Examination of the Antibacterial and Immunostimulatory Activity of a Wasp Venom Peptide

by

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Dissertation submitted in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy in the Department of
Molecular Genetics and Microbiology in the Graduate School of
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2013

ABSTRACT

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Abstract

Antimicrobial peptides (AMPs) are part of the innate immune system that is widely distributed in nature, acting as a defense mechanism against invading microorganisms. AMPs have potent antimicrobial activity against a range of microorganisms including fungi, bacteria, and viruses. In view of growing multidrug resistance, AMPs are increasingly being viewed as potential therapeutic agents with a novel mechanism of action. Mastoparan is a natural, highly positively charged AMP derived from the venom of wasps. It was originally of interest based on its inherent mast cell degranulation activity. Previously, mastoparan has been shown to exhibit antimicrobial activity *in vitro*; however, these studies have been limited in scope. Here we hypothesize that mastoparan possess the capacity to be a potent broad-spectrum antibacterial agent including activity against multidrug resistant bacteria.

We examined the scope of antibacterial activity exhibited by mastoparan using a variety of antimicrobial susceptibility tests and have utilized a bacterial skin infection (*Staphylococcus aureus*) model to determine the potential of mastoparan to serve as a therapeutic agent. We tested mastoparan against four

Gram-positive clinical isolates (e.g., *Staphylococcus aureus*, and *Enterococcus faecium*), nine Gram-negative clinical isolates (e.g., *Escherichia coli*, *Pseudomonas aeruginosa*, and *Burkholderia cepacia*), and four multidrug resistant clinical isolates (e.g., MRSA, ESBL *Escherichia coli*, and ESBL *Klebsiella pneumonia*). These studies reveal that mastoparan exhibits broad-spectrum activity against both Gram-negative (MIC: 1.9 – 125 μg/ml) and Gram-positive (MIC: 15.6 – 125 μg/ml) bacteria and against multidrug resistant bacteria (MIC: 7.8 – 125 μg/ml). We also demonstrated that mastoparan disrupts the bacterial membrane, exhibits fast acting antibacterial activity, and is highly effective against both multiplying and non-multiplying bacteria. Furthermore, we have shown that mastoparan demonstrates efficacy as a topical antimicrobial agent reducing lesion size by up to 79% and the amount of bacteria recovered from skin lesions by up to a 98% reduction. Based on these results we conclude that mastoparan is a highly effective antibacterial agent and is therefore a potential alternative to currently antibiotics. Mastoparan offers a promising new therapeutic option for treating bacterial infections.

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Chapter 1: Introduction

1.1 Bacterial Infections

Bacteria are a major cause of human death and disease and are responsible for infections such as tuberculosis, pneumonia, tetanus, typhoid, cholera, and foodborne illness. The World Health Organization (WHO) lists lower respiratory infections (pneumonia, tuberculosis and bronchitis) and diarrhoeal diseases (most commonly caused by *Escherichia coli* and rotavirus) as the top two leading causes of death in low-income countries and the top five causes of death worldwide [1]. Infection occurs when bacteria in the environment as a component of either the indigenous microbiota or as exogenous organisms invade the host.

Bacteria are classified as either Gram-positive or Gram-negative, broadly based on the structural differences of their cell membrane and the effects of these structural differences on the outcome of the Gram stain test established by Hans Christian Gram. Gram-positive bacteria have a thick peptidoglycan layer and a single membrane, while Gram-negative bacteria contain both an inner and outer membrane. Bacteria multiply at a rapid rate with generation times varying from several minutes to greater than 24 hours. This rapid generation typically occurs

via binary fission and can select for beneficial, spontaneous mutations that account for rapid evolutionary adaptation. These adaptations allow bacteria to utilize a variety of nutritional sources, which contributes to their ability to occupy habitats where few other forms of life can exist. Bacteria, which can infect humans, animals and plants, can develop a range of different relationships with a host including parasitic, free-living, and mutualistic. Bacteria have been known to infiltrate our bodies, food (e.g., crops and domesticated livestock), and water supply. Their metabolic diversity allows them to persist in a range of different habitats.

While bacteria are a major cause of disease, not all bacteria are pathogenic. Non-pathogenic bacteria play a vital role in ecosystems and in human society such as in waste breakdown, drug production, and environmental bioremediation. As a natural host for bacteria, humans possess a microbiome made up of bacteria in places such as the gastrointestinal tract, mucosal surfaces and skin. The bacteria that comprise the human microbiota are typically beneficial or harmless; however, a pathogenic association can develop if bacteria numbers grow beyond their typical ranges, the host has a compromised immune system, or bacteria are able to populate atypical or vulnerable areas of the body

[2-5]. In addition to these opportunistic pathogens, there are also obligate pathogens, which in contrast, are not typically a component of the normal microbiota and are only able to grow and reproduce within the cells of other organisms. These types of pathogens cause diseases such as chlamydia (*Chlamydia trachomatis*) and typhus (*Rickettsia* bacteria). Bacterial infections result from an imbalance of bacterial virulence and host defense mechanisms. Natural selection usually favors a predominance of less virulent microorganisms as it is typically more beneficial for bacteria to multiply rather than cause disease [6]. Death or severe impairment of the host caused by pathogenic infections compromises the survival of the infecting microbe, therefore; it is in the best interest of the bacteria to thrive in the host without causing significant damage [7, 8].

Approaches to treat bacterial infections constitute the major emphasis of this dissertation. Here we propose the use of an antimicrobial and immune stimulatory peptide as a potential antimicrobial agent against bacterial infections. The subsequent sections of this chapter describe the attributes of pathogenic bacteria, the host-pathogen relationship created during infection, the mechanisms by which microorganisms are able to cause infections, and ways

(e.g., using wasp venom antimicrobial peptides) in which we can treat bacterial infections. Focus is also given to the emergence of resistant bacterial pathogens and the global health problem that this presents.

1.1.1 Bacterial host-pathogen interaction in humans

The manifestation of the host-pathogen interaction depends on the interplay of bacteria virulence factors and the host response. The skin and mucosal surfaces act as a physical and chemical barrier, providing the first line of defense against colonization by bacterial pathogens [9]. In healthy individuals, the cellular and humoral mechanisms of the host are capable of clearing the bacteria that are able to penetrate these barriers. However, in individuals with a defective immune response, even less virulent bacteria are able to colonize and cause frequent, recurring infections. Elderly individuals and infants are especially susceptible to infections due to immune systems that are weakened or have not been fully developed [10-14]. Furthermore, the indigenous bacteria microbiota found on the skin and at mucosal sites, also functions as a line of defense against the colonization of exogenous bacteria [4]. Indigenous bacteria produce proteins, metabolites, and end products that function as specific and nonspecific antagonist against exogenous bacteria. If a bacterial pathogen is able

to overcome this first line of defense and breaches an anatomical barrier, then the potential for colonization is increased.

Colonization is the first stage of an infection and usually occurs in host tissues that are in contact with the external environment [15]. Once bacteria have penetrated the skin or mucosal surfaces, the host employs several mechanisms of growth restriction, such as cellular defenses (e.g., phagocytosis and inflammation) [16-18]. The innate immune system defends against pathogens by producing antibacterial enzymes (e.g., lysozyme) and rapid localization of macrophages and neutrophils to engulf and kill foreign cells [19-24]. The inflammatory response serves as a means to recruit cells (e.g., neutrophils, macrophages, and lymphocytes) and defensive components (e.g., antibodies, and complement) to the site of invasion [25, 26]. In addition to the activation of the innate immune system, the body may also utilize mechanisms of adaptive immunity to detect and attack bacterial pathogens. The adaptive immune system exhibits immunological memory involving antibody-mediated immunity (AMI) and cell-mediated immunity (CMI) that allows the body to react quickly if exposed to the same organism in the future. This systemically-acquired response serves to inhibit future invasion and damage and includes defense mechanisms

such as interleukin production by T cells and the production of circulating antibodies by plasma cells [27, 28]. Overall, the host utilizes a variety of resistance mechanisms to defend against bacterial pathogens; however, the pathogen also employs a number of strategies that allow for survival in the presence of these host defense mechanisms.

In order for a bacterial pathogen to establish an infection, several things must occur. The pathogen must be able to attach and enter the body, evade the host defenses, multiply to significant numbers, and transmit the infection so that the cycle can continue. Once inside of the host, bacteria utilize several mechanisms that allow them to evade the host defenses. Many bacteria can be encapsulated in mimics of host matrices allowing them to go unrecognized by phagocytes and protect them from phagocytosis [17, 29]. Gram-negative bacteria produce lipopolysaccharide endotoxins that can provide protection against host membrane attack complexes and can cause potentially lethal biologic effects (e.g., fever, lethal shock and sepsis) [30, 31]. Both Gram-negative and Gram-positive bacteria secrete exotoxins (e.g., cytotoxins, neurotoxins, and enterotoxins), extremely toxic substances that cause damage to the host [32]. The ability of bacterial pathogens to overcome and evade the extensive capabilities of the host

defense mechanisms serves as evidence that bacterial virulence is a highly sophisticated process.

The many factors that contribute to the outcome of a host-pathogen interaction, and the relationship between bacteria and the host, force their individual evolution to be inevitably linked. Much of the current knowledge about the human immune system has come through the investigation of host-pathogen interactions that occur for successful versus unsuccessful pathogens. As understanding of the intricacies of the immune system increases, there is a corresponding increase in the understanding of bacterial mechanisms that subvert the immune response. Additionally, increased understanding of the host-pathogen relationship on a molecular level also provides valuable insight on pathogens and pathogenicity. This information is useful in designing novel ways to counter bacterial infections. The infectious disease problem is a growing public health issue. Understanding of the host-pathogen interaction is pivotal in developing future technologies that increase host resistance to bacterial pathogens.

1.1.2 Bacterial skin infections

Bacterial skin infections are responsible for a variety of debilitating chronic clinical skin conditions including impetigo, cellulitis, folliculitis/furunculosis, abscesses, and ulcers [33-35]. Gram-positive bacteria (e.g., *Staphylococcus* and *Streptococcus*) characteristically cause bacterial skin infections; however, Gram-negative organisms (e.g., *Pseudomonas*) may also cause some skin infections. These infections have become a significant public health problem as they result in over 11 million outpatient and emergency room visits and ~500,000 hospitalizations per year in the United States [33].

The skin, which harbors a diverse array of bacteria that are typically nonpathogenic, serves an important role as a barrier at the host-environment interface. Initial colonization of the skin by bacteria occurs in the birth canal, and the normal microbiota is established within 48 hours after birth [36, 37]. It is estimated that one square centimeter of human skin is home to 1,000 – 10,000 different bacteria organisms [38-40]. Susceptibility to infection occurs when the skin barrier is breached, especially in situations such as burns, surgical wounds, and cuts. Treatment of these infections has been complicated by the widespread emergence of antimicrobial resistant strains such as methicillin-resistant

Staphylococcus aureus (MRSA) [41-45]. Even in instances where appropriate antimicrobials are applied, they are largely ineffective because many of the bacteria are in stationary phase and are typically not affected by antimicrobial agents [46-50]. As a result of these limitations in therapy, skin infections remain highly debilitating, especially in immunocompromised individuals. The studies outlined in this dissertation investigate an approach to potentially treat bacterial skin infections.

1.2 Antimicrobial Agents

In addition to the natural host immune response to bacterial pathogens, there are also antimicrobial agents that assist in the fight against these pathogens. The therapeutic use of antimicrobial agents has had a powerful impact on the treatment of infectious diseases. Ancient cultures used antimicrobial agents, even without understanding the underlying cause of the antimicrobial properties of these substances. Early Egyptians (around 1550 BC) used a mixture of honey, lard, and lint to dress wounds, and it is now known that honey contains hydrogen peroxide, which has antibacterial properties [51-53]. Other ancient civilizations used moldy bread, soil and plants to treat infected wounds [53, 54]. The therapeutic nature of these substances was most likely due to metabolites

and chemicals serving as raw forms of antibiotics, and these cultures believed that these remedies influenced the spirits or Gods associated with the illnesses [52].

Towards the end of the 19th century, the introduction of the germ theory of disease ushered in improvements in the defense against bacterial diseases by expanding understanding of the causes of disease and proposing that microorganisms are at the root of many infectious diseases [55-58]. This led to increased research into effective ways to remedy microbial infection. One such finding by E. de Freudenreich in 1888, was the use of a blue pigment from *Bacillus pyocyaneus* which demonstrated antimicrobial activity against bacteria and was later named "pyocyanase" by Rudolf Emmerich and Oscar Loew [52]. The discovery of penicillin by Alexander Fleming is stated to have initiated the modern era of antimicrobial agents. Fleming noticed that a fungus from the *Penicillium* genus inhibited the growth of bacteria in culture dishes, Ernst Chain and Howard Florey later purified this antibiotic [59-62]. Penicillin came into clinical use in the 1940s and is renowned for saving the lives of many soldiers during World War II.

In the decades following these discoveries scientist ushered in the golden age of antimicrobial development highlighted by the discoveries of chloramphenicol, tetracycline, streptomycin, and erythromycin. By the 1960s, advances in fermentation techniques and medicinal chemistry strengthened progress and led to the attainment of a broader antimicrobial spectrum and higher antimicrobial activity. Nevertheless, the development of antimicrobials with novel mechanisms of action has slowed in recent times, with the majority of recently introduced antimicrobials being modifications of previously discovered drugs [63, 64]. Infectious diseases continue to be a growing global issue with an increase in opportunistic infections, emerging and re-emerging infections, and resistant microorganisms.

Antimicrobial agents are classified based on several different characteristics including their spectrum of activity and their bactericidal or bacteriostatic effect. The spectrum of activity of an antimicrobial agent depends on the range of bacterial species that these agents are able to affect. An antibacterial that has a broad spectrum of activity is effective against a wide range of both Gram-positive and Gram-negative bacteria (e.g., fluoroquinolones and tetracyclines). A narrow spectrum antibacterial is only effective against

Gram-negative or Gram-positive bacteria (e.g., bacitracin, which is only effective against Gram-positive bacteria, and polymyxins, which are usually only effective against Gram-negative bacteria). Additionally, antimicrobial agents are classified by their ability to cause actual death of bacteria (bactericidal effect) or simply inhibit or delay growth and replication (bacteriostatic effect). Some antibacterial agents are both bactericidal and bacteriostatic depending on the state of the invading bacteria (e.g., log versus stationary phase), the duration of exposure, and the concentration [63, 65]. Bacteriostatic agents typically have a slower mechanism of action. However, bacteriostatic agents can be advantageous since they require collaboration with the host immune system as normal defenses of the host are ultimately responsible for destroying the microorganism [52, 65]. Thus, bacteriostatic agents are not very effective in immunosuppressed or immunocompromised individuals.

Antimicrobial agents are categorized as an antibiotic if it is a natural substance produced by microorganisms, a chemotherapeutic agent if it is chemically synthesized, and a semisynthetic if it is a hybrid substance that is produced from natural substances that have been modified by chemist [66]. Overall, in order for an antimicrobial agent to be clinically useful it must exhibit

a range of beneficial characteristics: a broad spectrum of activity, nonallergenic, minimal toxicity and side effects, chemically stable, inexpensive and easy to produce, able to reach the site of infection, and improbable microbial resistance development. Unfortunately, there is not a single agent that works best for all applications. Nonetheless, knowledge of the characteristics exhibited by an ideal antimicrobial can be useful in the development of future antimicrobial agents.

1.2.1 Antimicrobial agents' mechanism of action

In order for a bacterial infection to occur, bacterial cells must grow and divide, and this requires the bacteria to synthesize or incorporate many different biomolecules. Efficacious antimicrobial agents have the ability to inhibit specific processes that are necessary for bacterial growth and/or division. These mechanisms of action are based on the structure of the antimicrobial and their target site within the bacterial cell.

The bacterial cell wall is critical for the life and survival of bacteria. Human and animal cells do not have cell walls; therefore, antimicrobial agents can selectively target the cell wall and only affect bacterial species. As noted previously, bacteria are classified as Gram-positive or Gram-negative. Both Gram-positive and Gram-negative bacterial cell walls contain peptidoglycan.

Antimicrobial agents that inhibit cell wall synthesis generally inhibit a step in the synthesis of bacterial peptidoglycan [67-70]. Examples of antimicrobials that inhibit bacterial cell wall synthesis are penicillins, cephalosporins, bacitracin, and vancomycin.

The cell membrane plays an important role in separating the intracellular and extracellular environment of the cell and serves in regulating the flow of substances (e.g., water, ions and nutrients) in and out of the cell. Cell membranes are composed of lipids, proteins, and lipoproteins. Antimicrobial agents that inhibit cell membrane function and integrity usually cause disorganization of the structure of the membrane or loss of function. If the integrity of the cell membrane is compromised, leakage of cell content (e.g., ions and macromolecules) can occur, leading to rapid cell death. Both human and bacterial cells contain phospholipid cell membranes, and thus antimicrobial agents that interact with the cell membrane typically have a poor selective toxicity. However, some structural differences between bacterial and human cell membranes exists and include the presence of lipopolysaccharides (LPS) on the outer membrane of Gram-negative bacteria [71, 72], and the lack of sterols (except in mycoplasmas) [73-75]. Examples of antimicrobials that disrupt the cell

membrane are polymyxins, which have specificity for the lipopolysaccharide molecule [76-79].

Bacterial cells depend on protein synthesis for multiplication and survival. Many therapeutic agents inhibit bacterial protein synthesis. Protein synthesis inhibitors disrupt cellular metabolism, which inhibits growth and multiplication in bacteria. Eukaryotic and prokaryotic ribosomes differ in size and in relation to the amino acid composition of the ribosomes, which confers specificity upon this class of antimicrobials [80, 81]. The major antimicrobial agents exploiting this mechanism of action are aminoglycosides, tetracyclines, chloramphenicol, and macrolides.

Synthesis of DNA and RNA are key processes in all living organisms. Antimicrobial agents that inhibit nucleic acid synthesis exploit the differences that exist in relation to structure and sequence, between eukaryotes and prokaryotes. Antimicrobial agents of this nature can work by binding bacteria-specific components involved in replication and transcription of DNA (e.g., DNA or RNA polymerases), by binding directly to the DNA or RNA to prevent their function, or they can hinder the synthesis of nucleotides or interconversion. Bacterial organisms that are unable to replicate DNA or transcribe DNA into

RNA are compromised in their ability to multiply and survive. Examples of antimicrobial agents that inhibit nucleic acid synthesis are quinolones and rifamycins. Some antimicrobial agents specifically inhibit cellular processes that exist in the bacterium but not in the host. Chemically, these inhibitors are analogous to bacterial metabolites or growth factors that are necessary for bacterial metabolism. However, these mimics are unable to perform the metabolic function and competitively inhibit the function of the normal metabolite [82].

Antimicrobial agents have the potential to affect both the host and the microorganism but most have a mechanism of action that specifically inhibits processes and structures that are only found in bacterial cells. The therapeutic use of antimicrobial agents requires that we understand not only the pathogenesis of the infection but also the pharmacodynamics and efficacy of the drug. The selection of an antimicrobial agent should ultimately be based upon the incorporation of the antibacterial activity of the agent with its pharmacologic properties.

1.2.2 Bacterial resistance to antimicrobial agents

Antimicrobial substances are powerful therapeutic tools and have been critical in the global reduction of death and disease due to infectious diseases. Unfortunately, they have not eliminated bacterial diseases from human and animal populations and the development of antimicrobial resistant bacteria has significantly reduced their efficacy. The World Health Organization defines antimicrobial resistance as the "resistance of a microorganism to an antimicrobial medicine to which it was previously sensitive" [83]. Antimicrobial resistant bacteria have a higher minimum inhibitory concentration (MIC) than what was originally required. Multidrug resistant organisms are those that are resistant to more than one antimicrobial agent, typically from different classes. Antimicrobial resistant bacteria are able to survive and multiply in the presence of an antimicrobial agent due to some change in the population that abolishes the efficacy of the antimicrobial.

