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Effectiveness of a Novel Porphyrin Exhibiting Dark Toxicity against the Model Organism Mycobacterium smegmatis

Marissa C. Jama

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Abstract

Antimicrobial photodynamic therapy (PDT) utilizing porphyrins has emerged as a possible ancillary treatment against antibiotic resistant bacteria. Porphyrins, such a novel zinc containing porphyrin designated ZnP, create reactive oxygen species that are toxic to bacterial cells utilizing light by a mechanism that is not yet fully understood. Previous experiments have shown ZnP to be able to kill microorganisms in the dark which is unique to few porphyrins. The aim of this research is to understand the effect ZnP has on *Mycobacterium smegmatis*, a model organism for the pathogenic bacterium *Mycobacterium tuburculosis* which causes the lung infection tuberculosis. Further experimentation is to include understanding the effects of ZnP on *M. smegmatis* biofilm formation and disruption as well as antibiotic uptake in formerly antibiotic resistant cells.

UNIVERSITY of DAYTON

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Introduction

Antibiotic resistance has gained attention within the scientific and healthcare communities as the problem continues to worsen not just here in the United States but globally. The miracle of antibiotics has been met by the growing reality that new technologies must be developed to combat seemingly incurable infections. Drug-resistant strains of bacteria do not respond to traditional treatment with antibiotics which poses a threat to the lives of those infected and all in close proximity to them. There has been a collective push by researchers and healthcare professionals to begin developing alternative treatments to what are now being called "superbug" infections in addition to practicing antibiotic stewardship in clinical settings.

Infections caused by bacteria that are classified by the genus *Mycobacterium* have proven to be difficult to treat with loss of limb or life likely in patients without proper diagnosis and antibiotics. Common illnesses caused by the opportunistic infections from *Mycobacterium* species are the respiratory infection, tuberculosis, as well as skin infections such as leprosy and aquarium granuloma. According to the Centers for Disease Control and Prevention, the incidence rate of antibiotic resistant strains of the bacterium that causes tuberculosis has decreased slightly domestically; however, the number of known cases abroad has more than doubled (CDC, 2015). The necessity to develop ancillary treatments has become urgent as widespread antibiotic resistant infections reach epidemic proportions.

A possible treatment is photodynamic therapy which utilizes emerging technology to treat infections caused by bacteria that are resistant to other methods. In the past photodynamic therapy has attempted to harness the power of visible light in combination

with a photosensitizer chemical, to kill malignant and pathogenic cells by damaging their structure or metabolic pathways. The goal of my project is to gather data to advance the effectiveness and understanding of porphyrin technology utilizing a patented novel porphyrin under dark conditions as a possible antibacterial treatment against infections caused by *Mycobacterium* species.

Background and Significance

Antibiotic Resistance in Healthcare Settings

Antibiotic resistance in healthcare settings has the potential to be catastrophic if the trends that are being seen continue. During one year in the United States the Centers for Disease Control estimate that there are upwards for 2 million illnesses due to antibiotic resistance and as many as 23,000 deaths according to the most recent data released in 2015. Since the publication of this data by the CDC, the first pan-drug resistant infection was seen in a urinary tract infection caused by *Escherichia coli* in May of 2016 (The Washington Post, 2016). As the pharmaceuticals that are used as the last-line of defense begin to fail, common infections can become fatal without the development of alternative treatments.

A secondary issue that presents as antibiotic resistance continues worsen is the occurrence of virulent opportunistic pathogens causing harm for patients. Most notably are infections caused by *Pseudomonas auerginosa*, methicillin resistant *Stahpylococcus aureus* (MRSA), and *Clostridium difficle*. With symptoms ranging from uncontrollable diarrhea to tissue necrosis to the point of loss of limb, opportunistic pathogens create another costly outcome of antibiotic resistance. Disrupting the human microbiome with broad-spectrum antibiotics creates the perfect environment for bacteria that are present in normal to undergo a population explosion (e.g. *Clostridium difficle* infections).

According to the National Institutes of Health the use of broad-spectrum antibiotics is on the rise since new antibiotics are not being developed (Doron and Davison, 2011). A microbial imbalance can wreak havoc on a person's body, called dysbiosis, leading to a need for antibiotics that work with greater specificity. By initially targeting the

pathogenic microbe through alternative treatments, broad-spectrum antibiotics can be used less and lead to more positive outcomes following treatment.

As the list of antibiotic resistant species of microbes continues to grow there is a call for antibiotic stewardship at both the medical and consumer levels. Overuse and over prescription of antibiotics is a large proponent to the problem being faced by the medical community. With the implementation of stricter standards and tighter regulation, the occurrence of opportunistic pathogen infections will likely decrease as seen in Figure 2 (Talpaert, et al, 2011). Although a large portion of antibiotic use originates out of the medical community, other areas contribute to resistance such as agriculture and farming practices as well as the presence of antibiotics in household items such as hand soap. By increasing the amount of exposure to antibiotics in the daily lives of the average population there is an increased risk for the development or transfer of resistance genes which can have devastating impacts in the future. Some may argue that future is nearer that currently anticipated.

Porphyrin Technology as a Possible Solution

An emerging technology that is being explored as a possible alternative to antibiotics are treatments utilizing photosensitizers called porphyrins. Porphyrins are highly conjugated, heterocyclic compounds with a transition metal constituent central to the structure. Treatment with this type of compound is referred to as antimicrobial photodynamic therapy or APDT. In this therapy porphyrins (which are light sensitive) are activated by visible light adjusted to the appropriate wavelength to illicit excitation. Mechanism is seen in Figure 3. Upon excitation, the photosensitizer catalyzes a reaction

with the oxygen present in the aerobic environment to create reactive oxygen species (Banerjee, et al). The porphyrin itself remains unconsumed at the completion of the reaction. Changing the constituents attached to the ring structure or the transition metal in the center of the porphyrin have resulted in different APDT properties observed.

Current Clinical Uses of Porphyrin Technology

The current clinical uses of porphyrin technology are limited to infections or rapidly dividing cell conditions on surfaces that are able to be exposed to light. Trials done in the field of oncology in recent years have yielded positive results. Photosensitizer technology is beneficial in these cases by inciting rapid cell lysis in the vasculature supplying malignant tumors (O'Connor, et al, 2009). Another condition that APDT is proving useful for is macular degeneration. Research suggests that porphyrin treatment can restore visual acuity for extended periods of time in individuals with the age-related from of the condition (TAP Study Group, 2001). Photodynamic therapy has the possibility for specificity to target diseases involving rapidly dividing cells that lead to serious conditions.

