthermore, no reliable vaccine exists to protect populations against the pulmonary form of the disease. In 1921, Calmette and Guérin reported an attenuated *Mycobacterium bovis* strain following multiple passages on culture mediumThis attenuated strain has demonstrated highly-variable efficacy in preventing tuberculosis when applied as a vaccine.[14] The lack of a completely protective vaccine, combined with mounting drug-resistant strains and limited treatment options, urgently calls for attention to the need for new antimycobacterial drug development.

Drug development efforts incorporate new applications of approved drugs found to be effective against *M. tuberculosis,* including a focus on identifying new targets and amplifying host cell mechanisms.[29] Autophagy is a mechanism of particular interest when considering intracellular *M. tuberculosis,* because of the potential to link innate and adaptive immune responses and boost production of antimicrobial oxygen and nitrogen radicals.[11,27,29] Gutierrez *et*

al. demonstrated that induction of autophagy inhibits survival of intracellular bacteria, and Kim *et al*. reported that two, front-line, antimycobacterial agents, isoniazid and pyrazinamide, function to up-regulate host cell autophagy as the key mechanism to combat *M. tuberculosis.*[22,29] Previous studies into the mechanism of autophagic killing of *M. tuberculosis* focused primarily on the role of genes and cytokines in phagosomal maturation and lysosomal fusion to destroy the bacilli.[3,22,29] These findings are important for identifying potential host cell targets but provide no *in vivo* evidence of potential novel chemotherapeutic agents.[29]

In addition to studies mapping autophagic targets, other studies have investigated novel mechanisms of action for development of potential chemotherapeutic agents against *M. tuberculosis.*[30] McLean *et al*. reported a bioinformatic analysis of the *M. tuberculosis* genome, identifying a homologue of CYP P450, which is an enzyme required for sterol synthesis that is inhibited by azoles.[37] Ahmad, Sharma, and Khuller investigated the synergistic use of the antifungal azoles with known antimycobacterial agents.[2] Hawn *et al.* highlight host molecular targets and inhibitory small molecules as potential players to be modulated in synergy with antimycobacterial agents due to their roles in the macrophage, host inflammatory response, and pathology.[23] The researchers propose that modulating the host-pathogen interaction can shorten treatment duration as well as reduce the damage to the lung tissue.[23] While there are increasing efforts and interests devoted to discovering new chemotherapeutic agents to combat tuberculosis infections, the number of new drugs targeting novel pathways is insufficient, especially concerning the usual obstacles of antimycobacterial treatments: treatment duration, drug-resistant *M. tuberculosis* strains, and pediatric tuberculosis cases.[8]

(1-Decyl)triphenylphosphonium bromide (dTPP) is a 10-carbon alkyl chain linked to a triphenylphosphonium moiety.[33] The hydrophobic carbon chain travels easily across biological

membranes (such as the plasma membrane and mitochondrial membrane), and the triphenylphosphonium cation causes accumulation of dTPP in the mitochondria by several hundred-fold due to the high membrane potential across the mitochondrial membrane.[33,39,42] dTPP is a moiety of MitoQuinone (MitoQ), a widely-available antioxidant found in anti-aging skincare and supplements, as well as studies investigating treatments for age-related diseases.[12,16,35] Due to the similar molecular structure (dTPP simply lacks the quinone component present in MitoQ), dTPP is cited as a control treatment in studies investigating MitoQ with no adverse effects reported.[7,16,20,25,38,40] Interest in the potential of dTPP as an antimycobacterial agent arose following an unpublished study investigating the effects of MitoQ on *Caenorhabditis elegans.* dTPP served as the negative control in the study, and investigators observed paralysis or death of the nematodes following treatment (Wolstenholme, personal communication). However, *in vivo* treatment against *Trichuris muris* proved less effective due to oxygen tension levels in the intestine, where the parasitic worms are found (Sakamoto, personal communication). Interest then moved to the potential of treating respiratory pathogens, like *M. tuberculosis*, as oxygen tension in the lungs is similar to that of ambient air.

The purpose of this study was to investigate antimycobacterial efficacy and cytotoxic effects of dTPP in murine bone marrow-derived macrophages. We also tested the efficacy and safety of dTPP usage *in vivo* using a murine model to determine synergistic effects when combined with a known antimycobacterial agent to combat *M. tuberculosis* infection.

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CHAPTER 2

EFFECTS OF (1-DECYL)TRIPHENYLPHOSPHONIUM ON *MYCOBACTERIUM TUBERCULOSIS* INFECTION OF MURINE BONE MARROW-DERIVED MACROPHAGES

[1]Quattlebaum SE, Sellers L, Franklin J, Rada B, Quinn F, Sakamoto K. To be submitted to *Veterinary Pathology*.

ABSTRACT

Highly-virulent *Mycobacterium tuberculosis* is the pathogen responsible for the disease tuberculosis, which affects individuals across the world. *M. tuberculosis* bacilli are demonstrating a trend towards increasing levels of resistance to known antimycobacterial agents. Treatment regimens are long-lasting and expensive, and efforts are needed to investigate and develop new antimycobacterial agents which are effective, safe, and affordable. In this study, murine bone marrow-derived macrophages (BMMPs) were infected with *M. tuberculosis* as an *in vitro* model of tuberculosis pathogenesis. Infected cells were treated with three concentrations of (1-Decyl)triphenylphosphonium (dTPP) to investigate efficacy and safety of the compound as an antimycobacterial agent. Efficacy was measured by culturing *M. tuberculosis* colony forming units (CFUs), and safety was assessed by measuring lactate dehydrogenase (LDH) leakage (as an indicator of cytotoxic activity) from cells following treatment with dTPP. While CFUs were significantly reduced at the highest concentration of dTPP applied, the compound also exerted significant cytotoxic effects *in vitro.*

