2022

Analysis Of The Antimicrobial Activity Of The Novel Chemotherapeutic Drug, Tpp1, Against Pseudomonas Aeruginosa And The Hydrogel Delivery Of Water-Soluble Antimicrobial Compounds

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THE COLLEGE OF WOOSTER

Biochemistry and Molecular Biology

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A THESIS SUBMITTED TO THE FACULTY OF THE COLLEGE OF WOOSTER
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
THE DEGREE OF BACHELOR OF ARTS

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Wooster, Ohio

March 28, 2022

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ABSTRACT

Cancer is one of the leading causes of death in the world, and it is commonly linked with bacterial infections that often complicate treatments. Recently, chemotherapeutics have been developed that are able to act as anti-cancer agents using delocalized lipophilic cations (DLCs) that are able to specifically target mitochondrial membranes of cancer cells. TPP1 is a newly developed chemotherapeutic drug that has activity against bladder cancer and melanoma cell lines *in vitro*. In order to determine if TPP1 has antimicrobial activity, TPP1 was tested against a common bacteria, *Pseudomonas aeruginosa*, to determine if antimicrobial activity was present. This testing was done using the Kirby-Bauer disk diffusion method and the zones of inhibition were determined. TPP1 did not appear to have any activity against PAO1 at the tested concentrations. To explore the delivery mechanism of water-soluble antimicrobial compounds, testing was done to determine if these compounds are able to swell into a poly vinyl alcohol (PVA) hydrogel. The hydrogels were able to increase their mass by an average of 28.78% over eight hours. As well, the PVA hydrogel is an effective delivery method for water soluble antimicrobial compounds as they can have antimicrobial activity against PAO1 when swelled with silver nitrate

INTRODUCTION

The link between bacterial infections and cancer is very significant. Infections themselves can contribute to the formation of cancer and metastasis due to the bacterial infection cycle (1). Similarly, once a tumor has formed, there is an increased risk of bacterial infections located around the tumor and other regions of the body that can make cancer treatment more complex (2). For example, lung cancer causes a large increase for the risk of pneumonia and other pulmonary infections where up to 70% of patients with lung cancer are diagnosed with pneumonia (3). Likewise, infections in the bladder such as urinary tract infections increase the likelihood of developing bladder cancer, and early on, it is very difficult to differentiate between bladder cancer and bladder infections as they exhibit similar symptoms like frequent urination, discomfort when urinating, and blood in the urine (4). Certain chemotherapeutics such as isoniazid and its derivatives have the ability to double as antimicrobial agents. Isoniazid, for example, is able to treat lung cancer as well as tuberculosis. This dual ability allows for the treatment of cancer and any resulting infections to be easier and more efficient (5).

Throughout recent history, cancers have been primarily treated in four ways: surgery, radiotherapy, immunotherapy, and chemotherapy (6). The first step in treatment often involves surgery in an attempt to remove the tumor, but this only goes so far as there can be metastasis around the tissue or the tumor could be unable to be removed. Surgery is often paired with radiotherapy on the tissue from which the tumor was removed and has proven to be effective in some types of cancers. The early stages of breast

cancer regularly involve a surgery to remove the tumor followed by radiation (7). More recently, immunotherapy has been used and has helped to revolutionize cancer treatments. This treatment mainly is used to inhibit the immune response at different checkpoints or impede the function of cell receptors. Immunotherapy primarily functions through the lymphocytes and acts to increase the activity of T cells and B cells against foreign bodies and cancer cells. Although effective in some cases, immunotherapy is only effective in small subsets of cancer types (8). This brings up some of the big problems in cancer treatment: cancer is very complex, and much is unknown as to how and why cells metastasize. Not all treatments work for all cancers, so it is necessary for different treatments to be available to cancer patients based on cancer type. Chemotherapy is commonly used and is the chemical treatment of cells that is either done to cause cancer cell death and be curative or to inhibit the growth of new cancer cells (9).

There have been multiple chemotherapeutics developed in recent times that have been effective in specifically targeting cancer cells in certain types of cancer, but others, due to their chemical nature, can have a harmful effect on healthy cells (6). Chemotherapy and radiation can inhibit the normal functioning of healthy cells and even cause cell death. Some chemotherapeutics, such as delocalized lipophilic cations (DLCs) are able to specifically target cancer cells and avoid many of the harmful effects present in other chemotherapeutics (10).

Generally, DLCs often kill cancer cells by targeting their energy source: the mitochondria. Many different cancers are dependent on mitochondrial processes and oxidative phosphorylation which operate at normal or heightened levels in cancer cells (11). Previous work was done to look at DLC mechanism of action and to focus on the

mitochondrial membrane of cancer cells. Within most cancer cells, there is an increased mitochondrial membrane potential due to heightened activity of the electron transport chain and other oxidative phosphorylation pathways (11). In one experiment, two groups of lung cancer cells were treated. One group was treated with ATPase inhibitors to increase the mitochondrial membrane potential while the other group had p53 tumor suppressor genes inserted to decrease mitochondrial membrane potential. When DLCs were added into solution they were found to accumulate in the ATPase inhibitor group with the higher mitochondrial membrane potential. This experiment concluded that DLC accumulation is targeted to the areas of high membrane potential, and accumulation results in the selective targeting and inhibition of oxidative phosphorylation in the mitochondria of cancer cells (12, 13).

One common group among many DLCs is the triphenyl phosphonium (TPP) group (Figure 1). Mitochondria membrane targeting compounds such as dodecyl-TPP utilizes the TPP as it is non-toxic and has bioactive properties (14). Within the TPP group is three aromatic rings connecting to a positive phosphorous ion, and this can attract and react with other negatively charged ions.

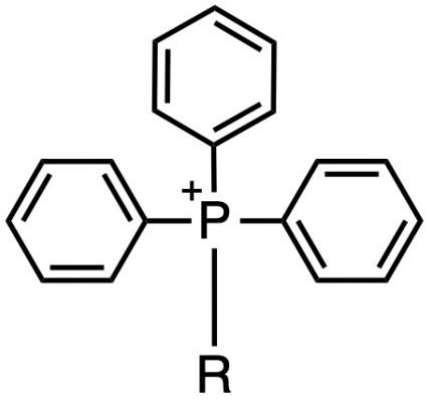

Figure 1: Representative image of the TPP group present in chemotherapeutic compounds.

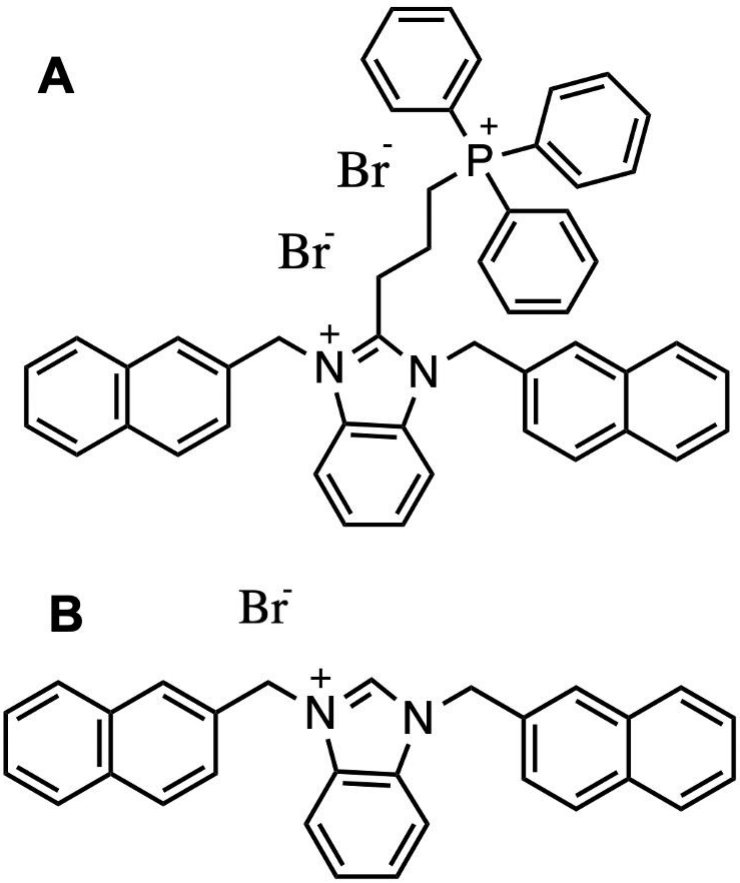

Figure 2: The compound TPP1 (A) consisting of an imidazole salt precursor (B) which the TPP group is bound to.

The TPP group has been known to have chemotherapeutic ability and is present within the drug TPP1. TPP1 is a compound that contains the TPP group and was synthesized through a substitution with an imidazolium salt (2-(3-hydroxypropyl)-1,3-bis(naphthalen-2-ylmethyl) benzimidazolium bromide) (Figure 2) (15). The apoptotic effect of TPP1 was tested with propidium iodide to determine whether cell death or growth inhibition took place. TPP1 had the ability to inhibit the proliferation of bladder cancer cell lines such as RT112 and J82, and TPP1 was also found to induce cancer cell apoptosis. In order to determine the extent of TPP1 action against cancer, TPP1 was tested in the National Cancer Institute's Developmental Therapeutics Program with 60 cancer cell lines. Through these tests, it was found that TPP1 had growth inhibition activity with some

cancer cell lines in melanoma, leukemia, and bladder cancers. In particular, TPP1 showed very promising inhibition results with the melanoma cell line LOX IMVI which is a human melanoma cell line (15). TPP1 was found to act similarly to other DLCs through testing with the use of a mitochondrial dye JC-1. As the mitochondrial membrane potential decreases, JC-1 changes color from red to green, and this depolarization color change corresponded with the initiation of apoptosis in bladder cancer cells (Figure 3). It was able to specifically target mitochondria that have increased mitochondrial membrane potential and induce an apoptotic effect on cancer cells.