Patented ZnP Porphyrin

Dr. Jayne B. Robinson Ph.D. of the biology department and Dr. Shawn Swavey Ph.D. of organic chemistry at the University of Dayton published a patent in February of 2016 for a novel porphyrin meso-5,10,15-tris(N-methyl-4-pyridyl)-20-(pentafluorophen-yl) porphyrinatozinc, (II), tris-p-toluene sulfonate, referred to as ZnP. Structure can be seen in Figure 4. ZnP is a transition metal porphyrin with a zinc ion constituent in the

center and has a fundamental characteristic specific to this porphyrin – dark toxicity. The ability for ZnP to be able to kill bacteria in the absence of light increases the potential use of porphyrin technology to inhibit infections under a wide variety of conditions. Testing has shown that ZnP is not harmful to adjacent healthy tissues and structures not the target of treatment making it an appealing alternative treatment for clinical infections. Due to the stability of the compound and ability to produce therapeutic results without harming eukaryotic tissues possible routes of administration are orally, intravenously, or by metered dose inhaler (Robinson and Swavey, 2016).

Dark Therapy Porphyrins and Hypothesized Mechanism

While the light mechanism for porphyrin photodynamic therapy is well-studied, hypotheses for the dark therapy mechanism are only beginning to be developed. Since toxicity is unrelated to photon excitation and creation of reactive oxygen species, the mechanism inhibits bacterial growth by another pathway. One possibility is that ZnP uptake causes perforations in the cell wall of a bacterial cell leading to the uptake of the porphyrin and the devastating effects of intracellular structures effluxing out of the cell. After ZnP is uptaken into the cell a second hypothesis supported by data recently gathered show evidence that ZnP intercalates with bacterial DNA to inhibit transcription (Figure 5).

Disruption of cellular function in this way may be dependent upon the composition of the cell wall which was proven to be null by ZnP having bactericidal effects again Gram positive and Gram negative bacteria *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Robinson, 2014). Having a cell-wall dependent hypothesis

creates interest in understanding the mechanism of uptake and bactericidal growth inhibition in the dark with *Mycobacterium smegmatis*. Comparison of bacterial cell walls can be seen in Figure 6.

Mycobacterium smegmatis as a Model Organism

The bacteria *Mycobacterium smegmatis* serves as a model organism for *Mycobacterium tuberculosis* since both of the microorganisms display similar physiological characteristics and morphology. Cells classified under the genus *Mycobacterium* have a characteristic cell wall with a high density of lipids which gives rise to the microbe's unique antibiotic resistant qualities (Parish, 2008). The advantage of working with *M. smegmatis* is that it is a non-pathogenic strain and is able to be safely used in laboratory facilities on the University of Dayton's campus. The decision to use *M. smegmatis* as the organism in question was two-fold with the first reason being porphyrin technology has not been tested on any species of its kind. The second reason is the domestic and international significance of the devastating effects of *Mycobacterium* infections cause and what that means for healthcare providers and patients alike.

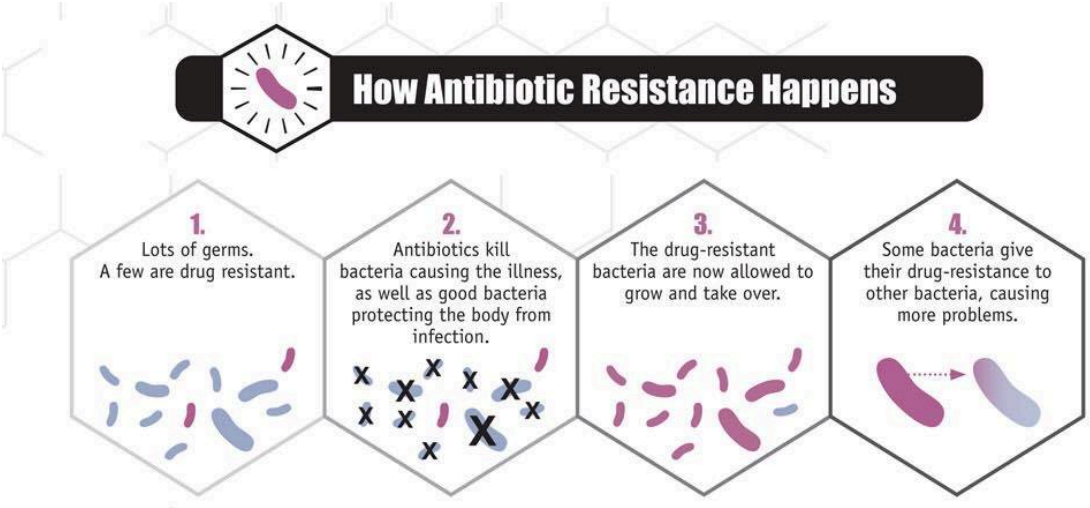


Figure 1

Diagram courtesy of the CDC.

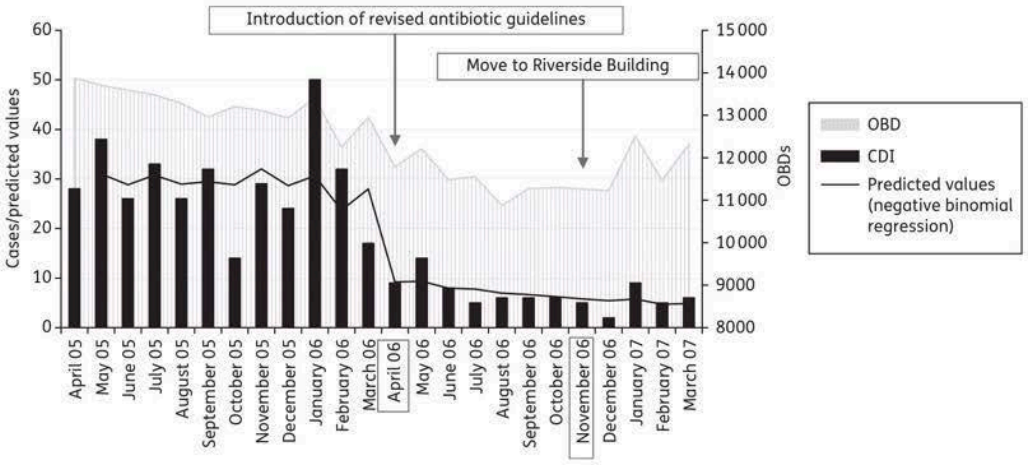


Figure 2

Data from Talpert, et al, 2011 showing the impact of the introduction of revised broad-spectrum antibiotic stewardship data on the occurrence of *Clostridium difficile* infections. Significant reduction in infections was seen post introduction of guidelines.