INTRODUCTION

Mycobacterium tuberculosis is the pathogen responsible for the widely-known disease tuberculosis, which is a serious disease with a long history and complex pathogenesis. Mycobacteria are acid-fast, Gram-positive bacilli, which appear as beaded, branching rods approximately 1-4 μm in length.[17] These organisms are slow-growing with a cell wall composed of mycolic acids, which makes them resistant to antibiotics and environmental stressors.[3] *Mycobacterium tuberculosis* bacilli are inhaled via aerosolized droplets expelled by an infected person and then travel through the respiratory tract until the bacilli make contact with alveolar macrophage surface receptors. The binding of pathogen and host cell receptors trigger phagocytosis, and infected macrophages are activated to secrete cytokines and chemokines, which recruit other immune cells to the site of infection and ignite the host immune response.[17] *M. tuberculosis* circumvents the destructive process within the macrophage by expressing virulence factors that delay the maturation and acidification of the phagosome.[17]

Many strains of *M. tuberculosis* are reported as multidrug-resistant and extremely drug-resistant Currently, treatment regimens for tuberculosis cases can last from six months to two years of cocktails consisting of multiple drugs, and concerns of expense and toxicity are always to be considered.[5,11,20] The lack of a reliable vaccine coupled with increasing levels of drug resistance in the face of limited treatment options calls for development of new antimycobacterial agents.[4]

(1-Decyl)triphenylphosphonium (dTPP) is a 10-carbon alkyl chain linked to a triphenylphosphonium moiety.[9] The hydrophobicity of the carbon chain allows it to travel easily across membranes, and the triphenylphosphonium cation causes accumulation by several

hundred-fold due to the high potential across the mitochondrial membrane.[9,16,19] As mitochondria are a main driving force for apoptosis (which would successfully kill intracellular *M. tuberculosis*), interest in the potential of dTPP as an antimycobacterial agent follows results of an unpublished study in which investigators observed paralysis or death of nematodes following treatment with dTPP as a control.[9] In addition, dTPP could be used in combination with known antimycobacterial agents, such as Rifampin, to boost killing of *M. tuberculosis*.

METHODS

Murine Bone Marrow-Derived Macrophages

A C57BL/6J mouse was euthanized, both femurs and tibias were removed, and bone marrow-derived macrophages (BMMPs) were harvested and cultured according Geisel *et al.*[4] Cells were scraped off the plate surface, transferred to a conical tube, and centrifuged at 200 x g for 5 minutes. The cell pellet was resuspended in BMMP medium (composed of L-cell medium, fetal bovine serum, L-glutamine, Penicillin-Streptomycin, sodium pyruvate, and Dulbecco's Modified Eagle Medium) and prepared for plate seeding for *in vitro* assays.

Cell Counting and Plate Seeding

Bone marrow macrophage cultures at 80% confluency were prepared for counting by removing medium, adding 10 mL 1X phosphate buffered saline (PBS), and incubating at 4°C for 15 minutes. Cells were scraped off of the plate surface, transferred to a 50 mL conical tube (one

tube per plate), and centrifuged at 200 x g for 5 minutes. Cells were counted using Trypan blue and standard methods.

Chemicals and Reagents

DTPP was acquired from Santa Cruz Biotechnology and resuspended in sterile PBS before diluting to 1.7, 17, and 170 nM concentrations in sterile BMMP infection medium (composed of L-cell medium, fetal calf serum, L-glutamine, Penicillin-Streptomycin, sodium pyruvate, and Dulbecco's Modified Eagle Medium) as determined by previous cytotoxicity assays to be the most effective working concentration *in vitro* with optimal mycobactericidal effects without compromising macrophage cell viability.

Survival Assay

BMMPs were plated in non-tissue culture-treated, 24-well plates with 5×10^5 cells in 1 mL medium per well. Plates were incubated at 37°C and 5% CO_2. *M. tuberculosis* strain H37RV was cultured in 7H9 broth at 37°C, measured by optical density (OD), and diluted so that the culture was OD600 = 0.5 the day before beginning the assay. On the day of the assay, the OD was used to determine the volume of *M. tuberculosis* culture used to infect to achieve a multiplicity of infection (MOI) equal to one (one bacillus for each macrophage).

For a MOI of 1, *M. tuberculosis* culture was added to 3 mL of uptake buffer, and centrifuged at 1800 x g for 10 minutes. The pellet was resuspended in 1 mL of uptake buffer and emulsified 9 times with a tuberculin syringe, and the volume of suspension required for a MOI of 1 was

added to infection medium. The medium was removed, and antibiotic-free medium was added to wells containing *M. tuberculosis* bacilli. Standard medium was added to control and dTPP-only treated wells . Plates were incubated at 37°C and 5% CO_2 for 1 hour. The medium was then completely removed, and wells were washed with 1 mL of wash buffer (composed of Dulbecco's Modified Eagle Medium and 5% fetal calf serum) to remove free extracellular bacilli. For the t=0 time point wells, the wash buffer was removed, replaced with 500 µL of lysis buffer, and incubated at 37°C for 10 minutes. After incubation, wells were scraped to fully remove the monolayer, and pipetted up and down to lyse cells. For all other time points, the wash buffer was removed and replaced with 1 mL of infection medium. Two-hundred and fifty µL of each lysed sample, as well as the original inoculum, was serially-diluted 1:10, 1:100, and 1:1000 in dilution buffer. Fifty microliters of each dilution was plated on 7H11 agar for quantitation, and after two weeks, colony counts between 30 and 300 were recorded, and total colony forming units (CFUs) were calculated.

