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ANTIMICROBIAL PEPTIDE AEROGELS AS AN INHALABLE THERAPY FOR DRUG-RESISTANT TUBERCULOSIS

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ABSTRACT

Antimicrobial peptides (AMPs) are cationic and amphiphilic molecules that are capable of selectively killing bacterial pathogens via membrane disruption. In this work, I focus on AMPs that kill *Mycobacterium tuberculosis* (Mtb), which is the causative agent of tuberculosis (TB). We hypothesized that by taking advantage of the membrane permeabilization effects of AMPs we can increase the efficacy of conventional TB antibiotics. In this study, we investigated the synergy between four AMPs and four TB antibiotics including rifampicin, moxifloxacin, ethionamide, and isoniazid. Ideal synergistic combinations are formed into aerosolize nanogels (aerogels) to form an inhalable therapy for drug resistant TB.

Peptides are prepared through solid phase synthesis and are further purified using reverse phase liquid chromatography. Antibiotic and AMP synergy was measured by performing combinatorial assays with attenuated cultures of Mtb (H37Ra). Synergy is determined by calculating the fractional inhibitory concentration index of each combination. Aerogels are formed by using a previously optimized electrospray technique. The method involves loading hyaluronic acid (HA) into a syringe and spraying it through a large electric field into a bath of peptide. The AMPs and HA then form an electrostatic cross assembly and form the aerogels. The size of the aerogels is determined using dynamic light scattering.

From the combinatorial assays, it was found that MAD1 and Moxifloxacin are a synergistic pairing while the other fifteen combinations were either additive or indifferent. I hypothesize this result is from MAD1's ability to permeabilize the membrane allowing for Moxifloxacin to more easily diffuse into the bacterium. In addition, all aerogel formations resulted in a diameter the size of 1 micrometer, which is the ideal size for an inhalable therapy.

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Chapter 1

Review of Tuberculosis

Current Status of The Disease and Its Treatment

.In 2017, 10 million new cases of Tuberculosis (TB) were reported globally. TB is caused by a bacterium called *Mycobacterium tuberculosis* (Mtb). When a person is infected, they can develop minor symptoms such as a cough and fever. Often, these symptoms are very mild which delays treatment. If an individual infected with TB goes untreated, they only have a 55% chance of surviving[1].

Currently, the treatment for TB is a lengthy multidrug chemotherapy regimen that lasts for at least 6 months with a concoction of four first-line drugs including rifampicin (RIF), isoniazid (INH), ethambutol (ETH), and pyrazinamide (PYR)[2]. If the infection persists due to resistance towards the first-line drugs, an alternate medication strategy is started. This plan usually consists of an additional 4 months of second-line drugs including cycloserine, aminoglycosides, terizidone, ethionamide, protionamide, capreomycin, aminosalicylic acid, and fluoroquinolones. The effects of these drugs are nonideal since they are more toxic and less effective[3]. Since drug-resistant strains pose a threat to our control of the disease, new treatment methods are in need. Antimicrobial peptides (AMPs) can provide a useful tool in maintaining our control as they have powerful antimicrobial effects, exploits bactericidal mechanisms that are not shared by conventional antibiotics. Additionally, they hold potential to increase the effectiveness of poorly permeable drugs by disrupting the bacterial cell membrane[4].

The rise of multidrug-resistant strains of Mtb is a threat to public health since it can nullify the treatments of many of the drugs used to treat TB. As mentioned, a solution to this is the use of AMPs, which are short sequences of amino acids that are cationic and amphiphilic giving them a strong capability to interact with bacterial cell membranes. The AMPs are capable of killing off bacteria rapidly through a variety of mechanisms, and the probability of bacteria acquiring resistance towards AMPs are low[5,6]. Therefore, AMPs are a favorable alternative to conventional antibiotics for drug development, particularly for multidrug-resistant strains[7].

Antibiotic Mechanisms of Action and Resistance

All of the antibiotic drugs act in unique ways to kill Mtb. Understanding their mechanisms has given information about how the bacterium has developed a resistance towards the drugs rendering them useless. The resistance of the antibiotics rises from chromosomal changes leading to mutations that shield them from the drugs[8]. Studying and understanding the molecular mechanisms of resistance that arise from these mutations will lead to more effective drug delivery development.

One of the most powerful chemicals in the frontline cocktail is RIF which has sterilizing abilities. The drug has a unique mechanism of action as it attacks the translational process in the bacterium. The enzyme DNA-dependent RNA polymerase (RNAP) is vital in the process of translating the DNA to RNA for the bacteria. The enzyme reads the DNA sequence and catalyzes the polymerization of the new complementary RNA chain. The enzyme has five different subunits to it, but the one that RIF binds to is the β-subunit. When introduced intracellularly RIF will bind to the β-subunit and physically block the elongation of the complementary RNA chain

after 2 or 3 nucleotides have been added. At this point, the cell can no longer undergo any of its essential processes and this eventually leads to cellular death[8].

Mtb can develop a resistance towards RIF through mutations in its genome. Specifically, the RhoB gene which codes for the β-subunit on the RNAP. Over 95% of the mutations occur in the three centers referred to as RIF-cluster I, II, and III. The mutation occurs chiefly through a single amino acid substitution. Studies have been shown that different mutant types of bacterium are responsible for facilitating low to high levels of RIF resistance. Although, any deviation from the wild type genotype results in a transformed conformation of RNAP which affects the binding affinity between RIF and the enzyme leading to a decrease in RIF efficiency **(Fig. 1)**. In the end, since the gene coding for the β-subunit is mutated it alters the enzyme geometry and does not allow for RIF to bind as it usually does, therefore, preventing RIF from stopping the translation process allowing the bacterium to survive[8].