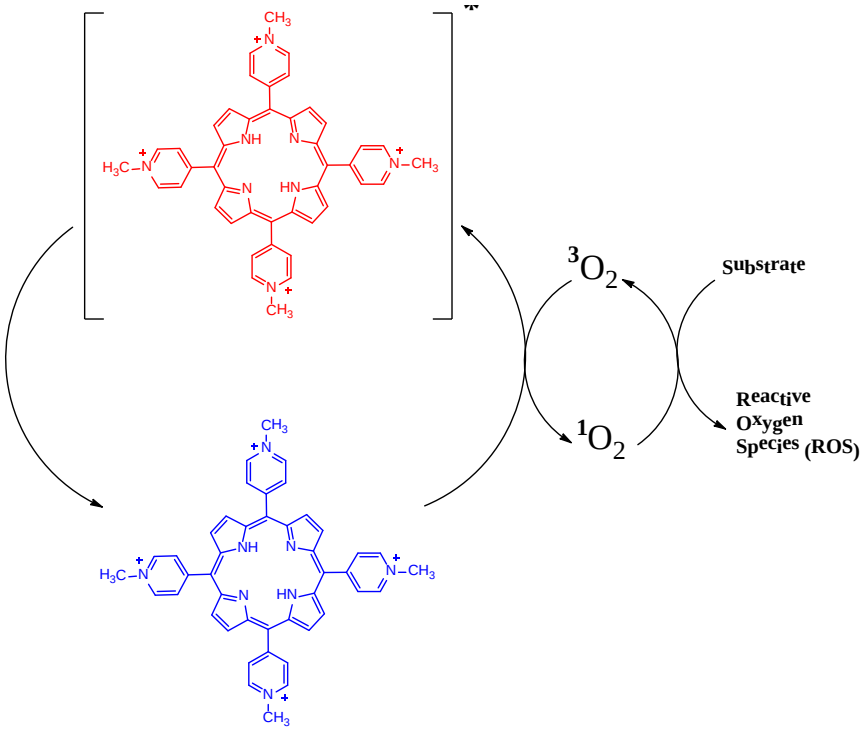


Figure 3

Diagram of the mechanism of light reactions utilizing porphyrins to create reactive oxygen species in aerobic environments which are toxic to bacterial species.

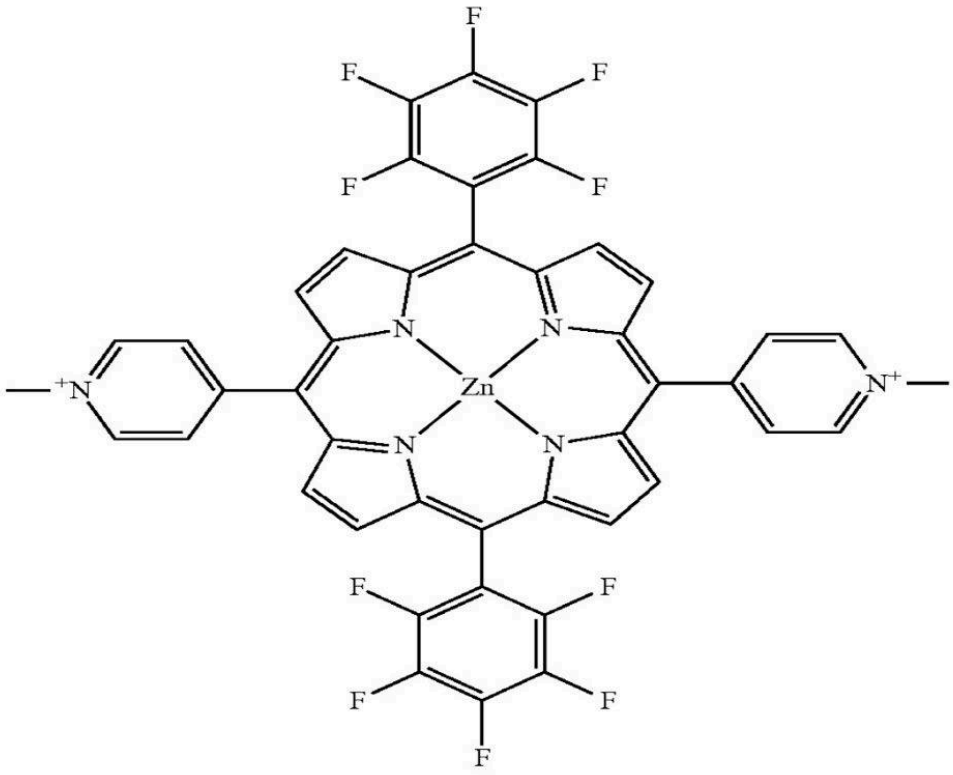


Figure 4

Structure of the novel Zn porphyrin patented by Dr. Jayne B. Robinson Ph.D. and Dr. Shawn Swavey Ph.D. (2016).

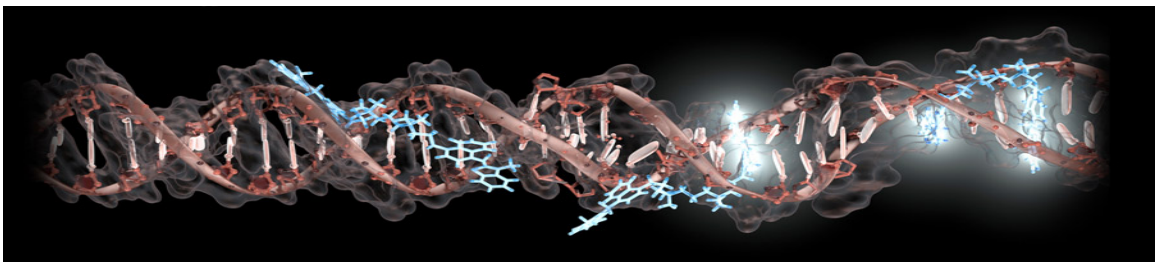


Figure 5

Visual representation of intercalation courtesy of The Perkins Lab at University of Colorado, Boulder. There is evidence that ZnP intercalates between base pairs disrupting bacterial cell transcription.

