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A Modular Approach to the Histone H2A Family of Antimicrobial Peptides

Alexandra Fischer
afische2@wellesley.edu

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Alexandra Fischer

Advisor: Donald E. Elmore

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ABBREVIATIONS

AMP – Antimicrobial Peptide
BF1 – Buforin I
BF2 – Buforin II
BF2+CTERM – Buforin II plus hipposin's C-terminal region
CD – Circular Dichroism
CTERM – Hipposin's C-terminal region
DesHDAP – Designed Histone-Derived Antimicrobial peptide
DNS-PE – 5-dimethylaminonapthalene-1-sulfonyl phosphatidylethanolamine
FRET – Fluorescence Resonance Energy Transfer
HIP – Hipposin
PARA – Parasin
PC – Phosphatidylcholine
PG – Phosphatidylglycerol
PI – Propidium iodide
TFE – Trifluoroethanol
TSB – Tryptic Soy Broth

Abstract

Hipposin is a naturally occurring 51-mer antimicrobial peptide (AMP) derived from histone H2A. Parasin (PARA), Buforin II (BF2), and Buforin I (BF1) are also naturally occurring AMPs, and share high sequence homology with the n-terminal region, the middle region, and the terminal and middle regions of hipposin, respectively. Two designed peptides, CTERM and BF2+CTERM, have the same amino acid sequences as the c-terminal region of hipposin, and BF2 plus the c-terminal region of hipposin, respectively. Their permeabilizing abilities, translocating abilities, and antimicrobial activities were assessed to determine if this set of peptides operates in a modular fashion. When combining peptides, the membrane permeabilizing ability of the new peptide appears to be dictated by the more permeabilizing of the parent peptides. For example, when PARA and BF2 are combined to make BF1, the resulting peptide has a membrane permeabilizing ability closest to PARA, which is more membrane permeabilizing than BF2. Translocating ability is preserved when two translocating peptides, CTERM and BF2, are combined. Future studies will attempt to determine whether mechanistic abilities are additive, and will test the effects of these peptide domains when added on to peptides outside the histone H2A AMP family.

Introduction

Previous Antibacterial Agents and the Beginnings of Resistance

The story of infectious bacteria and the drugs that treat them might be best illustrated by the history of the bacteria *Staphylococcus aureus*. Discovered in the late 1880's, *S. aureus* caused painful skin infections or oft-fatal infections in the bloodstream. However, due to the discovery of penicillin, infections of *S. aureus* could be treated fairly easily by the 1940's.[1] Yet before the decade was over, *S. aureus* had begun to develop resistance to penicillin, which prompted the introduction of methicillin, a penicillin derivative. Continuing its established pattern, methicillin-resistant strains of *S. aureus* (MRSA) were observed by the early 1960's, but not common in the United States until the 1980's.[1-2] The pharmaceutical industry countered with the development of fluoroquinolone agents, like ciprofloxacin, which at first seemed to be the end of the problem[2]. Unsurprisingly, the drug's effectiveness was short-lived and in one year alone, MRSA resistance to ciprofloxacin went from under 5% to greater than 80%.[3] In the United States today, nearly all strains of *S. aureus* are penicillin resistant, and there are over 90,000 invasive MRSA infections each year.[4] There is one antibiotic, vancomysin, that remains an effective treatment, but there are reports of MRSA strains with decreased susceptibility to vancomycin.[4] Clearly, there is a need for antimicrobial agents that attack bacteria in ways that minimize the development of resistance.

Traditional β-lactam antibiotics, like penicillin, kill bacteria by either damaging or inhibiting the synthesis of the bacterial cell wall through interfering with necessary enzymes.[5] Vancomycin also disrupts cell wall synthesis, but does so by binding to terminal residues of the nascent peptidoglycan chain, which prevents the cross-linking steps necessary for a stable synthesis of the cell wall.[5] Other antibiotics, like quinolones, will suppress the synthesis of nucleic acids by causing double strand breaks during DNA replication.[2, 6] As demonstrated by these examples, antibiotics typically have a specific

chemical target, through which they will inhibit growth or kill the cell. This property makes it fairly simple for an organism to develop resistance to a specific antibiotic.

Countering these antibiotics usually involves either a mutation of genes or acquisition of genes from other bacteria. For example, an organism could acquire genes that encodes for a β-lactamase enzyme, which would destroy a penicillin molecule before it could cause damage.[7] The organism could alternately pick up genes that ultimately eliminate the binding site of the antibiotic, preventing it from killing the organism.[7] As illustrated by *S. aureus*, organisms are developing resistance to antibiotics faster than new antibiotics can be developed. This calls for development of an antimicrobial agent that will remain active against its target for longer periods of time.

Antimicrobial Peptides

Antimicrobial peptides (AMPs) offer a promising alternative to traditional antibiotics, due to their enduring efficacy and vast variety. AMPS are a part of the innate immune systems of many plants[8], insects[9, 10] and animals, including humans.[11,12] They are characterized by a net positive charge and are fairly short, generally between 12 and 100 amino acids long. These peptides can be active against fungi, parasites, protozoa, viruses, and even cancerous cells.[13] However, these peptides are studied largely for their anti-bacterial properties, which will also be the focus of this thesis. Over 2000 naturally-occurring AMPs have been discovered, in addition to an untold number of designed peptides.[14]

As mentioned earlier, antimicrobial peptides are cationic, often carrying a net charge between +2 and +9. Electrostatic interactions play a very important role in AMP activity and selectivity. Bacterial membranes carry more negative charge in their membranes than healthy mammalian cells due to a higher proportion of negatively charged lipid head groups, like phosphatidylglycerol(PG).[15] Bacterial

membranes also have a higher negative membrane potential than eukaryotic cells. Additionally, Gram-negative bacteria have negatively charged lipopolysaccharide molecules in the outer leaflet of the membrane, which protects them from hydrophobic antibiotics, but bind strongly to positively charged AMPs.[16] These electrostatic properties also allow for the low eukaryotic toxicity of many naturally-occurring AMPs, a very desirable trait for therapeutic agents. At least in one case however, a peptide can be too positively charged, to the point that it becomes extremely hemolytic.[17] Thus, designers of AMPs need to keep in mind that there may be an optimal charge range that allows for effective prokaryotic membrane targeting, while sparing the healthy eukaryotic cells.

In terms of secondary structure, antimicrobial peptides are often alpha-helical, but they may adopt beta sheet, random coil, disulfide bridge loops, and polyproline extended helix conformations.[18] Alpha helical peptides are well studied, especially in relation to the arrangement of hydrophobic and hydrophilic regions.

Antimicrobial peptides have persisted as viable agents of many organisms' immune systems despite hundreds of years of bacterial evolution. Unlike the antibiotic agents discussed earlier, most peptides do not target a specific site in bacteria. This was shown in a study which compared peptides made of either D- or L-enantiomers.[19] If AMPs operated by binding or interacting with a specific target site, the D-enantiomer peptide would have completely different activity from the L-enantiomer peptides. However, the two peptides displayed the same activity against bacterial cells, indicating that AMPs do not operate using a specific chemical target.[19] Instead, they use integral features of bacterial membranes to attack the organism. This is further evidenced by the fact that AMPs are can permeabilize lipid vesicles, which indicates that there is no specific chemical target for many of these peptides. This property makes it difficult, but not impossible, for pathogens to evolve in such a way as to render AMPs ineffective. One familiar bacteria, *S. aureus*, has developed resistance against human AMPs by lowering

its overall surface charge, while other organisms have begun producing protease enzymes, which degrade peptides.[20] The latter tactic is of limited use, as bacteria also depend on peptides for many functions inside the cell, so a histone that destroyed AMPs would also destroy the cell's own peptides. Despite this, AMPs remain effective agents against a wide breadth of pathogens, offering a promising alternative to traditional antibiotics.