After 24 hours, the infection medium was removed from remaining wells and replaced with 1 mL of dTPP (suspended in PBS) in medium at various doses for treated cells, or 1 mL fresh infection medium for control cells and infected, untreated cells. Plates were incubated at 37°C and 5% CO_2 for 24 hours, after which medium from all wells was collected, filter sterilized, and stored in microcentrifuge tubes at -80°C. Control wells used for maximum cytotoxicity controls were lysed, while minimum control supernatants from unlysed wells were used to establish minimum lactate dehydrogenase (LDH) production. The process for lysing infected cells, diluting, and plating *M. tuberculosis* in supernatants for each well was repeated (as in the t=0 time point) for the 24-hour time point, and every 48 hours for 7 days.

Cytotoxicity Assay

A 96-well plate was used for the lactate deyhydrogenase detection/cytoxicity assay with 100 µL of each supernatant added to individual wells, and reagents were added according to manufacturer's instructions. Absorbance was read at 490 nm subtracting background, and cytoxicity was calculated by measuring lactate dehydrogenase with the Roche Cytoxicity Detection Kit (LDH). Supernatants collected from treated, infected, and control wells were tested to quantify cytotoxicity due to dTPP treatment according to manufacturer's instructions.

Statistical Analysis

The effects of the three dTPP concentrations upon colony forming unit counts were subjected to statistical analysis using the Dunn.Test (Version 1.3.2, 2016) package in R (R Core Team). Kruskal-Wallis tests were used to compare CFUs between treatment groups for each experiment separately. If significant differences were found, then paired comparisons were performed using Dunn's test. A significance threshold of 0.05 was used.

The effects of the three dTPP concentrations on lactate dehydrogenase production levels as an indicator of cytoxicity were analyzed using SAS V 9.4 (Cary, NC). Differences between treatment and no treatment, as well as differences between doses, were analyzed using two-way analysis of variance (ANOVA) and a significance threshold of 0.05 was used.

RESULTS

Survival Assay

Significant differences were observed in colony forming units (CFUs) of *M. tuberculosis* between the 1.7 nM dTPP group and the 170 nM dTPP group (Figure 1). The mean number of CFUs cultured from infected BMMPs treated with 170 nM dTPP for 24 hours was significantly lower than that of all other groups for all experiments. There was no significant difference in CFUs between infected, untreated cells and infected cells treated with 17 nM dTPP. These results indicate that dTPP demonstrates some antimycobacterial activity at 170 nM, but this activity is lost at lower doses.

Cytoxicity

Increased leakage of LDH is often considered a diagnostic indicator of cell death due to toxicity.[15,16] As the cells begin to die and lyse, LDH is released into the cell culture medium and available for detection. LDH release increased with increasing dTPP concentration (Figure 2). There were overall statistically significant differences in lactate dehydrogenase (LDH) levels between doses (dTPP concentration) but not between treatments (uninfected versus infected). No significant interaction was found between treatment and dose.

DISCUSSION

The number of CFUs cultured from infected BMMPs treated with 170 nM dTPP was significantly reduced relative to untreated cells or cells treated with 1.7 or 17 nM dTPP. While the CFUs were increased in cells treated with 1.7 nM dTPP compared to infected, untreated cells, the difference was not statistically significant. These results indicate dTPP reduces bacterial load at a concentration of 170 nM, however the significant cell death measured by LDH detection coincides with significant decrease in CFUs. These results suggest the mechanism of host cell death is responsible for the significant decrease in CFUs rather than apparent mycobacterium-killing activity of dTPP. As infected host cells began to die and lyse following dTPP treatment, they detached from the monolayer and were washed away during the wash step; intracellularly-dwelling *M. tuberculosis* was also washed away, thereby causing the apparent reduction in CFUs.

Experiments not included in this report investigated reactive oxygen species (ROS) production as a possible explanation for the observed cell death and found a directly proportional relationship between increasing dTPP concentration and ROS production. Production of ROS by the mitochondria is associated with oxidative damage to the cell, which can lead to cellular degeneration, and if the antioxidative efforts of the cell are overcome by the insult, then apoptotic pathways may be triggered.[2,23] In this case, it is possible that dTPP, like other compounds not native to the host and known to interact with the electron transport chain, increases the production rate of superoxide, which signals the mitochondria to initiate an apoptotic pathway.[22] Additionally, McManus *et al.* reported that dTPP does not display significant antioxidant activities like its MitoQ counterpart, which possesses the ubiquinone

component.[14] This mechanism of ROS production, if investigated and found to be occurring, would offer an explanation of the elevated rate of cell death. However, LDH leakage can be detected only when the plasma membrane is disrupted, so assays like the one used in this study usually indicate cellular necrosis and not apoptosis.[7,8,13] Necrotic cell death caused by dTPP treatment explains the large amount of cellular debris observed *in vitro* under light microscopy, and other assays should be employed to detect an indicator of mitochondrial-mediated apoptosis, such as caspase 3 to strengthen understanding of the mechanism of action.[11,13] Additionally, if dTPP exerts caspase-blocking activity like that observed by McManus *et al.*, then the cell is unable to carry out apoptosis and is pushed in the direction of necrosis.[11,14]

Furthermore, light microscopy of the cell cultures revealed cells to be visually viable under 1.7 and 17 nM treatments, but the 170 nM treatment cycle demonstrated visual loss of host cells, which coincides with the LDH-measured 65% cell death. Based on the percent of LDH detection, it is likely cells were also dying under the 17 nM treatment, but cell death was not as visually apparent or rapid as in the 170 nM treated cells. However, a study by Sheibani *et al.* reported that LDH can act as an inhibitor of cell death triggered by stressors, and Adinarayana and Kishore reported decreased LDH activity in yeast cells (mimicking mammalian cells) that were actively dying due to its role in glycolysis and metabolism.[1,19] Therefore, this study's report of increased lactate dehydrogenase could indicate increased cellular metabolism to support increased activity.