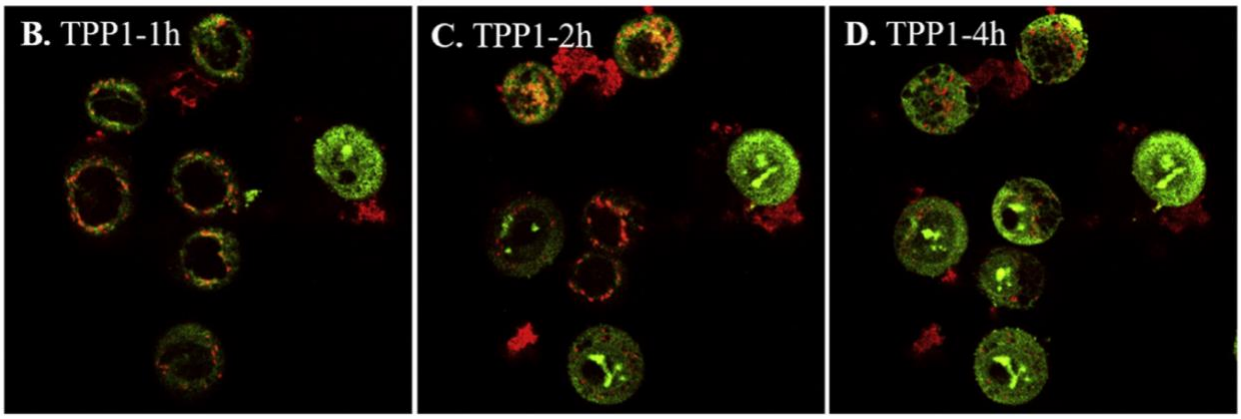

Figure 3: JC-1 mitochondrial membrane stain following treatment with TPP1 on RT112 bladder cancer cells. The red indicated high membrane potential, and the shift to green indicates a shift to low mitochondrial membrane polarization (15).

When it comes to the current treatments of melanoma and bladder cancer, multiple complications arise. Bladder cancer becomes very difficult to treat as it progresses. Its treatment in the past has been done with Bacillus Calmette-Guérin (BCG) immunotherapy. This treatment was shown to be effective in early-stage bladder cancer patients, but in recent time, there has been a worldwide shortage of BCG as it is also

used in the treatment of Tuberculosis (16). Even with BCG treatment, there is no guaranteed success. It has been found that with BCG treatment in later-stage bladder cancer patients, metastasis and death still commonly occur (17). Since the shortage, there has been a need of other effective treatments. Bladder removal has been common in recent time, but it presents to be a very challenging procedure for the patient without the guarantee of success. These problems present the necessity of new approaches and why chemotherapeutics can be effective. With TPP1 having chemotherapeutic ability towards bladder cancer cell lines, this offers another possible treatment for bladder cancer that could be less invasive than bladder removal and possibly more effective than BCG treatments. TPP1 could also be effective in that it could have antimicrobial activity, so it is important to determine how TPP1 acts on bacteria and if it is able to inhibit bacterial growth or induce an apoptotic effect.

Like different bladder cancer cell lines, TPP1's activity against human melanoma cell lines can prove to be very important. Melanoma is a common kind of skin cancer that is first characterized by swelling, redness, and misshapen moles. Melanoma is extremely prone to metastasis and is known to be the most deadly and aggressive form of skin cancer (18). Melanoma treatment has generally consisted of surgical removal of the tumor, chemotherapy, and immunotherapy. When melanoma is removed, this surgery is considered a dermatological surgery, and roughly 8.7% of all general dermatological surgeries result in infection (19). Even with the risk of infection, not all surgeries recommend antibiotic treatment following surgery. Low risk surgeries generally do not require the use of an antibiotics which have been found to be inappropriate and should be discouraged. But when a procedure is deemed high risk based on the location of the

surgery or patients at a higher risk of infection, the use of antibiotics is encouraged. The removal of cancer and melanoma is deemed a high-risk dermatological surgery because the patients are at a higher risk of infection, and these patients are often given antibiotics as treatment. This is commonly a topical ointment or cream that is applied to the site of incision. So, the dermatological removal of melanoma requires an antibiotic to prevent infection of the surgical site. If TPP1 is found to also have antimicrobial properties, it could be administered to the surgical site and act as a dual therapeutic to treat the cancer cells and potentially to stop any infections from forming (19).

In previous studies, there has been evidence to suggest that the TPP group can be included in the structure of antibiotics. One compound containing two separate TPP groups, mitocurcumin (MitoC), has shown antimicrobial activity against both gram-positive (thick peptidoglycan cell wall) and gram-negative (thinner peptidoglycan cell wall) bacteria through the disruption of the bacteria membrane (Figure 4) (20). Although there has been antimicrobial activity in some TPP containing compounds, the mechanism for these compounds is unknown meaning that that the TPP group is potentially not responsible for the activity. In bacteria, TPP is unable to act in the same mechanism that they kill cancer cells due to the fact that bacterial do not contain mitochondria, so TPP is unable to target the increased mitochondrial membrane potential. Another study investigated adding the TPP group to a known antibiotic, ciprofloxacin (CFX) and treating a variety of both gram-positive and gram-negative bacteria. The results were mixed in that the CFX-TPP (Figure 4) derivative lowered the antimicrobial ability against all gram-negative bacteria and some gram-positive bacteria but was more effective against some gram-positive bacteria such as *S. aureus* than CFX (21). It is hypothesized that the TPP

group is able to interact with the lipid membrane of bacteria, but with an unknown mechanism of action. As the TPP group may not be solely responsible for activity it is unknown as to whether chemotherapeutics containing the TPP compound such as TPP1 will be able to exhibit antimicrobial ability.

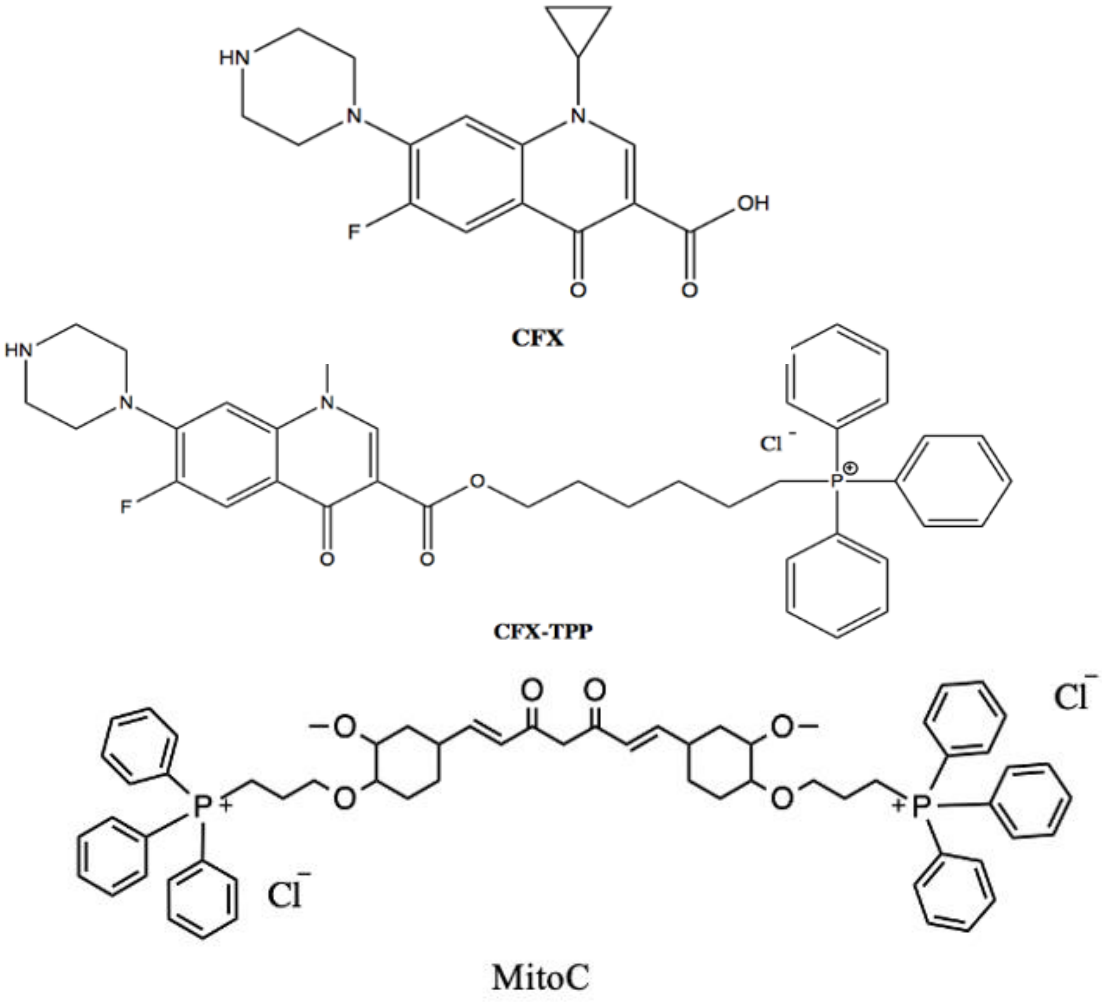

Figure 3: The known antibiotic CFX, the CFX derivative CFX-TPP, and MitoC (20, 21).

Similarly, imidazolium salt containing compounds have shown the ability to have antimicrobial activity. The compounds alkyl-3-methylimidazoliumhalides and alkyl-3-hydroxyethyl-2-methyli-midazolium chloride have activity against a broad range of

bacteria including *P. aeruginosa* (22). The imidazolium salt core allows for additional side groups to be added such as long carbon chains or the TPP group. When it comes to the TPP1 compound, the aromatic rings bonded to the imidazolium salt may be able to contribute to antimicrobial activity and the activity of TPP1 compound as a whole.