Antimicrobial resistant pathogens represent a large public health and economic burden. Individuals that are infected by antimicrobial resistant bacteria typically have prolonged illness, greater possibility of death, and an increased potential of spreading the resistant organism to others [84].

Antimicrobial resistant bacteria are responsible for increased cost of treatment and longer hospitalization, which burdens the entire health care system. The Centers for Disease Control and Prevention (CDC) reports that these pathogens cost an estimated $21 - $34 billion a year in health care costs and $35 million in other societal costs [85, 86]. Each year approximately 2 million Americans (5%-10% of all hospitalized patients) develop hospital-acquired infections which result in more than 95,000 deaths and the majority of these deaths are due to antimicrobial resistant pathogens [86, 87]. Of the bacteria causing infections in hospitals today, approximately 70% of them are resistant to at least one commonly used drug; given the current trend, it is suggested that within the next ten years we will no longer have effective treatments for the diseases associated with these pathogens [83, 88]. The Director-General of the World Health Organization spoke to the ensuing problem surrounding antimicrobial resistance in her keynote address at the 2012 "Conference on Combating Antimicrobial Resistance: Time for Action." The Director-General warned that if the present trend continues, then "things as common as strep throat or a child's scratched knee could once again kill" and that "some sophisticated interventions, like hip replacements, organ transplants, cancer chemotherapy, and care of preterm

infants, would become far more difficult or even too dangerous to undertake" [89].

Antimicrobial resistance has been an issue since the start of the antimicrobial era [90, 91]. Soon after penicillin began to be widely used, it was discovered that some bacterial strains had developed resistance to this antibiotic [92]. With the development and introduction of each new class of antimicrobial agents came the emergence of resistant microorganisms. This emergence is not surprising since pathogens, like all living organisms, are guided by natural selection. Resistance development in bacteria is abetted by their ability to evolve rapidly, their short generation time and their capacity to exchange genetic information encoding for resistance in an inter- and intra- species fashion [93]. Many bacterial organisms synthesize antibiotics, therefore, the genes for resistance development are ancient, given that these microbes have mechanisms to survive in the presence of the antimicrobials that they themselves produce [94-96]. Penicillin-resistant bacteria were in the environment before penicillin was used therapeutically, and penicillinase was recovered from dried soil in dormant *Bacillus licheniformis* endospores [97, 98]. These naturally occurring resistance

genes can be transferred from nonpathogenic bacteria to those that cause disease [95].

Bacteria are very adept at mounting resistance to antimicrobial agents, and there are a number of mechanisms that have evolved to confer resistance. The basic mechanisms of resistance include inactivation or chemical modification of the antimicrobial, altered entry or active export of the antimicrobial from the cell, alteration of the target site for the antimicrobial, development of a resistant metabolic pathway, or acquisition of an enzyme in exchange for the sensitive one [99-101]. Bacteria can develop antimicrobial resistance as an inherent/natural property or as a secondarily acquired mechanism [96]. Bacterial cells that are able to survive the effects of an antimicrobial agent can then pass the resistant genes on to other bacterial cells via vertical or horizontal gene transfer [85, 102]. Vertical transfer is the passage of resistance genes that result from chromosomal mutations from one generation to the next via DNA replication [96]. Horizontal gene transfer occurs via conjugation (direct transfer via cell-to-cell contact), transformation (via plasmids or transposons), or transduction (bacteriophage transfer) [96, 102]. Continued exposure to antimicrobials provides a selective

pressure that creates a situation in which the persisting bacteria are more likely to be resistant [85, 103].

Soon after the use of antimicrobial agents began, signs of resistance to these antimicrobials were observed. These antimicrobial agents allowed us to cure some common infections and control outbreaks of infectious diseases. Nevertheless, this victory was premature and it is now evident that microorganisms are not only capable of mounting resistance at an alarming rate, but there is a rapid increase in multiple drug resistance as well. This presents us with a frightening trend considering that most antimicrobials may no longer be effective for the generation to come, and common bacterial infections may once again become lethal. The fight against these resistant pathogens continues, and long-term management of the increase in microbial resistance will require a change in both behavior as well as the evolutionary view of microbes to devise innovative ways to combat infections.

1.2.3 Factors contributing to antimicrobial resistance

Evolutionary pressure exerted by an antimicrobial drives resistance development and the spread of resistance amongst bacterial species. In addition to understanding the scientific basis of resistance, it is also imperative to

understand the social and administrative practices that contribute to resistance emergence. One major driving factor behind antimicrobial resistance is the misuse or overuse of antibiotics [83, 93]. Each time an individual utilizes an antimicrobial agent the sensitive bacteria are killed; however, the resistant organisms are able to survive and multiply. An individual has a greater potential for antimicrobial resistance development for up to a year after taking a single dose of an antimicrobial agent [104]. Patient expectations, in which patients have come to expect to receive antimicrobial agents even when these drugs may not be effective, typically drive overuse. One study reports that antibiotics were prescribed in 68% of acute respiratory tract infections even though they were unnecessary in 80% of these cases [104]. The use of antimicrobial agents against microorganisms to which they are ineffective such as viral infections (e.g., common cold and the flu) is a leading force in the emergence of resistance [85].

In some countries, antimicrobial agents are sold without a prescription, which also promotes resistance development due to misuse. These drugs are typically poorly manufactured, counterfeit and lacking in full potency [93]. Some additional behaviors driving antimicrobial resistance are failure to adhere

to the recommended dosing information (e.g., premature discontinuation, and ill-timed dosing), inability to afford the full course therapy, and poor hygiene [93, 105, 106]. Hospitals are also responsible for the emergence of resistance with a combination of immunocompromised patients, extensive antimicrobial use, invasive procedures and close proximity of patients [93]. This leads to the outcome of many hospital-acquired infections that are extremely resistant.

The debate over the magnitude and nature of the antimicrobial resistance problem is riddled with controversy. Many reports contend that the major cause of antimicrobial resistance is the abuse and misuse of antimicrobial agents [107-110]. A move towards the judicious use of antimicrobials through optimal drug selection and appropriate dose and duration may slow the emergence of resistance development. Regardless of the cause, human ingenuity has not outsmarted microorganisms, and resistance has become a global public health challenge.

The Centers for Disease Control and Prevention estimates that 1 in 6 Americans (approximately 48 million people) contract a foodborne illness each year, resulting in 128,000 hospitalizations and 3,000 deaths [111]. Bacteria (e.g., *Salmonella*, *Escherichia coli*, and *Listeria*), *Toxoplasma* or norovirus typically cause

foodborne illnesses and treatment usually requires antimicrobial agents [111, 112]. Thus, antimicrobial resistance in foodborne pathogens is a major concern. Antimicrobial agents are intensively used in the food production industry to promote animal growth, protect animal resources from infection, minimize the increase in zoonotic bacteria into the environment and food chain, treat sick animals and contain epidemics that could result in loss of animal and human lives [112-116]. Additionally, the use of antimicrobials to promote animal growth came about after research showed improved growth in animals that received subtherapeutic amounts of antimicrobials [117, 118]. Soon after these experiments, the Food and Drug Administration (FDA) approved the use of antibiotics in livestock diets as a means of growth promotion and disease prevention [112, 119]. Approximately 40-70% of the total antimicrobial production in the United States is used in the livestock industry, and antibiotic use in animal husbandry represents the most abundant use of antimicrobials worldwide [93, 112, 120-122].

The use of antimicrobial agents in animals has many benefits. Nonetheless, along with these benefits comes a contribution to the increase in the emergence of antimicrobial resistance. The non-therapeutic use of antibiotics as

a food additive to promote growth and prevent infections reduces drug efficacy and promotes the growth of antimicrobial resistant bacteria that can be subsequently transmitted to humans [112, 113, 123-128]. For instance, nonpathogenic bacteria that are a part of the normal gastrointestinal flora of animals may be pathogenic in humans. Resistance development can be selected for when these animals ingest antimicrobial agents in their feed [114, 129]. Transmission to humans can occur via consumption of meat, direct contact with animals, or via the environment [130-132]. Antimicrobials are also used in plant (sprayed aerially) and fish (added directly into the water) farming and can easily contaminate the water and surrounding sediment, leading to antimicrobial resistant bacteria in the environment [133, 134]. Antimicrobial agents in the environment drive antimicrobial resistance, and these resistant bacteria can serve as a source of resistant genes that can be spread throughout the bacteria population [92, 135].

The growing issue of antimicrobial resistance requires the judicial use of antimicrobials in both human medicine and in animals. In order to preserve the efficacy of our antimicrobial agents in treating animals we must prevent the abuse of these drugs and work to minimize the risk of breeding resistant

microorganisms. Using our current knowledge, some antimicrobials have been banned from use in food animals, and other procedures have been put in place to promote the appropriate use of antimicrobials in both humans and livestock animals.

1.2.4 Limitations of current antimicrobial agents

In addition to the increased emergence of resistance, there are also other limitations to the use of currently available antimicrobial agents including limited efficacy against Gram-negative bacteria, and the ineffectiveness of most antimicrobials against non-multiplying (stationary phase) bacteria. Most worrisome is the increase in antimicrobial drug resistance among Gram-negative pathogens, especially given the paucity of effective treatments under development that specifically target these organisms [136-138]. Gram-negative bacteria are particularly difficult to treat because of the unique features of these bacteria including the presence of a double membrane (as opposed to Gram-positive bacteria, which only have an inner cell membrane). This double membrane makes it difficult to specifically treat Gram-negative bacteria because many antibiotics are unable to penetrate the double membrane or are degraded or modified in the periplasmic space [139, 140]. Additionally, Gram-negative

bacteria have increased toxicity due to the incorporation of lipopolysaccharide, an endotoxin that can trigger a range of events important in pathogenesis including high fever and a drop in blood pressure [139, 140]. Studies show that the most predominant Gram-negative pathogens are *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter* species, *Serratia* species, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* [141, 142].

In clinical infections, bacteria exist as both multiplying (logarithmic phase) and non-multiplying (stationary phase) bacterial cultures [48, 143]. Current antimicrobial agents are typically inefficient or ineffective against these non-multiplying bacteria because most of these agents have a mechanism of action that requires active cell growth and replication to be effective [46, 49]. These non-multiplying bacteria are problematic because they can serve as a continued source of infection as they cycle into a reproduction phase and generate multiplying bacteria [143]. This can lead to chronic, recurring infections that require repeated administration of antimicrobial agents and prolonged treatment [143, 144]. Frequent and prolonged periods of antimicrobial therapy contribute to the emergence of bacterial resistance.

1.2.5 Current perspectives on antimicrobial agent development

Bacterial infections and antimicrobial resistance are some of the most significant challenges of this century and continue to increase in scope and impact. The development of new antimicrobials that can serve as adequate treatment against these infections is a top priority. Recently, there have been a few new antimicrobials brought to market and several others in clinical trial [145]. Most of the drugs in the pipeline are minor alterations to currently available antimicrobials. Furthermore, none of these new antimicrobials are suitable combatants against resistance, considering that they all have limited activity and are only active against Gram-positive bacteria [146]. Since the identification of daptomycin in 1987, we have been in a "Discovery Void" with no new antimicrobials with a novel mechanism of action being developed [147]. The Infectious Disease Society of America has started a "10 x 20 initiative" calling for the development of 10 new antimicrobial agents by the year 2020 [148]. In order to combat resistance development and stimulate the establishment of new antimicrobials, there are several mechanisms being deployed including the screening of natural antimicrobial compounds and development of therapies that stimulate the immune defense mechanisms.

1.2.5.1 Natural antimicrobial compounds

Most of the existing antimicrobial agents were derived as bioactive natural compounds. As stated previously, the first major antimicrobial, penicillin, was discovered as a metabolite of *Penicillium notatum*. Screening natural compound libraries that contain compounds from bacteria, fungi, plants, insects and even higher animals can lead to the discovery of powerful antimicrobials [47, 149]. These natural antimicrobial compounds include chemical compounds and substances such as antimicrobial peptides, steroids, and polyenes [150]. Today the scientific literature is filled with the identification of natural antimicrobial compounds from a variety of sources including marine organisms and insects [149]. Only a small fraction of the Earth's biodiversity has been explored, and natural antimicrobial compounds represent a promising source of potentially novel antimicrobial agents.

1.2.5.2 Immune stimulatory compounds

Immunomodulatory therapy is a new, novel approach to combating bacterial infections. Stimulation of the natural host immune system can serve as a critical component of improved antibacterial therapy by boosting the natural defense mechanisms against infection. Many antibacterial drugs have been

developed to treat bacterial infections; however, antimicrobial agents are most effective when the infection is also being fought by healthy immune defenses. Immune modulators are superior to typical antibiotic therapies since they can potentially have broader applications (e.g., vaccine adjuvants) and reduced resistance emergence. This is because in contrast to antibiotics, immune modulators do not directly endanger the bacteria and consequently may not exert selective pressure [129, 151, 152].

1.3 Antimicrobial Peptides

The increase in antimicrobial resistant organisms, coupled with a decline in development of new antibacterial therapies is a growing global public health concern. Alternative approaches to combat these infections are needed and a promising approach is the use of antimicrobial peptides (AMPs). As demonstrated in the previous section on the current perspectives on antimicrobials agents, the screening of natural compounds is an effective measure for the development of new antimicrobial agents.

All forms of life are in a constant antagonistic relationship with some of the diverse organisms in their environment. Both prokaryotes and eukaryotes have developed strategies to help protect them against these potentially harmful

and competitive organisms, including the evolution of a complex assortment of constitutive and inducible AMPs. In humans, one of the most effective host defense measures against microbial attacks is the secretion of broad-spectrum cationic peptides by epithelial and immune cells. Defensins, for example, are AMPs induced in phagocytes to destroy phagocytosed pathogens [153-155]. Bacteria produce AMPs as a response to intra- and inter-species competition in the fight for niche and nutrient advantage [156-158].

More than 700 AMPs (e.g., defensins, cathelicidins, and magainins) have been discovered in humans, plants, insects, and animals [159-163]. A database of currently known AMPs can be found via the Antimicrobial Peptide Database (http://aps.unmc.edu/AP/main.php). These peptides are an important component of the innate immune system found in nearly every kingdom and phylum [163-166]. AMPs act as a defense mechanism against invading microorganisms and are produced, stored, and secreted in exposed tissues, or synthesized *de novo* upon exposure to a pathogen [167, 168]. They demonstrate a broad-spectrum of antimicrobial activity against bacteria, fungi, protozoa, and viruses [166, 169-172]. These peptides are small (typically between 6-350 amino

acids in length), positively charged, and amphipathic (containing hydrophobic and hydrophilic regions) [173-176].

Most AMPs have a direct mechanism of antimicrobial activity via selectively damaging the membranes of bacteria [174, 177-179]. This membrane interaction allows efflux of essential ions and nutrients, leading to cell death through the formation of pores, membrane perturbations, or cell lysis [174-176, 179-181]. AMPs are able to selectively target bacteria membranes due to fundamental differences between microbial and mammalian cells, such as the presence of lipopolysaccharide, and differences in transmembrane potential and polarization (e.g., absence of negatively charged lipids on the surface of eukaryotic cells and lack of strong membrane potential gradient). Additionally, some AMPs may inhibit bacteria growth through intracellular targets. Studies have shown that once they have translocated across the membrane these proteins can hinder components of cellular physiology by inhibiting DNA/RNA and protein synthesis, biosynthesis of the cell wall, cell division, translocation and protein folding [182-188]. Therefore, AMPs have a mechanism of action that may include several different targets, in addition to an interaction with an important physiological structure.

The broad-spectrum of activity, combined with their rapid mechanism of antibacterial activity, makes AMPs excellent candidates for development of therapeutic agents. There are a few AMPs currently in clinical use such as polymyxin B (from *Bacillus polymyxa*) and gramicidin S (from *Bacillus brevis*), which are both naturally produced by bacteria and have been clinically used in topical antimicrobials for years. Several additional AMPs currently in development as potential therapeutics include pexiganan for the prevention of diabetic foot ulcers and plectasin, which has demonstrated bactericidal activity against antimicrobial resistant bacteria [175, 189-191].

Although numerous AMPs have been identified, their utility as therapeutics has encountered two main drawbacks. One obstacle is proteolytic degradation of the peptides; however, this can be overcome with strategies such as chemical modifications, the use of peptidomimetic equivalents, and the substitution of D- or non-natural amino acids [174, 175, 192-197]. Additionally, AMPs are also precluded by rapid absorption (especially in the kidneys), due to their small size and highly charged nature [174, 189, 193, 198]. This hurdle has been overcome with strategies such as extension of the length (e.g., via attachment of polyethylene glycol) of the peptides to increase their size, direct

injection into the site of infection, and the use of these peptides in topical applications instead of systemic applications [175, 189, 199, 200]. Despite these shortcomings, AMPs continue to be favorable therapeutics due to the many advantages they have over conventional antimicrobial agents including their broad-spectrum of action, modest resistance induction, and their potential use both alone and in synergistic combination to enhance the effectiveness of other antimicrobials.

1.3.1 Immunomodulatory roles of antimicrobial peptides

As discussed previously, the innate immune response is the first line of defense against invading microorganisms. The cellular and molecular systems of the innate immune system recognize and eliminate pathogens utilizing a variety of signaling pathways (e.g., toll like receptors initiated pathways) that trigger the rapid deployment of a wide spectrum of biological responses, which are relatively nonspecific. Several studies have shown that in addition to their direct antimicrobial effects, some AMPs are important in triggering immunomodulatory responses [170, 201-207]. They include the ability to act as chemokines as well as to induce chemokine production, leading to recruitment of

leukocytes to the site of infection, promotion of wound healing, and the ability to modulate adaptive immune responses [201, 202, 204].

Certain similarities in structure between AMPs and chemokines have raised the possibility that AMPs have intrinsic chemotactic traits. Indeed human neutrophil peptide 1 (HNP-1) and human cationic antimicrobial peptide (hCAP) have been reported to bind CC-chemokine receptor 6 (CCR6) on neutrophils and monocytes, triggering chemotaxis [206, 208-212]. Additionally, AMPs can trigger chemotaxis of immune cells through eliciting the secretion of chemoattractants from various cells. Both HNP-1 and hCAP serve as chemotactic agents for mast cells and can trigger the subsequent release of histamine [206, 213, 214]. Overall, immune stimulatory molecules have the ability to enhance and modify the natural host response to infection while reducing and modulating harmful inflammatory effects.

1.3.2 Antimicrobial peptides in wasp venom

The order Hymenoptera includes *Apis* (e.g., bees), vespids (wasps, yellow jackets, and hornets), and ants. The stinging species of this order produce venom that consists of a mix of enzymes (e.g., hyaluronidases, and phospholipases), neurotoxins, peptides, and low molecular mass substances (e.g., ions and

neurotransmitters) [177, 215-219]. Venom enables the insects to protect against predators (paralyze and/or kill), and in some species, to subdue prey [177, 190, 215, 220]. The fast-acting mechanism of venom is evolutionarily advantageous since predators can cause tremendous damage to the individual insect, the colony, or the nest [177, 215, 219]. Venom serves a variety of purposes and the composition is optimized to reduce biosynthesis energy costs, while maintaining effectiveness both through minimized concentrations of single compounds and synergism between compounds [177, 215, 219, 221, 222].