It is ill-advised to reject dTPP as a potential treatment against *M. tuberculosis* infections on the basis of assumed cytoxicity displayed by lactate dehydrogenase detection. However, it should be considered that the 170 nM dTPP caused an apparent 65% cell death rate according to

the detected LDH. These results fail to support the hypothesis that dTPP is a safe and effective agent against the intracellular pathogen.

CONCLUSION

DTPP administered at a concentration of 170 μM could be considered a potential treatment for *Mycobacterium tuberculosis* infection based upon significant antimycobacterial activity. However, this study shows that cell death increases with increasing concentration of dTPP applied to BMMPs, and these findings call for further investigation into the molecular mechanism of action to potentially find a safe and effective application of dTPP against *M. tuberculosis* infections.

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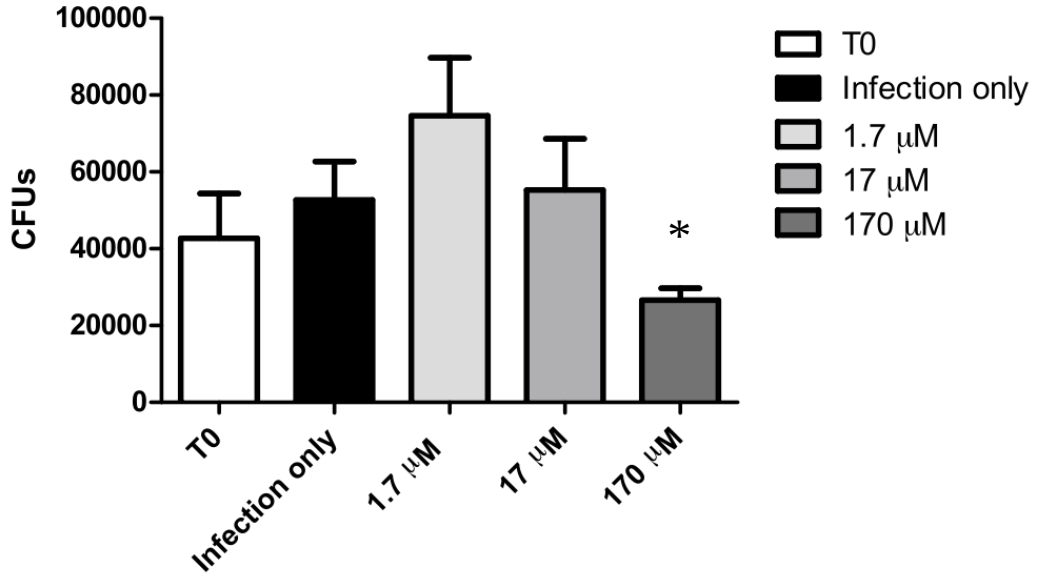

Figure 1. Mean number of colony forming units (CFUs) following treatments for T_0, Infected/untreated cells, 1.7 nM, 17 nM, and 170 nM dTPP. Asterisk denotes significant difference ($p<0.05$) from other treatment results.

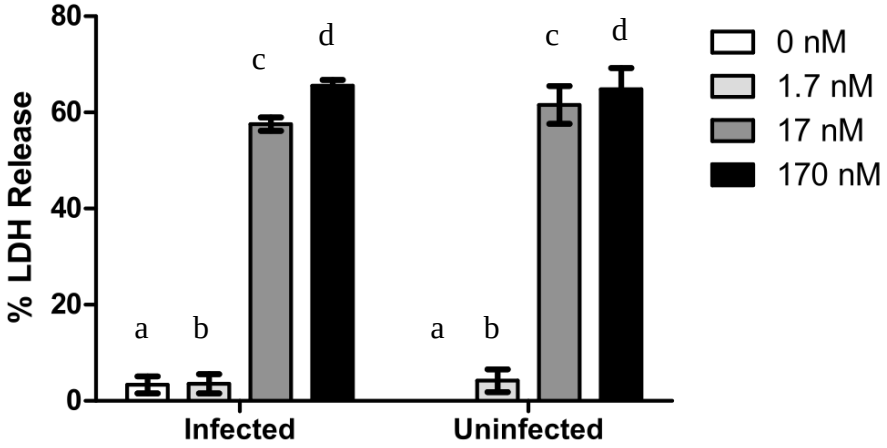

Figure 2. Percent of lactate dehydrogenase released from uninfected and infected cells following treatments of control, 1.7 nM, 17 nM, and 170 nM dTPP. Letters denote significant difference ($p<0.05$) between doses, with no significant difference between treatments.

CHAPTER 3

EFFECTS OF (1-DECYL)TRIPHENYLPHOSPHONIUM TREATMENT ON A MOUSE MODEL OF *MYCOBACTERIUM TUBERCULOSIS* INFECTION

[1]Quattlebaum SE, Sellers L, Helms S, Gupta T, Franklin J, Quinn F, Rada B, Sakamoto K. To be submitted to *Veterinary Pathology*.

ABSTRACT

Mycobacterium tuberculosis poses a threat around the world through a complex pathogenesis as well as mounting resistance mechanisms to known antimycobacterial agents. Researchers are urgently calling out for new investigations into developing treatments against the disease. Previous investigations into the anti-parasitic activity of (1-Decyl)triphenylphosphonium (dTPP) suggest that the compound could be a potential antimycobacterial agent to combat tuberculosis safely. In this study, mice were infected with *M. tuberculosis* and treated with a subcutaneous injection of dTPP, oral gavage of Rifampicin, or a combination of the two treatments. All mice treated with dTPP were found dead or presented with neurologic signs upon physical examination the next day. This is the first study reporting adverse effects of dTPP.