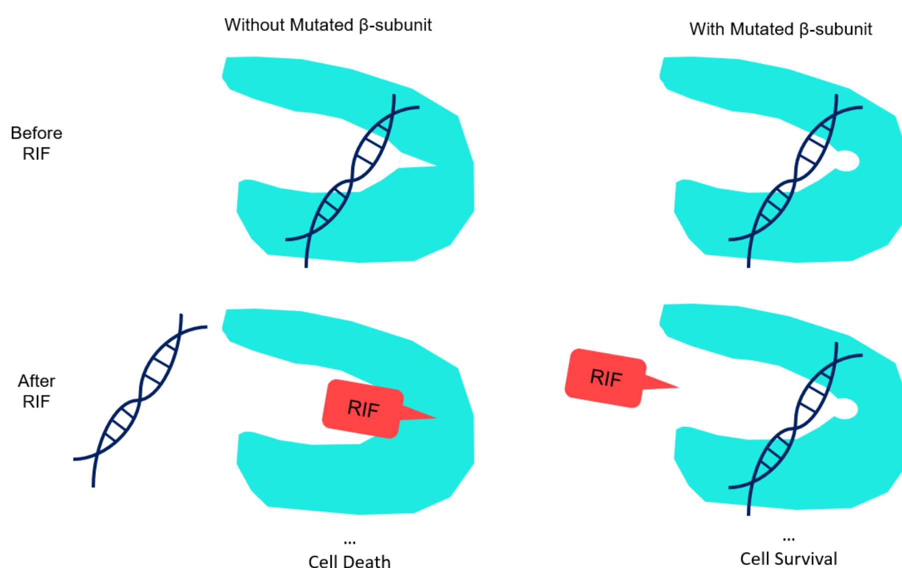

Figure 1: Mtb resistance towards RIF. Without the mutation RIF binds to RNAP preventing RNA polymerization. With the mutation RIF can no longer bind to RNAP which allows for RNA polymerization.

PYR is another one of the four main ingredients in the frontline chemotherapy for TB and the mechanism of killing for this compound is indirect. PYR itself is not the active form that prevents the growth of Mtb, rather its acidic form, pyrazinoic acid (POA), kills the bacterium. PYR enters the cell by passing through the membrane through the process of passive diffusion and once inside the cell a cytoplasmic enzyme, Pyrazinamidase (PZase), converts PYR to POA. POA is transported out of the cell through passive diffusion and efflux, but an acid enabled POA influx is stronger than the two mechanisms transporting POA out of the cell so there ends up being an accrual of POA inside the Mtb cell. The protonated form of POA causes an intracellular accumulation of protons which leads to cytoplasmic acidification. The acidification leads to the inhibition of vital enzymes which eventually leads to cellular death[8].

PYR is susceptible to becoming ineffective through the bacterium acquiring resistance from genomic mutations, and this can occur in a method that is similar to that of RIF. The mechanism is that the gene, pncA, that codes for the PZase enzyme gets a mutation at the binding site. Most of the mutations are a single amino acid substitution through a missense mutation on the portion of the gene that codes for the catalytic site. This, in turn, leads to a loss of PZase activity which inhibits the formation of POA and gives the bacterium resistance towards PYR. Overall, the mutation in the catalytic site of PZase prevents the transformation of PYR to POA which further prevents cytoplasmic acidification and cellular death[8].

INH is another drug that is used as a frontline chemotherapy treatment for Mtb. INH in some ways acts very similarly to PYR as INH is not the active form of the drug itself. INH must passively diffuse into the mycobacterium and once in the cell, it is converted into its active form by an enzyme called KatG. Once converted into its active form INH can act in a variety of ways that eventually kills the cells either through disruption of cell wall synthesis and nucleic acid

synthesis or inhibition of cellular respiration. Once converted to its active form, INH can form a complex with intracellular NADH. This complex is a strong inhibitor to the enzyme InhA. This enzyme is key to the synthesis of mycolic acids; without this enzyme the bacterium cannot properly synthesize its cell wall. It has also been shown recently that INH can bind to NADP and form a complex that can bind to the enzyme dihydrofolate reductase. This enzyme is a key player in the biosynthesis of nucleic acids for the bacterium. Another form of action for INH is that KatG can produce a radical form of INH, which this radical form can readily bind to key respiratory enzymes for the bacterium, therefore, shutting down its metabolic processes[9].

INH also becomes ineffective towards Mtb through genetic mutations that alter some of the key players in its antimicrobial pathway. The most common mutation is one that effects the KatG enzyme that converts INH into its active form. The mutation causes an inadequate INH product to form which will not bind to NADH, preventing it from performing its antimicrobial activities. Another mutation occurs in the InhA enzyme which can either result in overexpression of the protein or deformation in its active site, which either decreases the affinity and effectiveness of INH[10].

ETH is the fourth drug in the frontline cocktail used to treat TB and has a mechanism of action that targets an enzyme as well. In Mtb, the enzyme, EmbA, is essential as it is an arabinosyltransferase and contributes to the synthesis of arabinogalactan, which is part of the cell wall. EmbA is one of the three enzymes that ETH targets, with the other two being EmbB and EmbC. EmbC is also essential because it contributes to the synthesis of lipoarabinomannan, which is also part of the cell wall. ETH will bind to these enzymes and halt the production of the cell wall and inhibit the growth of Mtb[11].

Just as the other three frontline drugs became resistant due to a genetic mutation, so does ETH. The genes that code for the transferase enzymes, embCAB, will undergo a mutation which will decrease the affinity for ETH. The mutations are a result of an amino acid substitution and depending on which amino acid was substituted the binding affinity can range. Overall the mutations can lead to ETH being a less potent bacteriostatic drug by allowing important wall features to be synthesized[10].

Antimicrobial Peptides

AMPs are found in a wide variety of life from bacteria, insects, mammals, amphibians, and even plants, but with all these sources of AMPs, they all still have similar properties of being amphipathic and cationic[12]. With the wide variety of sources and the thousands of AMPs that have been identified comes a variety of mechanisms of bactericidal activity. The mechanisms of AMPs include the prevention of protein synthesis either through DNA or RNA inhibition, the inhibition of enzyme activity, or the inhibition of cell wall formation[13]. Another important mechanism is membrane disruption, which creates pores in the bacterial membrane[14].

In our study, we investigate synergistic combinations of short AMPs and TB antibiotics to then form aerogels that can be aerosolized for inhalation. We hypothesize that our peptides will form pores in the bacterial cell wall membrane that will allow for much greater permeabilization. This will make the drugs more effective and decrease the amount needed for a dosage to kill tuberculosis.

AMPs are still a relatively recent discovery with considerable potential to be a potent treatment for diseases. When AMPs were initially discovered they were observed to prevent

bacterial infections. As the library of known AMPs grew, so did the understanding of their mechanisms of action. This deeper appreciation has led to the development of potent AMPs against specific bacteriums, such as Mtb.

The initial findings of AMPs first occurred in 1939 by Dubos, where he isolated a bactericidal agent from a soil bacillus strain[15]. The agent was then tested *in vitro* and *in vivo* on gram-negative and gram-positive bacteria. In this study, virulent pneumococci were used as the gram-positive model and Klebsiella pneumoniae were used as the gram-negative model. When applying the bactericidal agent to the bacteria, it was found that the growth of gram-positive bacteria was inhibited while the growth of gram-negative bacteria was unaffected. The *in vitro* and *in vivo* results were comparable. Mice were used to model the *in vivo* effects, as they were injected with varying dilutions of bacterial culture. The mice were then be treated with the bactericidal agent and depending on the amount of agent and bacteria some of the mice would survive. In all cases, however, treated mice would always outlive the untreated mice. This was only true for mice infected with pneumococci, as mice infected with Klebsiella pneumoniae received no effect from the agent[16].