Most adult wasps do not feed upon captured prey and instead they obtain their nutrients from nectar. However, social wasps feed their brood pulpified prey (e.g., cockroaches and flies) and parasitic wasps lay their eggs (included as a component of their venom) inside or on the outside of their prey which serve as a microenvironment and food for the offspring [177, 215, 223-225]. Some wasp venom components affect the central nervous system of the prey and facilitate paralyzing or killing prey by allowing it to quickly reach cellular targets (e.g., high hyaluronidase content which increases tissue permeability and hydrolyzes animal connective tissue) (reviewed in [177], [215, 221]). Additionally, the prey may harbor a plethora of bacteria and fungi (including human and insect

pathogens) that represent a hazardous environment for both the wasp and its offspring [223, 226-231].

AMPs are a component of wasp venom and serves as a defense against microorganisms that may infect the venom gland and reservoir, due to ingestion of or direct contact with infected prey during venom injection [215, 226]. Venom AMPs may also play a role in protecting wasp larvae against the range of microorganisms that they may encounter as well as ensuring the successful development of their offspring [221, 226, 232, 233]. One recent study has shown that wasp larvae secrete a combination of AMPs to protect against microbes that they may encounter during growth and development inside cockroach hosts [223].

1.3.3 Mastoparans

The mastoparans are a family of peptides representing the most abundant group of peptides isolated from the venom of wasps [234]. These peptides typically share several characteristics including size (low molecular weight and 14 amino acids in length) and structural (hydrophobic and basic amino acids that form an amphipathic α-helical structure) characteristics [235-238]. Table 1 lists several peptides that have been identified from wasps [234, 238-250].

Table 1: Mastoparan peptides identified from wasps.

Peptide Name	Wasp Species	Peptide Sequence
Mastoparan	*Vespula lewisii*	INLKALAALAKKIL-NH_2
Mastoparan B	*Vespa basalis*	LKLKSIVSWAKKVL-NH_2
Mastoparan C	*Vespa crabro*	LNLKALLAVAKKIL-NH_2
Mastoparan M	*Vespa mandarinia*	INLKAIAALAKKLL-NH_2
Mastoparan X	*Vepsa xanthoptera*	INWKGIAAMAKKLL-NH_2
Polistes mastoparan	*Polistes jadwigae*	VDWKKIGQHILSVL-NH_2
Eumenine mastoparan	*Anterhynchium flavomar-ginatum micado*	INLLKIAKGIIKSL-NH_2

These peptides have several biological activities including mast cell degranulation [234, 238, 239, 243], activation of phospholipase A_2 [251, 252], and G protein activation [253]. Mastoparans have also demonstrated *in vitro* antimicrobial activity against both Gram-negative and Gram-positive bacteria [238, 241-243, 245, 254, 255].

The first identified peptide of the mastoparan family, Mastoparan, was isolated from *Vespula lewisii*. It has the typical family structure in that it is a natural, positively charged peptide with a nonpolar center and a mostly positive,

polar 3′ and 5′ end [239]. Mastoparan was first identified based off its capacity to activate mast cells [239]. Mast cells are increasingly becoming known for their critical role in the innate and adaptive immune responses to infection [256-258]. Additional studies have also shown that mastoparan has antibacterial activity against a few bacterial species including *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Acinetobacter baumannii* [255, 259]. The antimicrobial and immunomodulatory nature (via mast cell activation) of mastoparan may allow it to serve as a potential agent for the treatment of infections.

1.4 Impetus for This Work

Bacterial pathogens and the infectious diseases that they cause are a major global health problem. With the continued emergence of antimicrobial resistant pathogens and the ability of these pathogens to easily pass along resistance genes to other pathogens, commonly treatable infections may become deadly due to increasing resistance to available treatments. The lack of effective treatments against these pathogens (especially against Gram-negative bacteria) presents an even greater global health problem. As mentioned in a previous section, current investigations into new antimicrobial agents to combat these problems focus on the use of natural compounds such as antimicrobial peptides and immune

stimulatory molecules. In view of the previously published reports pointing to the intrinsic antimicrobial activity of mastoparan along with its mast cell and immune activating properties, we have embarked on studies examining the potential of mastoparan in anti-infective therapy. The rationale described in the proceeding sections sets the stage for the studies outlined in Chapter 2 of this document.

1.4.1 Evaluation of the antibacterial properties of mastoparan

Previously, mastoparan was shown to demonstrate antibacterial activity against a few bacteria species including *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Acinetobacter baumannii* [255, 259]. In light of the need for development of new antimicrobials, we wanted to broaden this spectrum and examine the scope of antibacterial activity exhibited by mastoparan. As described in an earlier section, some of the current limitations of available antimicrobials are their ineffectiveness against Gram-negative bacteria and non-multiplying populations of bacteria. Our initial observations demonstrated that mastoparan was effective against both Gram-negative and Gram-positive bacteria encouraging us to further investigate the spectrum of activity by testing a range of clinical isolates of bacteria including multidrug resistant strains.

Additionally, we tested mastoparan against both logarithmic phase (multiplying) and stationary phase (non-multiplying) bacteria. Studies were also performed to elucidate the mechanism of action exhibited by mastoparan. Chapter 2 of this document details studies that show that mastoparan has a broad spectrum of action and is therefore an ideal candidate for determining its future therapeutic potential.

1.4.2 Evaluation of the immunostimulatory properties of mastoparan

The ability to enhance the innate immune response is a potentially powerful way to prevent or treat bacterial infections. Studies from our lab (unpublished data), along with others [239, 241, 260, 261], have demonstrated the *in vitro* potential of mastoparan to activate mast cells. The notion of selectively activating mast cells during infection represents a marked advancement in scientific knowledge. Since their discovery over 150 years ago, mast cells have remained an enigma. Although they are well known to be powerful mobilizers of immune cells, their physiological function has largely been overlooked. This is partly attributable to their well-known contribution to the pathology of a number of common inflammatory disorders such as asthma, rheumatoid arthritis, and multiple sclerosis.

Several years ago, members of the Abraham laboratory reported that mast cells played a key role in immune surveillance by functioning as powerful recruiters of neutrophils and dendritic cells to sites of infection [262]. There is also a growing body of evidence pointing to mast cells as critical mediators of tissue remodeling and wound repair [258, 263]. These observations suggest that mast cells play a multi-faceted role in combating infections and that purposeful activation of mast cells at infected sites might be beneficial. The findings that mast cell activating molecules can be administered locally to boost innate and adaptive immune responses, without any accompanying toxicity [264] is supportive of this notion and has significant translational implications.

Given our initial *in vitro* observations of the ability of mastoparan to induce mast cell degranulation, we were led to further investigate the *in vivo* capacity of mastoparan to activate mast cells and stimulate neutrophil recruitment. This work involved the continuation of studies completed by a previous member of the Abraham laboratory, Dr. Joseph Onyiah, and includes a series of studies described in Chapter 2 that elucidate the potential of mastoparan to cause mast cell activation and neutrophil influx in the peritoneal cavity. In order to determine the amount of neutrophil influx encountered in

mice, additional studies were also performed, utilizing a bacterial skin infection model that included the use of both wild type and mast cell deficient mice. We incorporated the latter into our model to distinguish the contribution of mast cell activation. This work demonstrates the *in vivo* ability of mastoparan to stimulate the immune system and points to the important role mast cells play contributing to the immunomodulatory capabilities of mastoparan.

1.4.3 Evaluation of the therapeutic potential of mastoparan

Mastoparan was preferentially chosen as a prospective therapeutic for this study, based on several distinctive features. This peptide is potentially superior to other previously described AMPs that only exhibit antibacterial activity because it possesses potent immunomodulatory properties in addition to its antibacterial activity. This combination may therefore make mastoparan markedly more effective *in vivo*. Although AMPs were identified over a decade ago, they were not considered as therapeutic agents until recently, when pressure increased to develop novel approaches to combat resistant pathogens.

Previous sections have outlined the therapeutic limitations in the treatment of skin infections. Given that mast cells reside largely at skin surfaces, where Gram-positive *Staphylococcus* and *Streptococcus* species are the primary

pathogens, we sought to investigate whether the activation of mast cells during infection may stimulate the innate immune system leading to better clearance of these bacteria. We suspected that the direct bactericidal activity, combined with the ability to activate mast cells and mobilize innate immune response, would result in mastoparan being a more effective therapeutic (Figure 1). This work is described in detail in Chapter 2 and highlights the potential utility of mastoparan as a therapeutic against bacterial skin infections.

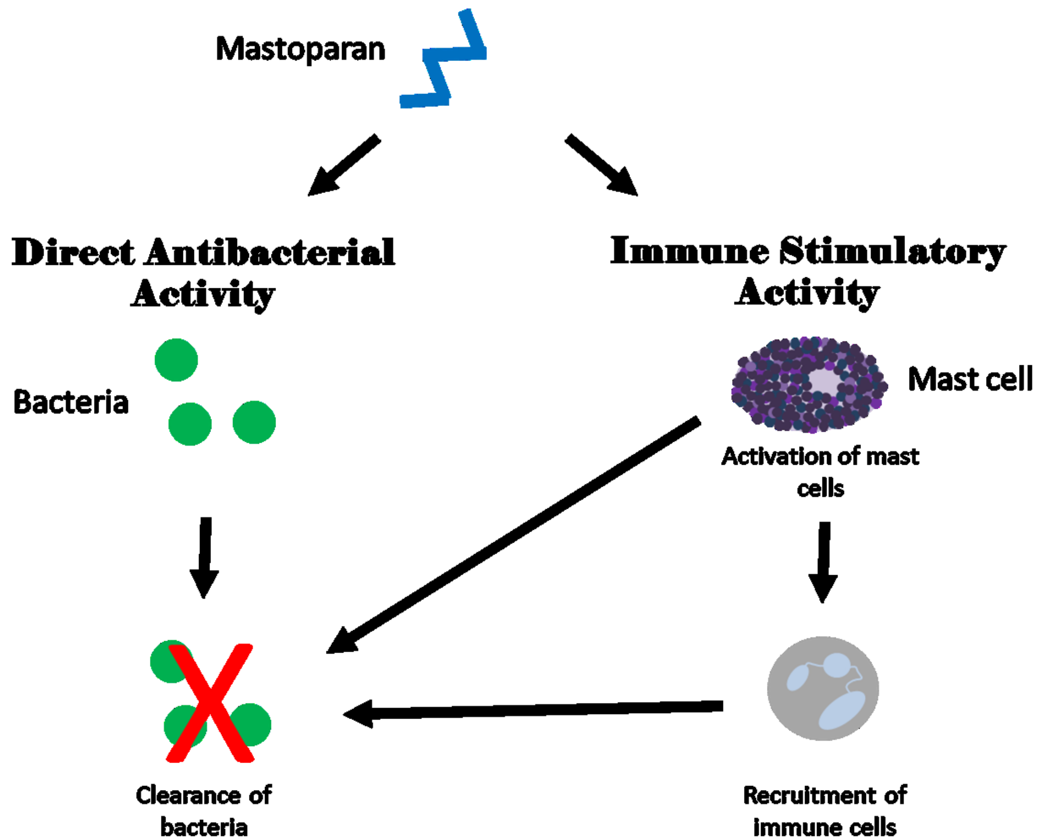

Figure 1: Potential antibacterial and immunomodulatory role of mastoparan. The ability of mastoparan to exhibit both antimicrobial and immune stimulatory activity may enhance its ability to cause bacterial clearance.

Chapter 2: Dual Action Immunomodulatory and Broad-Spectrum Antibacterial Activity of Wasp Venom Peptide

2.1 Introduction

The skin, which harbors a distinct and diverse microbiome, is a major site of microbial attack and infection. Bacterial skin infections are initiated when a break in the skin (e.g., from a cut, insect bite or post-operative wound) occurs, allowing one or more of the skin's colonizing bacteria to overcome the host immune responses leading to an infection of the skin or soft tissue. Bacteria are responsible for a variety of skin conditions such as abscesses, ulcers, impetigo and cellulitis [33, 35, 90, 265]. Bacterial skin infections have emerged as a significant public health problem. In the United States, skin and soft tissue infections are among the top conditions which require use of antimicrobial therapy and they account for 7% to 10% of all hospitalizations [33, 266, 267]. These high rates of incidences are because infections of the skin are notoriously difficult to eradicate with conventional antimicrobial agents for multiple reasons. First, there is the coexistence of multiplying and non-multiplying bacteria that exist in most infections [48, 143, 268]. Whereas the actively growing bacteria are cleared, their static or non-multiplying counterparts are usually unaffected by

current antimicrobial treatments [46-49, 269, 270]. Second, many antimicrobial treatments are ineffective against the growing emergence of multidrug resistant bacteria [43, 271-277]. Third, many antimicrobial agents are typically effective against only Gram-positive or Gram-negative bacteria and therefore lack broad-spectrum activity needed to be effective against different types of infecting bacteria. The ineffectiveness of existing therapeutics combined with the increase in the number of bacterial infections, represent a significant health threat and therefore we are in dire need of new antimicrobial agents to treat these infections.

Antimicrobial peptides (AMPs) are natural, evolutionarily conserved oligopeptides that contribute to innate immunity of the host and are found among all classes of life. These natural peptides, ranging from 6 – 350 amino acids in length [172, 174, 175, 278], typically work by inducing structural perturbations and increasing permeability of the microbial membrane phospholipid bilayer [176, 178, 279]. AMPs have been isolated from a wide range of animals and anatomical sites including venom sacs of wasps, bees, and scorpions, the skin of frogs, and epithelial mucosa and vascular compartment of humans and other mammals. These peptides have potent antimicrobial activity

against a wide range of microorganisms and are increasingly being viewed as potential therapeutic agents and viable alternatives for conventional antimicrobials [165, 166, 168, 170]. However, in spite of their *in vitro* effectiveness, their *in vivo* effectiveness has been surprisingly disappointing. The limited *in vivo* effectiveness of AMPs is attributable, at least in part, to their short half-life, as AMPs are susceptible to early degradation by host proteases. Additionally, these peptides are often positively charged, allowing them to be readily adsorbed by host cells whose surfaces are negatively charged [174, 175, 190, 280, 281].

Mastoparans are a family of peptides that are the most abundant AMPs in wasp venom [232, 234, 282]. AMPs in this family typically vary in sequence by one or two residues. The first mastoparan identified from this family of peptides, mastoparan (*Vespula lewisii*), is composed of 14 amino acid residues (INLKALAALAKKIL-NH_2), a nonpolar center, and mostly positive polar 3′ and 5′ ends [239]. Mastoparans are discharged from venom sacs when wasps lay their eggs inside or on cockroaches and flies. These AMPs presumably protect the emerging larvae from microbes at the necrotic sites surrounding the laid eggs

and functions to protect the venom gland and reservoir from invading microorganisms [215, 221, 226, 232, 233].

Interestingly, the mastoparans also reportedly possess a wide range of biological activity in eukaryotic cells including stimulation of some phospholipases [251, 252], and G proteins within various cells [253, 283-285]. These peptides are also known for their extensive degranulation of mast cells in higher animals [233, 236-239, 243, 251]. Since there is growing evidence that mast cells orchestrate the early innate and adaptive immune responses to infection, we hypothesized that in addition to their antimicrobial actions, mastoparans may possess additional traits for boosting host immunity. We reasoned that these dual capabilities of mastoparan might make it an especially potent agent for treating infections, such as skin infections, that are refractive to conventional antibiotics. Additionally, in spite of the literature [243, 254, 255, 286] describing the antibacterial actions of mastoparan, relatively few studies have undertaken an in depth examination of its scope, mechanism of action and therapeutic potential. Therefore, here we have examined the range and spectrum of antibacterial action of mastoparan and then evaluated its *in vivo* effectiveness against skin infections utilizing standard murine models.

2.2 Materials and Methods

2.2.1 Bacteria strains and growth conditions

All bacteria strains used in this investigation were clinical isolates selected for this study by the Duke University Clinical Microbiology Lab (Durham, NC). From frozen stock, bacteria were grown overnight via agar plating. Bacteria from two to three normal-appearing colonies were selected to prepare liquid cultures. Overnight liquid cultures were inoculated in either Luria Broth (LB; Becton, Dickinson and Company, Franklin Lakes, NJ) or Brain Heart Infusion (BHI; Becton, Dickinson and Company, Franklin Lakes, NJ) at 37°C, and these cultures were aerated at 250 rpm.

2.2.2 Antimicrobial agents

Synthetic mastoparan (*Vespula lewisii*, INLKALAALAKKIL-NH_2) was obtained commercially at >90% purity from BACHEM California, Inc. (Torrance, CA) or CPC Scientific (Sunnyvale, CA). The antibiotics: tobramycin, polymyxin B sulfate, neomycin, gramicidin and bacitracin; were all obtained from Sigma-Aldrich (St. Louis, MO). Neosporin® (Johnson & Johnson Consumer Companies, Inc.), which contains the active ingredients polymyxin, 5,000 units/g; bacitracin, 400 units/g; and neomycin, 3.5 mg/g, was purchased as an over-the-counter drug.

2.2.3 Mice

Female six to eight weeks old C57BL/6 mice obtained from the National Cancer Institute Animal Production Program (Frederick, MD) and mast cell deficient W^{sh}/W^{sh} (bred in the Duke University animal facility) were used for all mouse experiments in this study. Mice were bred and housed in community cages at the Animal Care Facilities at Duke University. All mouse experiments were done according to protocols approved by the Duke Division of Laboratory Animal Resources and the Duke University Institutional Animal Care and Use Committee.

2.2.4 Broth microdilution bacteria susceptibility test

The minimum inhibitory concentration (MIC) was assayed by the broth microdilution method according to procedures outlined by the National Committee for Clinical Laboratory Standards [287]. The initial inoculum was adjusted to 5×10^5 colony-forming units per milliliter. In order to monitor the validity and reproducibility of the assay, incubations with bacteria were carried out in parallel with decreasing concentrations of antibacterial agent. The concentration range assayed was 0.97 μg/mL – 2 mg/mL. A twofold serial dilution of each antimicrobial agent was prepared in the appropriate medium

(LB or BHI). To each well of a sterile polypropylene 96-well microtiter cell-culture plate (Sigma-Aldrich), 40 μL portions of each peptide dilution were added along with 40 μL of each bacterium. The microtiter plates were incubated at 37°C overnight, and each concentration was run in duplicate. Growth controls without antibacterial peptide were included in each test. The MIC was taken as the lowest concentration of antibacterial agent that completely inhibits visible bacteria growth. Values were obtained from 3-4 independent experiments.

2.2.5 Time-kill kinetic assay

A time-kill kinetic assay was used to measure bacteria viability or growth as a factor of time. This assay was performed following the National Committee for Clinical Laboratory Standards guidelines [288]. Cultures of *Pseudomonas aeruginosa*, *Escherichia coli*, and *Staphylococcus aureus* were incubated overnight at 37°C and then diluted 1/20,000. The bacteria were then plated into a 96-well microtiter plate and optical density (OD) readings (630nm) were taken every 15 minutes using a BioTek® absorbance microplate reader. Once the bacteria reached either log or stationary phase, mastoparan was added to the bacteria culture. In a parallel experiment, 10 μL of mixtures were serially diluted in media to minimize the carryover effect and then plated onto LB plates and

incubated overnight to obtain viable colony counts. Growth controls without any antibacterial agent added were performed in parallel. Data presented as the average of 2-3 independent experiments.

2.2.6 Fluorescence microscopy assay to measure cell membrane permeability

Cell membrane permeability was measured using the LIVE/DEAD® Bacterial Viability Kit (BacLight™). *Pseudomonas aeruginosa* was grown overnight at 37°C and then incubated for 4 hours in the presence of mastoparan (31 μg/mL), polymyxin (4 μg/mL), or tobramycin (1 μg/mL). These concentrations represent twice the demonstrated MIC (Table 3) for each respective antimicrobial. In addition, a control experiment was performed whereby bacteria were incubated without any antibacterial agent present. Equal volumes of the kits two dye components, SYTO 9 and propidium iodide, were mixed together, and added to the bacteria suspension (1.5 μL of dye mixture per 500 μL of bacteria suspension). The suspension was then incubated at room temperature in the dark for 15 minutes. After incubation, the bacterial suspension was fixed between a slide and coverslip using Prolong Gold antifade reagent (Molecular Probes) and viewed via fluorescence microscopy. The SYTO

9 dye is membrane permeable and labels all bacteria and propidium iodide is membrane impermeable and only labels bacteria cells with damaged membranes, as it is unable to penetrate healthy bacterial cells. Bacteria with intact cell membranes stain fluorescent green and those with damaged membranes stain fluorescent red.