Minocycline is a known antimicrobial agent that supports the hypothesis that drugs are able to act as both an antimicrobial and chemotherapeutic agent. It can act on bacteria through its ability to inhibit protein synthesis, and it can act as a chemotherapeutic compound through its ability to inhibit cancer cell metastasis and increase tumor cell death. Although lacking a DLC group like TPP1, it contains some structural similarities of ring complexes that could account for similar activity within bacteria (Figure 4). Overall, minocycline presents the possibility for a complex like TPP1 to be multifunctional as both a chemotherapeutic and antimicrobial agent (23).

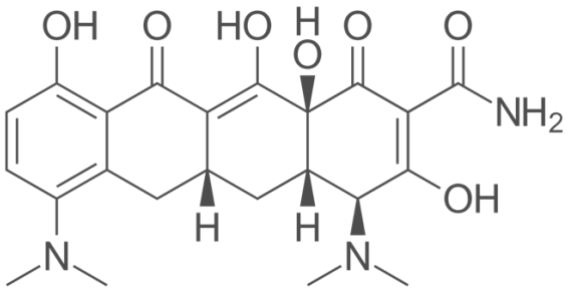

Figure 4: The structure of the antibiotic and chemotherapeutic compound minocycline.

The tests to determine antimicrobial activity of TPP1 will be done mainly using the Kirby-Bauer disk diffusion assay. This experiment will show whether TPP1 has any ability to reduce bacterial growth of PAO1 (24). If a compound has antimicrobial activity, then bacterial growth will be inhibited surrounding the treated disks showing a zone of inhibition. This assay will give insight into whether there is antimicrobial activity against *P. aeruginosa*.

P. aeruginosa is chosen as a model organism as it is very common in infections throughout the body, and it is a gram-negative bacterium. Overall, the majority of the antibiotic resistant bacteria are gram-negative bacteria, so this creates a need and challenge for antibiotics (25). When comparing gram-negative with gram-positive, gram-negative bacteria contain a complex outer membrane that makes diffusion through it more difficult. This membrane contains a rigid peptide cell wall as both an inner and outer membrane. If TPP1 were to have activity against the *P. aeruginosa*, that would highlight its antimicrobial activity and open the door for further testing with more complex or resistant bacteria.

In addition to the testing done on *P. aeruginosa* to determine if TPP1 has any kind of antimicrobial activity, another area of interest deals with the delivery of TPP1 and other water-soluble antibiotics to a site of infection or cancer. There are many different ways that drugs can be administered including orally, rectally, topically, and intravenously. Since TPP1 has a known activity against human melanoma cell lines and dermatological removal of melanoma is often treated with topical antibiotics, the potential for TPP1 to be delivered topically is of great interest. If TPP1 were able to be delivered topically and have antimicrobial activity, then it would be able to treat the surgical site by potentially killing remaining melanoma cells not removed by surgery and help to prevent infection. This is important as cancer causes people to be considered high risk when developing infections as their immune system may be weakened.

One common way that drugs are administered is topically, and many topical products are in the form of a hydrogel as they allow for optimized and controlled delivery of drugs (26). A hydrogel is a hydrophilic compound that is able to have absorptive properties that allow it to swell and absorb water and biological fluids. Hydrogels are also commonly used in drug delivery as they can absorb an active drug and be spread or applied topically onto the target site (27). Poly (vinyl alcohol) hydrogels (PVA) are often formed and used in drug delivery systems and is soluble in water through the hydroxyl groups branching off the carbon chain (Figure 5). PVA can improve the solubility of drugs as it is able to prevent crystal transformation through its density of reactive hydroxyl functional groups (28)**.** Due to its structure, PVA is also non-toxic and biodegradable making it a perfect candidate to administer TPP1 (29). A PVA hydrogel can be easily prepared by mixing and dissolving PVA in heated water. Following this, an overnight freeze thaw cycle is done to form the hydrogel and increase the general viscosity. Once formed, the hydrogel can swell with the water soluble compounds such as TPP1. Both TPP1 and PVA are water soluble allowing for the PVA hydrogel to be formed using water and for TPP1 to swell into the hydrogel. This would allow for the TPP1 PVA hydrogel to be tested on bacteria to determine if the hydrogel delivery method is a viable method (30).

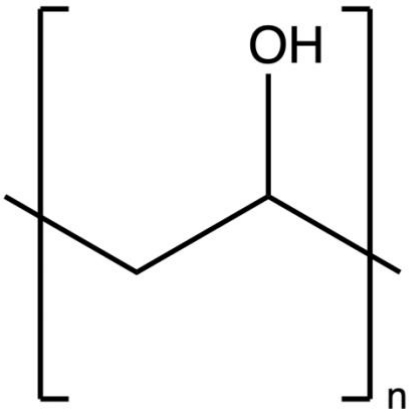

Figure 5: Structure of poly (vinyl alcohol) hydrogels. The length of the carbon chain in the polymer varies in length

This project will determine whether TPP1 will be able to exhibit antimicrobial activity in addition to its known chemotherapeutic ability. This will shed some light on the TPP group as a whole and open the door to determine its method of action towards

antimicrobial activity. Additionally, the research into the delivery method of TPP1 through a PVA hydrogel could present a viable option to the treatment of dermatological surgery and the removal of skin cancers such as melanoma. This research could lead to *in vivo* work to determine if TPP1 is able to have the same antimicrobial properties in an animal model and determine if it has an apoptotic effect on human melanoma cancer cell lines.

Overall, the primary objective of the research is to further understand the compound TPP1 which contains the TPP (triphenyl phosphonium) group. The TPP group is a DLC (delocalized lipophilic cation) that can specifically target the mitochondria of cancer cells due to their increased mitochondrial membrane potential. Overall, it is believed that the TPP group allows for drugs such as TPP1 to have a chemotherapeutic ability. In addition to treating cancer, some chemotherapeutics can also function as an antimicrobial agent. Bacterial infections and cancer are linked in many ways as infections have been found to contribute to cancer formation. Tumor and cancer treatments have been known to cause bacterial infections making overall treatment more difficult and less effective. Thus, the ability for chemotherapeutics to double as antimicrobial agents is very important as it will help to prevent infections and reduce complications that occur during cancer treatment. The specific aim of this research is to determine if the TPP1 compound is able to act as an antimicrobial agent *in vitro*.

Antimicrobial activity of TPP will be explored through different experiments on *Pseudomonas aeruginosa*. To first determine if the drug has any impact on bacterial growth, a Kirby-Bauer disk diffusion assay will be done. If TPP1 exhibits antimicrobial activity, then its delivery method can be examined with TPP1 along with the water-soluble positive control silver nitrate. These compounds will be swelled into PVA hydrogel, and their activity will be determined. If successful, then this would be the first insight into the

dual chemotherapeutic and antimicrobial activity of TPP1. Overall, this study will allow for further exploration into the TPP group, the chemotherapeutic complexes that contain it, and the medical use of chemotherapeutics and water-soluble antimicrobial agents in hydrogels.

EXPERIMENTAL PROCEDURES

General Considerations

Pseudomonas aeruginosa (PAO1) was obtained as a generous gift from Dr. Paul Hyman from Ashland University (Ashland, OH). Sigma-Aldrich was utilized to provide poly (vinyl alcohol) (molecular weights 85,000-124,000 and 30,000-70,000), silver nitrate, DMSO, LB broth, plate agar, and 10 mm sterile disks. TPP1 and the imidazole precursor were made by researchers in the Youngs lab at The University of Akron (Akron, OH). Procedures done were modeled after CLSI standard protocols. All activity requiring a sterile environment was done in a Labconco biosafety cabinet, and all lab space was used through the College of Wooster.

Culturing and Plating Bacteria

PAO1 was taken from -80º C and was allowed to partially thaw. Using a sterile swab, PAO1 was streaked on an agar plate using the quadrant method (Figure 6). After each pass, a new sterile swab was used to reduce the amount of total bacteria on the plate to allow for individual colonies. The plate was stored at 37º Celsius overnight. Single colonies were removed and used for future steps using an inoculation loop. The plate was then wrapped in parafilm and stored at -20º Celsius in the freezer.

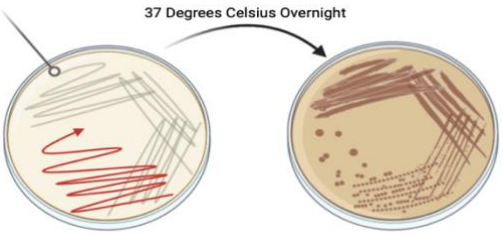

Figure 6: Streaking technique for PAO1

Creation and Comparison of Poly (vinyl alcohol) (PVA) Hydrogels

PVA of average molecular weights 30,000 – 80,000 KDa and 85,000 – 125,000 KDa were compared. The average molecular weights differ due to differences in PVA chain polymerization length. To make hydrogels, a 10% (w/v) PVA hydrogel solutions was made using DIH_2O. The DIH_2O and PVA were mixed in a beaker and placed on a stirring hot plate where the mixture was stirred and heated. The mixture was left on the hot plate until all solid PVA dissolved leaving a viscous solution. To remove air bubbles from the solution, stirring was continued for approximately 2 hours with the plate warmed around 30^o Celsius. The mixture was removed from the hot plate and was then poured into a 30 mm plate for gelation and was allowed to come to room temperature. For the hydrogel to form, overnight freeze thaw cycles were done where the PVA hydrogels would be placed in a -20^o Celsius freezer for 18 hours and then thawed at 20^o Celsius for 6 hours. The freeze thaw cycles were done 2 times. The PVA hydrogels of different molecular weights were compared in their viscosity and physical properties and swelling amount was determined. The gel that maintained a solid form and had the most swelling was used.

Swelling of the PVA Hydrogel

PVA hydrogels of molecular weight 85,000-124,000 were cut from the plate using a sterile 10 mL glass tube to cut a uniform segment out of the gel. The mass increase from swelling was calculated in triplicates where the PVA hydrogel was swelled with water. Segments of the gel were cut out using sterile 10 mL glass tubes, and then they were weighed and then placed into water solution for 1 hour, 3 hours, 8 hours, and 24

hours. The weights were measured following the swelling and the percent increase in mass was calculated to determine a timepoint where maximal swelling occurs.