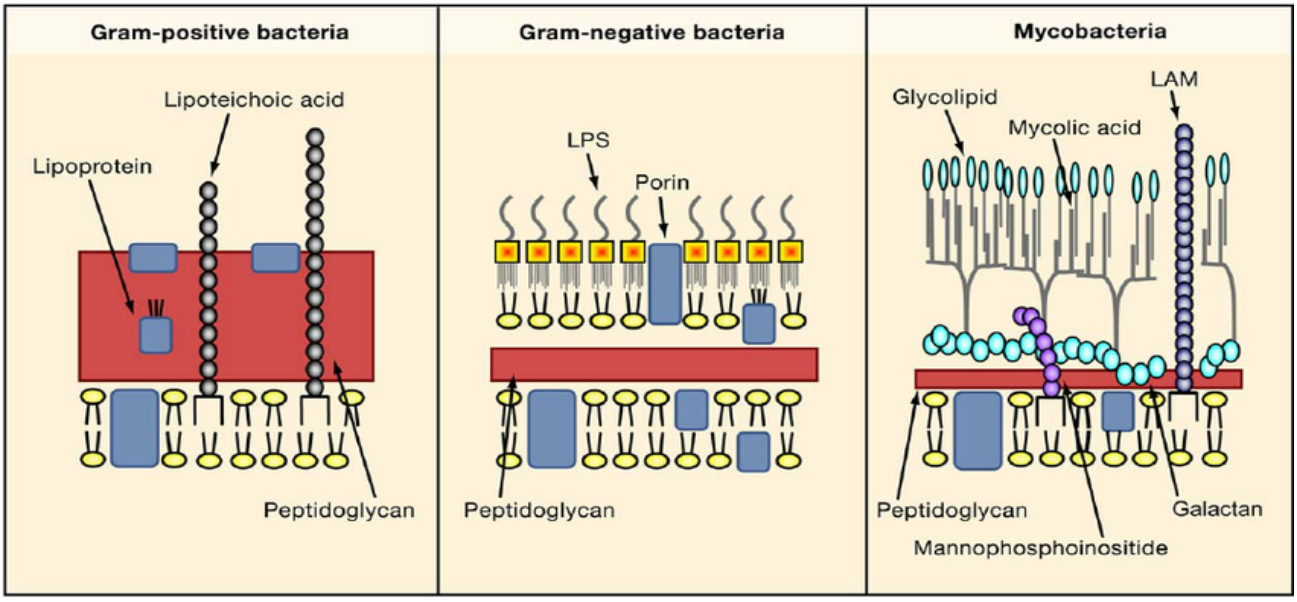


Figure 6

The diagram above details the components of different classifications of bacteria based upon cell wall constituents - Gram positive, Gram negative and *Mycobacteria*. Cell wall may play a role in the dark mechanism of ZnP.

Preliminary Data

One standard, and widely used porphyrin is 5,10,15,20-Tetrakis(4-methoxyphenyl)-21H,23H-porphine tetra-p-tosylate salt or TMP, structure can be seen in Figure 7. In past studies, TMP has shown a high rate of killing planktonic *Pseudomonas aeruginosa* cells in the light, however, TMP is unable to produce the same effect on *P. aeruginosa* cells in the dark (Robinson, 2014). The results found for TMP are in contrast to a second, novel patented porphyrin Zn PHPTMP, or ZnP. The advantage of using ZnP in contrast to TMP, is that in experiments using organisms such as *Pseudomonas aeruginosa* and *Staphylococcus aureus* not only did the porphyrin possess light toxicity but dark toxicity as well (Robinson, 2016).

Biofilms formed by *P. aeruginosa* in vitro were treated with TMP in light conditions and data collected (Figure 8) supported the claim that TMP was able to reach the bacteria through the biofilm layers (Collins, et al, 2010). A second experiment was done that tested the toxicity of TMP involving exposing a biofilm of *P. aeruginosa* to the porphyrin under dark conditions and then treating the experimental cultures with antibiotics. Live dead stains differentiate between live cells with intact cytoplasmic membranes that appear green and cells considered dead with a compromised membrane that appear red to show the effectiveness of antimicrobial treatments. After completing a live dead stain, it was revealed that although the biofilm was formed before being exposed to TMP, the antibiotics were able to reach the bacteria and kill the vast majority of cells growing as seen in Figure 9.