There are multiple mechanisms that AMPs can undergo to create its bactericidal effects. One of the mechanisms is membrane permeabilization. Once the AMPs have begun interacting with the bacterial membrane from electrostatic and hydrophobic interactions the AMPs accumulate and self-assemble on the surface. They then act through one of four models; the barrel stave model, the toroidal pore model, the carpet model, or the detergent-like model **(Fig. 2)**. In the barrel stave model, the peptides are first parallel with the lipid bilayer of the bacterial membrane but then insert themselves perpendicularly. A pore can then form through the help of peptide-peptide interactions. In the toroidal pore method, the peptides also insert themselves

perpendicularly but instead of peptide-peptide interactions, there are peptide-lipid interactions. These peptide lipid interactions cause membrane curvature leading to the disruption of the hydrophobic and hydrophilic arrangement. The pore becomes transient after disintegration and the peptides can enter the cytoplasm and target intracellular components. The carpet model is where the APMs adsorb to the lipid bilayer in a parallel direction until a threshold concentration is reached and the membrane becomes ‘carpeted’. Since the membrane is completely covered, this leads to undesirable interactions and loss of membrane integrity. The loss of membrane integrity can lead to the detergent-like model which is when the membrane collapses and forms micelles. AMPs can also kill bacteria without targeting the membrane; instead, they can either affect the cell wall or have intracellular targets. This works by having the AMPs interact with the cytoplasmic membrane then accrue inside the cell where they can inhibit critical cell processes. The AMPs will usually inhibit protein and nucleic acid synthesis or enzyme activity. When AMPs affect the cell wall, they do not bind to proteins involved in the wall synthesis, they typically bind to precursor molecules in the wall synthesis process[17].

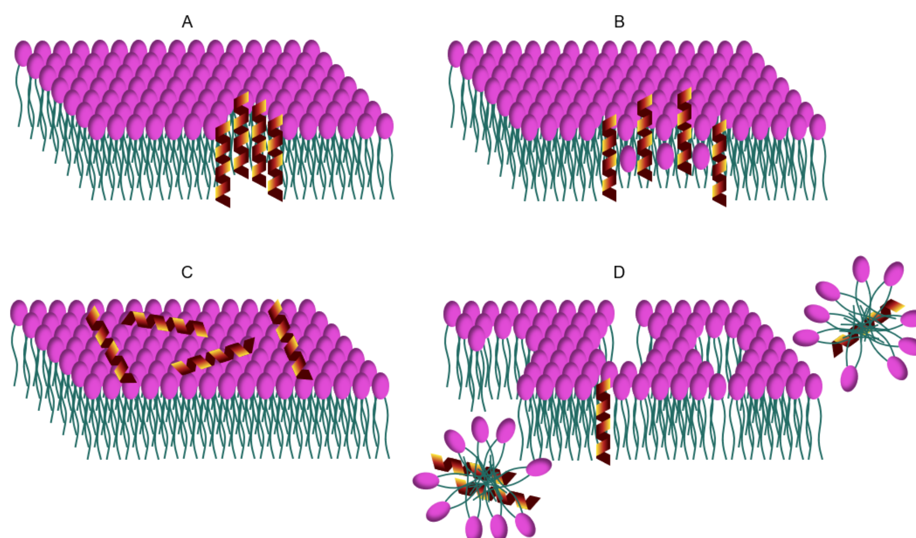

Figure 2: AMP membranolytic mechanisms; barrel stave (A), toroidal pore (B), carpet (C), and detergent like (D) mechanisms.

With the knowledge of AMPs and how they can have bactericidal effects, there is a lot of research on their effectiveness against diseases such as TB. Since their mechanism is known, we can formulate synthetic peptides to be produced for use against bacteria. One study showed that 12 of their artificial peptides had minimum inhibitory concentrations (MIC) values below 5 μM, with the lowest at 1 μM. They were also able to demonstrate that the peptides had low cytotoxicity against human THP-1 cells[18]. Another study has looked into the synergistic activity of peptides with other compounds. In this study, some peptides from the previously mentioned study by Ramon-Garcia were selected then coupled with cinnamic acid derivatives. This study found that peptides coupled with cinnamic acid derivatives were more potent against Mtb as they lowered the MIC value compared to just the free peptide[19].

In previous studies, the Medina lab has demonstrated that cationic peptides can form a crosslinking network with hyaluronic acid (HA) capable of being loaded with drugs for controlled delivery. In the study, the HA was put through a highly charged needle. This high charge caused the dissolved HA to disperse from the needle in extremely fine droplets. The droplets would then hit the peptide bath and begin the co-assembly of the polymers into an electrostatically bounded network that resembles a gel, which was termed an aerogel. The gels are composed of a non-covalent linked network, which allows for the particles to capture small molecules such as drugs. Depending on the drug, the best method of encapsulation will change. Proteins such as green fluorescent protein and β-galactosidase can be encapsulated by being dissolving in the peptide bath solution before the spay. On the other hand, compounds such as vancomycin are better loaded by dissolving in the HA solution. In the case that neither results in loading, such as for doxorubicin, the compound can be loaded by suspending the dry aerogel in DI water and dissolving the drug in DI water and allowing the two to mix and stir together. The

loaded aerogels can then be used for controlled release by causing swelling of the aerogel in physiological environments. This technique was proven to facilitate the delivery of cargo to target cells, in addition to improving the utility of the loaded cargo. The aerogels were successful at transporting therapies to the cytoplasm of cells that were considered membrane impermeable. This delivery system can lead to significantly more effective treatment of drugs, especially for drugs that certain cells might be resistant towards[20].