Mechanisms of Action

Antimicrobial peptides typically operate by one of three mechanisms. In the first of these mechanisms, the peptide disrupts the bacterial membrane nonspecifically, as described by the "carpet" model (Figure 1).[21] In this model, peptides arrange themselves parallel to the membrane surface, like a carpet, and when they reach high enough concentrations, the peptides will disrupt the membrane in a detergent-like manner, ultimately forming micelles, and destroying the membrane. Caerin 1.1, a peptide derived from the Australian green tree frog, appears to operate via this mechanism. The second mechanism also involves membrane disruption, but via the formation of regular pores. This mechanism is described by the "barrel-stave" and "toroidal pore" models. In the barrel-stave model, the peptides orient themselves such that their hydrophobic regions are associated with the lipid core of the membrane, while their hydrophilic regions are on the interior of the pore, forming a barrel-like structure in the membrane.[22] This structure allows water and other molecules to pass freely through the interior of the pore, causing cell death. Ceratotoxin, a 36-mer insect-derived AMP, appears to operate according to this mechanism, based on ion-channel experiments[23]. The toroidal pore model was proposed due to discrepancies surrounding the mechanism of one particular peptide.

The troublesome peptide was magainin II, a naturally occurring, 21-mer alpha-helical AMP, originally isolated from the skin of the frog *Xenopus laevis.*[24] Prior to a study by Ludtke et al., magainin's

mechanism was known to be membrane permeabilizing, but there was controversy surrounding the specifics of the mechanism. Previous studies using oriented circular dichroism had determined that the helical peptide oriented itself perpendicular to the surface of the membrane, which indicated a pore-like mechanism, not a carpet-like mechanism. However, several techniques, including Raman and NMR, showed that the peptides were associating with the head groups of the lipids in the membrane, which is not a feature of the barrel-stave model. Using their additional studies involving neutron in-plane scattering, Ludtke et al. proposed the toroidal pore model.[25] The toroidal pore model is very similar to the barrel-stave model, except instead of insertion into the membrane, the peptides cause the membrane to bend, such that the peptides are always associated with the lipid head groups.[21] This forms a pore lined with both lipid head groups and peptides, again allowing for the free movement of water and other molecules, lysing the cell. Of naturally derived peptides, very few have been found to operate via the barrel-stave mechanism, the majority appear to follow the toroidal pore model.[22]

Peptides that belong to the last category of mechanism, translocation, simply cross the bacterial membrane without disrupting it, and then go on to interfere with some intracellular process. One peptide that operates by this mechanism is buforin II. Buforin II (BF2) is a 21-mer alpha-helical peptide isolated from the Asiatic toad *Bufo bufo gargarizans.*[26] It has previously been observed that proline-arginine rich peptides, such as BF2, act differently than other AMPs which cause lysis. This is because proline breaks up alpha helical structures, and non-helical shapes can have trouble forming pores. It had previously been shown that the proline-rich AMP, PR-39, does not form pores, but instead interferes with macromolecular synthesis inside the bacteria.[27] In their study, Park et al. examined the lytic properties of BF2 and magainin II using absorbance and optical density of the bacterial solution as an indicator of lysis. They found that while both killed bacteria, buforin II showed minimal cell lysis, even when at five times the minimum inhibitory concentration (MIC), yet magainin showed significant cell lysis under the same condition.[28] The group then used confocal microscopy and fluorescently

labeled peptides to determine the areas of localization in the bacteria. Magainin II stayed outside or on the cell wall of the bacteria, while BF2 accumulated inside the bacteria. The group also found that BF2 binds to DNA and RNA over 20 times more strongly than magainin, indicating that RNA might be BF2's target inside the cell. Peptides like buforin II which translocate the membrane are of interest as potential drug delivery vectors.

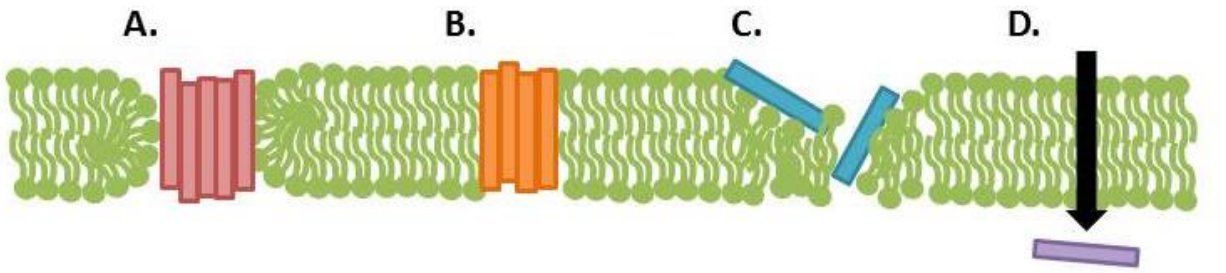

Figure 1. Common mechanisms of antimicrobial peptides. Peptides can interact with the bacterial membrane by inducing folding of the membrane, forming pores, but only interacting with the head groups of lipids, as in the toroidal pore mechanism (A), inserting perpendicularly into the membrane, forming regular pores and interacting with both head groups and tails of lipids, as in barrel-stave mechanism (B), laying parallel and generally disrupting the membrane, as in the carpet mechanism (C), or translocating the membrane without disruption (D).

Each of these mechanisms requires an amphipathic charge distribution about the helix of the peptide. In order for the peptide to interact with the water and the head groups of the lipids, the peptide needs hydrophilic regions. But the peptide must also be able to interact with the lipid tails, which are hydrophobic. For peptides which insert themselves into the membrane, conformational flexibility is required to expose hydrophilic and hydrophobic regions as necessary.[29] In the case of BF2, this flexibility comes from the presence of a proline residue, which gives a slight kink to its overall helical conformation. This proline has been shown to act as a 'hinge' which allows it to cross the membrane without disrupting it.[30] The position of this hinge is also important, as it alters the alpha-helical content and presumably the special relationship of the hydrophobic and hydrophilic regions.[31] Dathe et al.

created some model amphipathic peptides, and found that for surfaces with low negative charge, hydrophobic helix domains are more important than overall charge for binding and permeabilizing the membrane.[32] Yet in some helical peptides, such as V13K, when hydrophobicity is increased, hemolytic activity as well as self-aggregation increased.[33]

Methods for Mechanism Determination

Structural Measurements

The technique of oriented circular dichroism (OCD), mentioned earlier, can be used to determine the orientation of an alpha-helical peptide relative to the surface of the membrane. Circular dichroism (CD) involves left and right circularly polarized light. The left and right polarized light is then differentially absorbed by the chiral components of the peptide (often the alpha-helices or beta-sheets), and the difference in absorbance is measured as a function of wavelength. Purely alpha-helical and purely beta-sheet peptides give characteristic spectra, which can be compared to the spectra of the experimental peptide to determine approximate alpha-helical or beta-sheet content. In OCD, membranes with associated peptides are arranged in a multilayer array and sandwiched between two quartz plates.[34] Then the CD spectra are taken with light at both the normal and oblique angles with respect to the membranes. If and alpha-helical peptide is oriented perpendicular to the membrane, the normal angle spectra observed will be that of a characteristic alpha helix. As the peptide diverges from perpendicular, the normal angle spectra will be less and less like the characteristic shape. For the purposes of my thesis, I will be taking CD spectra, which will allow me to examine the effects of fragmentation on the secondary structure, and correlate this structure with mechanism and activity results. For example, if one fragment did not contain significant alpha-helical or beta sheet content, it might explain lowered activity if all other fragments were alpha-helical and displayed significant activity.

Determination of membrane permeabilization

Many peptide mechanism assays involve either bacteria (in vivo) or lipid vesicles (in vitro). Bacterial measurements better mimic actual peptide-pathogen interactions inside the body, but bacteria contain additional enzymes, proteins, carbohydrates, etc., that can interfere with the experiment. Lipid vesicles, on the other hand, are completely fabricated so the researcher can manipulate their contents. Of course, being artificial, lipid vesicles are not very accurate representations of pathogens. Often, these methods are used in combination.