2.2.7 Election microscopy

Pseudomonas aeruginosa was cultured overnight at 37°C and then adjusted for log phase of growth. Bacteria (5 x 10^5 cfu/mL) were then incubated for 3 hours in the presence of mastoparan. Mastoparan was used at a concentration of 15.6 μg/mL, which is its demonstrated MIC, and 31 μg/mL, a two-fold serial dilution above its MIC. Bacterial cultures were harvested, washed, and fixed with a fixation solution. Sample processing, sectioning, and examination by transmission electron microscope were performed by the Laboratory for Advanced Electron and Light Optical Methods at the College of Veterinary Medicine, North Carolina State University (Raleigh, NC).

2.2.8 Lactate dehydrogenase (LDH) cytotoxicity assay

Cytotoxicity was assessed using a LDH cytotoxicity kit (Sigma-Aldrich) according to manufactures instructions. Briefly, HeLa cells were seeded in a 96-

well tissue culture plate at a density of 1 x 10^4 cells per well and incubated overnight. After overnight incubation various dilutions of mastoparan (ranging between 0.49 μg/mL – 2 mg/mL) were added to the cells along with fresh media. Cell death was evaluated by the amount of LDH released into the culture medium after 4 hours of mastoparan treatment. The cell culture media from treated cells was transferred to a new microplate and mixed with the kit reagents. The change in absorbance of the samples was measured at 450 nm with an absorbance plate reader to determine LDH release. The amount of LDH released by cells killed with Triton X-100 was considered as total LDH release or maximal cell death.

2.2.9 β-Hexosaminidase assay for in vitro mast cell degranulation

As described previously by McLachlan et. al. [289], femurs from C57BL/6 mice were recovered from bone marrow, which was then cultured in complete RPMI 1640 medium containing 10% fetal bovine serum (FBS), 1% HEPES, amino acids, penicillin, streptomycin, and 10% recombinant stem cell factor supplemented with 2 ng/ml IL-3 for 4 weeks to obtain bone marrow mast cells (BMMCs). BMMCs were incubated for 1 hour at 37°C in the presence of various concentrations of mastoparan (ranging between 5 μM – 80 μM, 7.5 μg/mL – 120

μg/mL). The mast cells were centrifuged, the supernatant was then removed and mixed with the substrate p-nitrophenyl-N-acetyl-β-D-glucosaminide to test for the release of β-hexosaminidase by measuring the change in absorbance at 405 nm on an absorbance plate reader. Percent degranulation above baseline was calculated based on β-hexosaminidase release after corrections for spontaneous release from unstimulated cells was taken into consideration.

2.2.10 Mastoparan treatment of mice intraperitoneally

Mastoparan, at various concentrations, was injected into the peritoneal cavity of 6 to 8 weeks old C57BL/6 mice using a 1 mL syringe and a 30.5-gauge needle. At the specified time points, mice were sacrificed and a peritoneal lavage was performed with 3 mL of phosphate buffered saline (PBS). After 20 seconds of shaking, the peritoneal contents were recovered using a sterile pipette.

2.2.10.1 Microscopic analysis of peritoneal lavage

Following recovery of the lavage samples, a cytospin (Cytospin 3, Sandon Inc., PA) was used to fix the samples on slides. Samples were stained with 5% toluidine blue and viewed under a microscope to evaluate the percentage of mast cell degranulation. Mast cells were scored as fully granulated or having partial or complete granule loss. The number of degranulated versus the number of

non-degranulated mast cells were counted and the percentage degranulation reported.

2.2.10.2 Microscopic analysis of mesenteric mast cells

Specimens of mouse mesentery were prepared by dissection of the intestine and mesentery between the colon and root of mesentery from the peritoneal cavity of the mice treated with mastoparan or PBS as a control in a manner similar to previously described procedures [290, 291]. Briefly, samples were acetone-fixed, carefully spread on a microscope slide and slightly flattened. After the intestine was removed, samples were incubated in the presence avidin FITC (Molecular Probes) to allow for immunohistochemical study. Samples were then washed three times (10 min each) in PBS. Each sample was covered with ProLong antifade reagent (Molecular Probes) and a coverslip. Confocal images of stained tissue sections were obtained with a three-laser Nikon Confocal Laser Scanning Instrument (Nikon USA). Images were obtained under oil immersion with a Plan Fluor 20.0/0.75/.35 multiobjective and EZ-C1 Nikon software (Silver Version 2.01).

2.2.10.3 Myeloperoxidase assay for neutrophils

Neutrophil influx into the peritoneum was evaluated by measuring the amount of myeloperoxidase (MPO) present in the peritoneum following previously described methods [292]. Neutrophils have an abundance of the MPO enzyme and it has been shown to be a useful and reliable maker for neutrophil influx in inflammatory diseases [292, 293]. Briefly, 1mL of peritoneal lavage samples were centrifuged at 12,000 rpm for 10 minutes and the pellet was resuspended in 1 mL of potassium phosphate buffer (50 mM, pH 6.5) with 0.5% hexadecyltrimethylammonium bromide (HTAB) to release the MPO from the neutrophils. The samples were frozen and thawed twice and then centrifuged at 12,000 rpm for 2 minutes. Then 20 uL of the supernatants were analyzed in triplicate by transferring to a 96-well plate and adding 200 uL of 50 mM potassium phosphate buffer (pH 6.0) with 0.167 mg/mL o-dianisidine dihydrochloride and 0.0005% H_2O_2. The colorimetry of the supernatants was measured using an absorbance plate reader to measure optical density at 460 nm. MPO content was calculated using a standard curve, which was established using purified MPO.

2.2.11 Superficial skin colonization and infection models

The skin colonization and infection models were performed as described previously [143, 294] using C57BL/6 mice. Mice were anesthetized by intraperitoneal injection with 100 μL of a 1:3:15 mixture of 20 mg/ml xylazine, 100 mg/ml ketamine hydrocholoride, and sterile PBS. The backs of the mice were shaved (electric clipper) and hair removed by chemical depilation (Nair; Church & Dwight CO.). For the skin colonization model, log phase or stationary phase *Staphylococcus aureus* cultures were added to a 2 cm^2 area or the shaved skin, marked with a surgical skin marker (Covidien), and allowed to dry for 20 minutes. After drying, treatment with mastoparan or neosporin was performed by spreading the antibacterial agent on the 2 cm^2 area of skin with the aid of a pipette and utilizing the pipette tip to spread the antimicrobials to the entire infected area. Mastoparan was infused in a vehicle of olive oil (to enable the drug to stay in place on the skin) and DMSO (to encourage transdermal migration) to allow for transdermal passage.

For the skin infection model, C57B/6 mice or mast cell deficient mice (W^{sh}/W^{sh}) were injected (using a 25-gauge needle) intradermally with 100 μl of mid-logarithmic growth phase (A_{600}= 0.6, ~5 x 10^8 CFU) of *Staphylococcus aureus*

complexed to Cytodex (Sigma-Aldrich) beads as a carrier. Beginning at 12 hours after infection, mice were treated with either mastoparan or Neosporin every 12 hours. Lesion sizes were accessed daily by measuring the vertical and perpendicular diameters and calculating the area using the formula for an ellipse: (vertical diameter/2) × (perpendicular diameter/2) × π. At various time points, skin from the infection site was harvested using an 8mm biopsy punch, homogenized and plated for enumeration of colony-forming units.

To determine skin myeloperoxidase (MPO) levels, skin biopsies were homogenized an 1 mL of potassium phosphate buffer (50 mM, pH 6.5) with 0.5% hexadecyltrimethylammonium bromide (HTAB) to release the MPO from the neutrophils. The samples were frozen and thawed twice and then centrifuged at 12,000 rpm for 2 minutes. Then 20 uL of the supernatants were analyzed in triplicate by transferring to a 96-well plate and adding 200 uL of 50 mM potassium phosphate buffer (pH 6.0) with 0.167 mg/mL o-dianisidine dihydrochloride and 0.0005% H_2O_2. The colorimetry of the supernatants was measured using an absorbance plate reader to measure optical density at 460 nm. MPO content was calculated as units per gram using a standard curve, which was established using purified MPO.

2.2.12 Dermal toxicity study

The dermal toxicity of mastoparan was investigated using C57B/6 mice. Mice were anesthetized by intraperitoneal injection with 100 μL of a 1:3:15 mixture of 20 mg/ml xylazine, 100 mg/ml ketamine hydrocholoride, and sterile PBS. The backs of the mice were shaved (electric clipper) and hair removed by chemical depilation (Nair; Church & Dwight CO.). Mastoparan (50 μg or 100 μg) was administered every 12 hours via topical application or intradermal injection for 14 days.

Topical application of mastoparan was accomplished by applying 10 μL of mastoparan infused in olive oil and DMSO (to enable the drug to stay in place and facilitate transdermal migration) directly to a 2 cm^2 area of the shaved skin, marked with a surgical skin marker (Covidien). Mastoparan was applied to the skin with a pipette, using the pipette tip to facilitate spreading. Intradermal injection of mastoparan was achieved using a 25-gauge needle to inject 100 μL of mastoparan directly under the upper epidermal layer of the backs of shaved mice. Before and after each treatment, clinical signs of related adverse reaction were monitored including temperature, body weight, observable inflammation, and observable changes in behavior.

2.3 Results

2.3.1 Mastoparan exhibits broad-spectrum antibacterial activity against Gram-negative and Gram-positive bacteria

In order to explore the range of antibacterial efficacy demonstrated by mastoparan, we first screened randomly selected clinical isolates of Gram-positive (n=4) and Gram-negative (n=11) bacteria for mastoparan sensitivity using a broth microdilution method (Table 2).

Table 2: Mastoparan exhibits broad-spectrum antibacterial activity against Gram-positive and Gram-negative bacteria. Microbes (5 x 10^5 CFU per mL) were incubated overnight in the presence of decreasing serial dilutions of mastoparan and the minimum inhibitory concentration (MIC) was recorded as the lowest concentration needed to visibly inhibit 100% of bacterial growth. This data demonstrates that mastoparan has a broad-spectrum of antibacterial activity.

	Bacteria	Mastoparan (µg/mL)	Mastoparan (µM)
Gram-positive	*Staphylococcus aureus*	7.8	5.3
	Coagulase negative staphylococcus	1.95	1.3
	Enterococcus faecalis	125	84.5
	Enterococcus faecium	15.6	10.6
Gram-negative	*Escherichia coli*	7.8	5.3
	Pseudomonas aeruginosa	15.6	10.5
	Klebsiella pneumoniae	31.3	21.1
	Enterobacter cloacae	125	84.5
	Enterobacter aerogenes	31.3	21.1
	Citrobacter freundii	15.6	10.6
	Klebsiella oxytoca	15.6	10.6
	Serratia marcescens	125	84.5
	Stenotrophomonas maltophilia	15.6	10.6
	Proteus mirabilis	15.6	10.6
	Burkholderia cepacia	62.5	42.3

These clinical isolates were incubated in the presence of decreasing serial dilutions of mastoparan to determine the minimum amount of peptide required

to inhibit bacteria growth. The MIC of mastoparan against Gram-positive bacteria ranged between 1.9 – 125 μg/mL (1.3 – 84.5 μM). Against Gram-negative bacteria, the MIC ranged between 15.6 – 125 μg/mL (10.6 – 84.5 μM). This data indicates that mastoparan is active against clinical isolates of both Gram-negative and Gram-positive bacteria. The data presented here also demonstrates that there is no distinct difference in bacteria susceptibility between Gram-negative and Gram-positive bacteria since mastoparan demonstrated a similar range of efficacy against both types of bacteria. Our studies reveal that mastoparan demonstrates a broad-spectrum of antibacterial activity, and is therefore a favorable antibiotic candidate since most commonly marketed antibiotics have limited activity against only Gram-negative or Gram-positive bacteria.

2.3.2 Mastoparan has a broader range of activity against bacteria in comparison to selected marketed antibiotics

Many new antimicrobial agents do not make it to market due to the inability to demonstrate superiority of the new drug to current therapies. After determining that mastoparan is an effective broad-spectrum antibacterial, we sought to investigate the efficacy of mastoparan in comparison to six marketed antimicrobial agents in order to elucidate its potential as a superior anti-infective

therapeutic. We performed a broth microdilution test with mastoparan, tobramycin, neomycin, polymyxin, gramicidin, and bacitracin against *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* (Table 3).

Table 3: Mastoparan is more active against bacteria (both Gram-negative and Gram-positive) than selected marketed antibiotics. Microbes were incubated overnight in the presence of decreasing serial dilutions of the noted antimicrobial agents and the minimum inhibitory concentration (MIC) was recorded as the lowest concentration needed to inhibit bacteria growth. This data strongly suggests that mastoparan has a broader range of antibacterial activity in comparison to the other antimicrobial agents tested.

	Mastoparan		Tobramycin		Neomycin		Polymyxin		Gramicidin		Bacitracin	
	µg/mL	µM	µg/mL	µM	µg/mL	µM	µg/mL	µM	µg/mL	µM	µg/mL	µM
Escherichia coli	7.8	5.3	2	4.3	7.8	13	0.5	0.4	>2,000	>1,603	1,000	1,406
Pseudomonas aeruginosa	15.6	10.5	0.5	1.1	15.6	25	2	1.4	>2,000	>1,603	1,000	1,406
Staphylococcus aureus	7.8	5.3	>1,000*	>2,139*	125	203	125*	90.2*	>2,000	>1,603	500	351.5

Tobramycin and neomycin are both aminoglycosides, particularly effective against Gram-negative bacterial species by binding to the bacterial ribosomal subunit. They were selected for comparison due to their known efficacy against Gram-negative bacteria and their classification as an aminoglycoside. Additionally, neomycin was selected due to its inclusion as an active ingredient in Neosporin (Johnson & Johnson Consumer Companies, Inc), a commonly used topical antimicrobial agent. Polymyxin is a cyclic peptide

effective against Gram-negative bacteria through a cell membrane interaction via the lipid A component of the lipopolysaccharide [295]. It was selected for comparison due to its mechanism of action, peptide structure, and it is also an active ingredient in Neosporin. Both gramicidin and bacitracin are AMPs effective against Gram-positive bacteria via a direct interaction with cell membrane phospholipids [296-298]. They were selected for comparison to mastoparan due to their similar peptide nature and mechanism of action. Additionally, bacitracin was selected due to its inclusion as an active ingredient in Neosporin.

Tobramycin and polymyxin were only effective when tested against the Gram-negative bacteria. Neomycin demonstrated antibacterial activity against both Gram-negative and Gram-positive bacteria. However, it is active against *S.aureus* at a much higher concentration when compared to its demonstrated MIC against *E. coli*, and *P. aeruginosa*. Gramicidin did not demonstrate any activity at the concentrations (between 0.5 μg/mL – 2 mg/mL) we tested and bacitracin demonstrated activity against all three bacteria strains but at concentrations (500 μg/mL – 1 mg/mL) much higher than that demonstrated by mastoparan (7.8 μg/mL – 15.6 μg/mL). Mastoparan demonstrates activity against

both Gram-negative and Gram-positive bacteria with a similar MIC for both types of bacteria. Therefore, it appears that mastoparan has superior efficacy, when compared to gramicidin and bacitracin, and a broader spectrum of action in comparison to tobramycin, neomycin, and polymyxin.

2.3.3 Mastoparan exhibits antibacterial activity against resistant clinical isolates

Due to the growing trend in antimicrobial resistance and having observed the efficacy of mastoparan against standard clinical isolates, we sought to investigate if mastoparan is effective against resistant strains of bacteria. Researchers have identified some particularly problematic pathogens that include *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Acinetobacter baumanni*, *Pseudomonas aeruginosa*, and *Enterobacter* species ("ESKAPE pathogens") [138, 299, 300]. These pathogens cause the majority of nosocomial infections and are able to "escape" the actions of antibacterial agents as their resistance prevalence, transmission, and pathogenesis increases. In order to examine the ability of mastoparan to serve as a potential antibacterial against these pathogens, we tested the peptide against methicillin-resistant *Staphylococcus aureus*, extended spectrum beta-lactamase *Klebsiella pneumonia* and

Escherichia coli, and resistant *Pseudomonas aeruginosa* via the broth microdilution method. The data presented in Table 4 shows that mastoparan demonstrated antibacterial activity against all the resistant strains tested (MIC = 7.8 – 125 μg/mL) in a range similar to the MIC (Table 2) demonstrated against the standard clinical isolates tested in this study. This data suggests that mastoparan can serve as a powerful antibacterial agent against some resistant organisms and may therefore serve as a solution to the current rise in multidrug resistance.

Table 4: Mastoparan exhibits antibacterial activity against multidrug resistant bacteria. Multidrug resistant clinical isolates of *E. coli*, *P. aeruginosa*, *K. pneumonia*, and *S. aureus* were incubated overnight in the presence of decreasing serial dilutions of mastoparan. The MIC was recorded as the lowest concentration needed to visibly inhibit 100% of bacterial growth. These results show that these multidrug resistant strains are susceptible to mastoparan.

Bacteria	Mastoparan (μg/mL)	Mastoparan (μM)
E. coli (ESBL)	31	21
P. aeruginosa	62.5	42.3
K. Pneumoniae (ESBL)	125	84.5
S. aureus (MRSA)	7.8	5.3

2.3.4 Mastoparan disrupts bacterial membrane integrity

In light of studies demonstrating that most antimicrobial peptides similar in size and structure to mastoparan utilize a mechanism of action involving an

interaction with the cell membrane, we investigated whether mastoparan has an effect on cell membrane permeability. To test if the mechanism of action utilized by mastoparan involves an increase in membrane permeability, *P. aeruginosa* was incubated in the presence of mastoparan, polymyxin, or tobramycin at concentrations two-fold higher than their respective MICs. Polymyxin demonstrates antibacterial activity against Gram-negative bacteria through a cell membrane interaction whereby it binds to lipopolysaccharide and alters bacterial outer membrane permeability [77, 295], thus it is used in this assay as a positive control. Tobramycin was selected for comparison due to its efficacy against Gram-negative bacteria and because it is an aminoglycoside whose mechanism of action involves it binding to the bacterial ribosomal subunit therefore inhibiting the synthesis of proteins vital for bacterial growth [301].

We used a LIVE/DEAD® Bacterial Viability Kit (*Bac*Light™) to determine if mastoparan disrupts bacterial membrane integrity. The kit contains two dyes, SYTO 9, a membrane permeable dye that labels all bacteria and propidium iodide, a membrane impermeable dye, which only labels bacteria cells with damaged membranes. Bacteria with intact cell membranes stain fluorescent green and those with damaged membranes stain fluorescent red. The data

presented in Figure 2 shows that bacteria incubated in the presence of both mastoparan and polymyxin have green and red fluorescent staining, indicating that their membrane integrity has been disrupted. Bacteria incubated with tobramycin, as well as the control bacteria that were not incubated in the presence of any antibacterial, only fluorescence green indicating that their cell membranes are intact.