Pathogenesis of Mtb

Inside our bodies, Mtb becomes pathogenic by infecting itself inside our macrophage cells in the lungs. It has become such a successful pathogen due to its ability to infect many hosts, but only cause an active disease state in a few of those infected. Mtb can be found in neutrophils or the foci of caseous necrotic granuloma, but it mostly resides inside of the macrophage. The macrophage environment is hostile, yet the Mtb cell can alter their environment to better suit their survival[21]. Typically, when particles are engulfed by macrophages they are transported by phagosomes to the acidic and hydrolytic environment of the lysosome. To survive and proliferate, Mtb has developed strategies that will discontinue the maturation of the phagosome. In normal circumstances a phagosome becomes fully mature in about 12-15 minutes after formation, reaching an equilibrium pH of around 4.5. Mtb can prevent full acidification of the phagosome by stopping the pH at about 6.4, while this is still mildly acidic it is much more tolerable for the bacterium[22]. Mtb can prevent the acidification or acquisition of active hydrolases from the lysosome, yet at the same time, it has also been shown that it is still able to maintain access to cargo that was delivered to the cell through the

endosomal system[22] **(Fig. 3)**. Considering this, Mtb is not in a segregated compartment from the rest of the cell. Instead, it is fully incorporated into the endosomal system of the cell. There have even been studies that show how Mtb has been able to escape the phagosome and enter the cytosol of the macrophage, but this involves the pathology of later stages of the disease. Ultimately, the innate system will kill the pathogen by using multiple factors including increasing the production of nitric oxide, decreasing pH from the lysosome, and delivering AMPs[21]. Overall, Mtb is a tricky bacterium as it can undetectably spread from person to person, while only causing disease in a few. The fact that the bacterium can control its microenvironment dictates the need to form a robust drug delivery vehicle that can survive in the varying physiological conditions. In addition to that, the treatment must be able to effectively kill the Mtb without causing collateral cytotoxicity towards the host macrophage itself. In this light, AMPs provide a promising avenue of meeting all the criteria of eliminating the Mtb, especially when the bacterium is drug-resistant or extensively drug-resistant.

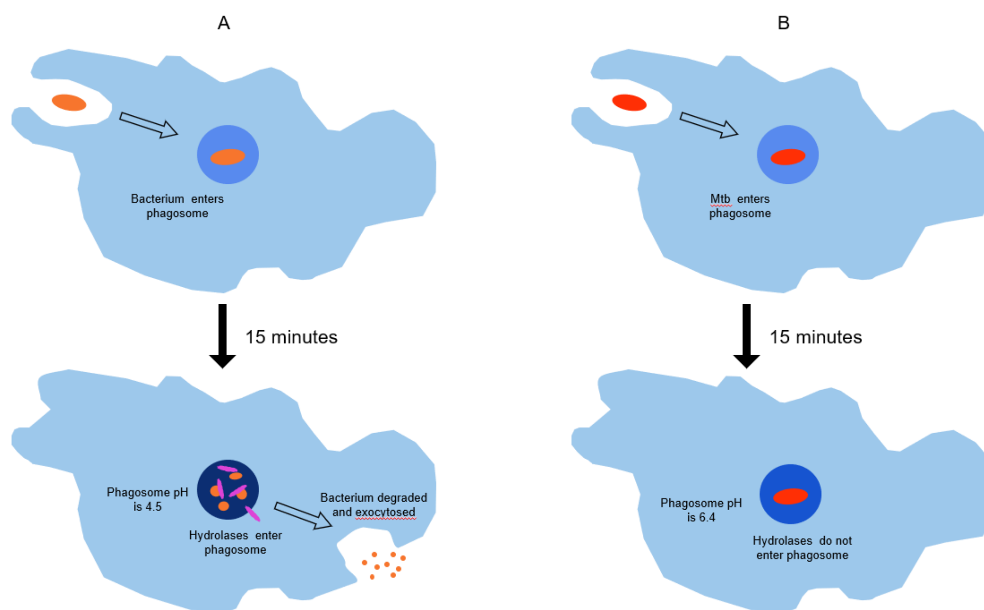

Figure 3: Macrophage phagocytosis of pathogens. (A) depicts a typical bacterium (orange) entering the phagosome to be degraded by the acidic environment and hydrolases (pink). (B) depicts Mtb (red) entering the phagosome and preventing full acidification and the presence of hydrolases allowing Mtb to survive.

The lung microenvironment is filled with mucus as a protective layer to remove anything that was inhaled from the environment such as toxic chemicals, particles, and pathogens. The mucus is composed of water and highly glycosylated proteins. The mucus is able to trap all of the foreign substances that were inhaled. The trapped toxins are then removed from the lungs via the ciliary beating and pushing them proximally until they are removed, or they can be removed via coughing. Cilia they can oscillate at a frequency of 12-15 times in a minute leading to a max velocity of 1 mm/s for the exiting mucus. As this process of transporting mucus is occurring, the body is continuously producing more layers to replenish what it had expelled. Although, this can be affected by the hydration of the individual as a well-hydrated person can experience mucus clearance at a faster rate[23]. Studies on human patients have revealed that from day to day there can be a variability in mucous turnover rate, which in turn shows that there is a wide range of a turnover rate. Although, when the peripheral airway becomes infected with bacteria, the kinetics of the mucus clearance is altered. When bacteria are present in the lungs, they can take up 6

hours to be cleared, assuming that it is optimal for bacterial growth with a doubling rate of 20 minutes. There have been studies that show when bacteria are present, factors that can suppress bacterial growth are produced. Interestingly, these endogenous antimicrobial factors have been identified as proteins such as lactoferrin and lysozyme as the major factors while defensins were only a minor fraction of the antimicrobial substances present[24]. When designing a delivery vehicle for transport to the lungs, the clearance time should be considered to create an effective therapy. If the lungs are able to completely clear all the foreign particles before all the drug is released, then the designed vehicle is rendered completely useless. In order to effectively gauge the usefulness a drug release kinetics assay will have to determine if an effective amount of drug is released.

Since the initial discovery of antimicrobial peptides, there has been a lot of promise that can be used to treat diseases. Understanding their mechanism of action has helped improve the selection of peptides used and the design of peptides synthesized. Recently, studies show promise that peptides are powerful bactericidal agents and can be even more effective when they are paired with other compounds. In the first articles reviewed, they presented the first finding of the antimicrobial activity of a peptide. This was extremely innovative as it opened a whole new area of research since they were able to prove the efficacy of the peptides *in vivo* and *in vitro*. Although the findings were novel, they were not able to purify the compound with certainty. In the study by Ramon-Garcia, they were able to create a miniature library of synthetic peptides that had low MIC values. This study was also able to show that the peptides had low cytotoxic effects on human cells. The next study by Silva was able to take this discovery even further and show that some of the peptides from the mini library had synergistic effects with cinnamic acid derivatives. In this study, three peptides from the Ramon-Garcia library will be tested for

synergistic combinations against Mtb that utilize the membrane permeabilization. We then want to form them into nanoparticles which can be aerosolized for delivery right to the lung **(Fig. 4)**.