One assay that is used to detect membrane leakage, not peptide orientation, is a fluorescence quenching assay involving 8-Aminonaphthalene-1,3,6-Trisulfonic Acid(ANTS) and *p*-Xylene-Bis-Pyridinium Bromide(DPX). ANTS is an anionic fluorophore, whose fluorescence can be quenched by cationic DPX. ANTS is encapsulated in lipid vesicles while the DPX and peptide are added outside of the vesicles, while fluorescence is monitored.[35] If the peptide permeabilizes the membrane, the ANTS will be able to leak out of the vesicle and combine with the quencher, DPX, which will reduce the overall fluorescence. There are many similar assays involving other fluorophore/quenching pairs, such as terbium/dipicolinic acid.[36]

Another membrane permeabilization assay uses bacteria, not vesicles, which are closer to *in vivo* conditions than synthetic vesicles. In this assay, *E. coli* bacteria have been modified to produce the enzyme β-galactosidase, which digests the substrate ortho-Notrophenyl-β-galactoside (ONPG), which are not produced inside the bacteria, and neither enzyme nor substrate can cross the bacterial membrane alone. The bacteria containing β-galactosidase are placed in solution with both ONPG and peptide. If the peptide permeabilizes the membrane, the enzyme will begin digesting the ONPG, producing a yellow product (Figure 2). The absorbance of the solution is monitored over the course of the experiment, and can be used to determine the degree of permeabilization.[36]

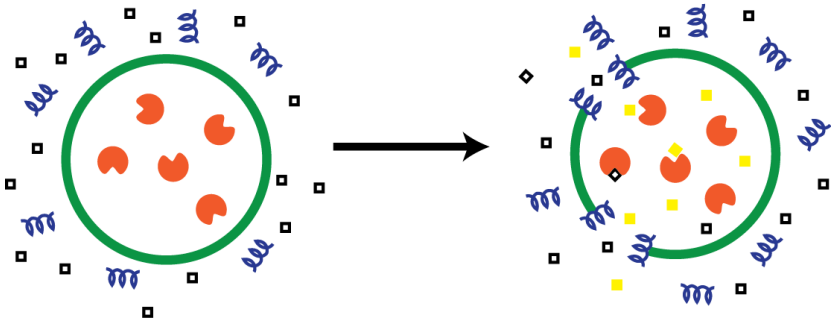

Figure 2. Schematic of β-galactosidase assay for membrane permeabilization. White squares represent undigested ONPG, the green circle represents the bacterial membrane, purple helices represent peptide, orange semi-circles are β-galactosidase enzyme, and yellow squares are digested ONPG.

Somewhat similarly, the propidium iodide (PI) leakage assay involves *E. coli* bacteria in solution with peptide and PI. PI is a DNA intercalator, which fluoresces when bound to DNA, but it cannot cross through the bacterial membrane. So if the peptide permeabilizes the membrane, the PI can get inside the cell and interact with its DNA, which results in an increase in fluorescence (Figure 3). The relative fluorescence increase is used as a quantitative measurement of membrane permeabilizing ability.[37]

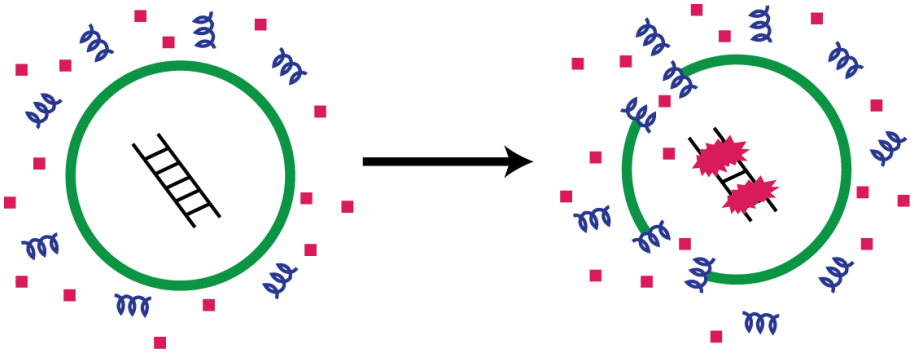

Figure 3. Schematic of propidium iodide assay involving a membrane permeabilizing peptide. Pink squares represent propidium iodide molecules, the green circle is a bacterial membrane, the ladder is bacterial DNA, and purple helices represent peptides.

Measurements of Membrane Translocation

Translocation can also be directly measured using a trypsin-digestion assay in which trypsin, an enzyme which cleaves peptide bonds, is encapsulated inside lipid vesicles. The lipid vesicles include with a fluorescently labeled lipid. These vesicles are placed in solution with a trypsin inhibitor, called Bowman-Birk inhibitor (BBI), as well as the peptide, containing at least one tryptophan. The inhibitor prevents the peptide from being digested by the trypsin outside of the vesicle. As the peptide associates with the membrane, fluorescence is measured as a function of the Foster Resonance Energy Transfer (FRET) between the tryptophan residues and the fluorescently labeled lipid. If the peptide operates via cell lysis, the peptide will remain associated with the membrane, and the fluorescence will remain constant. If the peptide translocates to the interior of the vesicle, it will be digested by the trypsin, and fluorescence will decrease.[38] The results from these vesicles are compared to those from a set of control vesicles, which are identical to the experimental vesicles, except they contain BBI both inside and outside the vesicle, to correct for incomplete inhibition of trypsin (Figure 4).

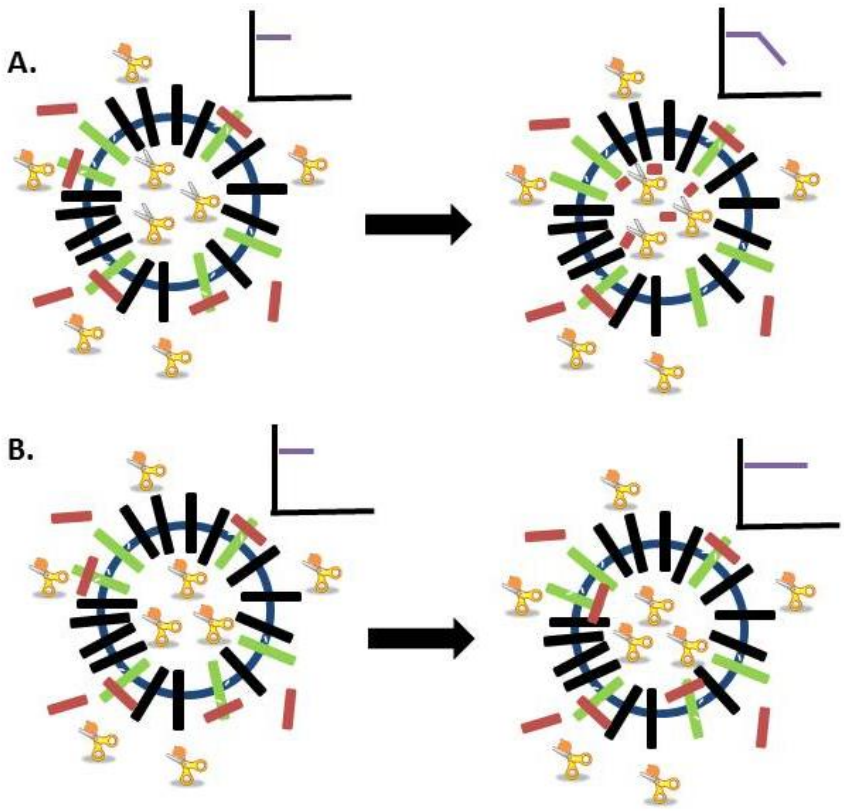

Figure 4. Schematic of trypsin digestion assay for translocation measurements. Row A represents the experimental condition, while row B represents the control condition. The small scissors represent trypsin, the red blocks represent peptide, the orange circles represent trypsin inhibitor, and the green squares represent DANS-PE in the lipid membrane. Small graphs represent fluorescence traces over the course of the experiment.

Translocation can also be observed in vivo using various microscopy-based methods. One such method involves confocal laser microscopy. In this assay, *E. coli* cells are incubated with biotin-labeled peptides. Streptavidin-FITC, which binds to biotin and fluoresces, is added then the cells are placed on a slide and fluorescence is examined using a confocal microscope. If the peptide is bound to the membrane, fluorescence will only occur along the membrane, and only the outlines of cells will be visible. However, if the peptide translocates, the peptide will have passed through the membrane to the interior of the cell, and the entire cell will fluoresce.

Modular Peptides

From a peptide-design perspective, having a set of modular peptides would be extremely useful. One could mix and match domains depending on the intended target or function. Lytic domains could be strung together to create extremely lytic peptides. One can also imagine scenarios where attaching a translocating peptide domain to a small molecule could allow for entry into a cell, or a peptide domain that inserts itself into the membrane could be attached to a fluorophore and used for cell tagging. Modular peptides would allow for customization not only of AMPs, but also many other therapeutic agents.

Antimicrobial peptides have previously been analyzed using a similar modular approach. One study by Yeaman et al. examined an AMP found in mammallian platelets, breaking it up by secondary structure into smaller 15-mer pieces. They found that one particularly active alpha-helical piece, the C-terminus, could act independently of the parent molecule when in a blood matrix. This domain appears to be easily accesible to proteases, which may indicate that the peptide could "release" that domain accoding to the environmental factors, allowing for continued antibacterial acivity, even in harsh conditions.[39] Hence, a modular approach could be used to create therapies that can remain active, even when proteases are present.