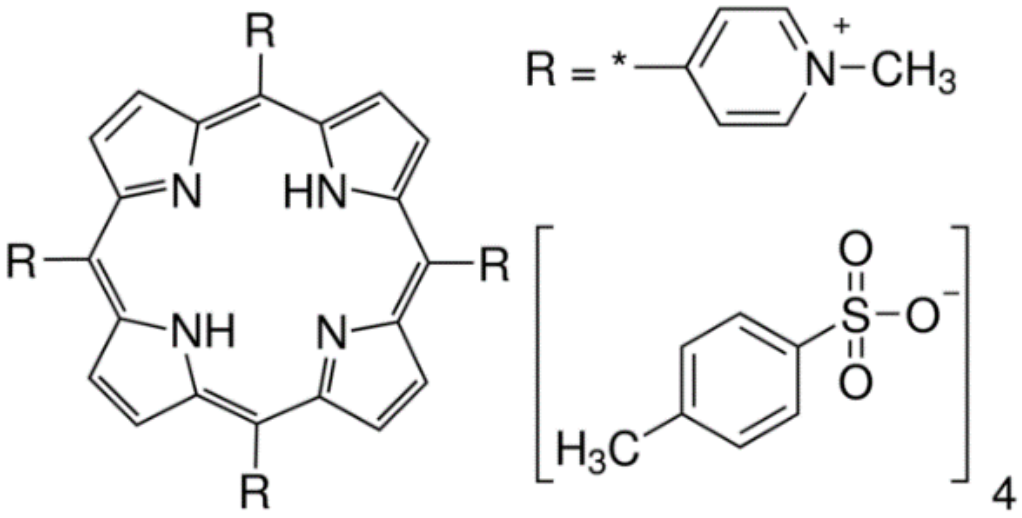
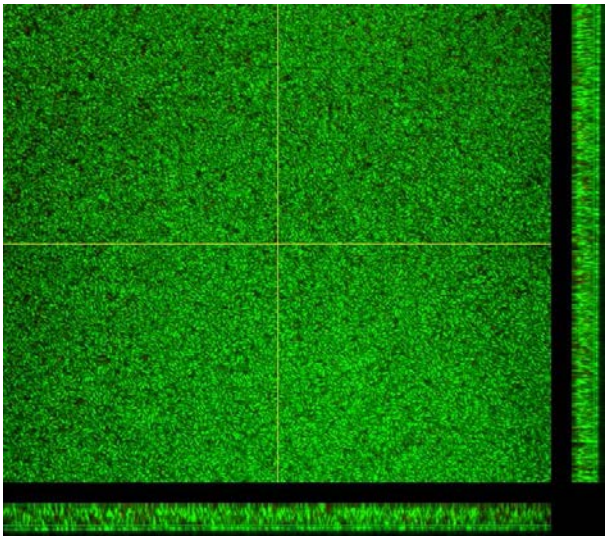
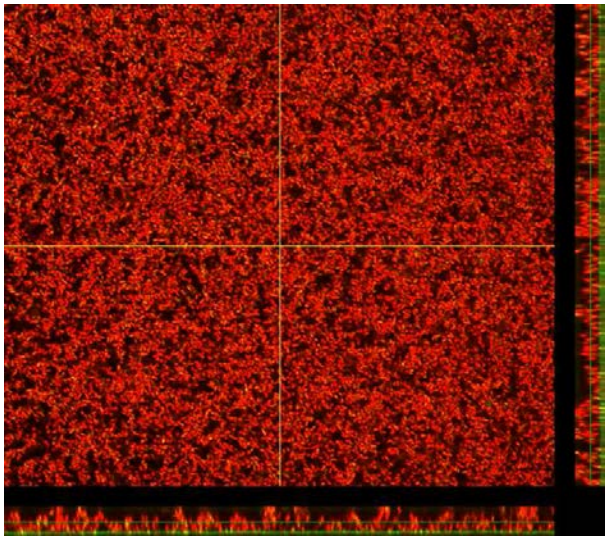


Figure 7

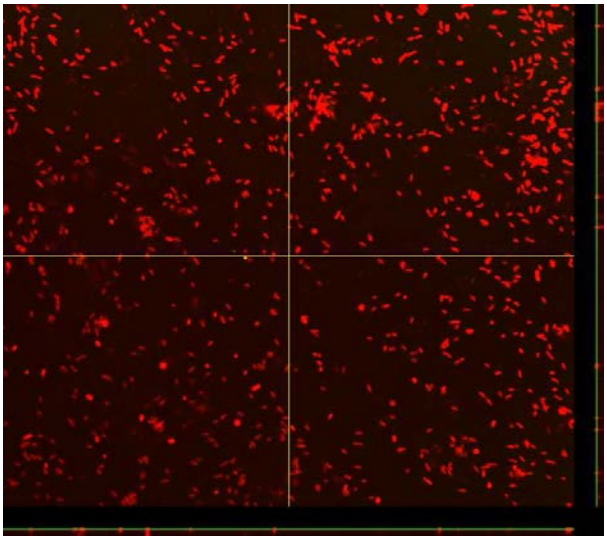
Structure of TMP exhibiting the highly conjugated carbon ring structure typical of porphyrins.



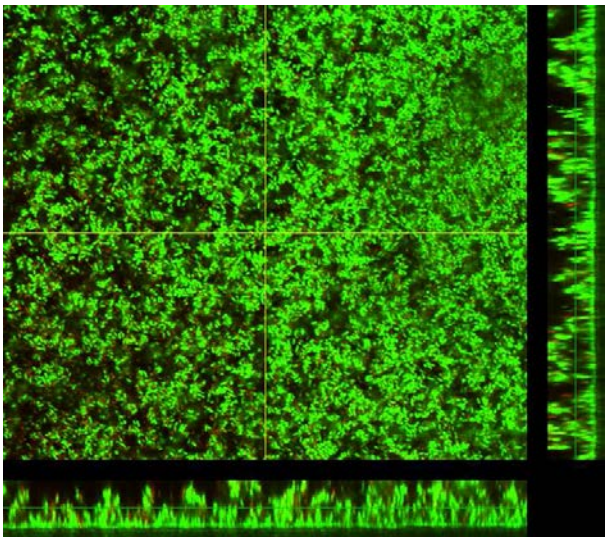
Light only



100 µM TMP + light



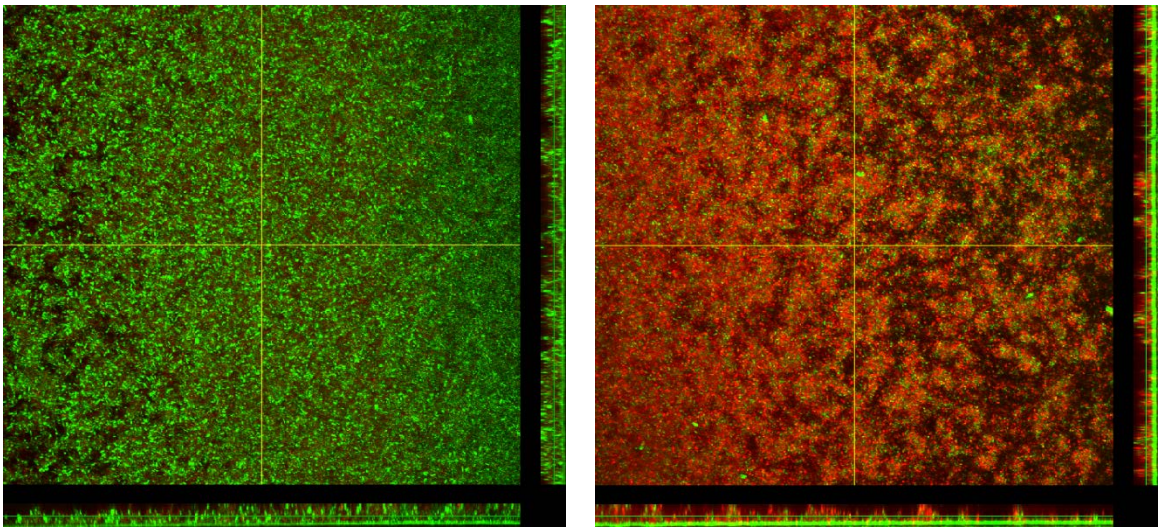
225 µM TMP + light



225 µM TMP (no light)

Figure 8

Live Dead Stain of *Pseudomonas aeruginosa* from a previous experiment published by Collins, et al. The cells fluorescing green are live cells, the cells fluorescing red are dead cells.