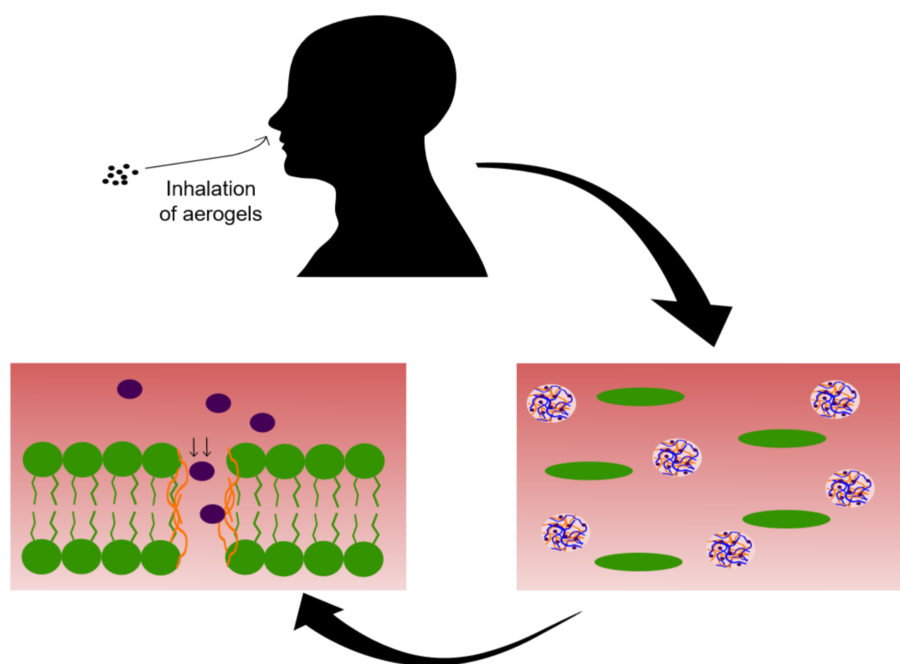

Figure 4: The design of the aerogels. The aerogels are designed to be inhaled so that they can be deposited to the lung environment. Once in the lung environment the APMs create pores in the bacterium membranes and allow for the transport of antibiotics across the membrane.

Chapter 2

Materials and Methods

Materials

Materials used in peptide synthesis were Fmoc protected amino acids, Rink Amide ProTide Resin, oxyma, diisopropylcarbodiimide (DIC), N,N-diisopropylethylamine, piperidine, dimethylformamide (DMF), dichloromethane, and a Liberty Blue Automated Microwave Peptide Synthesizer (CEM Corp., Matthews, NC). Peptide cleavage used trifluoroacetic acid (TFA), thioanisole, anisole, ethanedithiol, diethyl, ether, and FreeZone 2.5 Liter Benchtop Freeze Dry System (Labconco Corp., Kansas City, MO). Purification used trifluoroacetic acid (TFA), acetonitrile, formic acid, the Benchtop Freeze Dry System, and a high-performance liquid chromatography (Shimadzu, Kyoto, Japan). Aerogel formations used hyaluronic acid 100kDa (HA) and 300 kDa MWCO dialysis tubing. Toxicity assays used 96 well round bottom plates, RIF, MOX, ETH, INH, *mycobacterium tuberculosis*, 7H9 broth supplemented with OADC, glycerol, and tween 80.

Peptide Synthesis

Peptide synthesis of four different sequences **(Table 1)** was performed using Fmoc solid-phase synthesis using a Liberty Blue Automated Microwave Peptide Synthesizer. Before any amino acids are attached, the synthesizer was backflushed first to clean the lines. The amino acids were weighed out into a 50 ml conical tube and dissolved in DMF. Either vortexing or sonication was used for amino acids that had trouble dissolving. The oxyma solution was created

by weighing out pure oxyma into a 50 ml conical tube and dissolving it in DMF to create a 1 M solution. The DIC was prepared by pipetting DIC into a 50 ml conical tube and dissolving that in DMF as well. The TB1 and TB5 peptides used the mentioned process, but TB3 required a modified oxyma and DIC solution. The oxyma was prepared by having 0.1 M of N, N-diisopropylethylamine added and the DIC concertation was increased to become a 1 M solution. The piperidine solution was created by creating a 20% solution in DMF. For the main solvent pure DMF was used. The resin was massed out and put directly into the reaction vessel. Once all the solutions were prepared and properly attached to the Liberty Blue synthesizer, the correct method was run. Once the synthesizer was complete, the reaction vessel was removed the resin was put into a purification column and attached to an Erlenmeyer flask which was connected to a vacuum. The resin was put into the column and the vacuum was turned on to remove any solvent. The column was then rinsed with DMF and then dichloromethane to swell the resin beads. The column was then removed, capped, and wrapped with parafilm so that it could be out on the Benchtop Freeze Dry System to lyophilize.

After the completion of the synthesis, the peptides were cleaved from their solid support using a 10 ml cocktail of trifluoroacetic acid: thioanisole: ethanedithiol: anisole with a ratio of 90:5:3:2. The solution was made in a 10 ml glass graduated cylinder by adding the thioanisole, ethanedithiol, and anisole in first. The TFA was added in by using a glass pasteur pipette to fill the cylinder to the 10 ml line. The cocktail was mixed with the resin and a magnetic stir bar in a scintillation vial and the solution was stirred under argon for 3 hours at an RPM of 1500. After the three hours, the resin was dumped into a fritted filter funnel attached to vacuum in an Erlenmeyer flask and TFA was used to rinse off the resin. After the excess TFA was evaporated off, diethyl ether was used to precipitate out the peptide. An equal amount of the peptide was

then put into two 50 ml conical tubes and then put in a centrifuge at 2500 rpm for 5 minutes. The diethyl ether supernatant was evaporated and the crude peptide was collected in a scintillation vial. A Kimtech wipe was used to make a filter on the top of the vial and then was lyophilized.

Next, the peptide was purified using reverse-phase HPLC. 5 ml of methanol and 10 ml of standard A (0.1% TFA in water) was injected into the coils to clean the column after purging the line. The column was also cleaned by pumping each standard B (90% acetonitrile, 10% water, and 0.1% TFA) first and then standard A through for ten minutes. The crude protein was dissolved in standard A and then filtered through a polyethersulfone (PES) filter and injected into the coil. A linear gradient of standard B was pumped at 1% B per minute for TB1, at 1% per minute for 25 minutes then 0.5% per minute for another 40 minutes for TB3, and 2% per minute for 10 minutes then 1% per minute for 5 minutes and finally 0.5 % per minute for 40 minutes for TB5. The peptide was collected in a 15 ml conical tube when its peak would be displayed. Before the next run was started, the column was cleaned with running 10 minutes of standard B then 10 minutes of standard A. After the final run, the column was cleaned with standard B then standard A, but after standard A the column was run through with an additional 10 minutes of standard B so that the column could be stored in the organic solvent. All of the collected peptides were then put into a round bottom flask so that the volume of the peptide in the flask was no more than 10% of the total volume in the round bottom. The peptide was then frozen on the walls of the round bottom by rotating the flask in liquid nitrogen. The round bottom was then put on the Benchtop Freeze Dry System to lyophilize.