Histone-Derived AMPs

Given that BF2 shares high sequence homology with the histone H2A protein, BF2's RNA and DNA binding abilities are not very surprising. Histone proteins are an integral part of DNA storage in cells, acting as spools for strands of DNA. Similarly, BF2's DNA binding ability is also essential for its function. When crucial DNA-binding residues of BF2 are altered, there is a marked drop in antimicrobial

activity.[40] Other AMPs have been discovered that share high sequence homology with BF2, but do not seem to make use of its DNA binding capabilities as a part of their mechanism of killing.

One such peptide is hipposin, a 51-mer AMP isolated from the skin of Atlantic halibut (*Hippoglossus hippoglossus*).[41] Hipposin is also derived from histone H2A, and the middle section of hipposin shares high sequence homology with BF2 (Table 1). Yet, preliminary studies by myself and others in the Elmore lab indicate that, despite similar antimicrobial activities, the two peptides seem to operate by entirely different mechanisms. Buforin II, as we know, translocates the membrane and then binds to DNA, but hipposin appears to kill bacteria through one of the membrane permeabilizing mechanisms. Clearly, the other portions of hipposin somehow alter the mechanism of this peptide from translocating to permeabilizing.

HIP	KGRGKTGGKARAKWKTRSSRAGLQWPVGRVHRLLRKGNYAHRVGAGAPVWL
BF2	TRSSRAGLQWAVGRVHRLLRK
BF1	KGRGKQGGKVRAKWKTRSSRAGLQWPVGRVHRLLRKGNY
PARA	KGRGKQGGKVRAKWKTRSS
CTERM	GNYAHRVGAGAPVWL
BF2+CTERM	TRSSRAGLQWPVGRVHRLLRKGNYAHRVGAGAPVWL

Table 1. Primary sequences of hipposin, buforin II, buforin I, parasin, the c-terminal region, and buforin II plus the c-terminal region.

Several other naturally-derived peptides may shed light on this question (Figure 5). Buforin I (BF1) is a 39 residue AMP which, like BF2, was isolated from the asiatic toad[26], and shares high sequence homology with the first two-thirds of hipposin. Parasin, a 19-mer AMP from catfish[42] , is very similar to the first third of hipposin. Rounding out this set of peptides are two designed peptides, one analagous to the C-terminus of hipposin (C-term), and one comparable to buforin II combined with the C-terminus of hipposin. By characterizing the mechanism of action for all of these hipposin fragments, we can determine whether the mechanism of hipposin can be considered in a modular fashion. In other words,

do different parts of hipposin promote it working via a different mechanism. Taking a modular approach to investigating this family of peptides, peptide design could be greatly simplified. By identifying which portions of a peptide are associated with a particular mechanism, peptides could be designed for specific purposes. Drug-delivery peptides could be designed to translocate, while new antibiotics could be developed from extremely lytic as well as translocating peptides.

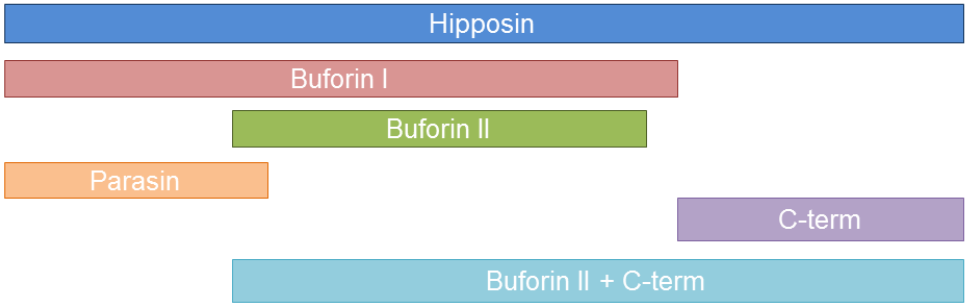

Figure 5. Schematic of a family of histone-derived peptides, aligned according to sequence homology.

This thesis will examine this family of histone-derived peptides using a modular approach. By examining their secondary structures, antimicrobial activities, and mechanisms, I examine the properties of each domain, as well as combinations of domains. A modular examinination of these peptides could lead to not only more rational peptide design, but allow for customization of other theraputics as well.

Goals of Thesis

In this thesis I examine the histone H2A family of antimicrobial peptides, including HIP, BF2, BF1, PARA, CTERM, and BF2+CTERM. I examined their secondary structures of BF2, BF1, and PARA using circular dichroism spectroscopy. I investigaged the membrane permeabilixzing abilities of all of these peptides using the propidium iodide assay, and explored permeabilizing abilities of BF2, BF1, and PARA further using the β-galactosidase assay. I also examined all of the peptides' translocating abilities using

the trypsin digestion assay. Lastly, I examined their antimicrobial activities against E. coli bacteria using the radial diffusion assay.

Materials and Methods

Peptides

Buforin II, buforin I, parasin, hipposin, as well as two hipposin fragments, described in table (1), were synthesized and purified to >95% purity by NeoBioSci (Cambridge MA.) Hipposin's two native tyrosine residues were mutated to tryptophan. Peptide concentration was determined using the average of three tryptophan absorbance readings at 280 nm. Peptides were dissolved in nanopure H_2O and stored at −20°C. Absorbance was measured using either a Thermo Scientific Nanodrop 2000 (Wilmington, DE) or a Biorad SmartSpec Plus spectrophotometer (Philadelphia, PA).

Circular Dichroism Spectroscopy

Circular dichroism spectra were collected using an Olis DSM 20 circular dichroism spectrometer (Bogart, GA) from 195 to 250 nm in 1 mm path-length quartz cuvettes (Atascadero, CA). Peptides were at a concentration of 25 µM in a 1:1 solution of trifluoroethanol : phosphate buffer (10 mM Na_3PO_4, 45 mM NaCl, 1 mM EDTA, pH 7.4). Reported spectra are an average of 3 scans collected at 25^{o}C with an integration time of 60s at a bandpass of 1.24mm.

Determination of Relationship between Colony-Forming Units and Optical Density

Bacterial colonies were picked from frozen stock and grown in either Luria broth (LB) (20% w/v, Sigma, St. Louis, MO) or Tryptic Soy Broth (TSB) (30% TSB w/v) overnight. The overnight culture was diluted 1:500 into new media and allowed to grow to an optical density between 0.2 and 0.5 at 600 nm. The solution was serially diluted into new media, and 100µL aliquots of the $1:10^4$, $1:10^5$, and $1:10^6$ dilutions were plated on LB agar (20%LB w/v, 10% bacto agar w/v, Sigma, St. Louis MO). The plates were incubated at 37°C overnight and colonies were counted and used to determine the relationship between optical density and colony forming units per mL. This process was repeated three times, and conversion

factors were averaged. The conversion factor of $2.5*10^8$ OD600/CFU for *Escherichia coli* (ATCC #2912) was used for all evaluations of bacterial concentrations[43].

Propidium Iodide Uptake Assay

E. coli (ATCC #2912) bacteria picked from frozen stock were grown overnight in TSB media (30%w/v TSB) at 37°C. The overnight culture was diluted 1:1000 in fresh TSB and allowed to grow 2.5 hours to mid-log phase. Bacteria were pelleted via centrifuge at 880 x g for 10 minutes at 4°C, washed once with 10 mM phosphate buffer (10mM Na_3PO_4, pH 7.4), pelleted again, and resuspended to an optical density of 0.5. Propidium iodide was added to a concentration of 20 µg/mL and the system was allowed to equilibrate. PI/DNA complexation was measured at an excitation of 535 nm and emission of 617 nm on a Varian Cary Eclipse fluorescence spectrophotometer (Agilent Technologies, Santa Clara, CA). Following equilibration, peptide solution was added to a concentration of 2 µM and fluorescence was monitored until new equilibrium was reached. Increase in fluorescence due to the peptide's presence was measured by comparing the fluorescence 5 minutes after peptide addition to the averaged fluorescence one minute before peptide addition. Averages of at least three independent experiments are reported. Comparisons were made using a one-tailed student's t-test.