Tobromycin Only **TMP + Antibiotic Tobromycin**

Figure 9

Live dead stain of *Pseudomonas aeruginosa* from a previous experiment published by Collins, et al (green = live cells, red = dead cells). The porphyrin TMP is shown to increase the effectiveness and killing capacity of the antibiotic tobramycin.

Materials and Methods

Culturing Specifics

The cultures of *Mycobacterium smegmatis* were taken from -60° F freezer stocks and grown initially on sterile Luria broth (LB) agar plates, a nutrient rich and non-selective media. Cultures were grown aerobically in a 37° C incubator for three days per the average growth time and specifics for *M. smegmatis* (Philly study). Isolate colonies were selected using a sterile loop and used to inoculate specified liquid media for further experimentation.

Ananerobic Killing Assays

M. smegmatis colonies were selected from LB agar plates and grown in 10mL liquid minimal salt glucose media (MSG). Cultures were places on a shaker at 180 RPMs in a 37° C incubator for 3 days. The liquid culture was diluted to an optical density (OD) of 0.15 using sterile 0.1% peptone solution in an autoclaved 150mL flask and placed back on the shaker in the incubator for a duration of one hour. Using three sterile 1mL centrifuge tubes, a control and two subsequent concentrations (25mM and 50mM) of ZnP porphyrin were prepared. The control contained 900 µL of 0.15 OD *M. smegmatis* culture suspended in peptone and 100 µL of sterile H_2O to equal a total volume of 1 mL. The first experimental trial had a concentration of 25 µM of ZnP. The porphyrin was filter sterilized and suspended in sterile water. 900 µL of 0.15 *M. smegmatis* was added to a 1 mL sterile centrifuge tube. To achieve the proper concentration, 50 µL of ZnP porphyrin stock with a concentration of 0.5mM was added in addition to 50 µL of sterile H_2O to achieve a total volume of 1mL. The final experimental trial had a concentration of 50 µM

of ZnP porphyrin. 900 μL of 0.15 *M. smegmatis* was added to a 1 mL sterile centrifuge tube. To achieve the proper concentration, 100 μL of ZnP porphyrin was added to achieve a total volume of 1mL. A 96 well plate was used to complete seven 10^{-1} serial dilutions. The dilutions were then plated in 10 μL aliquots on LB media agar plates at time points zero, seven and twenty-four hours. The plates were grown aerobically in a 37° C incubator for three days. Between time points the centrifuge tubes were kept in static conditions in the incubator and plated directly. Qualitative morphology results and plate count data were gathered after three days of growth. Plate count data was graphed to track the effect of bacterial growth inhibition due to the porphyrin.

Aerobic Killing Assays

The same protocol was followed as for the anaerobic killing assay however between time points the centrifuge tubes were aerobically grown on a shaker at 180 RPMs in a 37°C incubator. The serial dilutions were plated in 10 μL aliquots on LB media agar plates at time points zero and twenty-four hours. Qualitative morphology results and plate count data were gathered after three days of growth. Plate count data was graphed to track the effect of bacterial growth inhibition due to the porphyrin.

Resistant Colony Isolation

The aerobic killing assay experimental trials data revealed a few individual colonies growing on the 50 μM ZnP porphyrin LB plate for the twenty-four hour time point. The colonies were selected using a sterile inoculating loop and streaked on Lownstien-Jensen

selective media that is selective for *Mycobacterium* species. Qualitative morphology results were gathered after three days of growth.

Fluorescent Microscopy

Fluorescent microscopy was used to visualize the uptake of ZnP over a set of time point in a culture of *M. smegmatis* using an Olympus BX51 fluorescence microscope. The cultures were grown under conditions identical to the killing assay trials. When the control, 25 μM and 50 μM centrifuge tubes were properly prepared, 10 μL aliquots of each were placed on microscope slides and secured with glass cover slips to be viewed. Images were taken at time point zero, after 45 and 120 minutes. High power objectives viewed under oil immersion. The fluorescence filter used was Texas Red at an excitation wavelength of 561 nm and an emission wavelength of 615 nm (Abramowitz and Davidson, 2012).

Results

Anaerobic Killing Assay

Plate count data (CFUs/mL) gathered after three days of incubation showed insignificant reduction over the 24 hour time period. The data can be seen in Figure 10. Standard error bars can be seen. Qualitative findings revealed a change in the morphology of colony forming units, however, bactericidal effects were not apparent. When isolate colonies of the altered morphology were streaked on to new sterile LB agar plates, wild type rescue was not seen suggesting a true alteration of *M. smegmatis* morphology and no reversion back to wild-type growth.

Aerobic Killing Assay

Altering the protocol to keep the bacteria exposed to ZnP growing under aerobic conditions drastically changed the plate count data gathered. At the 24 hour time point there was near-complete clearing of both 25mM and 50 mM ZnP concentration trials. Data is presented in Figure 11. Standard error bars are present. Qualitative findings revealed the few single colony isolates that appeared on the 50 mM ZnP concentration LB agar plate to be phenotypically similar with wild-type *M. smegmatis.*

Resistant Colony Isolation

Single colony isolates from 50 mM ZnP trial were streaked using a sterile inoculating loop on LJ medium and revealed substantial growth after an incubation at 37° C for a period of 3 days.

ZnP Porphyrin Uptake Visualized with Fluorescent Microscopy

Control images taken of MSG liquid media and initial *M. smegmatis* culture revealed no significant findings. Time point zero showed faint fluorescence but definition of single cells present in the visual field. After 45 minutes elapsed individual cells could be more clearly seen emitting a red fluorescent glow which was retained for all time periods between 45 minutes and 120 minutes post exposure to ZnP. Images show evidence of porphyrin uptake rather than simple adherence to the exterior cell wall of individual bacterium. Images can be seen in Figure 12.