INTRODUCTION

Tuberculosis is a disease with a complex pathogenesis and poses a serious threat across the globe. The World Health Organization reports an estimated 9 million people developed tuberculosis in 2013, and 1.5 million deaths are attributed to tuberculosis infections. *Mycobacterium tuberculosis* is the pathogen responsible for tuberculosis in humans, and the disease prevalence and mortality rates make this bacillus the single greatest cause of infectious disease by a single agent.[9,11] *M. tuberculosis* is spread from infectious persons to naive persons by expressing aerosols containing the bacteria; one droplet can contain up to 400 bacilli, and the infectious dose is reported to be as low as 1 to 4 bacilli.[1] Bacilli are inhaled and travel down the respiratory tract, where they interact with surface receptors on alveolar macrophages to induce phagocytosis and maintain intracellular residence, evading immune system detection and preventing destruction through the expression of multiple virulence factors.

Beyond the evolution of virulence factors, many strains of *M. tuberculosis* are emerging as multidrug-resistant and extremely drug-resistant. Current treatment regimens for drug-resistant tuberculosis cases involve treatment cocktails consisting of multiple drugs, and regimens can last from six months to two years, making available treatments expensive with concerns of toxicity.[12,19,26] In addition, no vaccine exists to fully and reliably protect adults against the pulmonary form of the disease.[8] The mounting trend of bacterial resistance to the limited treatment options, coupled with the lack of a completely protective vaccine, calls for urgent efforts to investigate development of new antimycobacterial agents, which are safe and cost-effective.[11]

The mouse is a popular animal model of TB pathogenesis given cost-effective husbandry as well as widely-available reagents and similarity of immunological mechanisms to those observed in humans, with the exception of caseous granuloma formation, which the murine model lacks.[14,24] Comparative studies investigating experimental treatments to combat *M. tuberculosis* are first carried-out in the murine model.[4]

(1-Decyl)triphenylphosphonium bromide (dTPP) is a 10-carbon alkyl chain linked to a triphenylphosphonium moiety.[18] dTPP is a moiety of MitoQuinone (MitoQ), a widely-available antioxidant found in anti-aging skincare and supplements as well as studies investigating treatments for age-related diseases.[6,10,21] Due to the similar molecular structure (dTPP simply lacks the quinone component present in MitoQ), dTPP is cited as a control treatment in studies investigating the application of MitoQ, with no adverse effects reported.[10,13,22,23,25] Interest in the potential of dTPP as an antimycobacterial agent arose following an unpublished *in vitro* study investigating the effects of MitoQ on *Caenorhabditis elegans.* dTPP served as the negative control in the study, and investigators observed paralysis or death of the nematodes following treatment (Wolstenholme, personal communication). However, *in vivo* treatment against *Trichuris muris* proved less effective due to oxygen tension levels in the intestine, where these parasitic worms are found (Sakamoto, personal communication). Interest then moved to the potential of treating respiratory pathogens, like *M. tuberculosis*, as oxygen tension in the lungs is much closer to that of ambient air. This study investigates the safety and antimycobacterial efficacy of dTPP when used in tandem with Rifampicin in the widely-accepted murine model of *M. tuberculosis* infection.

METHODS

Chemicals and Reagents

DTPP was acquired from Santa Cruz Biotechnology and resuspended in sterile phosphate-buffered saline in an 8 mg/mL solution to be administered via 250 µL subcutaneous injection.

Rifampicin was purchased from Sigma and resuspended in syrup acquired from the University of Georgia Veterinary Hospital Pharmacy in a 10 mg/mL solution to be administered via 200 µL oral gavage.

Animals

Forty-nine, female, BALB/c mice were acquired from Harlan and housed in a specific-pathogen-free animal facility at the University of Georgia, College of Veterinary Medicine. Mice were weighed following a 7-day acclimation after receipt. All mouse experiments were performed following protocols approved by the UGA Institutional Animal Care and Use Committee.

Infection and Treatment

Forty-nine mice were used to test the efficacy and safety of (1-decyl)triphenylphosphonium (dTPP) against *Mycobacterium tuberculosis* infection according to Table 1. Upon receipt, animals were acclimated for 7 days, after which groups specified below as infected with *M.*

tuberculosis strain Erdman were dosed with approximately 100 bacilli each via Madison chamber. Twenty-four hours post-infection, three mice were euthanized and necropsied to determine baseline infection status by homogenizing the lung, serial dilution, and plating for colony-forming units (CFUs). Post-infection, mice were weighed and checked daily for the development of clinical signs. Twenty-one days post-infection, three mice were euthanized and necropsied to determine baseline infection prior to treatment as described above. Remaining treatment groups were to be treated every day for seven days according to Table 1. dTPP was administered via 250 µL subcutaneous injection at 100 mg/kg of body weight per the recommendation of previous investigators.[18] dTPP concentration reached 10 µg/g of lung tissue after 12 hours, and this level was reported to be non-toxic to the mouse.[22] Rifampicin was administered via 200 µL oral gavage at a concentration of 10 mg/kg of body weight, which has previously been reported as a recommended dose in a rodent model.[5,16,28] At necropsy, one lung was collected for histopathology and one for culture. Tissues were scored according to changes observed in the lung: percentage of lung affected, distribution of lung lesions, number of lung granulomas, perivascular cuffing (PVC), and number of liver granulomas.

Statistical Analysis

This experiment was designed as a blinded study to observe and score pathological changes associated with dTPP treatment against *M. tuberculosis* infection, and measure *in vivo* efficacy of the antimycobacterial activity of dTPP when used in tandem with Rifampicin. The effects of dTPP as measured by histopathology scores were subjected to statistical analysis using SAS V 9.4 (Cary, NC). Kruskal-Wallis tests compared variables between groups. If significant

differences were found, then paired comparisons were performed using the Dunn.Test (Version 1.3.2, 2016) package in R (R Core Team). A significance threshold of 0.05 was used.