Purity was determined by analytical LCMS. The LCMS was prepared by first purging the line then running the column with 10 minutes of standard B (90% acetonitrile, 10% water, and 0.1% formic acid) and then 10 minutes of standard A (0.1% formic acid in water). The peptide

was prepared by dissolving at a concentration of 1 mg/ml in standard A. The solution was then filtered through centrifugation and then put into an insert which then put into a vial. The vile was then placed inside a slot with a designated number on a tray. 50 µl were injected into the column and then ran with an increasing gradient of 1% B per minute.

Table 1. AMP Sequences

AMP	*Sequence*
TB1[18]	WKWLKKWIK
TB3[18]	KRWWKWRR
TB5[18]	RRWWRWVVW
MAD1	KRWHWWRRHWVVW

Aerogel Formation

Peptide aerogels were made by using an electrospray technique **(Fig. 5)**. HA was dissolved in DI water at 28.7 mg/ml and filtered through a 0.2 µm PES filter into a syringe. The peptide bath was made to a concentration of 100 µM and put into a petri dish with a grounding wire. The syringe with the filtered HA solution was attached to a 28G needle. The syringe was then put into a syringe pump set to eject a volume of 0.667 ml at a rate of 0.1 ml per minute. Once in the pump, the needle had an alligator clip attached to it. A high voltage power supply then charged the needle to 24 kV and the spray was then started. After the spray, the aerogel was incubated for 1 hour at 37 °C, and in parallel, the dialysis tubing was allowed to soak in DI water before use. The gels were then put into the dialysis tubing and allowed to sit overnight in a DI water bath.

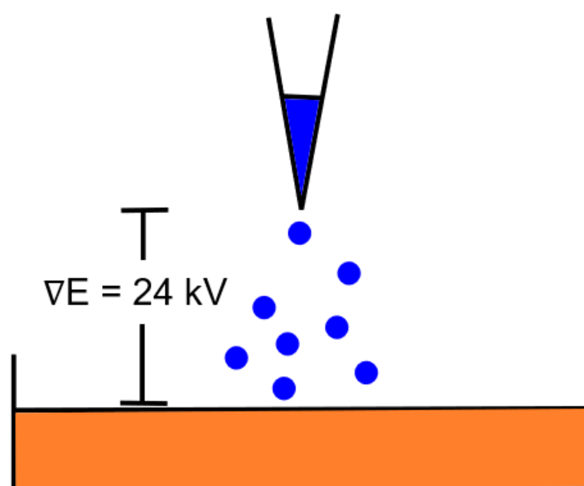

Figure 5: Electrospray schematic. The HA (blue) is being sprayed into the peptide bath (orange) creating a crosslinked network.

Aerogel particles were characterized by dynamic light scattering using a Zetasizer Nano ZS. Aerogel particles were diluted 1:10 in DI water and 1 ml of solution was put into a cuvette to be analyzed. Three independent measurements were taken per sample at a 175° angle with a 25°C with a 1-minute equilibration time.

Toxicity Assay

Mtb was cultured in 7H9 broth consisting of 4.7 g/l of broth powder and supplemented with 10 % OADC, 0.5% glycerol, and 0.05% tween 80. The bacterium was cultured at 37 °C in a shaking incubator at 200 RPM. Toxicity was assessed by performing combinatorial assays against the Mtb **(Fig. 6)**. Treatments of peptide and antibiotic were all prepared 40X in water and 10% DMSO then further diluted 10-fold in the broth before treatment. The plates were designed so that there were two replicates per plate, with the peptide concentration decreasing two-fold

down the column and the drug concentration decreasing two-fold down the rows. To prepare the plate, the first and seventh columns were left blank and 25 µl of broth was added to the rest of the columns. 50 µl of the 4X peptide solution was then added to the first and seventh column and then serially diluted. On a separate plate, the first row was left empty and the rest of the rows were filled with 25 µl of broth. The first row was then added with 50 µl of the 4X drug and serially diluted down. The solution in each of the wells on the drug plate was transferred to the peptide plate. The highest concentration of each treatment was 0.008 µM for RIF, 12.5 µM for ETH, 0.8 µM for INH, 0.3 µM for MOX, 40 µM for TB1, 40 µM for TB3, and 80 µM for TB5. Once the drug and peptide treatments were prepared on the plate, a 0.002 optical density of Mtb was added to each well. Plates were incubated for four days and then checked for any visual growth.

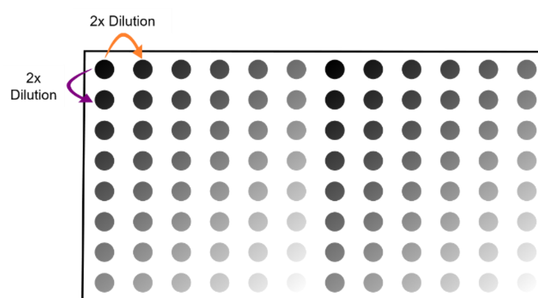

Figure 6: Combinatorial assay schematic. The peptide and drug are being diluted down the columns and rows respectively creating a two-dimensional gradient.

Aerogel Loading

Aerogels were formed with the same method as previously mentioned above. Dissolved HA was pumped through a 24 kV electric field into a bath of peptide solution. The bath was then put into dialysis tubing and dialyzed overnight in a water bath. To load the aerogels with an

antibiotic the drug was dissolved with either the HA solution or the peptide bath solution. The drug was loaded at a concentration of 5 mg/ml in the peptide bath and 15 and 30 mg/ml in the HA solution **(Fig. 7)**. The amount loaded was then characterized by allowing the particles to swell and burst in a 10X PBS bath for 1 hour. A small aliquot was taken and analyzed with UV-Vis spectrophotometry at a wavelength of 360 nm. Using a calibration curve, the amount loaded was then calculated.