β-galactosidase Assay

E. coli ML-35 cells (ATCC #43827) picked from frozen stocks and grown overnight in TSB media (30%w/v TSB) at 37 °C. The overnight culture was diluted 1:1000 in fresh TSB and allowed to grow approximately 2.5 hours to an OD600 of 0.55-0.65, then pelleted and resuspended in 10 mM sodium phosphate buffer at pH 7.4, which was repeated. The cells were then pelleted and resuspended in buffer containing 1.67 mM *ortho*-nitrophenyl-β-galactoside (ONPG) (Sigma Aldrich, St. Louis, MO), 10mM Sodium Phosphate, at pH 7.4. Absorbance was then measured at 420 nm every 30 seconds over the course of 45 minutes using an Agilent 8453 UV-Vis spectrophotometer (Agilent Technologies, Santa Clara, CA). At ten minutes,

peptide is added to a concentration of 2 µM. Permeabilization is reported as the slope five to six minutes after peptide is added, divided by the slope over the first five minutes of the experiment. Results displayed are averages of at least three separate measurements. Comparisons were made using a one-tailed student's t-test.

Large Unilamellar Vesicle (LUV) Preparation and Trypsin Digestion Translocation Assay

Phospholipids dissolved in chloroform were obtained from Avanti Polar Lipids (Alabaster, AL). Mixtures of phosphtidylcholine (POPC), phosphatidylglycerol (POPG), and 5-dimethylaminonapthlene-1-sulfonyl phosphatidylethanolamine (DNS-POPE) were made in a 50:45:5 ratio. Chloroform was evaporated using a nitrogen gas stream. Lipid cakes were desiccated overnight. The vesicles were rehydrated in either HEPES buffer (10mM HEPES, 45mM NaCl, 1 mM EDTA, pH 7.4) containing 0.2mM porcine trypsin (Sigma Aldrich #T-0303, St. Louis, MO) for the experimental condition or in HEPES buffer containing both 0.2mM porcine trypsin as well as 2.0mM Bowman-Birk trypsin inhibitor (BBI) (Sigma Aldrich #T-9777) for the control. Vesicles were then subjected to 5 freeze-thaw cycles before extrusion through a nucleopore track etch membrane with 0.1 µm pores (Whatman) for uniform vesicle size.

To remove phosphorus salts from control vesicles, an estimated 0.3µM of control vesicles were spun at 13,000rpm for ten minutes three times in Pall Corporation Nanosep 10K Omega™ microcentrifuge tubes and rinsed with HEPES buffer after each spin.

Vesicle concentration was determined by measuring phosphorus content in triplicate. Six standards of 0.0 µmoles. 0.0325 µmoles, 0.065 µmoles, 0.114 µmoles, 0.163 µmoles, and 0.228 µmoles of phosphorus (KH_2PO_4), as well as an estimated 0.1 µmoles of vesicles were added to 450 µL of 8.9M H_2SO_4 (Fisher Scientific, Pittsburg, PA) then heated for 25 minutes at 175-200°C. Afterwards, 150 µL of H_2O_2 was added to each solution, then all were headed for an additional 30 minutes at 175-200°C. After cooling briefly, water, ammonium molybdate, and ascorbic acid were added to each solution. Solutions

were then boiled in a water bath for 5-6 minutes. Absorbance at 280nm was determined for each solution, and a linear standard curve was found using the linear fit mode of Microsoft Excel. The standard curve was then used to determine the phosphorus content of the vesicles.

Vesicles were then diluted to 0.25 mM in a solution with trypsin inhibitor at ten times the trypsin concentration. 2 µl of peptides prepared at $2.5x10^4$ M was added to each well of an opaque 96 well plate (VWR, Radnor, PA). 200 µL of vesicle solution was added to each well, and fluorescence was monitored for 25 minutes with an excitation wavelength of 280nm and an emission wavelength of 525nm, using a SpectraMax M3 multi-mode microplate reader (Molecular Devices, Sunnyvale, CA). Percent fluorescence decrease was determined by dividing the average fluorescence over the final minute of the experiment by the fluorescence at t=0. Translocation of the experimental condition over the control condition was calculated using the following formula[43]:

$$\text{Translocation ratio} = \frac{\frac{F_{control}\ \text{final minute}}{F_{control}\ \text{initial}}}{\frac{F_{exp}\ \text{final minute}}{F_{exp}\ \text{initial}}}$$

Radial Diffusion Assay

The radial diffusion assay was performed using one gram-negative bacteria (*Escherichia coli* ATCC # 25922). Cells picked from frozen stocks were incubated overnight at 37°C in 30% w/v tryptic soy broth. The overnight culture is then diluted 1:500 in new TSB and grown for 2.5h. Bacteria were pelleted via centrifuge at 880 x g for 10 minutes at 4°C, washed once with 10 mM phosphate buffer (10 mM Na_3PO_4, pH 7.4), then pelleted again and resuspended. 4 $x10^6$ CFU of bacteria are mixed with 10 mL of molten agarose gel (10 mM Na_3PO_4, 1% TSB v/v, 1% agarose w/v, pH 7.4) and allowed to solidify on a petri dish. Wells were formed in the solid media using a pipette attached to a bleach trap, 2µL of 1 x 10^{-4}M peptide

solution was added to each well, and plates were incubated at 37°C for 3 hours. 10mL of overlay gel (30% w/v TSB, 1% w/v agarose) was poured over the underlay gel and incubated for 12-18 hours at 37°C. The diameter of bacterial clearing around each well was measured at 7x magnification. Data was collected from at least three independent plates. Comparisons were made using a one-tailed student's t-test.

Results and Discussion

In addition to the naturally-derived peptides BF2, BF1, and parasin (PARA), the peptide hipposin was broken modularly, resulting in two peptides resembling the C-terminal region (CTERM), as well as the C-terminal region connected to buforin II (BF2+CTERM) (Fig. xx). These two peptides, in addition to full hipposin peptide (HIP), contain mutations from tyrosine to tryptophan for use in translocation assays and to allow for more straightforward quantification. The mechanisms and the antimicrobial activity of these peptides were assessed.

Circular Dichroism Spectroscopy

In order to determine the secondary structure of the peptides, circular dichroism spectra of the peptides in a solution of 50% TFE and 50% phosphate buffer. BF2, as previously determined[30], appears to be largely alpha-helical (Figure 6). PARA on the other hand, seems to be at least partially alpha-helical, also corroborating with previous studies.[44] When the two peptides are combined to make BF1, the alpha-helicity appears to be an average of PARA and BF2, and very closely matches the weighted average of the PARA and BF2 spectra. This seems to indicate that, at least a secondary structure level, peptides will not interfere with each other to a great extent. The secondary structure of the BF2 portion of BF1 will have similar structure to BF2 alone. Thus, in terms of secondary structure, the peptides do seem to behave as modules.

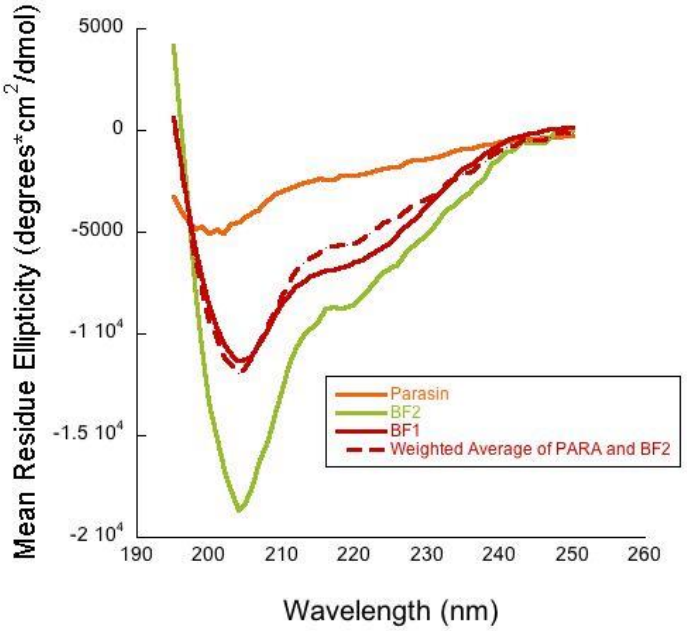

Figure 6. Circular dichroism spectra of peptides taken in a 50:50 TFE/phosphate buffer solution. CD spectra of PARA (orange), BF2(green), BF1 (red), and the weighted average of PARA and BF2 spectra (red dashes).