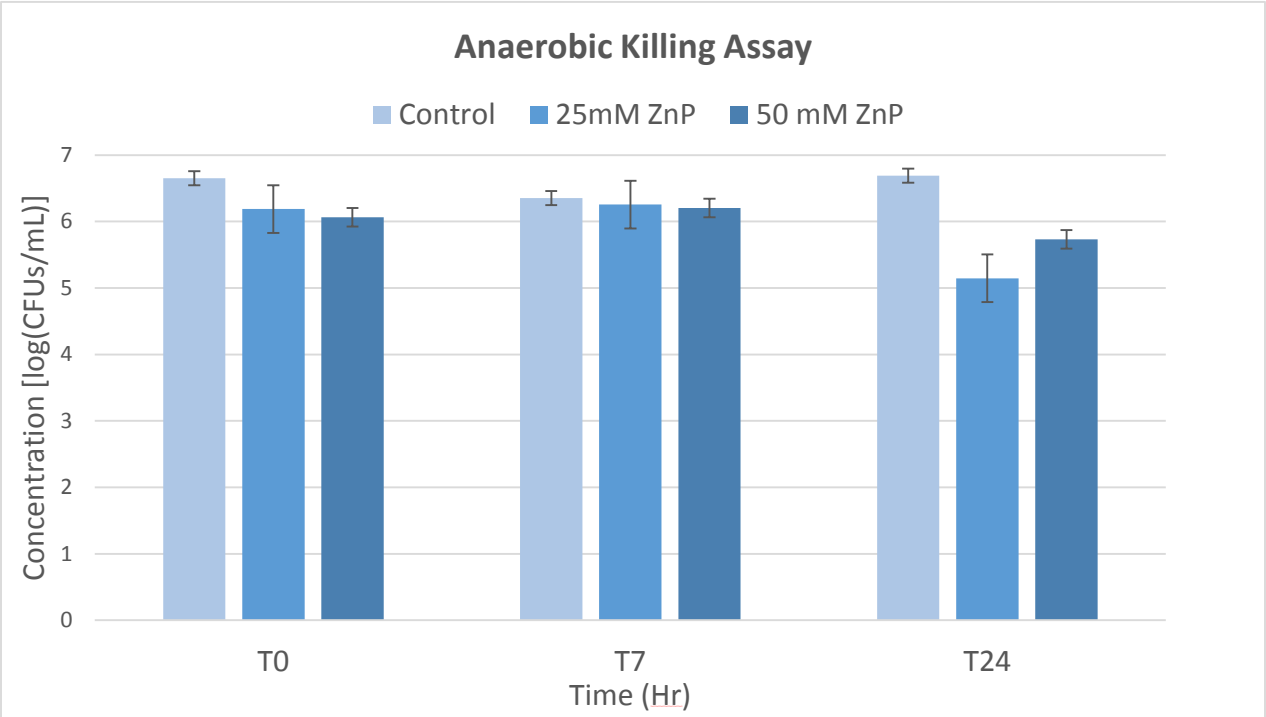

Anaerobic Killing Assay
Control
25mM ZnP
50 mM ZnP
Concentration [log(CFUs/mL)]
7
6
5
4
3
2
1
0
T0
T7
T24
Time (Hr)

Figure 10

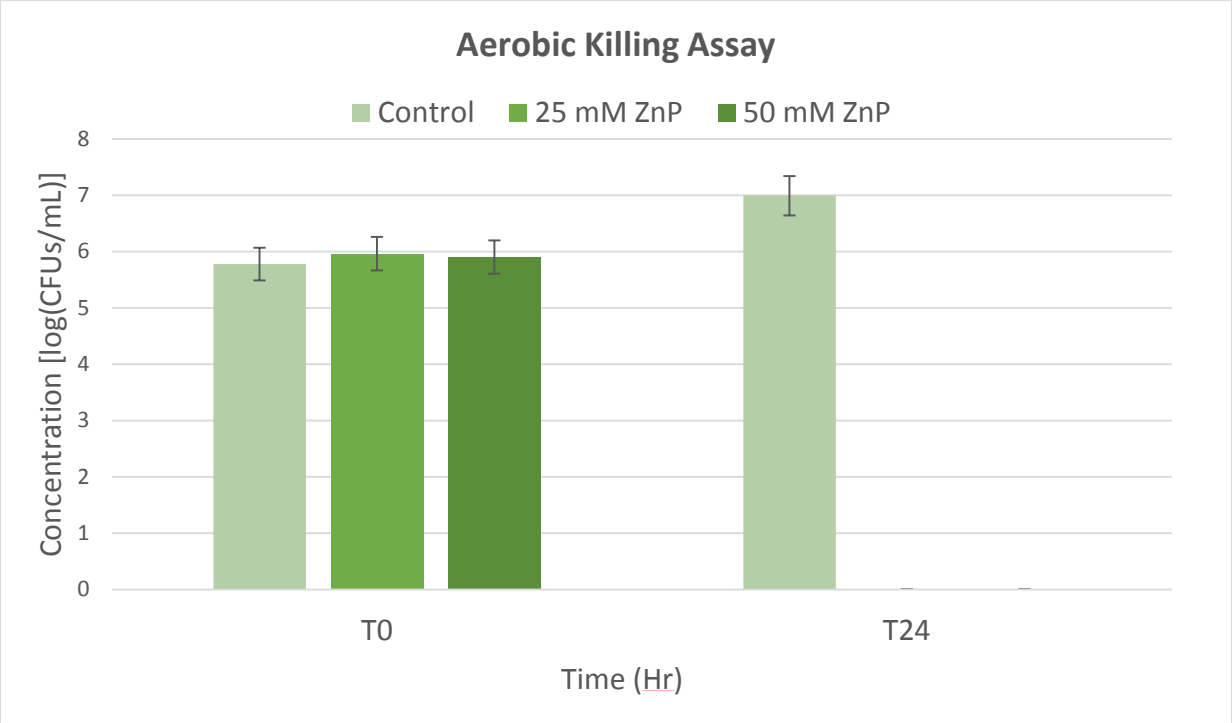
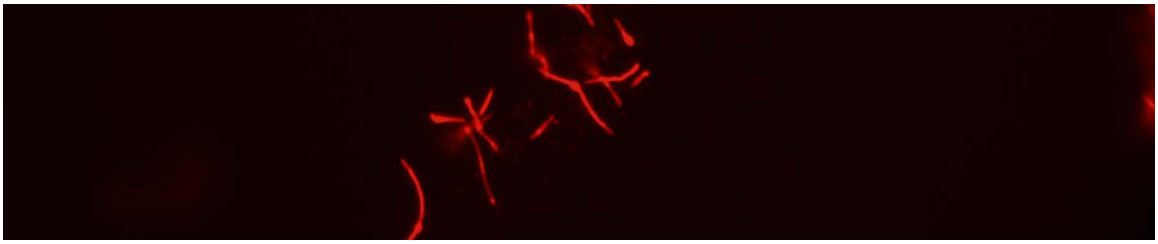
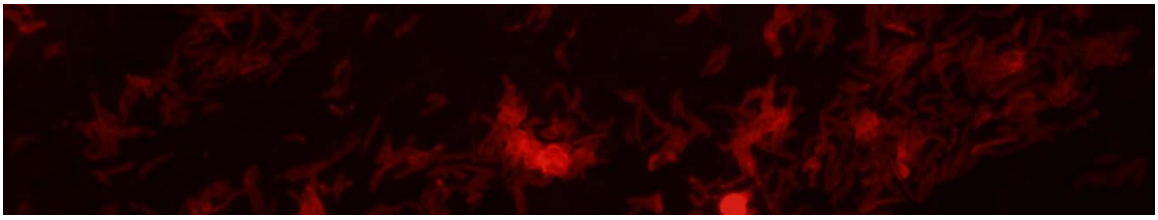