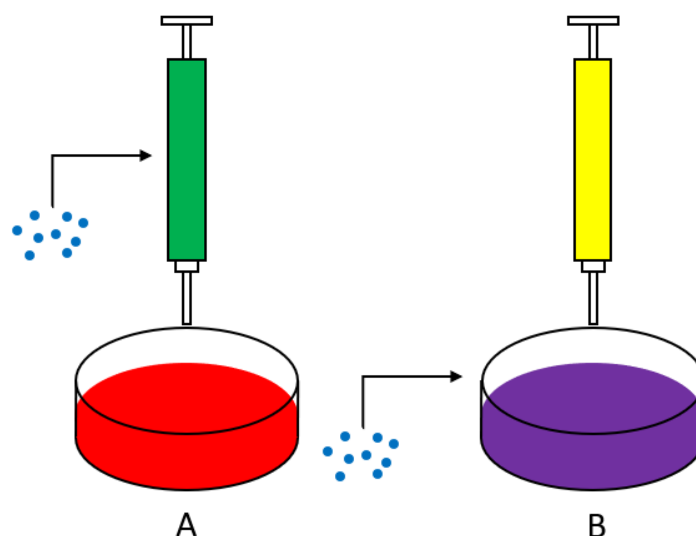

Figure 7:Drug Loading Schematic. The most efficient and highest loading method was determined by dissolving MOX (blue) in either the HA (yellow) solution (A) or in the peptide (red) bath (B).

Statistical Analysis

Every experiment that was conducted was run with triplicates to confirm the data. The average and standard deviation was taken from the particles that were analyzed by DLS.

Chapter 3

Results

Aerogel Formations

After the aerogels were synthesized using the electrospray technique mentioned above, they were taken for DLS sampling (n=3) **(Fig. 8)**. The TB1 aerogel had an average diameter size of 310.8 ± 95.49 nm with a peak that was monodispersed. The TB3 aerogel had an average diameter of 735.4 ± 219.6 nm and it also had a fairly monodispersed peak. The TB5 aerogel had an average diameter of 886.9 ± 226.2 nm with a monodispersed peak as well. MAD1 had an average diameter of 790.6 ± 175.9 nm with a moderately monodispersed peak. In the end, all of the peptides were able to form particle sizes on the same order of magnitude.

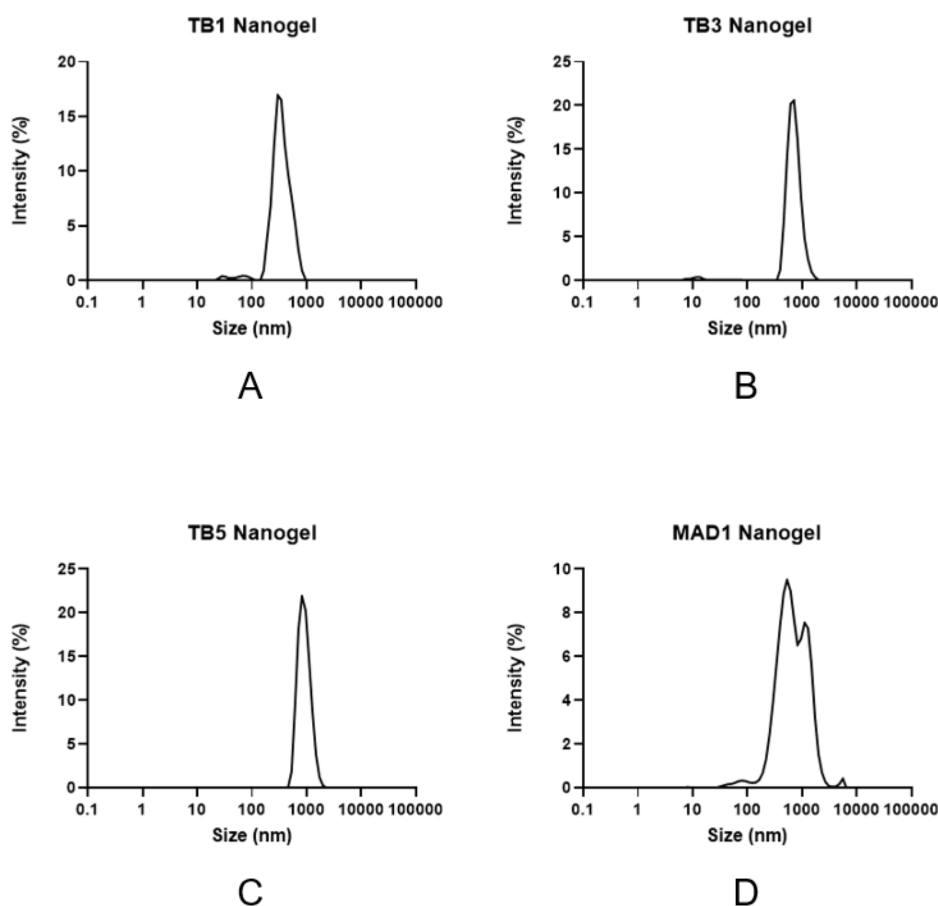

Figure 8: Particle size characterization of aerogels. They were prepared from TB1 (A), TB3 (B), TB5 (C), and MAD1 (D).

Combinatorial Toxicity

In order to determine the most suitable peptide to form the aerogel, as well as the best drug to load the aerogel with, mycobactericidal combinations were tested. To quantitatively determine the best combination, a fractional inhibitor concentration (FIC) score was given based on how well the compounds performed in combination compared to by themselves (n=3) **(Fig. 9)**. The formula used to calculate the FIC is: $FIC = \frac{C_A}{MIC_A} + \frac{C_B}{MIC_B}$ where C_A and C_B is the inhibitory concentration of drug A and B respectively in combination and MIC_A and MIC_B is the

inhibitory concentration of drug A and B respectively by itself. A FIC value of <0.5 is considered synergistic, a value from 0.5-1 is additive, and any value >1 is considered indifferent.

MAD1 and MOX were the only peptide and drug combinations that had a FIC score of less than 0.5, which was 0.28. RIF, ETH, and INH had scores of 0.53, 0.625, and 0.75 respectively, which puts them all in the additive range. TB1 had scores of 1, 2, 1, and 0.625 with MOX, RIF, ETH, and INH respectively. All of TB1's combinations were additive except RIF which was indifferent. TB3 had scores of 1, 0.75, 2, and 0.75 with MOX, RIF, ETH, and INH respectively. Again, three of the combinations were additive except ETH this time, which was indifferent. TB5 had scores of 1.52, 2.5, 1.48, and 3 for MOX, RIF, ETH, and INH respectively.