As results were found for the experimental and control groups, the water-soluble compounds with antimicrobial activity were swelled into the PVA hydrogel. Based on the average increase in mass from the swelling trials, an antimicrobial solution of 2.85 mg/mL was used in order for the gels to reach 30 µg. The gels were left allowed to swell for eight hours. As the disks were taken out of the solution, the excess liquid was removed by lightly pressing the gels against the inside of the tube and were allowed to air dry for a short time in a sterile environment.

Colony Forming Unit Count

PAO1 was cultured and streaked using the quadrant streak method on LB Agar plates. The plate was incubated overnight at 37^{o} Celsius. Using an inoculation loop, a single colony was isolated and placed into a glass tube of 5 mL MH media. A second tube of 5 mL MH media without the PAO1 bacteria was also used as a negative control. These tubes were placed in a 37^{o} Celsius shaker for around 1 hour. After incubation, MH media was added to match the cultured bacteria to the 0.5 McFarland standard. This was done visually through a side-by-side comparison checking the opacity of the culture. Once matched to the McFarland standard, the culture was serially diluted taking 50 µL of the stock and transferring it into glass tubes containing 450 µL PBS. Seven serial dilutions were done. Each dilution was then placed onto blood agar plates in the amount of four 10 µL drops spaced out in a line along the top of the plates. The plates were tilted around

70° Celsius so the bacteria is able to be spread and form four lanes. The plates were placed in the incubator at 37° Celsius overnight. Following incubation, the individual colonies were counted. The amount of bacteria (CFU/mL) was then calculated based on the average of the four columns on one plate.

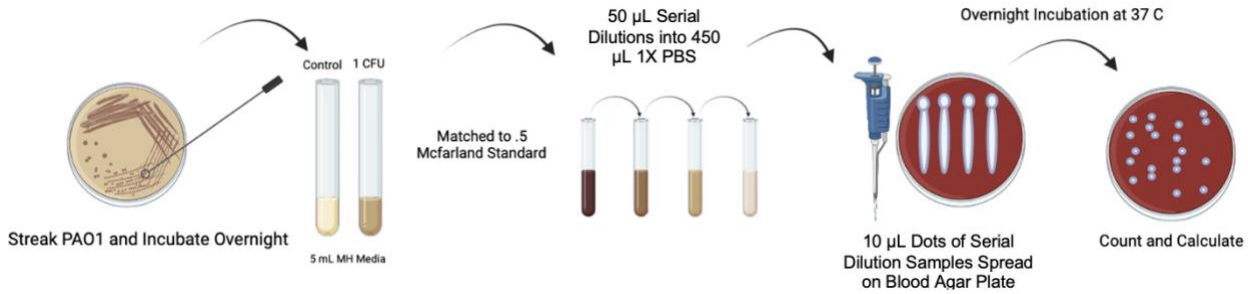

Figure 7: Methods diagram of the CFU counting methods.

Kirby-Bauer Disk Diffusion

The Kirby-Bauer disk diffusion method for calculating the zone of inhibition was modeled after CLSI protocols. PAO1 stored at -80° Celsius was partially thawed and plated as described above. Following the overnight incubation of the plate, using an inoculation loop, one colony was placed into 5 mL of MH media and was placed at 37° C to match to the 0.5 McFarland standard. Once matched, a sterile swab was used to spread PAO1 thoroughly over the surface of the LB agar plate. This was done by dunking the swab into the PAO1 mixture and spreading it across half of the plate. Then the plate was quarter turned and the swab was dunked again and spread across half the plate. This process was repeated until the entire plate was covered. Then disks and gels were added to the plates and treated.

There were two experimental conditions: compounds swelled into PVA hydrogels and compounds soaked into 10 mm sterile disks. In each experimental condition, there are two experimental groups, one positive control, and one negative control. The positive control exhibits antimicrobial activity where the negative control exhibits no bacterial inhibition. The positive control was sterile disks containing silver nitrate and PVA hydrogels swelled with 30 µg silver nitrate. The negative control was PVA hydrogels swelled with water and the sterile disks with water. One experimental group was sterile disks soaked with TPP1, and the other experimental groups included the sterile disks containing the imidazole precursor solubilized in DMSO, and a vehicle control of DMSO. In total, 20 µL of each solution was added to the sterile disks allowing for disks containing silver nitrate, TPP1, and the imidazole precursor to be tested with 10 µg and 30 µg of each compound.

Each of the control and experimental groups were placed in the center of the agar plates coated with PAO1 and they were left to incubate overnight. If there was an antimicrobial effect, then there was a circle of no bacterial growth surrounding the PVA hydrogel or sterile disk. The area of the circle can show how efficient of an antimicrobial agent the drugs are, so the diameter of the circles was measured in mm. The compounds that express antimicrobial activity were swelled into hydrogels, and the zone of inhibition was determined with the swelled gels.

RESULTS

In order to determine the ability of an antibiotic agent to be able to kill bacteria, the bacteria must first be standardized, and its relative amount must be quantified in the form of colony forming units (CFU). After the PAO1 was cultured, it was matched to the 0.5 McFarland standard where it was then serially diluted 6 times and segmented off into sections on blood agar plates beginning with the stock 0.5 McFarland standard PAO1 (Figure 8). After the serial dilutions were dropped onto the plates, they were grown overnight and counted. Among serial dilutions in each of the three trials from the stock to dilution number 4 after incubation, the colonies were too numerous to count, so the CFU was unable to be calculated. Among the 5th dilutions of each of the three trials, there was found to be an average of 14.25 colonies per 10 µL section equating to a CFU count of around 1.425×10^8 PAO1 (Table 1). With the 6th dilutions of the three trials, there was an average of 1.833 colonies per 10 µL section which was used to calculate an estimated amount of 1.833×10^8 PAO1 (Table 1).

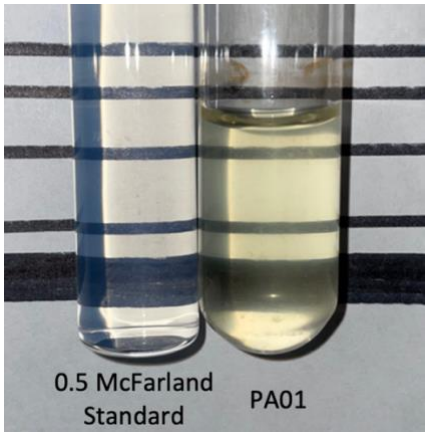

Figure 8: Cultured PA01 in MH media matched and compared to the 0.5 McFarland Standard.

Table 1: Colony forming unit counts of PAO1 matched to the 0.5 McFarland Standard. Each of the sections represents the 20 µL drops on the blood agar plates. The CFU count was calculated using the equation: $(Colonies\ Seen) \times (100) \times (10^n)$ where n is the dilution number. Dilution number 0 represents the stock solution matched to the 0.5 McFarland Standard.

Dilution Number	CFU/mL in Section 1	CFU/mL in Section 2	CFU/mL in Section 3	CFU/mL in Section 4	Average CFU/mL
Run 1:					
0-4	TNTC	TNTC	TNTC	TNTC	-
5	1.7×10^8	2.3×10^8	1.4×10^8	1.3×10^8	1.675×10^8
6	3.0×10^8	2.0×10^8	2.0×10^8	2.0×10^8	2.25×10^8
Run 2:					
0-4	TNTC	TNTC	TNTC	TNTC	-
5	1.1×10^8	9.0×10^7	1.2×10^8	1.3×10^8	1.125×10^8
6	1.0×10^8	1.0×10^8	1.0×10^8	4.0×10^8	1.50×10^8
Run 3:					
0-4	TNTC	TNTC	TNTC	TNTC	-
5	1.3×10^8	1.2×10^8	1.7×10^8	1.7×10^8	1.475×10^8
6	3.0×10^8	1.0×10^8	1.0×10^8	2.0×10^8	1.75×10^8
Average CFU/mL Dilution 5	1.425×10^8				
Average CFU/mL Dilution 6	1.833×10^8				

After determining the CFU count of PAO1, zone of inhibition testing via the Kirby-Bauer disk diffusion method was done with PAO1 grown and matched to the 0.5 McFarland Standard. Among three trials of zone of inhibition testing, the water negative control maintained no activity with bacteria and did not cause a zone of inhibition to form. When it came to the experimental groups, there was a clear distinction between the

positive control, silver nitrate, and all the experimental groups of the imidazole precursor and the TPP1 compound. The sterile disks containing TPP1 showed no impact on the bacteria as no zone of inhibition formed after overnight treatment with both 10 µg and 30 µg of TPP1. The imidazole precursor showed the same result as TPP1 where there was no inhibition of bacterial growth indicating no antimicrobial activity in each of the trials with 10 µg and 30 µg samples. Similarly, the vehicle control for DMSO showed no effect on the bacteria and did not form a zone of inhibition (Figure 9)

The positive control of silver nitrate that was tested on the sterile disks in the amount of 10 µg and 30 µg was found to show different activity depending on the concentration used on the disks. After three trials, the average zone of inhibition for 10 µg silver nitrate was 12.667mm and the 30 µg silver nitrate had an average zone of inhibition of 15.667mm. These results were found to be significantly different from each other indicating that 30 µg silver nitrate has a higher zone of inhibition than 10 µg silver nitrate ($T= -10.392$, $df=2$, $p=0.005$) (Figure 9).