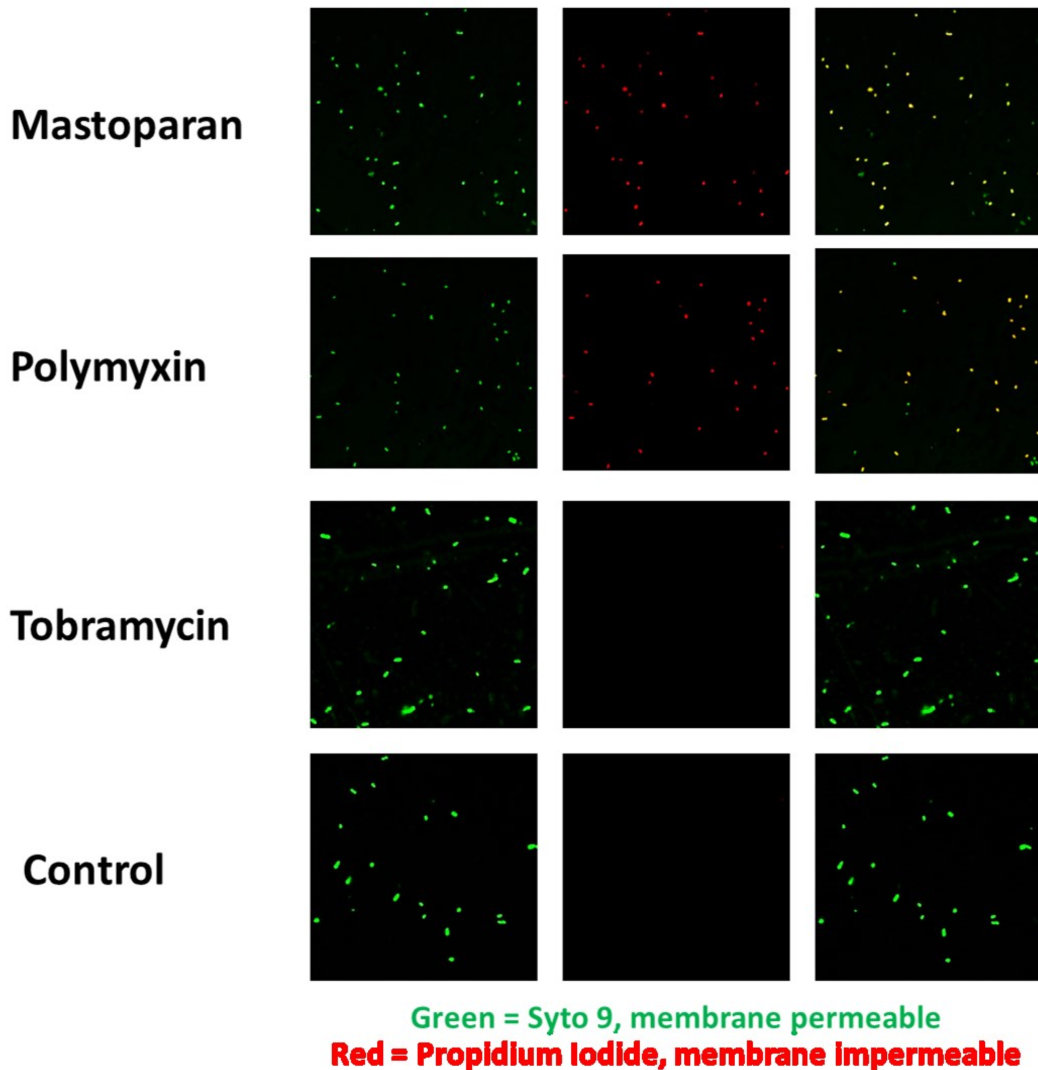

Figure 2: Mastoparan disrupts bacterial membrane integrity as demonstrated by fluorescence microscopy. *P. aeruginosa* was incubated for four hours in the presence of mastoparan (31 μg/mL), polymyxin (4 μg/mL), or tobramycin (1 μg/mL) and then stained using a LIVE/DEAD® Bacterial Viability Kit (BacLight™). After staining, the bacteria were mounted on a slide using Prolong Gold antifade reagent (Molecular Probes) and viewed via fluorescence microscopy. This data shows that mastoparan affects the membrane integrity of bacterial cells.

We also examined the ultra-structure of *P. aeruginosa* by transmission electron microscopy after treatment with mastoparan (Figure 3). At its minimum inhibitory concentration (15.6 μg/mL), mastoparan caused cell membrane disruption exhibited by extensive membrane blebbing, ruffling and detachment (Figure 3C & D). Furthermore, reduced electron density was demonstrated in bacteria cultures treated with mastoparan (Figure 3C-F) in comparison to the control (Figure 3A-B) signify a loss in cytoplasmic contents. At a two-fold higher concentration (31 μg/mL), we noticed that treatment with mastoparan led to ruptured membrane and expelled cellular contents (Figure 3E & F). Cumulatively, these data strongly suggests that mastoparan causes extensive membrane permeability and utilizes a cell membrane interaction as its mechanism of bactericidal action in a concentration dependent manner.

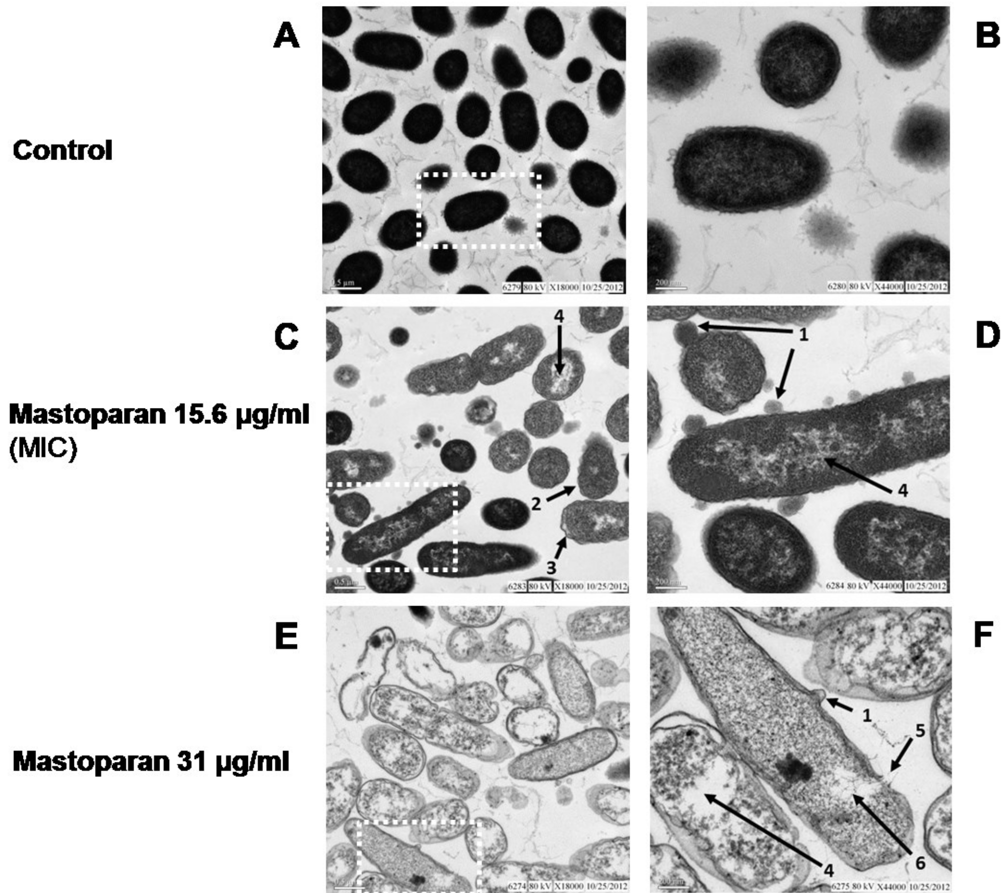

Figure 3: Mastoparan disrupts bacterial membrane integrity as demonstrated by transmission electron microscopy. *P. aeruginosa* after incubation in the presence of mastoparan via transmission electron microscopy. (3A) Control *P. aeruginosa*, no antibacterial added. (3B) Control bacteria at a higher magnification demonstrating electron dense bacteria with a continuous membrane. Mastoparan at 15.6 µg/mL (3C, higher magnification 3D), and mastoparan at 31 µg/mL (3E, higher magnification 3F) shows bacteria incubated in the presence of mastoparan demonstrated: 1: membrane blebbing, 2: membrane ruffling, 3: membrane detachment, 4: loss of electron density, 5: ruptured membrane, 6: release of cytoplasm. Left panels size bars represents 0.5 µm. Right panels size bars represent 200 nm. This data shows that mastoparan affects the membrane integrity of bacterial cells and causes loss of cellular contents.

2.3.5 Mastoparan does not elicit cytotoxicity at the concentrations necessary for its antibacterial activity

Many AMPs that were previously studied with a goal to exploit their antimicrobial activity have been restricted from clinical use due to their toxicity profiles. AMPs' proclivity for a mechanism of action that involves membrane interaction increases the likelihood of cytotoxicity. However, AMPs can selectively target bacterial membranes based on differences between human cells and bacteria cells including the presence of cholesterol, and differences in membrane potential. Having observed the potent activity of mastoparan on bacterial membrane permeability, we sought to determine the potential cytotoxicity of mastoparan in relation to human cells.

We tested the cytotoxicity of mastoparan by measuring the amount of lactate dehydrogenase (LDH) released from HeLa cells incubated in the presence of various concentrations (ranging between 0.49 μg/mL – 2 mg/mL) of mastoparan. LDH is released by cells with damaged membranes and has been used to evaluate toxicity of cells [302]. The data presented in Figure 4 shows that mastoparan does not demonstrate cytotoxicity at concentrations between 0.49 μg/mL – 250 μg/mL. At these concentrations, the minimal amount of LDH

activity seen is similar to that of the controls (cells alone and media alone). These concentrations are within the range necessary for mastoparan's antibacterial activity, which is between 1.9 μg/mL – 125 μg/mL. At high concentrations above 500 μg/mL, mastoparan does exhibit some cytotoxicity. This data suggests that mastoparan does not exhibit cytotoxicity against human cells at the concentration required for the killing of bacteria.

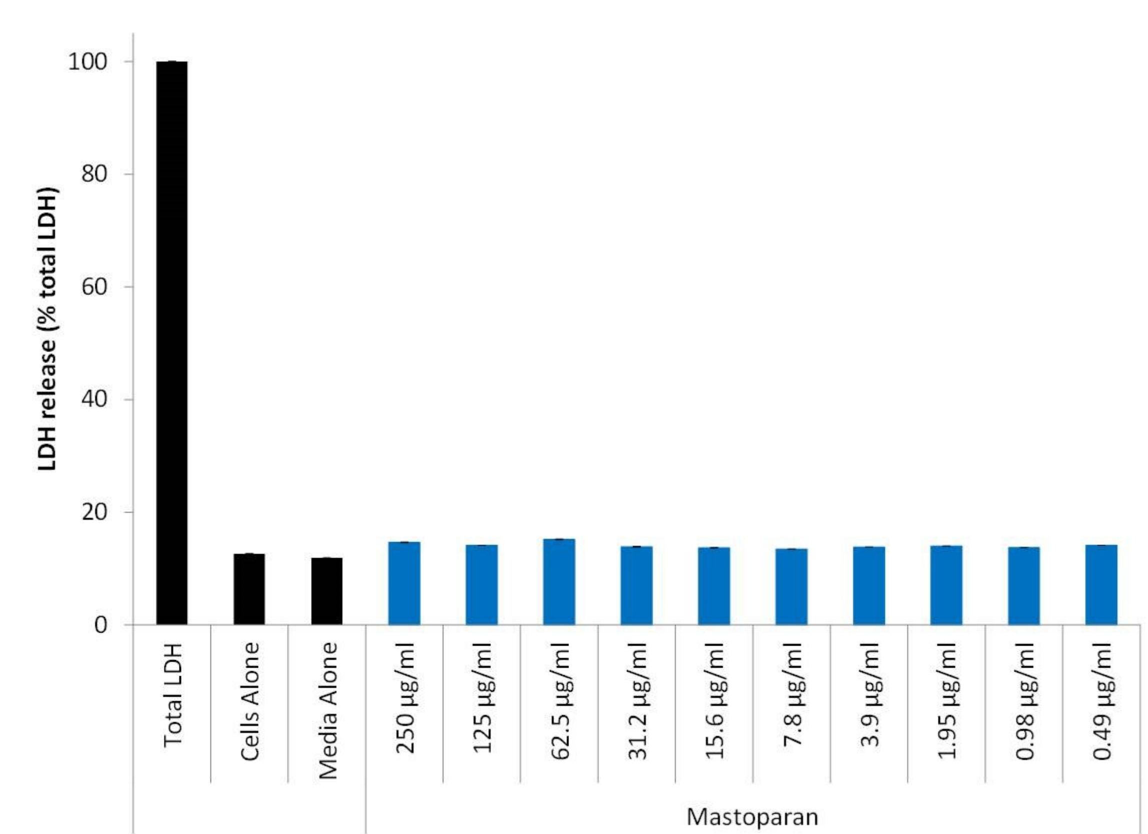

Figure 4: Mastoparan does not exhibit cytotoxicity to HeLa cells at concentrations similar to those needed for its antibacterial activity. HeLa cells were incubated in the presence of mastoparan at the noted concentrations. A LDH activity assay was performed to determine the amount of LDH released from these cells. This data shows that at concentrations between 0.49 μg/mL – 250 μg/mL, there was no release of LDH in comparison to the cells and media alone controls.

2.3.6 Mastoparan exhibits a kinetically rapid mechanism of bacteria growth inhibition

During infection, bacteria exist in both log and stationary phase. Thus, ideal antimicrobial agents will exhibit activity against bacteria in both log and stationary phase. We investigated the antibacterial activity of mastoparan against log phase *P. aeruginosa* cultures in which bacteria were undergoing replication. We also tested mastoparan against stationary phase *P. aeruginosa*, which have minimal replication, and growth. Mastoparan was added to bacteria cultures in each of these two growth phases and we assessed the impact of mastoparan using optical density (OD) and agar plating to determine viability counts.

Figure 5A & B reveals that addition of mastoparan prevented the increase in OD of log phase bacteria and markedly reduced colony viability count within half an hour after addition of mastoparan.

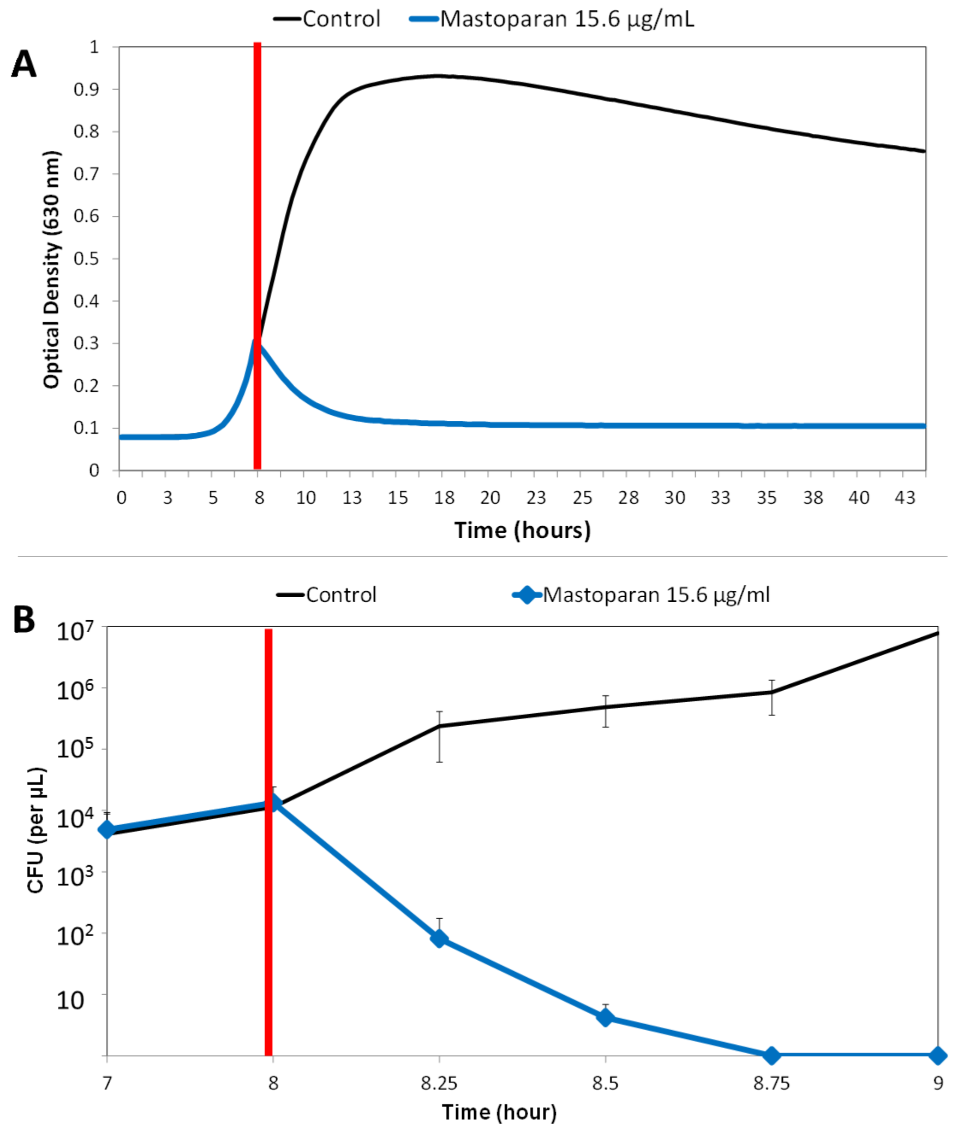

Figure 5: Mastoparan is effective against log phase bacteria. *P. aeruginosa* was incubated in the presence of mastoparan at the noted concentration. (A) Optical density and (B) colony-forming unit measurement of bacterial growth inhibition using a log phase culture of *P. aeruginosa*. Red line demonstrates when antibacterial was added. This data shows that mastoparan rapidly kills log phase bacteria. The same experiment was performed using *E. coli* and *S. aureus* with similar results (data not shown).

Interestingly, addition of mastoparan to stationary phase bacteria did not affect OD; however, the culture exhibited a drastic and significant reduction in colony viability count (Figure 6A&B). These findings are consistent with the idea that mastoparan is able to rapidly kill both log and stationary phase bacteria. Similar results were observed when mastoparan was added to *E. coli* and *S. aureus* cultures in log and stationary phase (data not shown). These observations imply that mastoparan exhibits a kinetically rapid and direct mechanism of bacterial killing and it is effective at killing bacteria in both log and stationary phase.