RESULTS

Upon physical examination less than 24 hours later, all mice dosed with dTPP were dead or presented with neurologic signs which required euthanasia. Figure 3 shows the mean histopathological scores of observed changes in percentage of affected lung tissue, number of lung granulomas, alveolar lesions, perivascular cuffing (PVC), and liver granulomas. All variables (percentage of affected lung, number of lung granulomas, alveolar lesions, perivascular cuffing, and number of liver granulomas) were significantly different between control mice and *M. tuberculosis*-infected mice, as well as between untreated control mice and infected mice treated with Rifampicin. Average scores for percentage of affected lung, alveolar lesions, and PVC were all zero for uninfected mice treated with dTPP only, indicating no noticeable tissue changes occurred in response to dTPP treatment for the five pulmonary parameters measured. No significant differences among variables were found between infected mice and infected mice treated with Rifampicin (Figure 3).

DISCUSSION

This is the first study reporting adverse effects following dTPP treatment. However, all three mice treated with dTPP were noted by the pathologist as displaying mild, diffuse, neutrophilic, interstitial pneumonia, which is suggestive of systemic inflammation, and the third mouse had

additional autolysis, likely to be due to time lapse between death and detection. Figure 4 shows photomicrographs of lung tissue collected. Lung tissue from control, untreated mice showed no significant changes. Infected mice treated with Rifampin showed usual pulmonary lesions and aggregates of macrophages and neutrophils associated with murine tuberculosis. Lung tissue from mice treated with dTPP showed infiltration of neutrophils and evidence of interstitial pneumonia.

It is hypothesized that the systemic inflammatory response is due to the molecular mechanism of action of dTPP at the mitochondrial membrane. Huang *et al.* reported high doses (noted to be 10 μM in their study) of dTPP applied to pancreatic acinar cells caused an increase in mitochondrial depolarization.[15] The triphenylphosphonium ion causes dTPP to embed in the mitochondrial membrane (Franklin, personal communication) and, as a result, cytoplasmic fatty acyl chains accumulate in the mitochondria.[15] This accumulation is documented as having the effect of uncoupling oxidative phosphorylation leading to a loss of NADPH and ATP as well as increased rate of necrosis.[2,3,15] These effects support our findings of elevated rates of necrotic cell death indicated by increased LDH leakage. Furthermore, Wilkins *et al.* reported release of mitochondrial DNA triggered neuron-driven inflammation, and increased mRNA levels of TNFα, IL-8, and MMP-8 as well as total NFκB protein.[27] If dTPP is permeating the mitochondrial membrane and interrupting vital processes, then damage suffered by the plasma membrane would cause mitochondrial contents to be leaked and exposed to the extracellular environment where they could come in contact with neuronal cells. Finally, Kulka *et al.* reported that rodent Mast cells express receptors for neuropeptides, and can undergo degranulation upon binding to vasoactive intestinal polypeptide and substance P.[17] Therefore, it is possible that, in this study, the high concentration of dTPP disrupted the mitochondrial membranes in cells of

mice treated with dTPP which led to necrotic cell death and leakage of mitochondrial DNA. This danger-associated molecular pattern (DAMP) then triggered neuronal-driven inflammation and release of neuropeptides, vasoactive intestinal polypeptide and substance P, which bound to receptors on Mast cells and caused degranulation. As a result, we observed a systemic inflammatory response similar to a Type 1 hypersensitivity reaction without prior exposure.[15,17,27]

The previous, unpublished study, on which our dose was based, administered dTPP through subcutaneous injection, tail vein injection, or oral gavage.[18] This study reported no adverse reactions in mice following subcutaneous injection throughout the study period of 12 hours. However, no pathology was performed. Consequently, based on our study, it is suspected that dTPP, when delivered subcutaneously, results in systemic inflammatory response not observed when given orally or intravenously.

In another study conducted by McManus *et al*. that investigated neuropathology in a mouse model of Alzheimer's disease using mutagenic mice expressing several mutant genes, including amyloid precursor protein, MitoQ was the treatment of interest, and dTPP served as the control.[22] Mice treated with dTPP yielded 300% more lipid peroxide and 200% more caspase levels than mice treated with MitoQ.[22] Additionally, Lowes *et al*. reported 50% loss of cell viability following *in vitro* treatment of human Achilles' tendon cells with dTPP associated with oxidative stress, while MitoQ maintained a viable cell count.[20] The question remains, is dTPP inactive against oxidative stress, or does it cause the oxidative stress?

It is important to continue pursuing autophagy-inducing mechanisms, as these responses seem to boost bacterial killing by promoting bacilli encapsulation within the phagosome.[29] Future studies, now armed with the results of our experiments, should focus on balancing induction of

host cell autophagy with mechanisms to protect the host cell from excessive oxidative stress (i.e. any stress beyond what is necessary to destroy the intracellular bacilli).

CONCLUSION

The subcutaneous injection of 100 milligrams of DTPP per one kilogram of body weight is not currently a recommended treatment for *Mycobacterium tuberculosis* infection. This study shows that neurological signs and even death are observed within 24 hours of treatment, and these findings call for further investigation into design and development to utilize dTPP to combat resistant and multidrug-resistant *Mycobacterium tuberculosis* infections.

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Table 1. Experimental design according to treatment group.