Membrane Permeabilization

Propidium Iodide

In order to examine the membrane permeabilizing ability of these peptides, Top 10 *E. coli* bacteria were grown to mid-log phase and washed repeatedly before the addition of propidium iodide (PI) , a DNA intercalator. The bacteria and PI were allowed to equilibrate for five minutes while fluorescence was monitored. Finally peptide was added and fluorescence was monitored for another 35 minutes. The relative fluorescence was determined by dividing the average fluorescence five minutes after peptide addition by the average fluorescence of the minute before peptide addition. Representative traces of the assay show a more membrane permeabilizing peptide (BF1) compared to a less membrane permeabilizing peptide (BF2) (Figure 7).

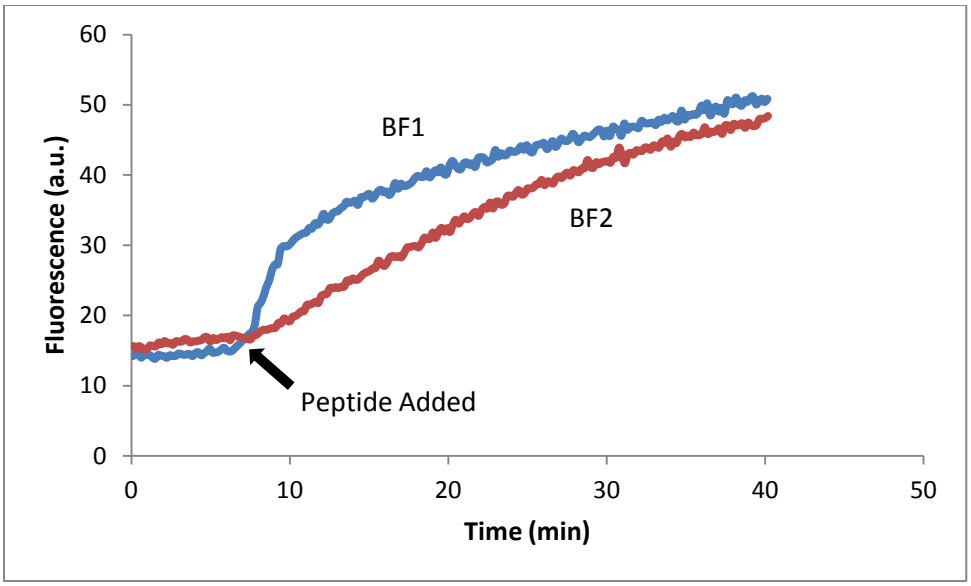

Figure 7. Representative traces of fluorescence from the propidium iodide assay done with two peptides, BF1 and BF2. Fluorescence increase is calculated by taking the average of the fluorescence five to six minutes after peptide addition divided by the average fluorescence before peptide addition.

BF2, the middle section of HIP, displays less membrane permeabilizing ability than magainin (MAG) ($p<0.11$), a well-characterized membrane permeabilizing peptide, but more than water ($p<0.005$) (Figure 8). PARA, the N-terminal region of HIP, shows much higher membrane permeabilizing ability than MAG ($p<0.07$). When the two are combined to make BF1, the membrane permeabilizing ability closely resembles PARA ($p<0.39$), not BF2 ($p<0.002$). This could mean that when combining two peptides, the new peptide will adopt the mechanism of the most membrane permeabilizing peptide.

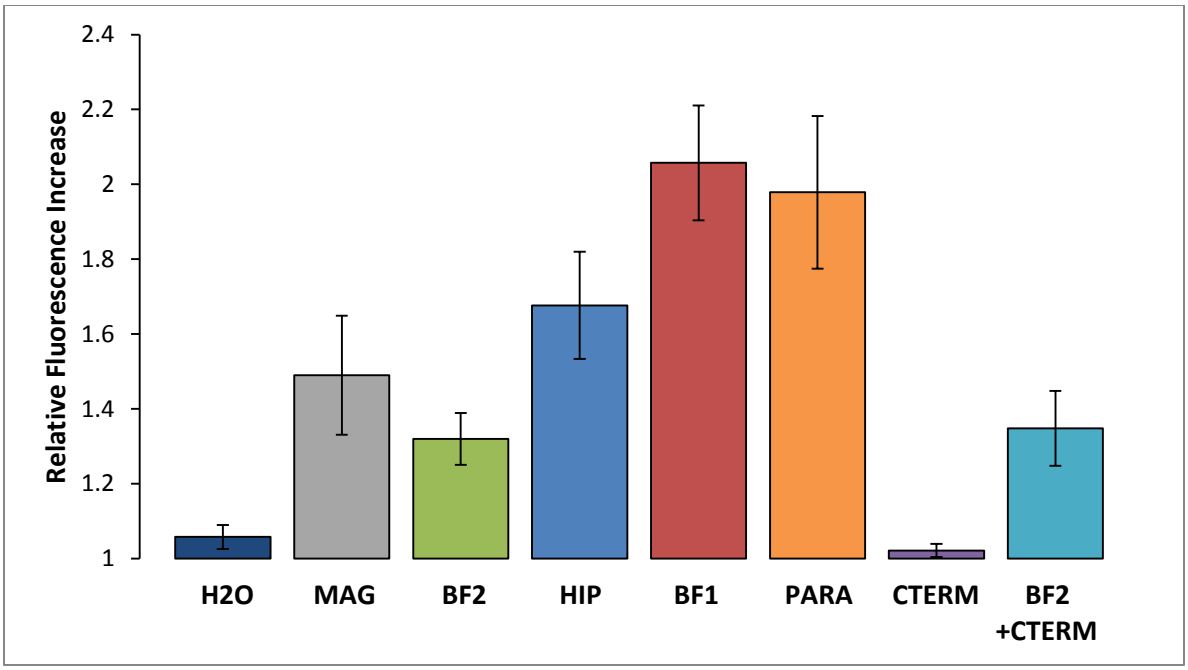

Figure 8. Membrane permeabilizing ability was measured using a propidium iodide uptake assay. Relative fluorescence increase is reported as the average fluorescence five minutes after peptide was added divided by the average fluorescence the minute before peptide was added. Average of at least three trials is shown with errors provided as standard error

This theory is reinforced by looking at the case of CTERM and BF2. Alone, CTERM displays no more membrane permeabilizing ability than water ($p > 0.17$). Yet when CTERM and BF2 are combined, the resulting peptide (BF2+CTERM) displays a membrane permeabilizing ability similar to that of BF2 ($p> 0.39$), the more lytic of the two peptides. This also appears to mean that a peptide's membrane permeabilizing ability cannot be 'dampened' through the addition of a non-membrane permeabilizing peptide.

These trends generally hold for HIP, although somewhat less quantitatively. HIP can be considered as a combination of PARA and BF2+CTERM or of BF1 and CTERM. If we look at HIP as a combination of BF1 and CTERM, we can see that while HIP does have a membrane permeabilizing ability closer to that of BF1 than of CTERM, it is somewhat lower than the permeabilization of BF1 ($p<0.07$).

Similarly, if we think about HIP as a combination of PARA and BF2+CTERM, the membrane permeabilizing ability is closer to PARA, but somewhat lower ($p<0.14$). Nonetheless, the resulting peptide's membrane permeabilizing ability appears to more closely resemble the more membrane permeabilizing fragment, but does not quite match it.

As can be seen in Figure 8, the propidium iodide assay is not very precise in determining the relative membrane permeabilizing abilities of more membrane permeabilizing peptides. We are unable to determine whether PARA or BF1 has higher membrane permeabilizing abilities ($p<0.39$), or even say with statistical certainty that HIP is lower in membrane permeabilizing ability than PARA ($p<0.14$). To try to more precisely differentiate between membrane permeabilizing abilities of peptides, we decided to employ the β-galactosidase assay.

β-galactosidase Assay

In order to determine more precisely the membrane permeabilizing abilities of our AMPs, *E. coli* ML-35 bacteria containing the enzyme β-galactosidase was grown to mid-log phase, washed repeatedly, then ONPG , a substrate of β-galactosidase, was added. The bacteria and ONPG were allowed to equilibrate for five to ten minutes while fluorescence was monitored, finally peptide was added and fluorescence was monitored for a total of 45 minutes. Relative absorbance slope increase is calculated by taking the slope of the absorbance from the fifth to tenth minutes after peptide addition, divided by the slope of the absorbance in the minutes before peptide addition (Figure 9). Rather than simply comparing average absorbance, slopes are compared in order to decouple the rate of membrane permeabilization from the rate at which the enzyme processes substrate.