Aerobic Killing Assay
Control
25 mM ZnP
50 mM ZnP
Concentration [log(CFUs/mL)]
8
7
6
5
4
3
2
1
0
T0
T24
Time (Hr)

Figure 11

A



B



C

Figure 12

A) *M. smegmatis*, 45 min post exposure, 25mM ZnP, Texas Red, Fluorescent Microscope, 100X, Oil immersion **B)** *M. smegmatis*, 45 min post exposure, 50mM ZnP, Texas Red, Fluorescent Microscope, 100X, Oil immersion **C)** *M. smegmatis*, 120 min post exposure, 50mM ZnP, Texas Red, Fluorescent Microscope, 100X, Oil immersion

Discussion and Future Directions

Porphyrin technology has the potential for being an alternative treatment for antibiotics as they become ineffective and research starts to move away from developing new antibiotic pharmaceuticals. ZnP in particular is an important compound due to its ability to effectively kill a range of bacterial organisms each with distinct characteristics. The additional benefit of porphyrins being able to increase the therapeutic effect of current antibiotics provides hope that common infections may not become deadly in coming years.

The results gathered from exposing *Mycobacterium smegmatis* to ZnP provides evidence of the potential clinical use of this compound against lung infections like tuberculosis. Being able to better define the effects of ZnP in aerobic and anaerobic conditions advances the understanding of how it would perform in vivo since the lungs have sites of characterized by different levels of oxygenation. To gather data in vitro that explores the possibilities of ZnP's therapeutic effect can lead to more well defined and efficient procedures in future studies.

The finding that ZnP is successful in killing *Mycobacterium smegmatis* provides a foundation for future testing to further quantify the therapeutic effects of porphyrin dark therapy. Due to the specificity of ZnP the promise of its use in clinical settings on human patients is almost tangible. Seeing as that is a hope for the far future there is still data that needs to be gathered to be able to move forward with implementation. A minimal lethal concentration (MLC) must be defined in order to most effectively use resources when treating infections caused by *M. smegmatis*. To better understand the possible impact that mycolic acid and other constituents of the cell wall of *Mycobacterium* species, the *M.*

smegmatis MLC can be compared to Gram positive and Gram negative MLC data. If dark therapy porphyrins do in fact have a mechanism dependent upon the bacterial cell wall then analysis of the data may reveal trends based upon cell wall makeup.

Another area that can be more well defined is not only the rate of ZnP uptake in wild-type *M. smegmatis* cells but the uptake of additional substances post-exposure. The importance of this data would be to see if antibiotics or other pharmaceuticals can be transferred to the intracellular environment for a therapeutic effect. Mycolic acid is fairly difficult to penetrate with current therapies which using the theory that ZnP is able to pass through the cell wall and possibly leave openings to allow exchange. In the event ZnP does interact with the cell wall this way, porphyrin dark therapy could increase the effectiveness of additional bactericidal therapies that exist but have low efficacy under current conditions.

Antibiotic resistance being a pressing issue and horizontal uptake of resistance genes causing troublesome outlooks for the future of infectious disease, porphyrin dark therapy may prove useful. Data gathered on the effect of ZnP exposure on antibiotic resistant strains of bacteria has the possibility increasing the therapeutic effect of current pharmaceuticals. Increased uptake of additional substances can lead to using technology already as a more efficient use of resources.

Whether or not the isolate colonies seen on 50mM aerobic killing assay LB agar plates were truly resistant is another area that will take additional trials. Of the trials, isolate colonies like these were only seen in one of the replicates per trial. If truly resistant, frequency of resistance must be determined and further experimentation includes

exposing subcultures to ZnP at varying concentrations higher than 50mM to see how often and under what conditions do similar colonies appear.

The final aspect that is to be explored that was beyond the scope of this project is to determine the effectiveness of either porphyrin (TMP or ZnP) on biofilm formation of the model organism. Previous experiments have shown the ability of porphyrins to penetrate the seemingly impenetrable external matrix produced by biofilms. Historically bacterial biofilms have prevented antibiotics from being effective against established biofilms due to the secretion of polymeric substances.

Acknowledgements

First I would like to thank my mentor, Dr. Jayne Robinson, for her guidance and support throughout this project. To start in her microbiology lab during the fall of my freshman year and continue through graduation has given me the opportunity to learn and grow as a researcher and individual in ways I never thought possible. I also want to thank Neha Patel, the Ph.D. candidate currently in our lab as well for her ability to answer my array of questions to be able to produce accurate results. Thank you to the Honors Program for serving as the foundation for my Honors Thesis and financial support. The College of Arts and Sciences Office of the Dean selected me to be a part of their 2016 cohort of the Dean's Summer Scholar's program which provided housing and financial assistance to complete work during the summer term. I cannot forget to mention how important the support from my family, friends and other peer undergraduate researchers has been throughout this process to complete an Honors Thesis.

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