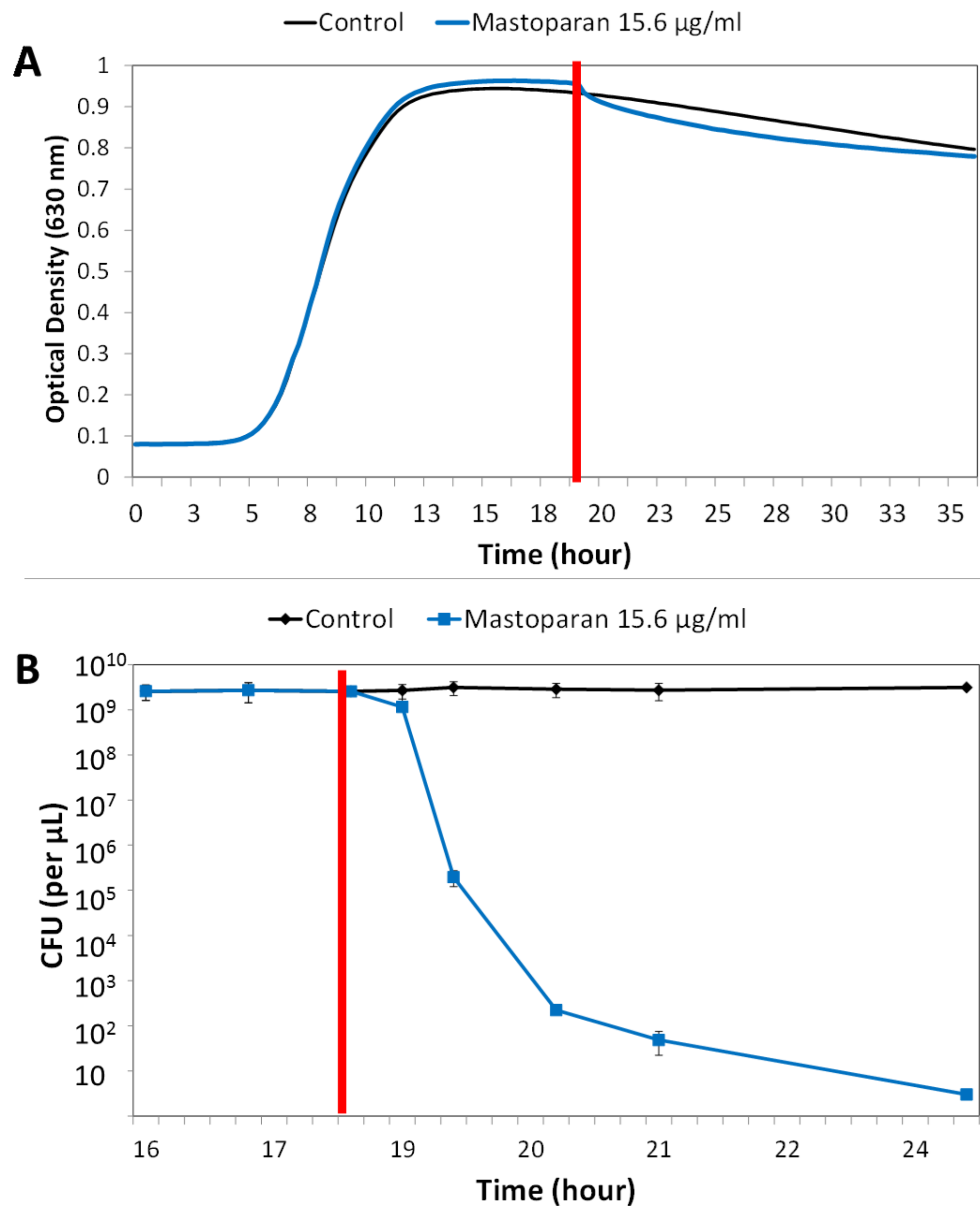

Figure 6: Mastoparan is effective against stationary phase bacteria. *P. aeruginosa* was incubated in the presence of mastoparan at the noted concentration. (A) Optical density and (B) colony-forming unit measurement of bacterial growth inhibition using a stationary phase culture of *P. aeruginosa*. Red line demonstrates when antibacterial was added. This data shows that mastoparan rapidly kills stationary phase bacteria. The same experiment was performed using *E. coli* and *S. aureus* with similar results (data not shown).

2.3.7 Mastoparan is a mast cell activator *in vitro* and *in vivo*

Given the ability of mastoparan to activate mast cells [239], we hypothesized that along with its antibacterial activity, mastoparan may function as an immunomodulatory molecule. To explore the capabilities of mastoparan to function as an immune stimulatory molecule, we first sought to confirm its ability to cause mast cell degranulation *in vitro* and *in vivo*. Consistent with previously reported studies [239, 260, 303-305], we observed that mastoparan is a mast cell activator *in vitro*. Following incubation of mouse bone marrow derived mast cells in the presence of varying concentrations of mastoparan, we found that mastoparan caused significant mast cell degranulation, in a dose-dependent manner, between a concentration of 5 to 80 uM (Figure 7).

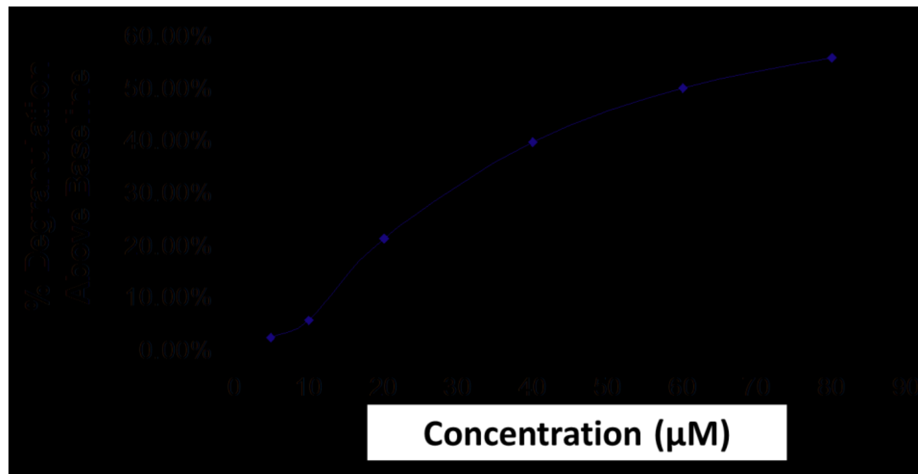

Figure 7: Mastoparan is an *in vitro* mast cell activator. Mouse bone marrow derived mast cells cultured from the femurs of C57BL/6 mice were incubated in the presence of various concentrations of mastoparan. Mastoparan causes significant mast cell degranulation at concentrations between 5 to 80 µM. Experiment performed by Joseph Oniyah.

Having confirmed its ability to activate mast cell *in vitro*, we then sought to demonstrate the *in vivo* mast cell stimulation activity of mastoparan. In order to investigate the capacity of mastoparan to cause mast cell degranulation *in vivo*, mastoparan was injected into the peritoneum of mice. We preferentially chose the peritoneum as the body site to investigate as it is a contained environment, which allows for convenient isolation of resident mast cells and recruited immune cells from the lavage.

After intraperitoneal administration of mastoparan, peritoneal contents were collected at varying time points up to 24 hours after treatment. Cells were fixed to a slide and stained with toluidine blue, a dye that selectively stains mast

cell granules. We observed that mastoparan displays mast cell degranulation beginning at the 1 hour after treatment time point and peaking at 12 hours after treatment (Figure 8A). As shown in Figure 8A, following the 12 hour time point, mast cell degranulation had decreased at the 24 hour time point. This observation suggests that after the 12 hour time point, the mast cells had either replaced their original granules or that this decrease was due to the migration of mast cells out of the peritoneum considering that there were fewer visible mast cells present in the field of view during this time frame (data not shown).

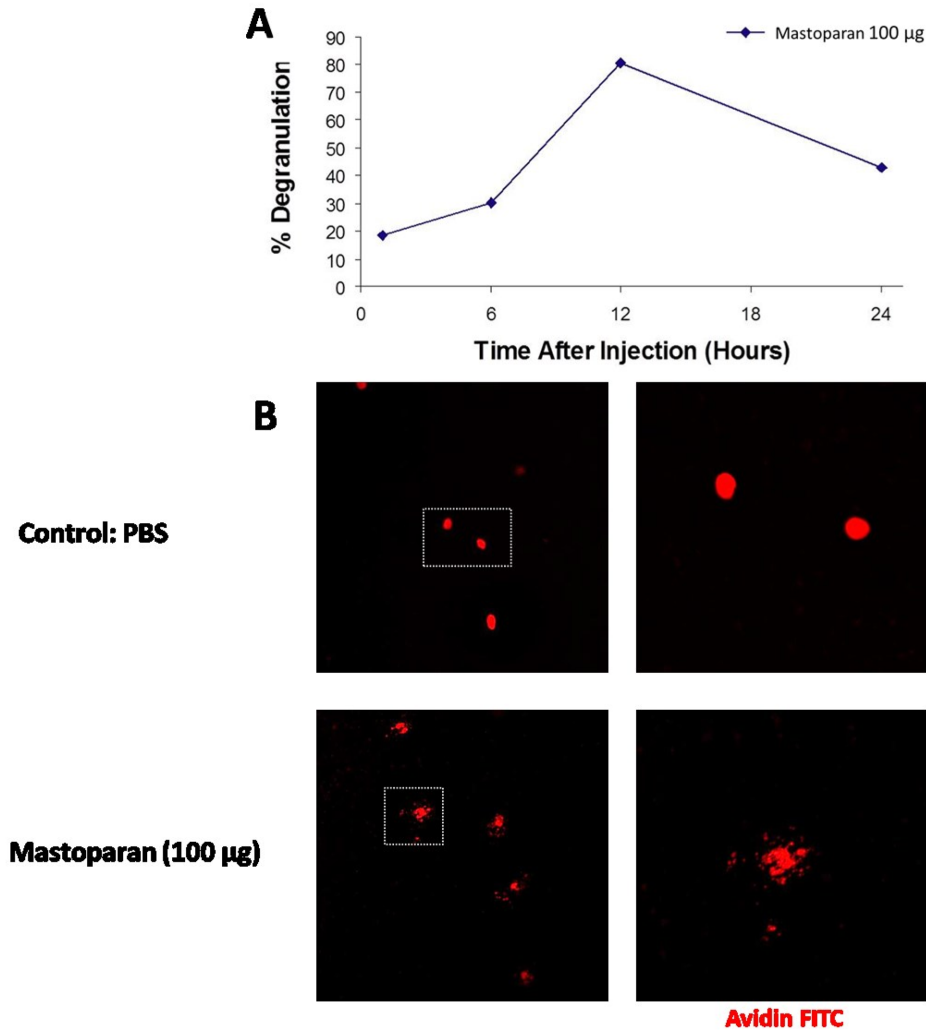

Figure 8: Mastoparan is a potent mast cell activator *in vivo*. (A) Mastoparan was injected into the peritoneum of mice and peritoneal lavage samples were collected and assayed for microscopic viewing of the mast cells present at the site of injection. Mastoparan causes mast cell degranulation peaking at 12 hours after treatment. The results represented in Figure 8A are from experiments performed by Joseph Oniyah. (B) The mesentery of the mice were also collected and stained with avidin FITC. Top two panels are representative of mesenteric mast cells from control mice, which display a clear cell boundary and abundant cytoplasmic granules. Bottom two panels represent mesenteric mast cells from mice treated with mastoparan, which have extruded and dispersed granules. Mastoparan elicited visual degranulation of mesentery mast cells.

To further investigate *in vivo* mast cell degranulation, we examined tissue-associated mast cells in the mesentery of mice following intraperitoneal administration of mastoparan. We collected mouse mesentery samples from C57BL/6 mice treated with mastoparan and stained them with avidin FITC, which binds selectively and distinctly to mast cell granules. As shown in Figure 8B, mastoparan elicited mast cell degranulation in the mesentery of mice. The mesentery of control mice (Figure 8B, top panels) treated with PBS revealed inactivated mesenteric mast cells that were visualized as tight granulated cells. In contrast, the mesentery of mice treated with mastoparan (Figure 8B, bottom panels) exhibited degranulated mast cells with dispersed granules around activated mast cells. Together, these data suggest that mastoparan is highly effective at causing dose-dependent mast cell degranulation *in vivo* and is therefore a potential immunostimulatory molecule.

2.3.8 Mastoparan treatment elicits the recruitment of neutrophils

Activated mast cells produce chemotactic factors that can modulate host innate immune response via the recruitment of pathogen clearing immune cells such as natural killer cells and neutrophils. Given the prominent role of mast cells in the immune response and having shown that mastoparan elicits

activation of mast cells *in vivo*, we hypothesized that this mastoparan elicited mast cell activation leads to increased recruitment of immune cells, such as neutrophils, to the site of treatment. To test this hypothesis and further investigate the potential of mastoparan to serve as an immunomodulatory molecule, we measured the amount of myeloperoxidase (MPO), a neutrophil marker enzyme, found in peritoneal lavage samples collected from mice treated with varying dosages of mastoparan. Mastoparan demonstrated dose-dependent neutrophil recruitment (Figure 9A) similar to the response displayed in relation to its *in vivo* mast cell activation (Figure 8).

Furthermore, we explored the timing of neutrophil recruitment elicited by mastoparan by evaluating the MPO activity of peritoneal lavage samples of mice treated with mastoparan (50 μg) at different time intervals. We found that mastoparan elicited the recruitment of neutrophils to the peritoneum beginning at six hours after treatment and peaking at twelve hours after treatment (Figure 9B). These studies suggest that mastoparan causes recruitment of neutrophils to the site of injection. Additionally, these data strongly suggest that this neutrophil recruitment is caused by the mastoparan induced mast cell

degranulation at this site since the neutrophil recruitment response parallels the mast cell degranulation response.

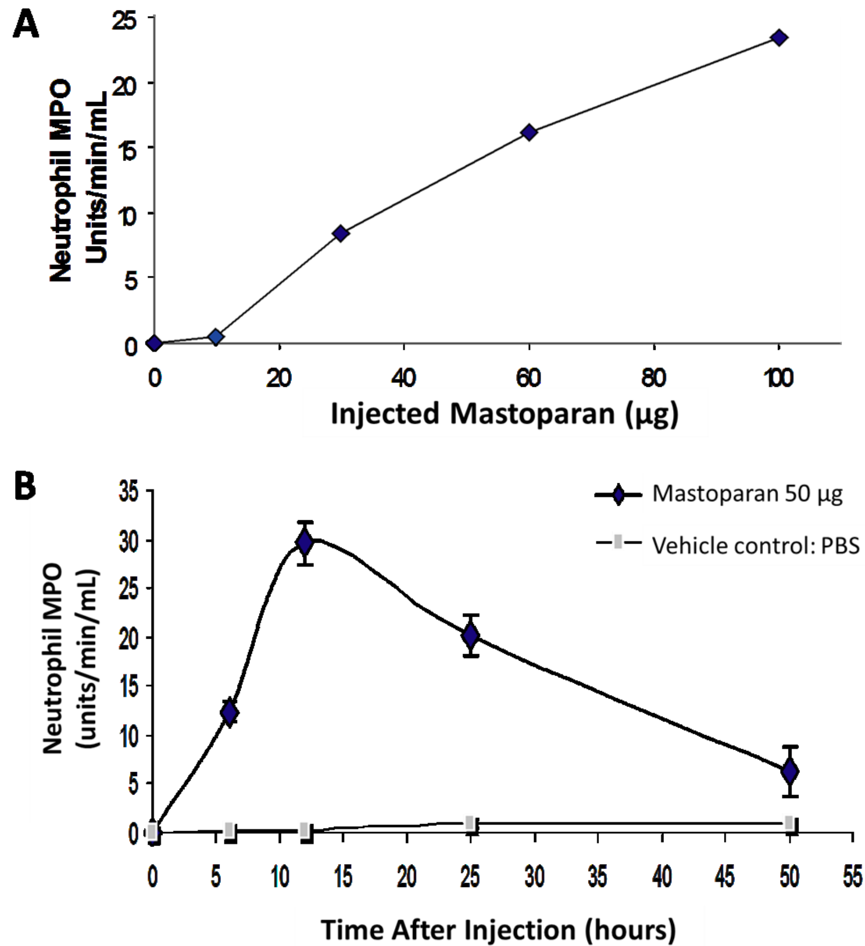

Figure 9: Mastoparan elicits the recruitment of neutrophils. (A) Various concentrations of mastoparan were injected into the peritoneum of mice and peritoneal lavage samples were assayed for MPO. Mastoparan causes significant neutrophil recruitment at concentrations between 10 to 100 µM. (B) At various time points, mice injected peritoneally with mastoparan (50 µg) were sacrificed and peritoneal lavage samples were assayed for MPO Activity. Instillation of mastoparan into the peritoneal cavity causes neutrophil recruitment into the site of injection. Experiments performed by Joseph Oniyah.

2.3.9 Mastoparan is effective as a topical antibacterial agent utilizing its dual action direct antibacterial and immunomodulatory activities

Globally there is an increase in bacterial skin infections, especially those caused by multidrug resistant bacteria. There is a lack of effective treatments against these infections, particularly treatments to eradicate infections consisting of stationary phase bacteria. We sought to determine the efficacy of mastoparan as a topical antibacterial treatment against skin infections utilizing a mouse skin colonization and infection model. We employed a skin colonization assay to test the ability of mastoparan as a topical antibacterial treatment against both log and stationary phase bacteria applied to the shaved intact skin of mice. For comparison, we used Neosporin a common topical antimicrobial agent whose active ingredients are neomycin, polymyxin, and bacitracin. The results of this experiment are summarized in Figure 10, which shows that mastoparan significantly reduced the number of colony-forming units recovered from the skin of mice colonized by log phase *S. aureus* with a 95% reduction and stationary phase *S. aureus* with a 99% reduction. In contrast, Neosporin was effective at reducing the number of colony-forming units recovered from the skin of mice colonized with log phase bacteria by 85%; however, it showed no activity against stationary phase bacteria.

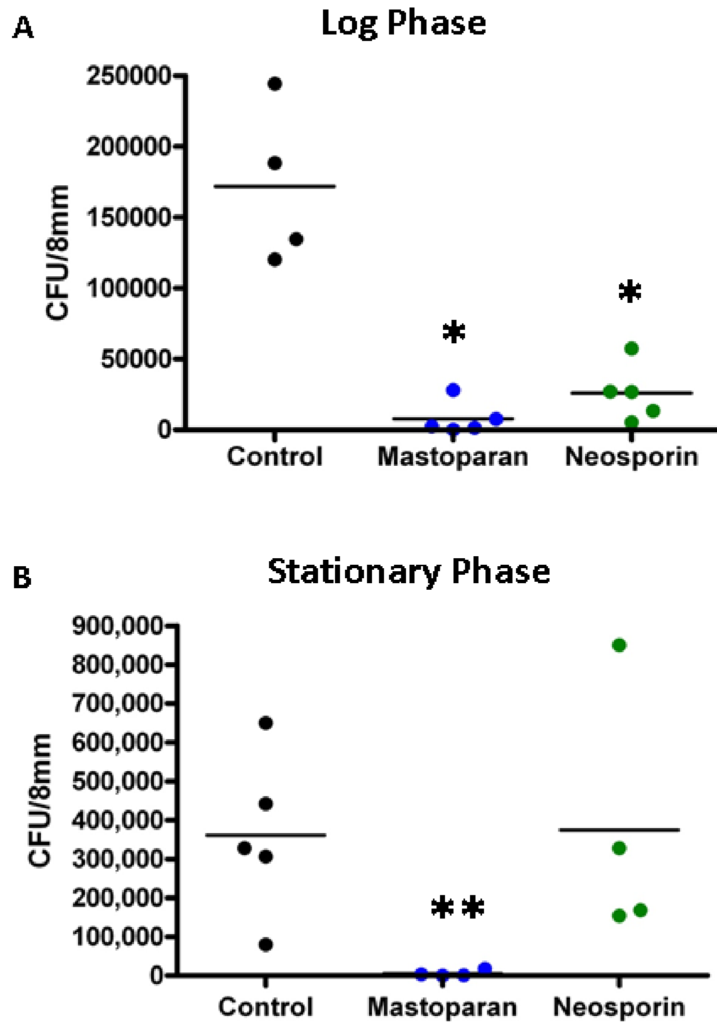

Figure 10: Mastoparan reduces the number of colony forming units (CFUs) in a superficial skin *S. aureus* colonization model. Enumeration of *S. aureus* recovered from mice following a superficial skin infection. The backs of mice were shaved and hair was removed by chemical depilation. (A) Log or (B) stationary phase bacteria were applied to the backs of the mice, and then treated 20 minutes later with mastoparan or Neosporin. Skin tissue was excised with an 8mm punch biopsy, homogenized, and CFU counts determined via agar plating. This data demonstrates that mastoparan is able to significantly reduce the number of colony-forming units present with both log and stationary phase bacteria. Statistical analyses were performed using the unpaired two-tailed Student's t-test. *$P < 0.05$, **$P<0.0005$

This data agrees with the studies presented by other researchers [46-49, 269, 270] demonstrating that most antibiotics in current use are unable to significantly affect stationary phase bacteria typically encountered in clinical infections. Importantly, we found that mastoparan is a potent topical antibacterial agent exhibiting activity against both log and stationary phase bacteria.