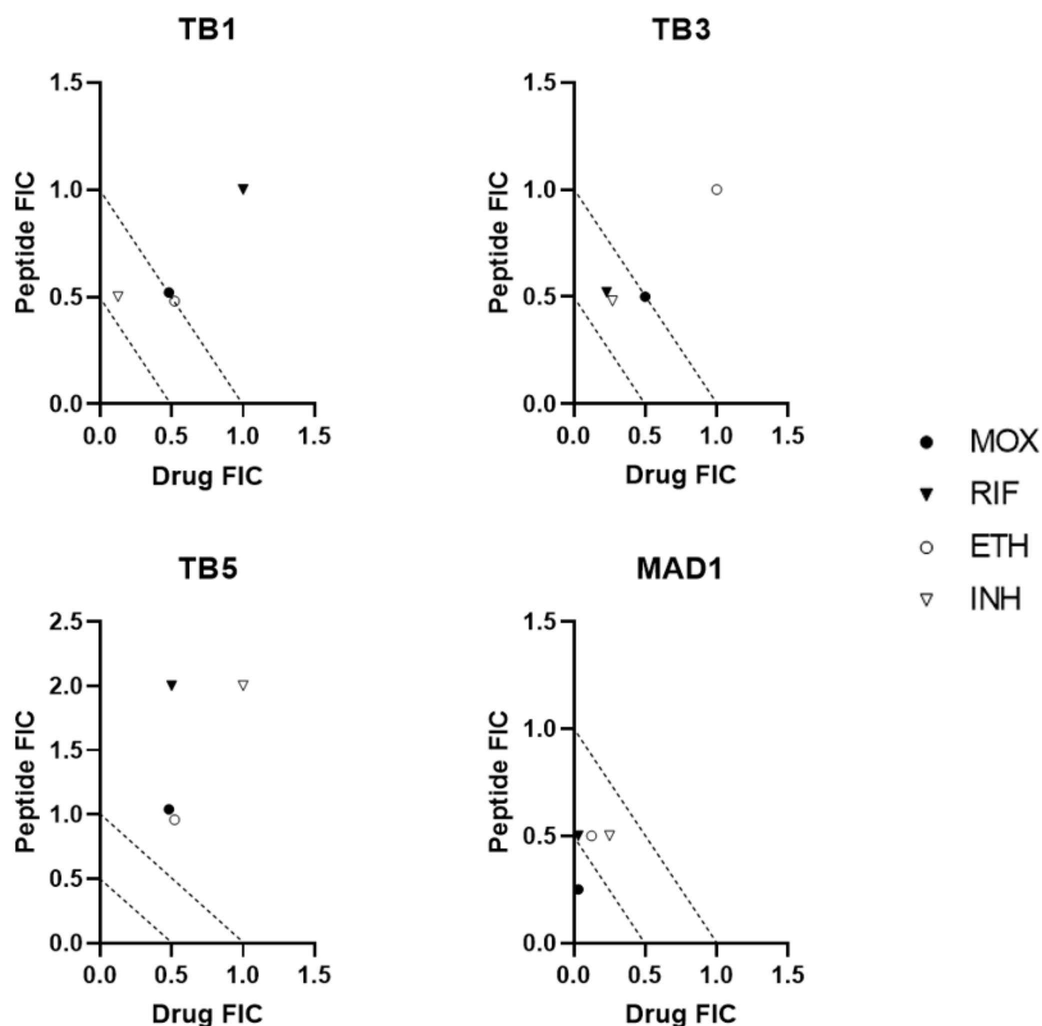

Figure 9: FIC scores for MOX, RIF, ETH, and INH each with TB1 (A), TB3 (B), TB(5), and MAD1 (D). Markers in the aera between the axes and the lower line are synergistic, markers in between the two lines are additive, and markers above the upper line are indifferent

Aerogel Loading

The loaded drugs were characterized to determine the amount they were able to load and the efficiency at which they did so **(Table 2)**. To load the gels, MOX was dissolved in either the MAD1 bath or the HA spray (n=1). In the MAD1 bath, the MOX was dissolved in at a concentration of 5 mg/ml which yielded a loaded mass of 3.21 mg and an efficiency of 6.41%.

When dissolving the MOX in the HA spray the efficiency increased nearly 4-fold. When dissolving at a concentration of 15 mg/ml the mass loaded was 2.79 mg with an efficiency of 27.87%. The loading concentration was then doubled to 30 mg/ml and there was a marginal decrease in efficiency to 23.7%, however, the amount loaded was increased to 4.74 mg. The 15% loss in efficiency was made up for by an increase of 70% mass loaded into the aerogels.

Table 2: Loading of MOX in Aerogels

	MOX in MAD1 (5mg/ml)	*MOX in HA (30 mg/ml)*	*MOX in HA (15 mg/ml)*
Loading Efficiency [%]	6.41	23.70	27.87
Mass Loaded [mg]	3.21	4.74	2.79

Chapter 4

Discussion

Overall, this project has demonstrated the capability for producing a novel drug delivery system using AMPs for drug resistant TB. The combinatorial assays have shown that MOX and MAD1 are the most synergistic combination. This is because MOX has a very difficult time permeabilizing through mycobacterium membranes, but through MAD1 creating a pore in the membrane, it helps with the influx of the drug. We were also able to demonstrate the ability to form aerogels with MAD1 and HA and subsequently load them with MOX. The peptides TB1, TB3, and TB5 were able to demonstrate that forming aerogels can be done using any AMP. The loading of the aerogels was more successful when the MOX was dissolved in the HA. The higher the mass loaded in the aerogel, the better as it allows for the use of less aerogel to deliver the same amount of drug.

Although this work was promising there is still more that needs to be addressed in the future. One thing still needs to be addressed is how well aerogels will be taken up by macrophages. This is key as Mtb thrive inside the macrophage where they are shielded from danger. In addition to a macrophage uptake study, a study testing for the MIC of bacterium inside a macrophage would need to be assessed. This will inform us about how potent the aerogels are towards Mtb that has entered the macrophages in our lungs. Finally, an *in vivo* study would provide a more complete picture. All of this would be able to support the implications this study has on the human body.

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- Organized and planned protocols for experiments to determine mechanical integrity of tendons
- Awarded funding to research over summer 2018 by the Erickson Discovery Grant
- Awarded funding to research through fall 2018 semester by College of Engineering Research Initiate REU

Undergraduate Research Assistant
The Medina Group – University Park, PA (January 2019 – May 2020)
- Researched chemically engineered peptides to use as precision therapeutics for anti-TB treatments
- Awarded funding to research through summer 2019 by the Multi Campus REU

Additional Experience

Clinical Engineer Intern
UPMC Hamot – Erie, PA (July 2018 - August 2018)
- Repaired malfunctioning equipment and performed preventative maintenance

Customer Service
South Shore Wine Company – Northeast, PA (May 2017 - August 2017)
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- Organized activities including club picnics and intramural sports throughout each semester

SHO Time Orientation Leader, *Mentor (August 2018, August 2019)*
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