In order to investigate the compound TPP1 and its antimicrobial properties, further testing was done. TPP1 overall was not completely soluble in cold or room temperature water. When TPP1 was mixed with water at a room temperature of around 20^{o} C at a concentration of 3 mg/mL, the product was a white cloudy and foamy solution where solid particle of TPP1 were visible and would gather toward the bottom of the solution. When TPP1 was mixed into a water solution and heated to around 56^{o} Celsius for 15 minutes, the TPP1 appeared to solubilize into solution. The product was clear, and no solid particles were visible. TPP1 was again placed onto 10mm sterile disks at an amount of

20 µL, but they were tested at a higher concentration, and to ensure proper solubility, TPP1 was tested with DMSO as a solvent. Unlike TPP1 with water as a solvent, TPP1

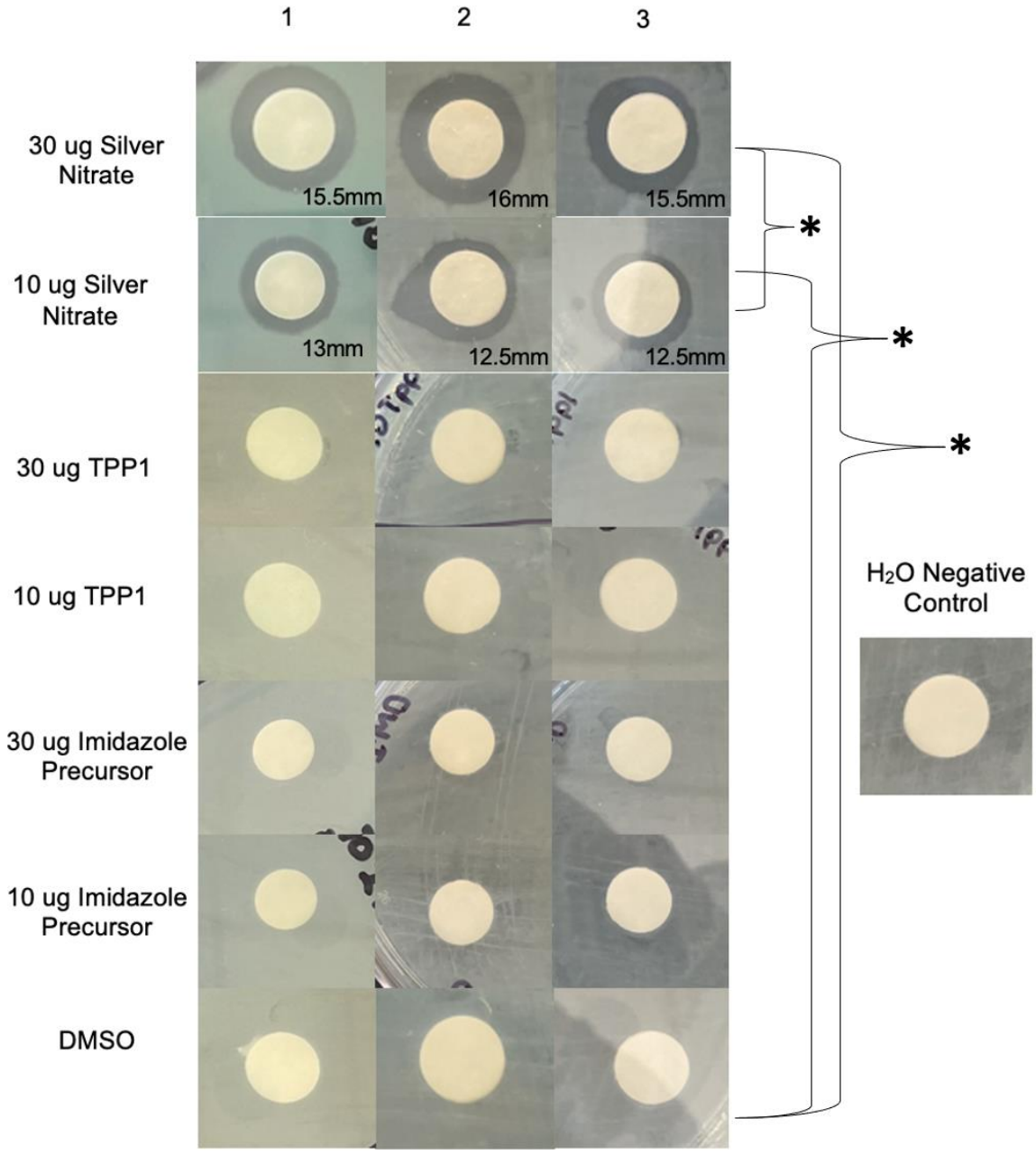

Figure 9: Representative images of three zone of inhibition trials of 10 mm sterile disks containing 20 µL of silver nitrate, TPP1, imidazole salt precursor, DMSO, and H_2O. The asterisk on the image indicated that there was a significant difference in zone of inhibition between. There was significant difference between 30 µg silver nitrate and 10 µg silver nitrate (P=0.005). Similarly, TPP1, the imidazole salt precursor, DMSO, and H_2O were significantly different from with the both the 30 µg silver nitrate and 10 µg silver nitrate as no zone of inhibition formed (P=0.001, P=0.001). The zones of inhibition measurements are shown. Significant difference indicated when $P<0.05$. N=3

dissolved very quickly at room temperature with DMSO as a solvent and formed a clear solution. The vehicle control DMSO again showed no zone of inhibition, and the 10 µg silver nitrate positive control again showed interaction with bacteria through a zone of inhibition of 13.5 mm. TPP1 at 60 µg did not have any visible inhibition of bacterial growth as no zone of inhibition formed. Similarly, 30 µg of TPP1 in the DMSO solvent also had no inhibition of bacterial growth. Both the 30 µg TPP1 in DMSO and 60 µg TPP1 are significantly different from the positive control of 10 µg silver nitrate. Following these results, TPP1 appears to have no activity against PAO1 in both water and DMSO as a solvent and increasing the concentration of TPP1 appeared to have no affect (Figure 10).

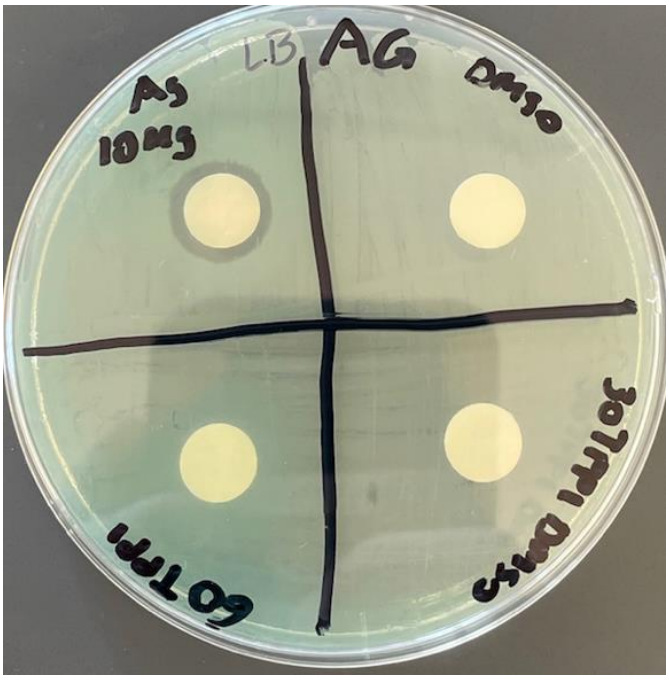

Figure 10: Image of test plate containing 20 µL of DMSO, 60 µg TPP1, and 30 µg of TPP1 dissolved in DMSO.

TPP1 and the precursor imidazole were also tested with *Candida albicans* to give more insight into the compounds and to determine if they had activity with any other microorganisms. One replicate was done for this testing. 30 µg Silver nitrate was again used as a positive control and had an average zone of inhibition size of 17.25 mm among the two treated disks. The imidazole precursor treatment appeared to have some inhibiting activity, but a clear zone of inhibition was not apparent (Figure 11). Similarly,

compared with the negative controls of DMSO and H_2O, all TPP1 treatments seemed to have a very slight impacts on *C. albicans,* but no apparent zone of inhibition was formed.

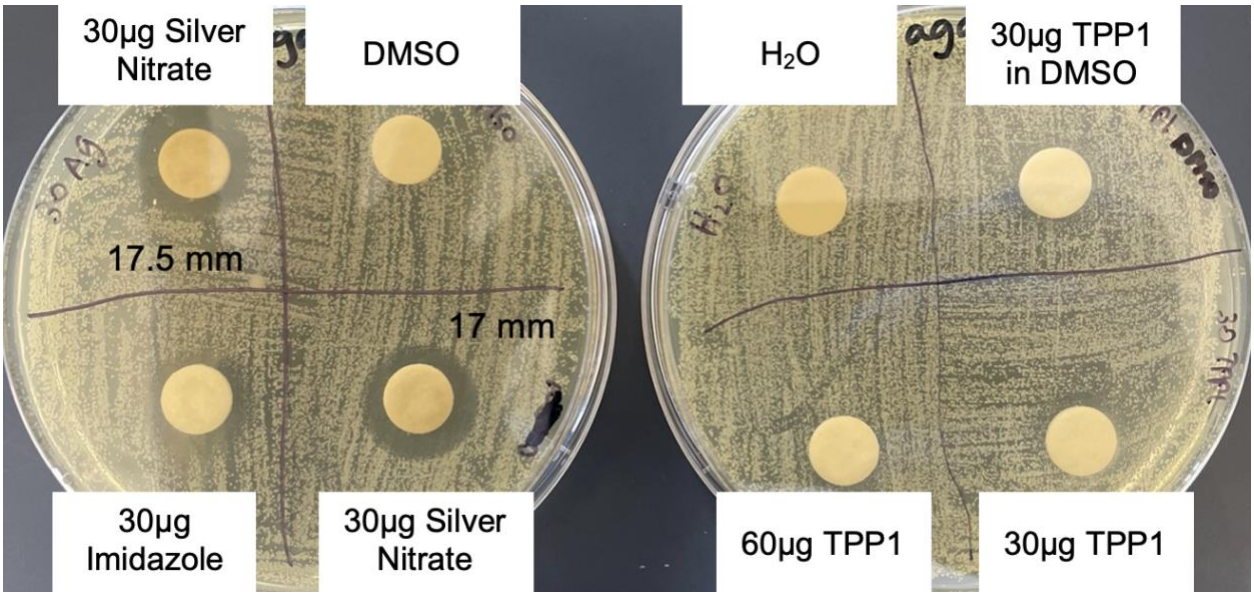

Figure 11: Sterile disk image of *C. albicans* treated with 30 µg silver nitrate, DMSO, 30 µg imidazole precursor, 30 µg TPP1, 60. µg TPP1, 30 µg TPP1 in DMSO, and H_2O. Treatments with a clear zone of inhibition are labeled with zone diameter. N=1.