Group	Number of animals	Treatment	Euthanasia date
1	3	Infect with *M. tuberculosis* (Mtb)	24 hours post-infection
2	3	Infect with Mtb	21 days post-infection
3	8	None (control)	Day 30
4	8	Infect with Mtb, treat with dTPP	Day 30
5	8	Infect with Mtb, treat with Rifampin	Day 30
6	8	Infect with Mtb, treat with dTPP + Rifampin	Day 30
7	3	Treat with dTPP only	Day 30
8	8	Infect with Mtb	Day 30

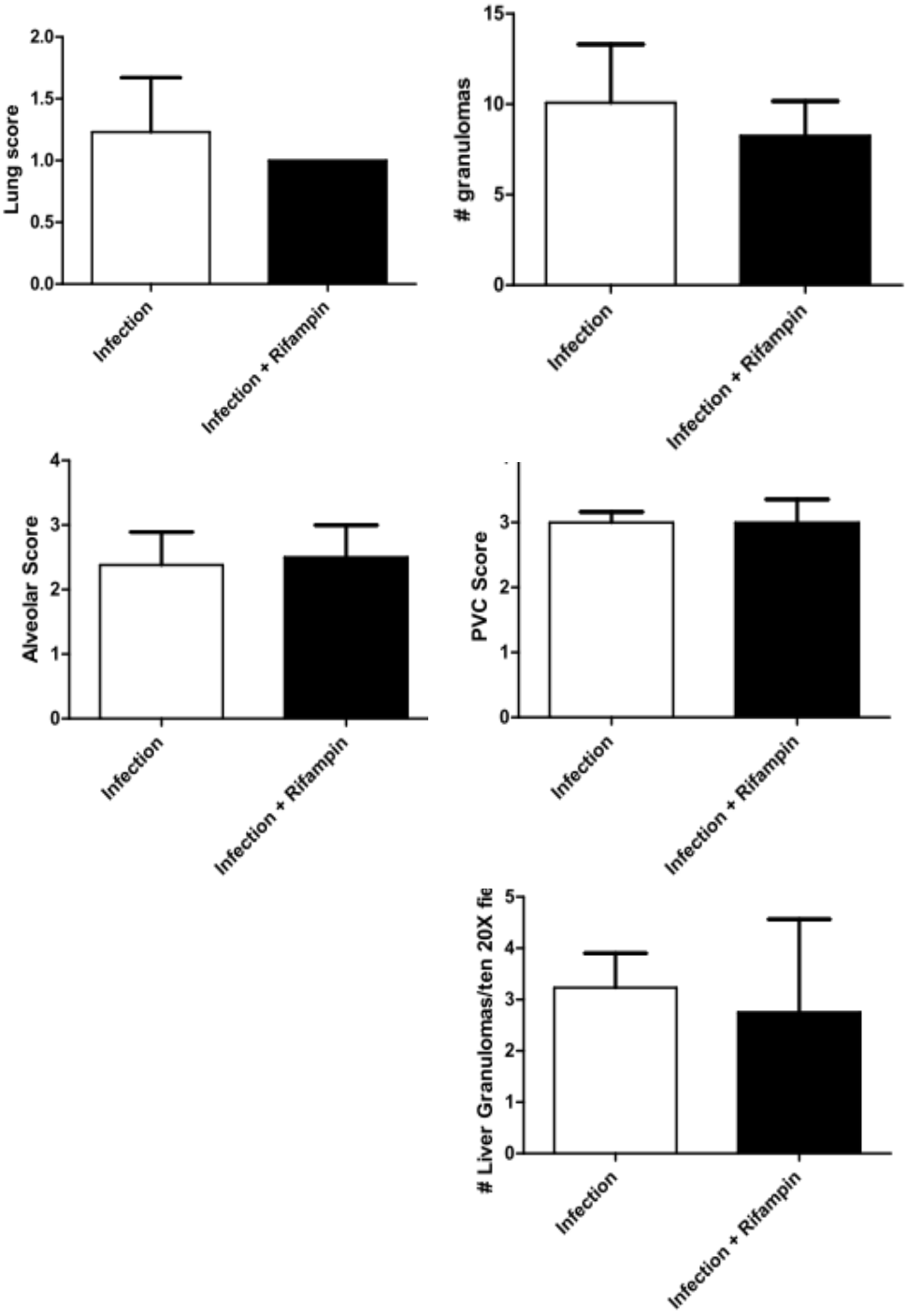

Figure 3. Mean histopathological scores of observed changes in percentage of affected lung tissue, distribution of lung lesions, number of lung granulomas, perivascular cuffing (PVC), and number of liver granulomas. No significant differences among variables were found between infected mice and infected mice treated with Rifampicin.