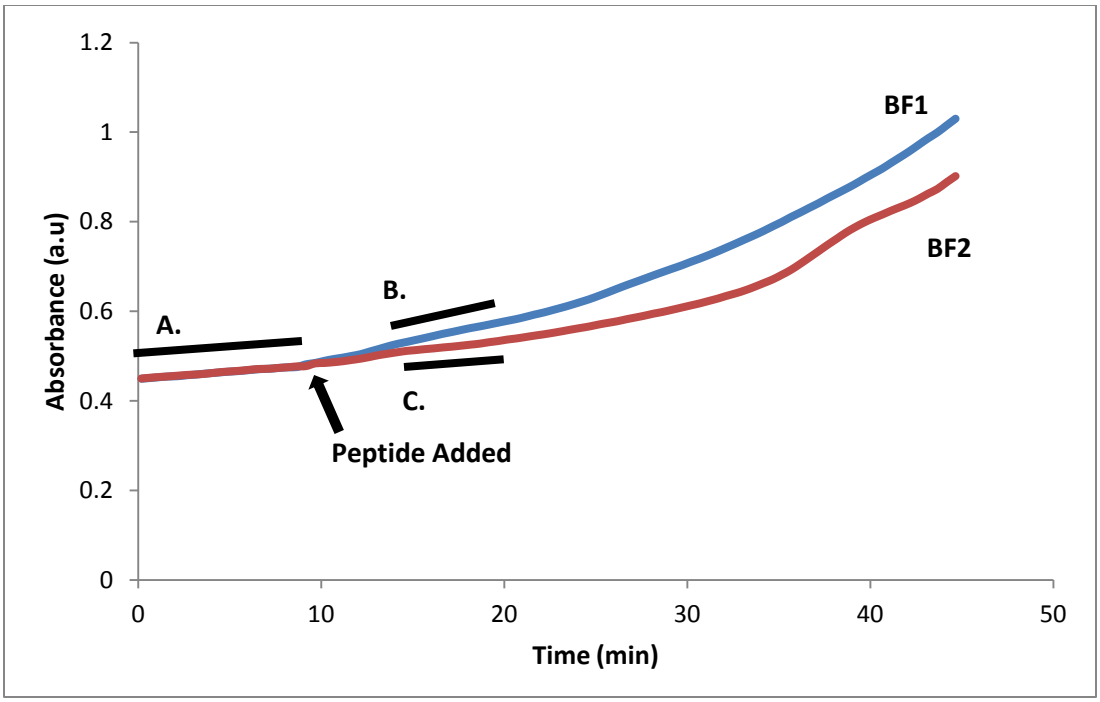

Figure 9. Representative traces of absorbance during a β-galactosidase assay, using peptides BF1 and BF2. Line A represents the slope before peptide is added, lines B and C represent the slopes over a five minute interval five minutes after peptides BF1 and BF2 are added, respectively. Results are reported as slope B or C divided by slope A to show increase in permeabilization due to peptide addition.

As expected, the results of the β-galactosidase assay showed similar trends to the propidium iodide assay (Figure 10). Combining BF2 and PARA to make BF1 still resulted in BF1 retaining the membrane permeabilizing ability of PARA. The method also provided slightly more precise determination of membrane permeabilizing abilities, but not significantly more. For example, instead of a p value less than 0.39 when comparing BF1 and PARA, the p value for the pair using this assay is less than 0.21 (Figure 10). Meanwhile, comparing BF2 and BF1 using the data from the propidium iodide assay gives a p value less than 0.002, but the same test using the β-galactosidase assay data gives a p value of less than 0.02(Figure 10), a full order of magnitude larger. However, this is less significant, given the fact that both represent a very high confidence level.

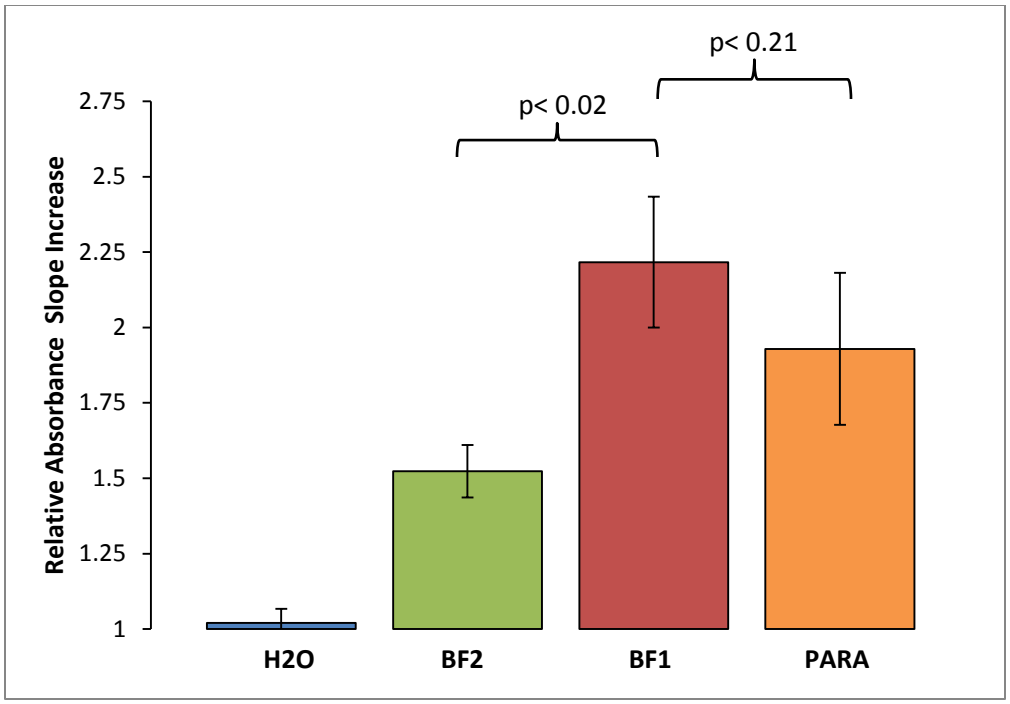

Figure 10. Membrane permeabilizing abilities of several AMPs as measured by the β-galactosidase assay. Relative absorbance slope increase is calculated by taking the slope of the absorbance from the fifth to tenth minutes after peptide addition, divided by the slope of the absorbance in the minutes before peptide addition. Results are the average of at least three trials, with standard error.

Thus, this assay is an improvement over the propidium iodide assay in terms of its ability to make comparisons between highly membrane permeabilizing peptides, but it if far from ideal. More replications as well as further investigation of data processing methods will allow us to further improve this assay.

Translocation

In order to determine the translocating ability of these peptides, a trypsin digestion assay was used. Trypsin, a peptide digesting enzyme, in encapsulated inside vesicles containing fluorescent lipids. Trypsin outside the vesicle is inhibited. Peptides are added and begin to associate with the fluorescent lipids in the membrane, causing high initial fluorescence through a FRET response between the

tryptophan residues in the peptide and the fluorescent lipids. If the peptide translocates, it will move to the inside of the vesicle and will be destroyed by the trypsin, decreasing the fluorescence. If the peptide remains associated with the membrane, as is usually the case for membrane permeabilizing peptides, it will not be destroyed by the trypsin, and the fluorescence should remain high. To control for incomplete inhibition of trypsin, control vesicles are prepared with inhibited trypsin both inside and outside the vesicle. Thus, any fluorescence decrease in this situation would result from the residual activity of inhibited trypsin inside the vesicle. The translocation ratio is determined by taking the decrease in fluorescence of the control vesicles and dividing it by the decrease in fluorescence of the experimental vesicles. In this assay, a translocation ratio greater than one is indicative of translocation. MAG, the membrane permeabilizing peptide that is known not to translocate[45], was used as a negative control.

Prior to this study, a translocation ratio greater than one had indicated translocation, while a translocation ratio of less than one indicated that the peptide did not translocate[46]. In one study by Pavia et al. examining the effects of proline to alanine mutations on designed peptides, a peptide called DesHDAP1 appeared to operate via a translocating mechanism, indicated by a translocation ratio of 1.13$\pm$0.08. The proline mutant of another designed peptide called DesHDAP3 P13A displayed significant membrane permeabilization, and minimal translocation, with a translocation ratio of 0.982$\pm$ 0.005[46]. These results show that the translocation assay can be used to demonstrate non-translocating behavior in membrane permeabilizing peptides.