Following disk diffusion, hydrogels were tested using a similar method. Comparison between the two PVA hydrogels of differing molecular weights portrayed large differences throughout the gelation process. The PVA of a lower molecular weight (30,000-70,000) was more of a powder and dissolved into the liquid quickly. Overall, it behaved much more as a liquid while being heated and stirred. Once it was stirred, heated to 90^o Celsius, and the powder was dissolved into the water, the final product was a clear adhesive slightly viscous fluid. Following two freeze thaw cycles, there was no noticeable change in the viscosity and gelation of the 30,000-70,000 molecular weight PVA. With three freeze thaw cycles, the finished product gained some viscosity, but remained a clear fluid in nature and was unable to be shaped and maneuvered for fluid swelling. The PVA

of a higher molecular weight (85,000-124,000) was crystalline and larger than the lower weight PVA. Once mixed with water, it was difficult to dissolve into solution. The heating and stirring process lasted 3 hours and the solution was an extremely viscous and adhesive gel like mixture. After two freeze thaw cycles, the resulting product was a cloudy solid hydrogel that was able to be swelled with fluid.

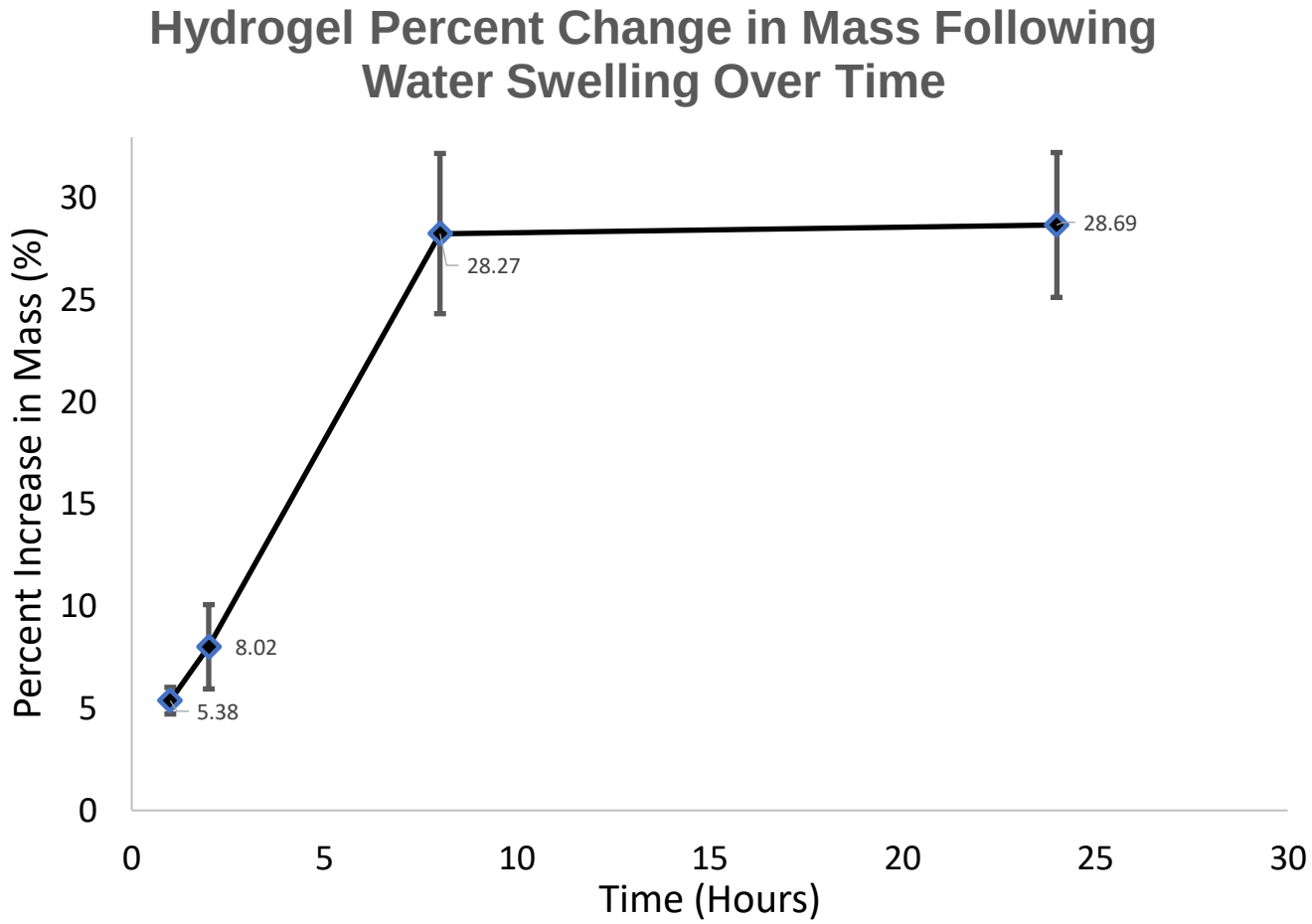

Figure 12: The percent change in PVA hydrogels of molecular weight 85,000-124,000 following swelling with DI H_2O (n=3). The initial masses of the gels are shown in table 2. Standard error is indicated in the table through the error bars. The average percentages are indicated next to each data point.

Table 2: Swelling of molecular weight 85,000-14000 PVA hydrogels in three trials measured after 8 hours using the control of water and the experimental 30 µg silver nitrate. Percent increase in mass was calculated for each trial using the equation: $\frac{\text{Final Mass} - \text{Initial Mass}}{\text{Initial Mass}} \times 100 = \text{Percent Increase in Mass}.$

Experimental Condition	Hydrogel Starting Mass (g)	Hydrogel Final Mass after 8 hours (g)	Percent Increase in Mass after 8 hours
Water Swelled Trial 1	0.156	0.196	25.64%
Water Swelled Trial 2	0.122	0.162	32.79%
Water Swelled Trial 3	0.144	0.182	26.38%
30 µg Silver Nitrate 1	0.230	0.287	24.78%
30 µg Silver Nitrate 2	0.215	0.273	26.97%
30 µg Silver Nitrate 3	0.222	0.284	27.92%

The molecular weight 85,000-124,000 PVA hydrogels were placed in water to determine if they would be able to swell with water and potentially function as a delivery mechanism for water soluble compounds. Three gels were swelled with DI water over timepoints of 1 hour, 2 hours, 8 hours, and 24 hours to determine the time of optimal swelling. Overall, the gels were able to swell with water over time, and this was observed through the increase in the mass of the gel as they were weighted before and after each timepoint. The average percent increase in mass was calculated for each timepoint (Figure 12). After one hour of swelling with water, there was an average mass increase of 5.38% and after 2 hours, there was an average increase of 8.02%. The next timepoint of 8 hours had an average mass increase of 28.69% which was similar to the timepoint

of 24 hours which had an average mass increase of 28.27% (P=0.211). This indicates that following eight hours, there was an observed plateau in the swelling of the PVA hydrogels. As well, the percent increase in mass for the eight-hour timepoint was significantly different from both the one hour and two-hour timepoints (P=0.006 and P=0.014). For the experimental group using silver nitrate, molecular weight 85,000-124,000 PVA hydrogels were swelled with silver nitrate for 8 hours.

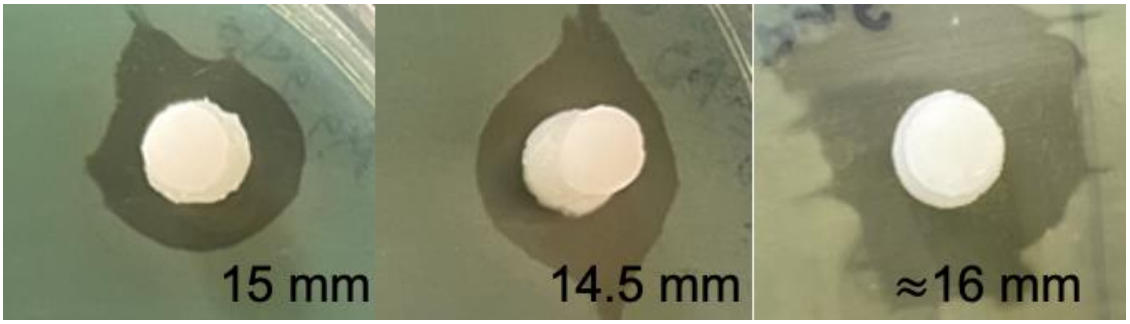

Figure 13: Three trials of PVA hydrogels swelled with 30 µg silver nitrate with PAO1 on LB agar plates. The measurements represent the calculated zone of inhibition surrounding the gels where it was circular. N=3.

Once swelled with silver nitrate, excess solution on the PVA hydrogels was removed and the hydrogels were plated with PAO1 to run zone of inhibition testing. The control PVA hydrogel swelled with DI water did not show any interaction with bacteria and formed no zone of inhibition (Figure S2). This indicated that the PVA hydrogel itself does not appear to inhibit bacterial growth. The PVA hydrogel swelled with 30 µg silver nitrate appear to have an inhibitory effect on PAO1 as a zone of inhibition was formed surrounding the PVA hydrogel (Figure 13). This indicates that the hydrogels are able to be successfully swelled with water soluble silver nitrate and can deliver the solution and have an inhibitory interaction with PAO1. The zone of inhibition for the 30 µg silver nitrate gels was an average of 15.2 mm which is statistically similar to the 30 µg silver nitrate sterile disks (P=0.239).