Next, we developed an intradermal skin bacterial infection model, where we injected *S. aureus* (10^8) directly under the upper epidermal layer of the backs of shaved mice. Mastoparan (10 μg) was infused in a vehicle of olive oil and DMSO to enable the drug to stay in place on the skin and to allow for transdermal migration. Mastoparan was applied topically (with the aid of a pipette tip to spread it across the infected area) to the site of infection every 12 hours with the first treatment taking place 12 hours after infection. As shown in Figure 11, mastoparan reduced the bacterial load of the infection by up to 98% (in comparison to the vehicle control of olive oil/DMSO).

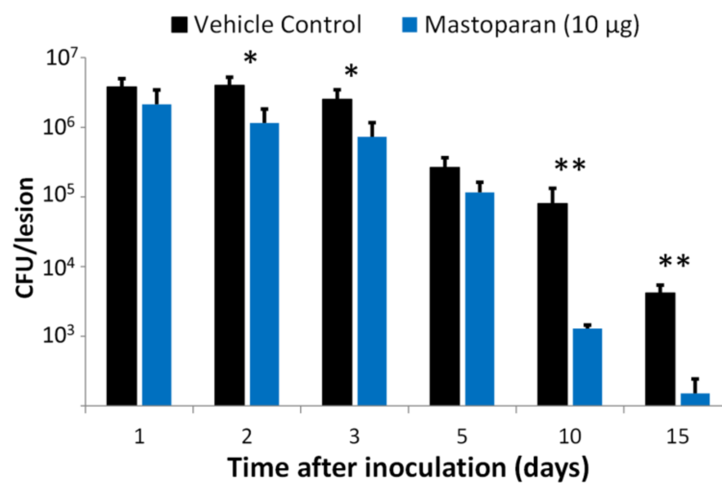

Figure 11: Mastoparan reduces the number of colony forming units (CFUs) of *S. aureus* recovered from excised skin lesions in an intradermal skin infection model. Enumeration of *S. aureus* recovered from mice (n = 8) treated with mastoparan or vehicle control (olive oil/DMSO) were sacrificed on days 1, 2, 3, 5, 10, and 15 following an intradermal skin infection. The backs of mice were shaved and hair was removed by chemical depilation. To establish an intradermal skin infection the mice were injected subcutaneously with 100 μL of *S. aureus* (10^8) complexed to Cytodex beads as a carrier. Mice were treated with mastoparan every 12 hours after infection (beginning at 12 hours after the bacteria were injected). Statistical analyses were performed using the unpaired two-tailed Student's t-test on log (10) transformed data. $*P < 0.05$, $**P<0.005$

Furthermore, we hypothesized that the ability of mastoparan to activate mast cells will allow it to be a more effective antibacterial agent through the recruitment of inflammatory cells (e.g., neutrophils) to enhance and increase host defense locally at the site of infection. In order to test this hypothesis, we excised 8mm punch biopsies of the skin at the site of infection and performed a MPO assay to determine neutrophil recruitment. We found that mastoparan is able to

cause a significant increase in the number of neutrophils recruited to the site of infection at the day 5 and day 10 time points (Figure 12). Overall, we have demonstrated that mastoparan is able to cause a significant reduction in the number of colony-forming units recovered in a colonization and infection model as well as increased neutrophil recruitment. Taken together, this data for the first time implicates mastoparan as a highly effective topical agent against bacterial skin infections having both direct antibacterial activity and increased immune stimulatory activity.

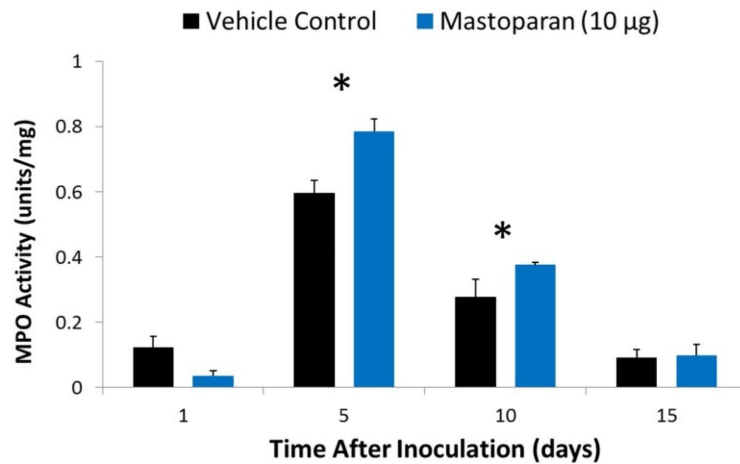

Figure 12: Mastoparan causes significant increase in neutrophil recruitment during a *S. aureus* intradermal skin infection model. MPO activity of mice (n = 8) treated with mastoparan or vehicle control (olive oil/DMSO) every 12 hours after infection (beginning at 12 hours after the bacteria were injected). Treatment with mastoparan caused an increase in the number of neutrophils recruited to the site of infection at the day 5 and 10 time points. Statistical analyses were performed using the unpaired two-tailed Student's t-test. *$P < 0.05$

2.3.10 Mastoparan does not exhibit dermal toxicity

To further elucidate the potential of mastoparan to serve as a topical antibacterial agent, we studied the possibility of mastoparan elicited dermal toxicity. To investigate mastoparan for potential dermal toxicity, mice were treated with mastoparan for 14 days via topical or intradermal application. We tested for dermal toxicity using a mastoparan dose of 50 μg and 100 μg, which represents 5 and 10 times the dosage used in the skin colonization and infection studies. Throughout the 14 days of administration, no treatment related adverse events were observed such as change in body weight, body temperature, observable behavior (e.g., distress, discomfort, and activity level), irritation, mutilation of the procedure site, or inflammation (data not shown). These observations imply that mastoparan does not exhibit dermal toxicity at a dose up to 100 μg.

2.3.11 Mastoparan promotes mast cell-dependent wound healing in mice

Interestingly, during our observation of skin lesions in the intradermal skin infection assay, we noticed that mastoparan treatment caused significant morphological changes in the appearance of the skin lesion. We found that mastoparan treated mice exhibited decreased lesion size implying faster healing.

Thus in addition to reducing bacterial numbers and increasing neutrophil recruitment mastoparan was evoking a third component of the immune defense, wound healing. Figure 13 shows pictorial representations of the morphological changes observed including progressed wound healing and scab formation with more advanced skin maturation and remodeling as the scab has begun to separate from the underlying tissue (inner circle of dried tissue at wound edges). These visual differences in lesion size between mastoparan treated mice and control mice, was confirmed by quantitative lesion size measurements (vertical and perpendicular diameters) which showed that treatment with mastoparan led to an up to 79% reduction in the size of the lesions (Figure 14).

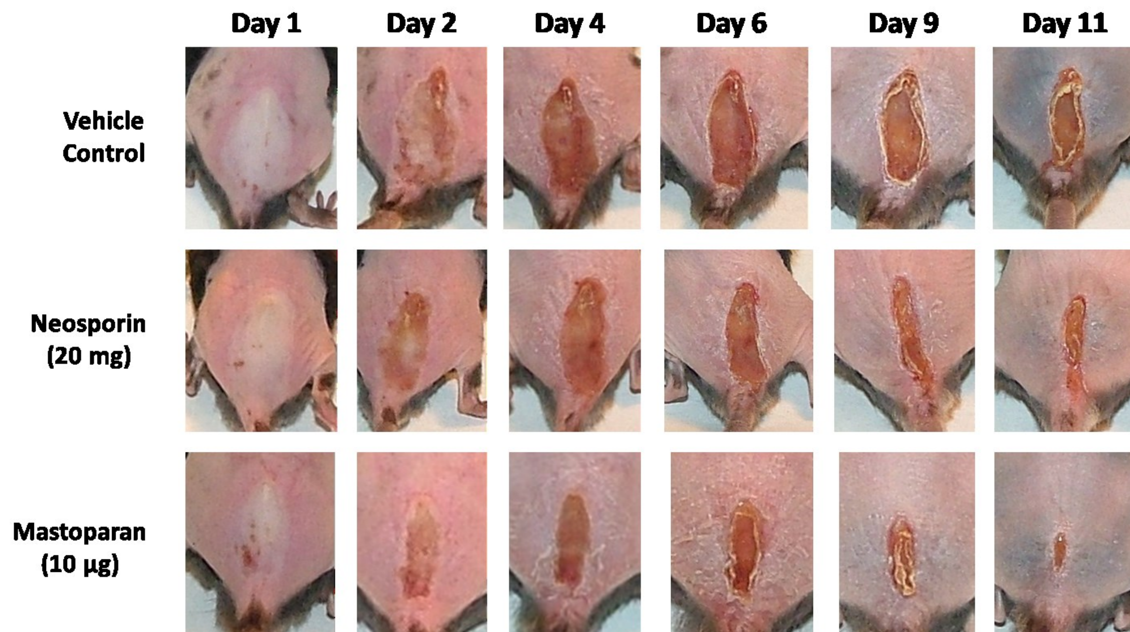

Figure 13: Pictorial representation of mice treated with mastoparan during a *S. aureus* intradermal skin infection. The backs of mice (n = 4-28) were shaved and hair was removed by chemical depilation. To establish an intradermal skin infection, the mice were injected intradermally with 100μL of *S. aureus* (10^8) complexed to Cytodex beads as a carrier. Mice were treated with mastoparan every 12 hours after infection. Pictorial representation of skin lesions of mice treated with Olive Oil/DMSO (vehicle control) as compared to mice treated with Neosporin or mastoparan. Mastoparan treated mice have progressed wound healing and accelerated wound closure in comparison to control and Neosporin treated mice. The pictures shown are representative of all studies in which similar differences were observed.

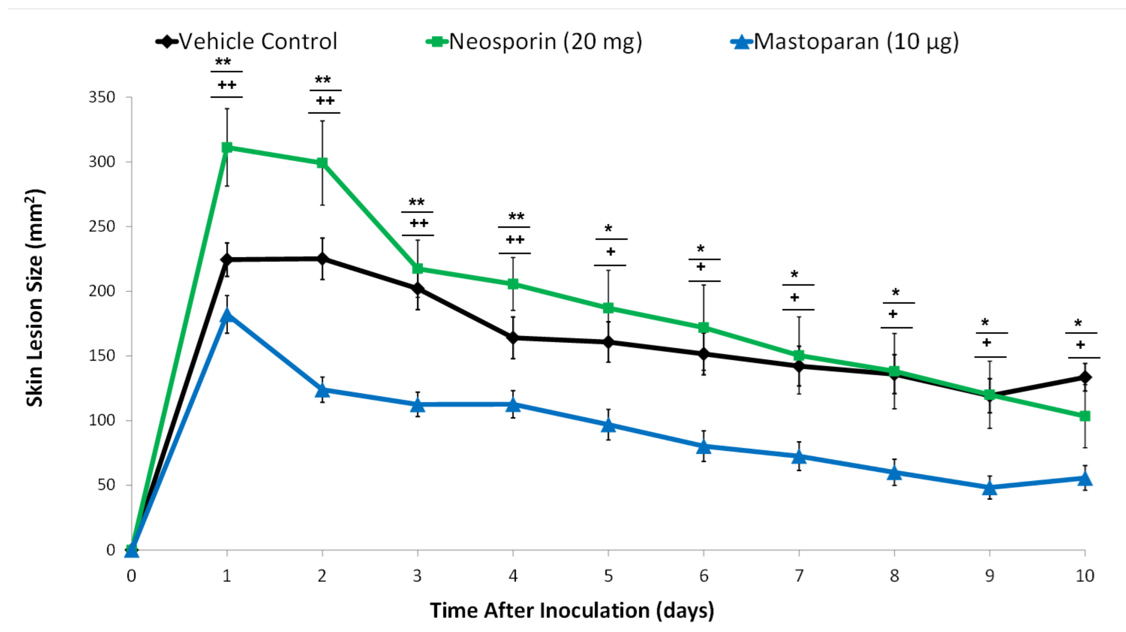

Figure 14: Mastoparan significantly reduces the size of lesions formed during a *S. aureus* intradermal skin infection. The backs of mice (n = 4-28) were shaved and hair was removed by chemical depilation. To establish an intradermal skin infection, the mice were injected intradermally with 100μL of S. aureus (108) complexed to Cytodex beads as a carrier. Mice were treated with mastoparan every 12 hours after infection. Lesion sizes were measured daily. Mastoparan treated mice have markedly smaller lesions as compared to untreated mice in a S. aureus skin bacterial infection model. Statistical analyses were performed using the unpaired two-tailed Student's t-test. *P < 0.05, ** P <0.005 (Mastoparan compared to vehicle control: DMSO/Olive Oil. +P<0.05, ++P<0.005 (Mastoparan compared to Neosporin).

To highlight the wound healing actions of mastoparan and distinguish them from its antibacterial actions, we compared these results to those obtained with Neosporin treated mice. We found that Neosporin, in spite of its known antibacterial actions, did not cause significant improvement in lesion size. Indeed, at certain time points the lesion size measurements for Neosporin treated

mice were greater than the lesion size measurements for the control mice. These observations suggest that the topical instillation of mastoparan at the site of infection increases bacterial clearance, enhances neutrophil recruitment, and promotes wound healing.

The enhanced wound healing finding was an unexpected result so we sought to investigate if this activity was linked to mastoparan elicited activation of mast cells. It is noteworthy that mast cells have previously been implicated in the healing of skin wounds [306-308]. Mast cells have been reported to accelerate wound closure and their release of histamine is functionally important for the healing of cutaneous wounds [309, 310]. Based on this, we hypothesized that mastoparan elicited mast cell activation could have contributed to the observed acceleration in wound healing. To test this notion we reasoned that mastoparan treatment of intradermal skin infections in mast cell deficient (W^{sh}/W^{sh}) mice would predictably show no obvious enhancement of wounds when compared to control mice. Utilizing the intradermal skin infection model, we undertook this experiment and compared lesion sizes following *S. aureus* infection of mastoparan treated and untreated mast cell deficient mice. We found that in the absence of mast cells, there appeared to be no discernible difference in the lesion

size of mice treated with mastoparan in comparison to the control (Figure 15). This data suggests that the observed promotion of wound healing by mastoparan is mast cell dependent.

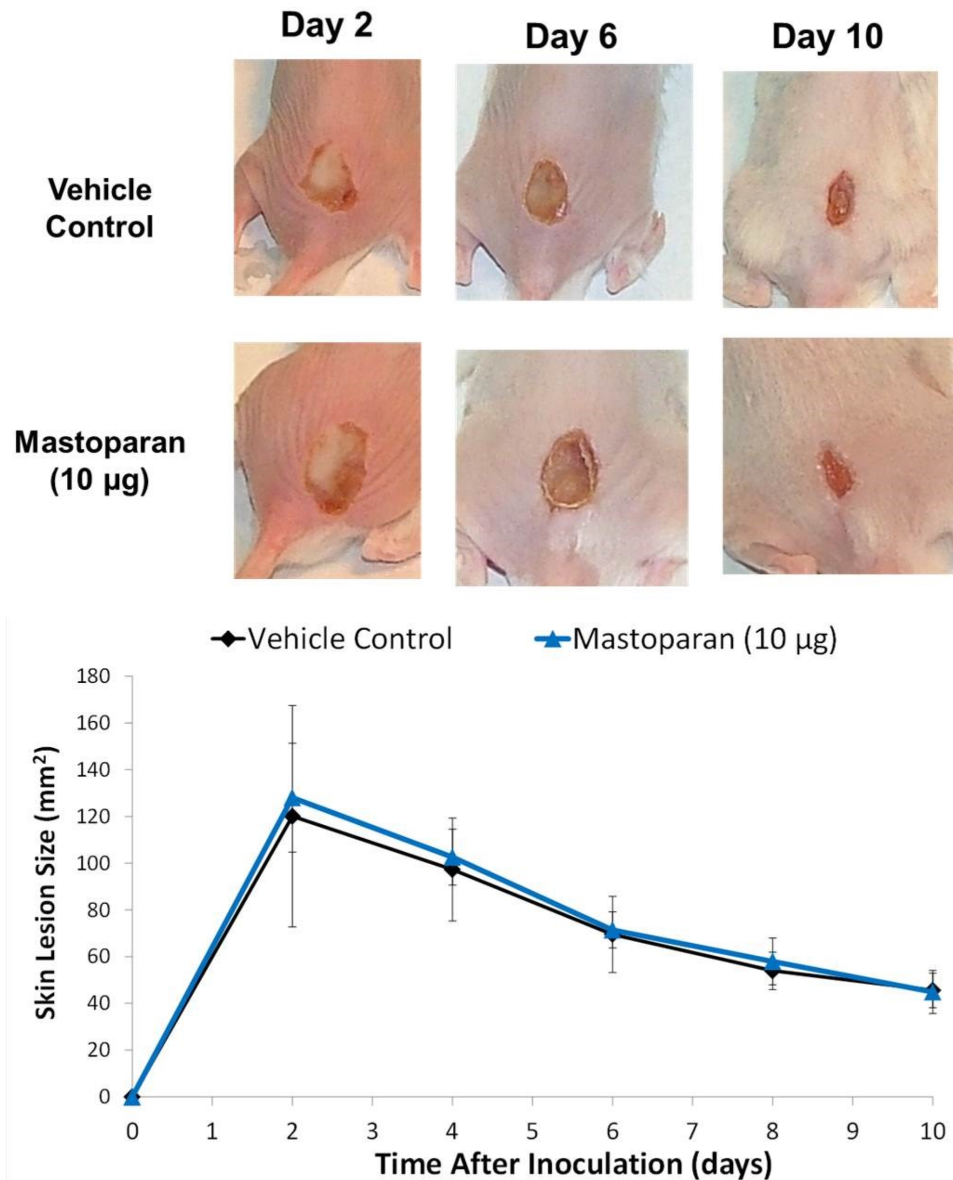

Figure 15: Mast cells are required for the promotion of wound healing by mastoparan. The backs of mast cell deficient (W^{sh}/W^{sh}) mice (n = 6) were shaved and hair was removed by chemical depilation. They were injected intradermally with 100µL of *S. aureus* (10^8) complexed to Cytodex beads as a carrier. Mice were treated with mastoparan every 12 hours after infection. Lesion sizes were measured daily. This data demonstrates that there is no significant difference in the lesion size of mice treated with mastoparan in comparison to the control.

2.4 Discussion

Antimicrobial peptides are a group of small peptides found amongst almost every class of life. These peptides typically have a potent and direct killing mechanism of action. Another group of peptides, innate immune modulators are an important component of host immune defenses via immunostimulatory activities including chemoattraction of immune cells and promotion of angiogenesis and wound healing. Together, antimicrobial and immune modulating peptides represent a new field of exploration for successful anti-infective therapies. Peptides with both abilities, antibacterial and immunomodulatory, have the capacity to be novel therapeutics against bacterial infections. The primary aim of this study was to demonstrate the antibacterial and mast cell activation capabilities of mastoparan and to show that these properties can be harnessed for the treatment of bacterial skin infections. In this study, we have successfully shown the dual action exhibited by a wasp venom peptide through its ability to stimulate the innate immune mechanisms and directly kill bacteria in a bactericidal manner (Figure 16). Furthermore, our observations strongly support the potential of mastoparan to promote epithelial wound healing.