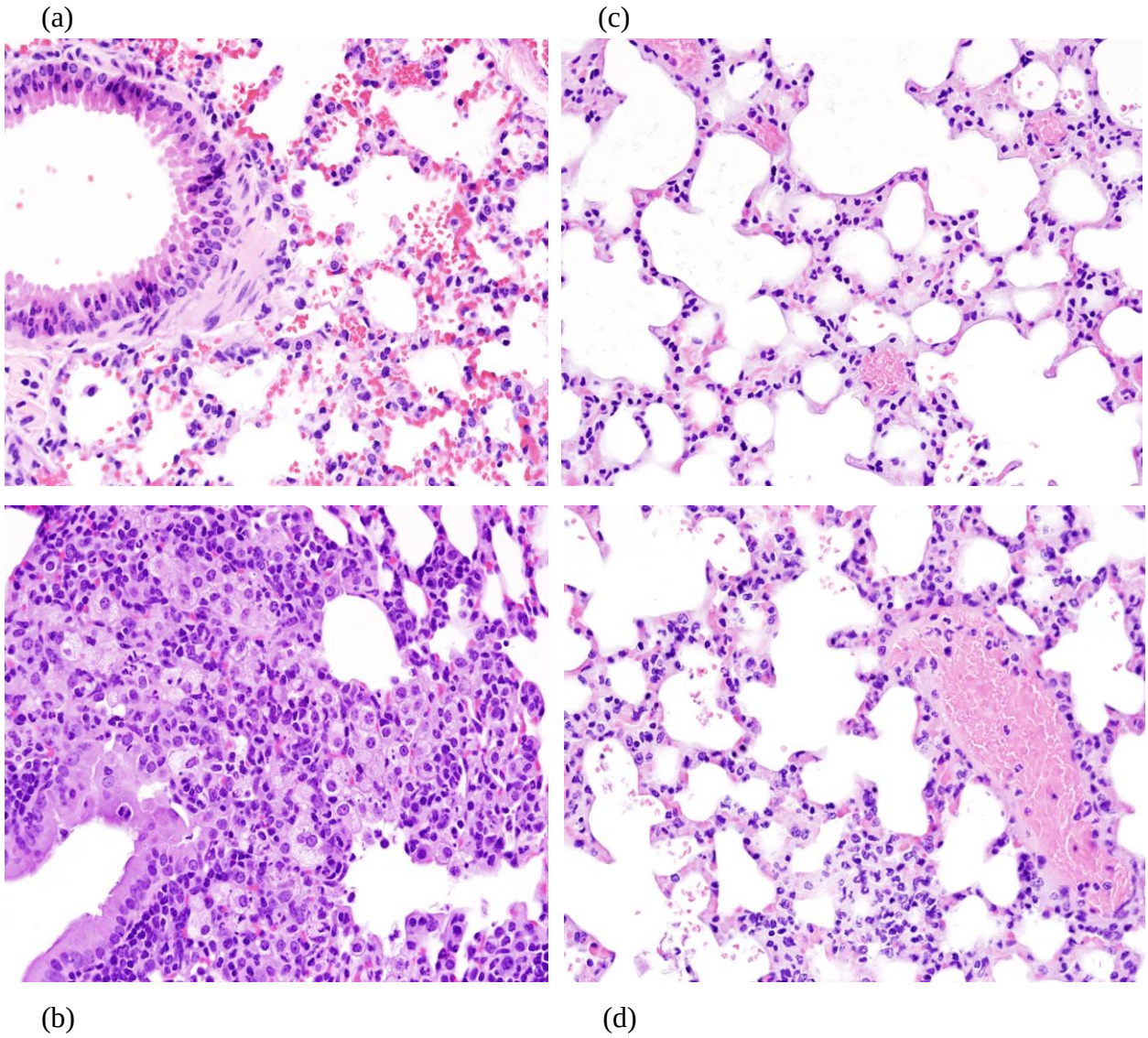

Figure 4. Lung tissue stained with Hematoxylin and Eosin. (a) Control mouse, untreated, day 28. (b) Mtb-infected mouse treated with Rifampin for 7 days, day 28. (c) dTPP-treated mouse, 24 hours post-treatment, day 22. (d) dTPP-treated mouse, 24 hours post-treatment, day 22.

CHAPTER 4

CONCLUSIONS

(1-Decyl)triphenylphosphonium (dTPP) demonstrates possible bactericidal activity against *Mycobacterium tuberculosis* when applied to infected BMMP cell cultures at a concentration of 170 µM; however, cell death increases with increasing concentration of dTPP applied to BMMPs. At the concentration required to see significant antimycobacterial activity, the measure of cell death approached 65% of the cell cultures. These findings call for further investigation into the molecular mechanism of action to potentially find a safe and effective application of dTPP against *M. tuberculosis* infections.

The subcutaneous injection of 100 milligrams of dTPP per one kilogram of body weight is not a recommended treatment for *M. tuberculosis* infection in the mouse model. Neurological signs and even death are observed within 24 hours of treatment, and these findings call for further investigation into why dTPP, which is typically used as a control for MitoQuinone, causes neurologic signs and death in mice.

A possible explanation for the systemic inflammatory response we observed in these dTPP-treated mice is due to the molecular mechanism of action of dTPP at the mitochondrial membrane. It is hypothesized the high dose of dTPP administered subcutaneously caused an increase in mitochondrial depolarization. The triphenylphosphonium ion caused dTPP to embed in the mitochondrial membrane and, as a result, cytoplasmic fatty acyl chains accumulated in the mitochondria. This accumulation uncoupled oxidative phosphorylation, leading to a loss of

NADPH and ATP, as well as an increased rate of necrosis which supports our findings of elevated rates of necrotic cell death indicated by increased LDH leakage. Furthermore, dTPP's permeation of the mitochondrial membrane interrupted vital processes, and damage suffered by the plasma membrane would cause mitochondrial contents to be leaked and exposed to the extracellular environment, where they could come in contact with neuronal cells. Mitochondrial DNA, a danger-associated molecular pattern (DAMP), was released upon cell lysis and triggered neuron-driven inflammation, which lead to increased mRNA levels of TNF-α, IL-8, and MMP-8, as well as total NF-κB protein. Additionally, neuron-driven inflammation triggers a release of neuropeptides, vasoactive intestinal polypeptide and substance P, which bound to receptors on Mast cells and caused degranulation. This mechanism could result in a systemic inflammatory response similar to a Type 1 hypersensitivity reaction without prior exposure.

This research provides the first report of adverse effects following dTPP treatment. While dTPP is not currently recommended for diagnostic or therapeutic use, it is widely used in studies investigating application of MitoQuinone in animal models. The next step in this research is to further investigate dTPP effects *in vitro* before subjecting any more animals to lesser-known adverse effects like the ones reported in this research. If results like those reported in this research are frequently missed in other investigations on histopathology (or lack thereof) or difference in dTPP dose and treatment intervals, then many animals may be suffering avoidable effects. Better understanding the chemical's mechanism of action and preferred route of administration will promote safe and effective use of dTPP in future studies as efforts continue to protect laboratory animals.