DISCUSSION

The study began by analyzing PAO1 through counting and confirming the amount of bacteria present. To do this, a colony counting assay was done in three separate trials to determine the CFU/mL of PAO1. Within the experiment, each of the six serial dilutions decreased in concentration ten-fold beginning with the stock solution which was matched to the 0.5 McFarland standard. Once plated, the concentrations of the first 4 dilutions were too high, so in each of the 10 µL section on the plate, there were too many colonies to count as they formed a solid strip stretching down the plate. In dilutions 5 and 6 however, colonies were able to be counted and calculated in each section of the plates. The 6th dilution was found to be 1.833×10^8 CFU/mL while the 5th dilution was determined to be 1.425×10^8CFU/mL. In previous studies using both *P. aeruginosa* and *E. coli*, the 0.5 McFarland standard has been found to 1.5×10^8 CFU/mL (Table 1) (31). Based on this information, the 5th dilution was deemed to be more accurate as it is close to the value 1.5×10^8 CFU/mL value. As well, there were less colonies in the 6th dilution causing much less of a sample size overall. This can cause the value of the calculated CFU/mL to change rapidly with only a small difference where the 5th dilution had a larger number of colonies per plate and per section. The McFarland standard is important to maintain throughout the experiment as it acts as a way to control bacterial growth and ensure that each experiment is done with bacteria around the PAO1 concentration of 1.5×10^8 CFU/mL (32). This allows for the experiment to remain consistent throughout and eliminate variation.

After standardizing and confirming the concentration of PAO1 with the 0.5 McFarland standard, zone of inhibition testing was done using sterile disks. Sterile disks were used as a part of the disk diffusion protocol and allowed for 20 µL drops of solution to be placed on each disk once they were placed on the plate. If the solution used on the disks has antimicrobial or inhibitory activity with bacteria, then the zone of inhibition will form around the disk and its diameter is measured. Silver nitrate was used in this experiment as a positive control as it has known activity with different microorganisms including *P. aeruginosa*. Silver nitrate can induce apoptosis by increasing the formation of catalases and by causing DNA fragmentation (33). Similarly, silver nitrate is an ionic compound which allows it to have improved solubility in water at room temperature. The positive controls of silver nitrate were tested in the amounts of 10 µg and 30 µg and both formed zones of inhibition around the sterile disks (Fig 9). Generally, concentrations beginning at 10 µg and ending around 30 µg are used in zone of inhibition studies with *P. aeruginosa* (32). Based on previous studies of silver nitrate in the amount of 10 µg, the zone of inhibition size was expected to be around 13mm $\pm$1 with *P. aeruginosa*, and results from the 10 µg silver nitrate in this experiment had an average zone of inhibition of 12.67mm which fits within the range (34). The 30 µg silver nitrate had an increased zone of inhibition with an average of 15.67 mm which is likely due to the higher concentration of the antimicrobial silver nitrate as size of the zone of inhibition generally correlates to stronger antimicrobial activity (35).

The primary experimental testing was on the TPP1 compound. Previous findings indicated that the addition of the TPP group onto the imidazole salt allowed for increased solubility in water to 8mg/mL from the lipophilic imidazole precursor (15). When

solubilizing TPP1 during the experiment, at room temperature there was little solubility in water at concentrations of 0.5mg/mL up to 3mg/mL. Once heated to 56^{o} C, TPP1 exhibited solubility in water. Overall, the cationic TPP group is thought to give the increased solubility compared to the other non-polar and neutral groups (Figure 1). Within a shortened time, it did not appear that TPP1 was able to achieve aqueous solubility under room temperature conditions. Unlike previous findings previous findings where TPP1 was water soluble with extended vortexing, TPP1 was also able to be solubilize with higher temperatures around 50^{o} Celsius (15).

Overall, the TPP1 compound appeared to have no inhibitory or antimicrobial activity towards PAO1 as no zone of inhibition formed around the disks (Table 1). TPP1 was tested at three concentrations: 10 µg, 30 µg, and 60 µg. The concentration of 60 µg was used as an upper bound to rule out the possibility that 10 µg and 30 µg were too low of concentrations for inhibitory activity. Since TPP1 had a limited solubility with water at room temperature and shorter mixing times, dimethyl sulfoxide was used as an alternate delivery method. TPP1 dissolved into the DMSO solution immediately. TPP1 did not have inhibitory ability with either water or DMSO. Based on previous experiments by Kumari et al., the compound mitocurcumin (MitoC) that contains two separate TPP groups has activity with gram-negative and gram-positive bacteria such as *E. coli and B. Subtilis* (20). The backbone of MitoC contains more oxygen species that may allow it to be more reactive than the non-polar and hydrophobic backbone of TPP1. MitoC and other TPP containing compounds such as CFX-TPP are thought to be able to have bactericidal activity through the disruption the bacterial cell membrane (20, 21). TPP1's chemotherapeutic ability also resides among membranes but is thought to be linked to

mitochondrial membrane potential through the cationic TPP group (12, 14). Bacteria such as *P. aeruginosa* do not contain mitochondria so this interaction would not occur, and there does not appear to be interaction that inhibits bacterial growth.

Similarly, the precursor imidazole, the backbone of TPP1, did not have any activity towards PAO1 (Figure 2, Figure 9). The structure of the imidazole is primarily hydrophobic with multiple benzene rings. This partially contrasts from the structure of the antibiotic minocycline which contains a benzene ring and other carbon ring structures, but also is hydrophilic and more reactive (15). From this contrast and the lack of inhibitory activity, it appears that hydrophobicity has a role in antimicrobial ability. TPP1 is a hydrophobic compound where MitoC and minocycline exhibit antimicrobial activity, and both compounds contain hydrogen bonding atoms and are more hydrophilic as a whole with the TPP group also increasing MitoC's solubility (20, 36).

In order to further investigate TPP1 and the imidazole precursor, a test plate was run on *C. albicans*. *C. albicans* was used as it differs from *P. aeruginosa* in that it is eukaryotic and is a gram-positive fungus rather than gram-negative bacteria. It contains mitochondria and this was thought to be of significance as TPP1 was identified to act as a DLC that targets increased mitochondrial membrane potential (11). Similarly, it commonly causes infections across the world, and the positive control of silver nitrate has similar antimicrobial activity as it does to *P. aeruginosa* (37). The positive control silver nitrate treatment had an observable zone of inhibition with *C. albicans*, and the imidazole precursor appeared to have some inhibitory activity as well (Figure 11). A clear zone of inhibition measurement was unable to be measured, but there was a reduced growth surrounding the treated disk, and this indicates that the imidazole precursor potentially

has antimicrobial activity. Similarly, when comparing each of the TPP1 test groups with the negative controls, there is a slight difference in *C. albicans* growth surrounding the disks. This could also indicate that there is potentially minimal activity against *C. albicans* that inhibits its growth. Only one replicate was performed on *C. albicans* so no clear conclusions can be drawn based on these results, but this testing opens up the possibility that TPP1 and the imidazole precursor may be able to have antimicrobial activity against different microorganisms.

Overall, TPP1 did not appear to have significant antimicrobial activity against the gram-negative PAO1 or the gram-positive *C. albicans.* Previous findings indicate that antibiotic resistance has been a large problem in the development of new antimicrobial compounds, and both gram-negative and gram-positive microorganisms contribute to this (25). Through the comparison of the imidazole precursor with silver nitrate against *C. albicans*, the potential antimicrobial activity presents the possibility that the TPP group actually may prevent antimicrobial activity (Figure 11). This could be due to several factors including decreased membrane permeability and decreased activity of membrane proteins (38). Since TPP1 contains additional charges, there may be difficulty in its diffusion through cell membranes, so it would never be able to impact the internal environment of bacteria. The imidazole precursor may be more likely to enter the cell as it is generally hydrophobic.

To further the investigation of bacterial infections and cancer treatment, PVA hydrogels were made as a possible delivery method. PVA is a hydrophilic compound that is able to dissolve into water once heated and stirred (Figure 5). Freeze thaw cycles are done to cause increased cross linking between PVA molecules without additional

additives to the solution. They increase and improve the mechanical properties of the PVA hydrogels, and the more freeze thaw cycles undergone, the lower the swelling (39). The 85,000-124,000 molecular weight hydrogels were used for swelling as they were able to form stable solid gels following two freeze thaw cycles. Similarly, 10-12% (w/v) PVA hydrogels appeared to swell aqueous solution the best, so 10% PVA hydrogels were used (38). Since PVA hydrogels are able to swell with water through its hydrogen bonding hydroxyl groups and silver nitrate exhibits solubility in water, testing was done to determine if PVA hydrogels were able to be swelled with aqueous silver nitrate. The results of an average mass increase of 28.7% after eight hours of immersion appears to be consistent with studies showing that the degree of swelling is near 25% following a 9-hour immersion (40). Following 8 hours of immersion, the swelling appeared to cease and no longer increase in a linear manner (Figure 12). After eight hours, the gel likely reached its maximum capacity and became fully saturated to the point where it was not able to swell anymore.

Based on the results of PVA hydrogel swelling with silver nitrate, it appeared that the gels were able to swell with aqueous silver nitrate and maintain antimicrobial activity against *P. aeruginosa.* The zone of inhibition diameters were similar to that of the sterile disks swelled with 30 µg silver nitrate, and there did not appear to be a drop off in activity. The gel was able to swell with both water and silver nitrate, and this is likely due to both the PVA and silver nitrate's hydrophilic natures. As a result, the gels were able to adequately swell with approximately 30 µg of silver nitrate. One observation is that the surrounding zone of inhibition around the gels was not perfectly circular as the zone of inhibition circles surrounding the disks were. A limitation to this study was that the plates

were not able to be turned upside down in the incubator as the gels did not fully adhere to the LB plates even as direct contact was made. This allowed for condensation to build up on the gels early in the incubation period and for liquid buildup on the plate and is likely the reason for the misshaped circles that make up the zones of inhibition surrounding the gels (Figure 13). As well, when gels were exposed to room air conditions the gels dry out relatively quickly, so there is a possibility that the gels partially dried out in the later stages of the overnight incubation and that may affect the results of PVA hydrogel zone of inhibition. Some of these problems could be potentially overcome by increasing the contact area of the hydrogels with the LB agar so there can be more adhesion.