Mast cells are preferentially located at the host-environment interface (e.g., skin and mucosal sites). They are activated by and can detect bacteria and recruit inflammatory cells such as neutrophils to the site of infection, thereby contributing to the innate immune response. Previous studies have shown that mast cells are functionally important in the induction of the host defense response during bacterial skin infections (reviewed in [263]). Mastoparan was initially discovered based on its ability to activate mast cells *in vitro* [239]. Here we have demonstrated that mastoparan is a potent mast cell activator (Figure 8), and stimulates the recruitment of neutrophils *in vivo* (Figure 9). Furthermore, we found that mice infected with bacteria and treated with mastoparan, demonstrate increased neutrophil recruitment (Figure 12) along with increased bacterial clearance (Figure 10and 11).

Several of the peptides in the mastoparan family have been shown to exhibit antibacterial activity *in vitro* [238, 239, 255, 286]. Our studies complement and extended these works by showing that mastoparan has antibacterial activity against a broad-spectrum of bacteria, both Gram-negative and Gram-positive strains (Table 2) and has a broader range of action and efficacy in relation to some marketed antibiotics (Table 3). Additionally, mastoparan has activity

against multidrug resistant bacteria (Table 4), which has become a growing global concern.

Our studies examining the mechanism of action exhibited by mastoparan show that it achieves its bactericidal effect via increased membrane permeability allowing efflux of cytoplasmic material (Figure 2 and 3). Furthermore, mastoparan exhibits a rapid mechanism of bacteria growth inhibition and has the ability to reduce bacteria colony forming units in less than 30 minutes, suggesting that has a direct killing method (Figure 6). Mastoparan is also able to significantly reduce bacteria numbers in both stationary and log phase bacterial cultures (Figure 6). This is in contrast to a method of inhibition that requires cell growth and replication such as that shown by marketed antibiotics. Mastoparan's rapid mechanism of killing combined with the broad-spectrum of action and efficacy against both stationary and log phase bacteria, leads us to conclude that mastoparan is an excellent candidate for development as a therapeutic agent

A particularly striking finding from our work is that mastoparan treated mice showed a significant reduction in the lesion sizes of mice infected with *S. aureus*, suggesting increased wound healing. Natural wound healing is a

dynamic process involving several different cell types and soluble mediators. These processes include inflammation, tissue remodeling, cell proliferation, and migration [311-315]. A key component of the inflammation process includes the recruitment of neutrophils to the site of injury [316-318]. As stated previously, our data demonstrates that mastoparan increases neutrophil recruitment and mast cell degranulation. We have also shown that mice treated with mastoparan have significantly smaller lesions as compared to untreated mice suggesting that mastoparan, through the activation of mast cells and the recruitment of neutrophils, may modulate tissue responsiveness and accelerated wound healing.

Mastoparan shows significant and vast potential for use as a therapeutic due to its demonstrated effectiveness against bacterial skin infections. Here, we have for the first time successfully illustrated the dual ability of mastoparan to stimulate the immune system through the activation of mast cells and it has direct antibacterial activity via membrane permeabilization. Together, these abilities contribute to its effectiveness against bacterial skin infections and makes it an excellent target as the next line of antimicrobials.

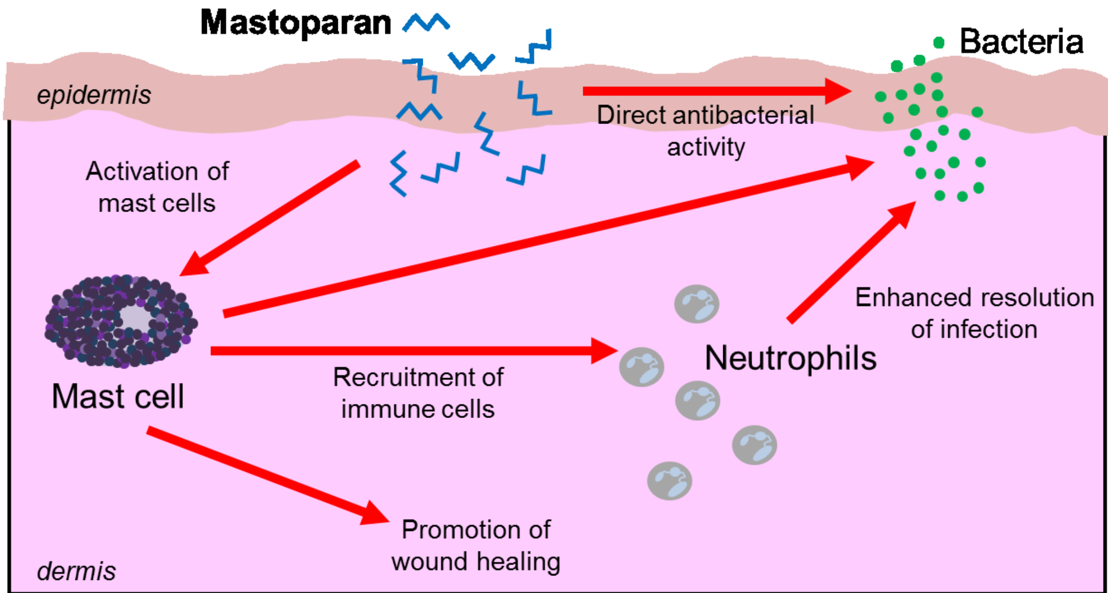

Figure 16: Summary of the biological activities of mastoparan.

Chapter 3: Perspectives and Conclusion

Bacterial infections are a major cause of death, disease, and increasing financial burden on the health care industry. Due to the growing threat of multidrug resistant bacteria, previously confined to health care settings (e.g., hospitals and clinics) but now seen more frequently in the community, bacterial infections are becoming even more difficult to treat. Multidrug resistance is now common and the increased accumulation of these pathogenic bacteria has greatly limited therapeutic options. In the pharmaceutical industry, antimicrobial drugs represent the third most profitable class of drugs behind those dealing with central nervous and cardiovascular systems [319]. However, there has been a lapse in the development of innovative antibacterial drugs in the past decade. Antimicrobial agents found in wasp venom have recently gained attention as novel natural products capable of combating multidrug resistant pathogens.

The work presented here provides experimental data that broadens our understanding of the potential utility of the wasp venom peptide, mastoparan, as an in vivo antibacterial therapeutic employing mouse models of infection. Prior contributions to this field have focused exclusively on demonstrating the general ability of mastoparan to act as an antibacterial or mast cell activator *in vitro* [239, 255, 259]. The current study has confirmed the ability of mastoparan to work

against natural pathogens and shown its efficacy against multidrug resistant bacteria, while also determining the *in vivo* biological significance of mastoparan. This dissertation investigates the ability of mastoparan to both directly kill bacterial pathogens and stimulate the natural host immune system via mast cell activation. Immunomodulatory therapy is a novel anti-infective approach that may enable us to selectively enhance the host immune response to infection. Due to the growing evidence that mast cells orchestrate early innate and acquired immune responses to infection, we believe that the bactericidal activity of mastoparan combined with its immunomodulatory activity via mast cell activation make it an effective but largely overlooked therapeutic.

3.1 Broad-spectrum antibacterial and immunomodulatory activity exhibited by mastoparan

The development of new antimicrobial agents to combat the growing incidence of bacterial infections and bacterial resistance is a primary global concern. Most antimicrobial agents in the pipeline for development have a limited range of action and are unable to work against stationary phase bacteria. Here, we have demonstrated that mastoparan is effective against a wide range of Gram-negative, Gram-positive, and clinically resistant bacterial pathogens. The data presented here demonstrating that mastoparan is effective against

multidrug resistant bacteria *in vitro* suggests its potential utility against these pathogens *in vivo*. Further studies to determine if mastoparan is effective against these resistant pathogens *in vivo* are necessary. Due to the increased need for new anti-infective therapies, further studies investigating the utility of mastoparan against a range of different microorganisms (e.g., viruses, parasites, and fungi) may be useful since previous studies have shown that other antimicrobial peptides similar to mastoparan also have antiviral, antifungal, and/or anti-parasitic activities as well [174, 175, 226, 320, 321].

We have presented multiple lines of experimental evidence to show that mastoparan utilizes a mechanism of action that involves interaction with the bacterial cell membrane similar to other antimicrobial peptides [174, 177-179]. Here, we show that mastoparan interacts with the cell membrane via a mechanism that causes structural perturbations such as blebs and pores, leading to the release of bacterial cytosolic contents. Our experimental data demonstrates a rapid (in many instances less than 15 minutes) mechanism of action that significantly reduces the number of colony-forming units. Interestingly this reduction in colony-forming units occurs even in the presence of stationary phase (non-multiplying) bacterial cultures. This suggests that the

main mechanism of action for mastoparan is via an interaction with the bacterial cell membrane.

Previous studies have revealed that some antimicrobial peptides may also have a mechanism of action that includes an intracellular target in addition to their interaction with the cell membrane [182-188, 322, 323]. Stationary phase bacteria require minimal cellular metabolic processes such as DNA/RNA synthesis and protein synthesis. Most intracellular targets hinder such components of cellular physiology, and therefore if mastoparan is able to reduce colony-forming units for stationary phase bacteria, this suggests that it has a killing mechanism of action that does not require bacterial growth and reproduction. Future studies to determine if mastoparan has the ability to utilize an intracellular target could be beneficial.

Although we have not noticed any resistance development, additional benefit can also be gained from an investigation of mechanisms that bacteria utilize to develop resistance to mastoparan. To this end, given that antimicrobial peptides are a component of wasp venom that aid in combating invading microorganisms, we can utilize this information to search for microorganisms that have been able to successfully circumvent this natural system in wasps to

help reveal information about the type of systems that bacteria can exploit to develop resistance. This will allow us to more efficiently devise methods to reduce the development of resistance with strategies such as combination therapy.

Immune stimulatory molecules have an enormous amount of potential utility as antimicrobial agents, due to their ability to boost the natural host response (e.g., mast cell activation and neutrophil recruitment) to infections. There are several natural molecules such as small peptides and CpG DNA that have been shown to stimulate the immune response to control infection; therefore, it may be possible to use similar molecules to boost the natural immune response to infection [205, 324-326]. Our studies, along with the work of other investigators, have shown that mastoparan is a potent mast cell activator *in vitro* [239].

Mast cells are specialized, granulated innate immune cells involved in bacterial clearance. Mast cells assist in the clearance of pathogens (i.e., parasites, bacteria, and viruses) and are able to directly and indirectly recognize pathogens including those bound by host defense proteins. They can rapidly degranulate, releasing pre-stored inflammatory mediators, and can synthesize *de novo*

mediators and inflammatory cytokines. Mast cells have the ability to directly kill bacteria through phagocytic actions and the release of antibacterial peptides (e.g., cathelicidins) of their own [257, 327-335]. However, the majority of their antibacterial activity is due to their ability to mediate the recruitment of other immune cells such as neutrophils and antigen presenting cells to the site of infection [332, 336-339]. The studies presented here have demonstrated that instillation of mastoparan in the peritoneal cavity causes mast cell activation and neutrophil recruitment. This suggests that mastoparan is a potent immunomodulatory agent *in vivo*. In future studies, we hope to determine whether mast cells are the only immunomodulatory cell type activated by mastoparan.

Mast cells are not only known to play a role in innate immunity but they are also known to contribute to acquired immunity leading to long-lasting recognition of pathogens, which increases eradication and clearance speed during subsequent infection. Studies have shown that mast cells facilitate the acquired immune response via mechanisms such as the secretion of products, antigen presentation, and the mobilization of effector cells (reviewed in [332], [339]). In future studies, we hope to confirm that mastoparan induced mast cell

activation enhances the acquired immune response to infection in addition to the increase in innate immunity that we have demonstrated.

3.2 Mastoparan is a highly effective therapeutic agent against bacterial skin infections

Skin and soft-tissue infections account for more than 14 million outpatient visits in the United States each year and have an estimated prevalence of 7-10% among all hospitalized individuals [266, 267]. Furthermore, skin infections are the third most common diagnosis in emergency care settings, behind chest pain and asthma. These infections are becoming more complicated due to the emergence of multidrug resistant organisms such as methicillin-resistant *Staphylococcus aureus* (MRSA) [340, 341]. As a complex organ, human skin has many natural protective mechanisms to ensure protection from microbial attack. However, when the skin barrier is breached via damage such as burns, cuts, or wounds infection can occur. Here, we have shown that topical application of mastoparan is able to significantly reduce the number of colony-forming units recovered from a skin infection model in mice.

As discussed, we believe that the ability of mastoparan to serve as an immunomodulatory stimulator via mast cell activation leading to neutrophil recruitment, in addition to its direct antibacterial capabilities, makes it a more

powerful and effective topical antimicrobial agent. Having determined that mastoparan is able to activate mast cells and stimulate neutrophil recruitment in the absence of infection, we sought to determine if these same capabilities were present or enhanced during bacterial skin infections. Previous studies have shown that the activation of mast cells and the recruitment of neutrophils are required for the clearance of bacterial skin infections in mice [342]. We have shown that mice treated with mastoparan have increased neutrophil recruitment in the presence of a bacterial skin infection. This is strong evidence that the immune stimulatory activity of mastoparan may promote and enhance its antibacterial activity.

In future studies, we hope to determine how mast cells modulate the enhanced antibacterial activity that we observed and if there are other cell types involved. Additionally, information determining whether the observed increase in neutrophil recruitment is due to direct activation of mast cells or to increased inflammation caused by the direct killing of bacteria by mastoparan would give further insight into distinguishing the contribution of the antibacterial and immunomodulatory activities of mastoparan. The data presented here demonstrating that mastoparan is effective against multidrug resistant bacteria *in*

vitro suggests its potential utility against these pathogens *in vivo*. Further studies to determine if mastoparan is effective against these resistant pathogens *in vivo* are necessary.

Most antimicrobial peptides have demonstrated limited *in vivo* capabilities due to host toxicity, protease degradation, and absorption within the body due to their small size and high charge [174, 175, 192, 193]. Thus, their therapeutic utility is typically limited to topical applications or direct injection into the site of infection. We have presented multiple lines of experimental evidence demonstrating that there is no detectable toxic effect with mastoparan at a dosage of up to 100 μg (10 times the amount used in our skin infection model). In future studies, we hope to determine whether mastoparan can be used in a systemic model (parenteral and oral) of infection. Systemic use may require that we make chemical modifications to the structure of mastoparan (e.g., produce a peptidomimetic equivalent or extend its length via attachment of polyethylene glycol) in order to reduce the likelihood of protease degradation or minimize cellular absorption. Additionally, future studies to determine an appropriate topical formulation (many topical ointments contain a petroleum jelly base) for mastoparan are necessary along with studies to determine its stability in such a

base formulation. Recent studies have demonstrated the effectiveness of incorporating antimicrobial peptides into materials such as polyelectrolyte multilayer films, formulation into liposomes or nanoparticles, and freeze-dried antimicrobial wafers to improve stability, permit prolonged drug release and extended application [190, 321, 343-345].

3.3 Mastoparan promotes wound healing

Treatment of chronic wounds costs over 25 billion dollars annually and more than 6.5 million people are affected by chronic wounds in the United States [346, 347]. The number of individuals affected by these wounds and the amount of associated health-care costs is steadily increasing due to such factors as the drastic increase in our elderly population and a global increase in the number of individuals suffering from diabetes and obesity [348]. Wound infections have led to almost 100,000 leg amputations, which severely compromise quality of life and increased mortality [348, 349]. Additional concerns surround post-surgical wound care (more than 34 million surgeries are performed each year), acute emergency wound care, and skin scarring [348, 350]. Wounds (caused by burns, surgery, etc.) pose significant global health, economic, and social problems

presenting the need for the development of new approaches for the treatment of these wounds.

Prior contributions to this field have demonstrated the potential of small peptides to promote wound healing and angiogenesis due to their ability to mediate innate and acquired immune responses. Human cathelicidins (hCAP18/LL-37) have been shown to be expressed after wounding and activate epidermal cells and fibroblasts as chemoattractants for wound healing components such as macrophages, fibroblasts, and keratinocytes [351-353]. Similar peptides have also been shown to inhibit tissue injury through the inhibition of proteases [205]. In attempting to understand the contribution of mastoparan to the reduction of colony-forming units in a bacterial skin infection model, we have also addressed its ability to promote wound healing. In doing so, we found that treatment of wounds with mastoparan resulted in up to a 79% reduction in lesion size. We have also shown that the promotion of wound healing by mastoparan is mast cell dependent via studies utilizing mast cell deficient mice (Kit^{W-sh}/Kit^{W-sh}). In future studies, we hope to further elucidate the role that mast cell activation plays in this promotion of wound healing, especially

in the absence of infection, which will allow us to gain further insight into how mastoparan facilitates wound healing.

3.4 Conclusions

There is a need for new therapies to treat bacterial infections especially infections involving multidrug resistant bacteria for which the incidence of resistance is steadily increasing, mostly due to antibiotic misuse or overuse. One major area for future development to combat these infections is the use of antimicrobial peptides that promote immunomodulatory activity. These molecules have the broad potential not only to be utilized as an anti-infective agent but also as adjuncts to support other treatments including wound healing, vaccine adjuvants, and anticancer drugs. Mastoparan is a potential anti-infective with a broad-spectrum of activity that includes efficacy against resistant bacteria (e.g., MRSA, ESBL E.coli, and ESBL K. pneumonia). In addition to its strong antibacterial properties, it is also an effective immunomodulatory molecule, which increases its versatility and overall efficacy. This work makes important contributions to the general understanding of antimicrobial peptides and immune stimulatory molecules and their potential as anti-infective agents. The use of immune stimulatory molecules is a novel approach to combat bacterial

infections and we can exploit the immunomodulatory properties of these molecules to create more effective anti-infective therapies. Furthermore, increased understanding of the fundamental processes involved will present new approaches and innovative technologies for managing immune responses in a clinical setting.

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Biography

Yuvon Rondreise Mobley was born and raised in High Springs, Florida. After graduating from Santa Fe High School, Yuvon attended Princeton University in Princeton, New Jersey where she received her Bachelor of Arts degree in Ecology and Evolutionary Biology and a certificate in African American Studies in 2004. Yuvon then spent two years teaching seventh grade science at Deady Middle School in Houston, Texas as a member of the Teach for America Corps. Following her time teaching, she moved to Durham, North Carolina and completed the Post-Baccalaureate Research Education Program (PREP) at Duke University. In the fall of 2008, Yuvon began the pursuit of her Ph.D. at Duke University in the Department of Molecular Genetics and Microbiology. Under the guidance of Dr. Soman Abraham, she has spent the past five years investigating the antibacterial and immunostimulatory activity of a group of antimicrobial peptides originally identified from the venom of wasps.

Publications & Presentations

Y. Mobley, J. Oniyah, H. Staats, S. Abraham. *Dual Action Immunomodulatory and Broad-Spectrum Antibacterial Activity of Wasp Venom Peptide*. In preparation.

Y. Mobley. *Antibacterial Activity of Wasp Venom Peptide*. Young investigator oral presentation. American Society for Microbiology, 113th General Meeting, Denver, Colorado (May 2013).

Y. Mobley. *Taking the Sting Out of Skin Infections - Wasp Venom Peptide as a Topical Antimicrobial Agent*. Oral Presentation presented at the Duke University Department of Molecular Genetics & Microbiology Departmental Seminar, Durham, NC (February 2012).

Y. Mobley, A. Hofmann, L. Harrell, H. Staats, S. Abraham. *Determining The Therapeutic Use of Wasp (Vespula lewisii) Venom Peptide Against Bacterial Pathogens*. Poster session presented at the Annual Biomedical Research Conference for Minority Students (ABRCMS), Charlotte, NC (November 2010).

Y. Mobley, A. Hofmann, L. Harrell, H. Staats, S. Abraham. *A Novel Antimicrobial Against Resistant Gram-Negative* Organisms. Poster session presented at the Keystone Symposium Meeting Antibiotics and Resistance: Challenges and Solutions, Santa Fe, NM (February 2010).

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