Summary

Overall, the chemotherapeutic compound TPP1 did not exhibit antimicrobial activity against PAO1 or *C. albicans* as seen through the Kirby-Bauer disk diffusion methods. The sterile disks containing TPP1 in various concentrations of 10 µg, 30 µg, or 60 µg. Similarly, to ensure that the use of water as a solvent was not inhibiting activity, DMSO was used as a solvent and TPP1 still appeared to lack antimicrobial activity. Although the experimental group of TPP1 seemed to lack activity, the water-soluble drug delivery study was able to proceed using silver nitrate. PVA hydrogels were able to be formed through freeze-thaw methods, and then the hydrogels were able to be swelled with water and silver nitrate in water solution. On top of that, through a modified Kirby-Bauer disk diffusion method, PVA hydrogels swelled with 30 µg silver nitrate were able to

antimicrobial activity against PAO1 that was similar to the results of the sterile disks swelled with 30 µg silver nitrate.

In order to further investigate the antimicrobial activity of TPP1 testing can be done on additional types of bacteria that are both gram-negative and gram-positive. One of the most common bacteria that is commonly associated with soft tissue infections is *Staphylococcus aureus* (41). *S. aureus* is a gram-positive bacterium that would be a good model for the testing of TPP1 due to its relevance in the field of medicine and resistance to antibiotics. Similar commonly occurring bacteria could also be tested with TPP1. As well, if TPP1 were to have antimicrobial activity with other microorganisms, then minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) testing can be done (24).

When it comes to the swelling of PVA hydrogels with water soluble antimicrobial compounds, the experiment can be expanded to other bacteria such as *S. aureus.* First, the methods can be improved to improve upon limitations such as the build-up of condensation. The sterile disks can be made thinner and wider so that more surface area is exposed to the plates and allows them to adhere more. This would allow for the plates to be turned upside-down in the incubator and limit condensation from reaching the agar. As well, PVA hydrogels can be swelled with DMSO and other solutes. This would allow for a wider range of solubilized compounds that are not water soluble to swell into the hydrogel and be delivered to the area of need.

ACKNOWLEDGEMENTS

I would like to thank my advisor Dr. Marie Southerland for all the time she spent preparing me to carry out this study. She did an amazing job educating me on different assays and working with me to guide myself throughout the process both during lab work and when writing. Dr. Mark Snider was also very helpful to me as he helped to prepare me for this research through many classes leading up and through Junior Independent Study. I would also like to thank the College of Wooster for the lab space and resources to make this research happen. My family also played a big role as they gave me the opportunity to attend Wooster and take advantage of the Independent Study as well as numerous words of encouragement along the way. I would also like Ben Kennedy, Kylie Keller, and Macy Bischoff as their collaboration and teamwork was greatly appreciated. I would also like to thank my friends Tyler Chumita, Ben Gbur, Dom Stilliana, and Angelo Petracci along with the Wooster baseball team as their encouragement and positive attitudes helped me to continually work throughout the year.

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Appendix

Table S1: Raw data count from CFU count assay. The numbers of colonies seen in each dilution section are listed. TNTC indicates that their bacterial colonies were too numerous to count.

Dilution Number	Colonies in Section 1	Colonies in Section 2	Colonies in Section 3	Colonies in Section 4
Run 1:				
0-4	TNTC	TNTC	TNTC	TNTC
5	17	23	14	13
6	2	2	2	2
Run 2:				
0-4	TNTC	TNTC	TNTC	TNTC
5	11	9	12	13
6	1	1	1	1
Run 3:				
0-4	TNTC	TNTC	TNTC	TNTC
5	13	12	17	17
6	3	3	3	2

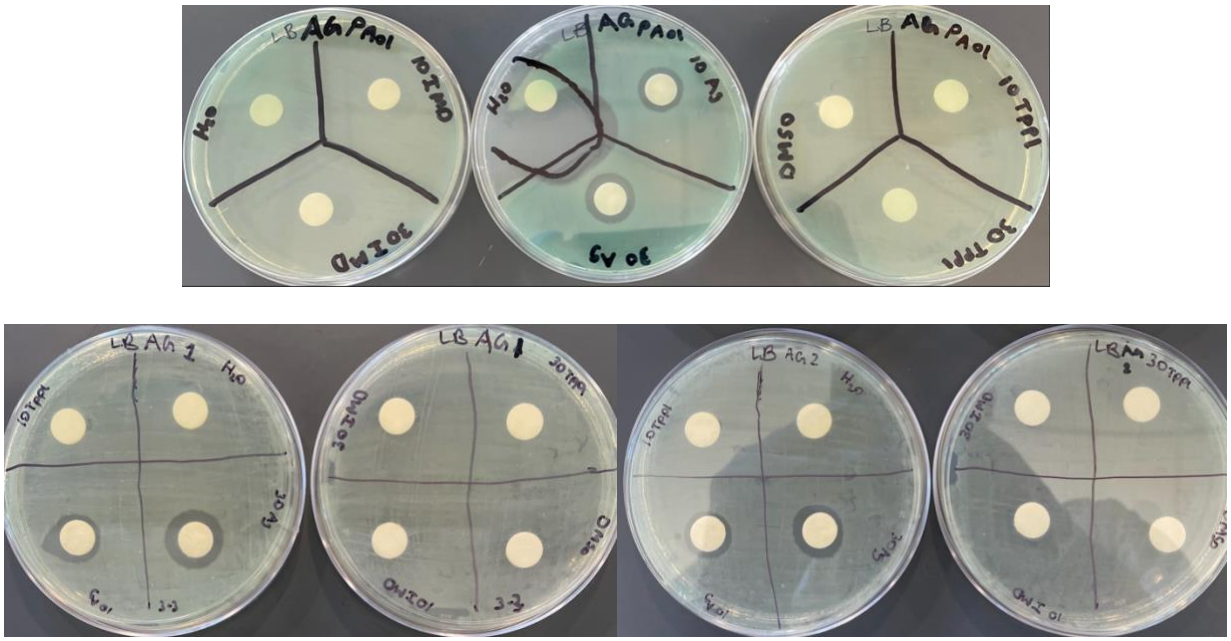

Figure S1: Three trials of LB disk diffusion plates represented in figure 1. Plates are labeled with their experimental group: Water (H_2O), DMSO, 10 μg silver nitrate (10 Ag), 30 μg Silver Nitrate (30 Ag), 10 μg TPP1, 30 μg TPP1, 10 μg imidazole precursor (10 IMD), and 30 μg imidazole precursor (30 IMD). The water control on the upper middle plate was eliminated due to 30 μg silver nitrate dropping onto the agar.

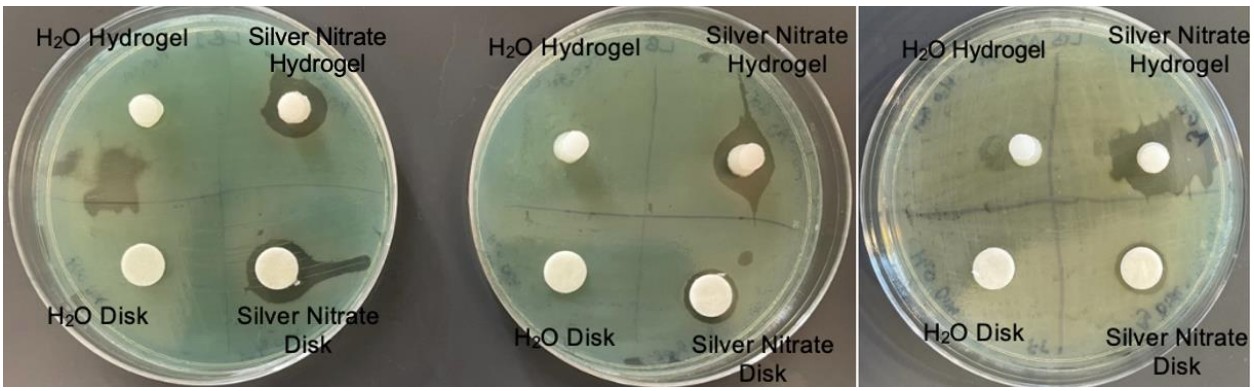

Figure S2: Three trials of swelled PVA hydrogels and sterile disks with water and 30 μg silver nitrate.

Pairs	Mean	Std Dev	Std. Error Mean	Lower	Upper	t	df	p-value
30 μg Silver Nitrate and 10 μg Silver Nitrate	-3.00	0.50000	0.28868	-4.24207	-1.75793	-10.392	2	0.005
TPP and 10 μg Silver Nitrate	-12.67	0.28868	0.16667	-13.38378	-11.94956	-76.000	2	<0.001
Imidazole Precursor and 10 μg Silver Nitrate	-12.67	0.28868	0.16667	-13.38378	-11.94956	-76.000	2	<0.001
Water and 10 μg Silver Nitrate	-12.67	0.28868	0.16667	-13.38378	-11.94956	-76.000	2	<0.001
TPP and 30 μg Silver Nitrate	-12.67	0.28868	0.16667	-16.38378	-14.94956	-94.000	2	<0.001
Imidazole Precursor and 30 μg Silver Nitrate	-15.67	0.28868	0.16667	-16.38378	-14.94956	-94.000	2	<0.001
Water and 30 μg Silver Nitrate	-15.67	0.28868	0.16667	-16.38378	-14.94956	-94.000	2	<0.001
8 Hour Swelling and 24-Hour Swelling	-.00427	0.0073901	0.0042667	-0.0226247	0.0140913	-1.000	2	0.211
1 Hour Swelling and 8-hour swelling	-.2288	0.0454858	0.0262613	-0.3417930	-0.1158070	-8.712	2	0.006
2 hour swelling and 8 hour swelling	-.2024	0.0590415	0.0340876	-0.3491006	-0.0557660	-5.939	2	0.014
30 μg Silver Nitrate Gel and 30 μg Silver Nitrate Disk	-.500	1.0000000	0.5773503	-2.9841377	1.9841377	-0.866	2	0.239

Table S2: Paired T test results run through the program SPSS to calculate the significance between paired groups.