However, the data for peptides in this study includes some extremely lytic peptides like BF1 and PARA showing evidence of translocation (Table 2). It may be that some of the peptides have dual mechanisms, but it is more likely that these peptides permeabilize the lipid membrane quickly and to such an extent that there is no longer any separation between the trypsin and the inhibited trypsin. This could also be due to the degree of permeabilization observed for some of these peptides, like PARA

(Figure 8). In the Pavia et al. study, the translocation assay demonstrated non-translocation for lytic peptides such as DesHDAP3 P11A, however that peptide had a permeabilizing ability on the order of MAG, not on the order of PARA or BF1[46]. Thus, this trypsin digestion assay for measuring translocation might not be suitable to assess translocation of extremely lytic peptides.

Peptides	Translocation Ratio
MAG	0.98 ± 0.01
BF2	1.08 ± 0.07
HIP	1.09 ± 0.06
BF1	1.26 ± 0.04
PARA	1.07 ± 0.06
CTERM	1.06 ± 0.09
BF2+CTERM	1.17 ± 0.11

Table 2. Translocating ability was measured using the trypsin digestion assay. Results displayed as the average translocation ratio ± range/2; n=2.

However, this assay is still a viable option for assessing the translocating abilities of peptides with lower membrane permeabilizing abilities, like buforin II and the C-terminus. Translocation appears to follow a similar trend to that seen for permeabilization for these peptides. Using the translocation assay, BF2 seems to translocate through the membrane, as reported in previous studies (Table 2).[28,30] CTERM also shows evidence of operating via a translocating mechanism, despite the large error. When the two peptides are combined into BF2+CTERM, the translocating ability seems to be preserved. The error range does not permit specific comparisons, but it seems to be true that combining two translocating peptides will not eliminate translocating ability. Because of the concerns about using this assay with extremely membrane permeabilizing peptides, we cannot address the effects of combining a permeabilizing and translocating peptide. More replications are needed before fully conclusive statements can be made about the translocating abilities of BF2, CTERM, and BF2+CTERM.

Antimicrobial Activity

In order to determine the antimicrobial activity of these peptides, a radial diffusion assay was used. Top 10 *E. coli* bacteria were grown to mid-log phase, washed repeatedly, then combined with a molten agarose mixture and allowed to gel. Wells were punched in the gel and filled with peptide or water, the agar/peptide layer was covered with another agarose layer containing TSB and incubated overnight. The diameter of clearance around these wells was measured, with a larger diameter indicating an ability to kill bacteria at a lower concentration.

Unlike physiological conditions, this assay takes place in a solid medium, which can hinder molecule movement. Bacteria are also in an anaerobic environment, which is not ideal for bacteria growth. Thus, the results might not predict the true *in vivo* effectiveness of these peptides. Additionally, though solid agarose is porous, it is a polar molecule, and might interfere with peptides' mechanisms of action. However, it is a good way to learn about the relative antibacterial efficacy of these peptides.[47]

BF2 has the highest diameter of clearance of all the peptides studied, which means that it can kill bacteria at the lowest concentration (Figure 11). Given its low membrane permeabilizing ability as well as its ability to translocate, BF2 appears to kill bacteria primarily though translocation, as seen in previous studies[28].

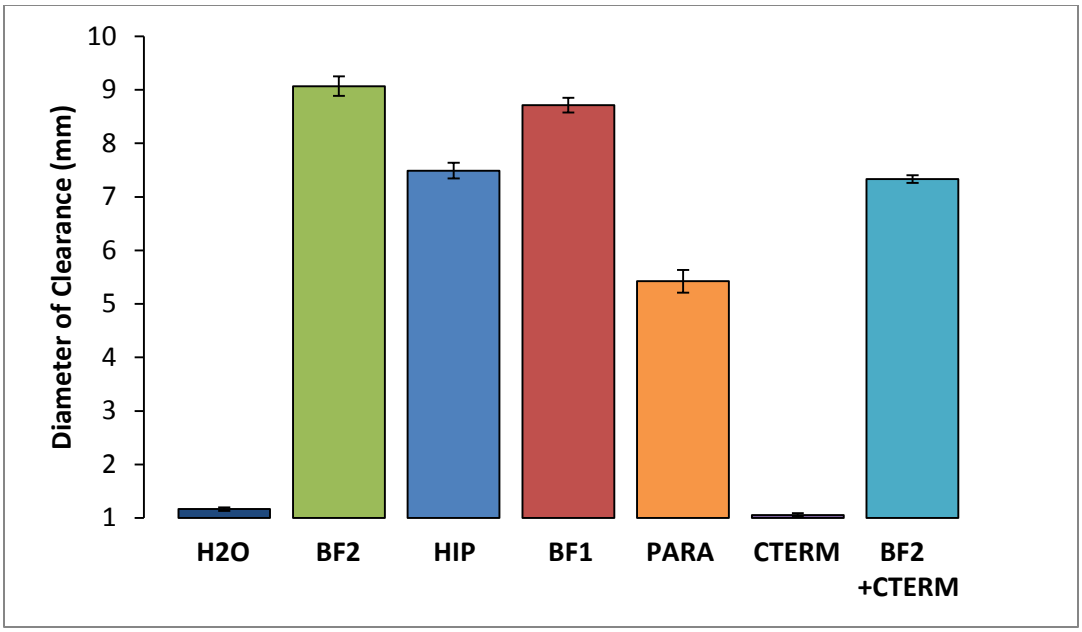

Figure 11. Antimicrobial activity of peptides was assessed against *E. coli* using a radial diffusion assay. Average of at least nine wells per peptide over three independent days. Error bars represent standard error.

While the membrane permeabilizing data had shown that PARA has a similar membrane permeabilizing ability to BF1, however, they display differing levels of antimicrobial activity. This could indicate one of two things. Either BF1 has a secondary mechanism, like translocation, or PARA and BF1 operate by differing membrane permeabilizing mechanisms and BF1's mechanism allows for more bacterial killing at a lower concentration. For example, if the two peptides operated via a pore-forming mechanism, since BF1 includes twice as many residues as PARA, it might be able to form pores with fewer peptides than PARA, which would allow it to kill the bacterial cells at lower concentrations. It could also be the case that BF1 is able to form larger pores than PARA, which would increase cell leakage.

The other possibility, that BF1 operates through a combination of translocation and membrane permeabilization, would not be measured through our current set of assays, as the translocation assay

may produce a false positive for extremely lytic peptides. One alternative method involves confocal microscopy. This type of microscopy allows the researcher to examine individual layers on a slide. This means that bacteria can be imaged in lateral slices, as opposed the through-and-though images obtained in conventional microscopy. This can be applied to AMP mechanisms by taking peptides labeled with biotin, then allowing the peptides to equilibrate with bacterial cells, then adding avidin, which binds to the biotin on the peptides, and fluoresces. By looking at the localization of the fluorescence, one can determine the general mechanism of the peptide. If the fluorescence is only along the membrane of the bacteria, the peptide is inferred to be membrane-permeabilizing. If the fluorescence fills in the entire bacteria, the peptide is inferred to be translocating. Other studies in the Elmore lab have shown that BF1 nearly always appears localized in the membrane of bacteria, which would indicate a solely membrane permeabilizing mechanism.[48] However, this method is only qualitative, not quantitative. In the future, we hope to develop a quantitative assay that would be able to measure translocation and permeabilization simultaneously so that we could determine if a peptide had dual mechanisms.

Somewhat similarly to BF1, HIP also might operate using a combination of mechanisms. HIP is slightly less membrane permeabilizing than PARA, but it is much more active. This means that though HIP appears to operate primarily via membrane permeabilization, it might also be able to translocate, or like BF1, it could simply have a membrane permeabilizing mechanism that allows it to kill bacteria at a lower concentration than PARA. Looking at HIP's activity from a modular standpoint, it appears that activity preservation behaves similarly to permeabilization preservation. When BF1 and CTERM are combined to make HIP, the activity of HIP is much closer to BF1, but it is significantly less than BF1 ($p<0.00001$). This might imply that activity can be diminished by adding an inactive region to an active peptide. Similarly, if HIP is considered as the combination of BF2 and PARA, the resulting activity also seems to be an average of the two.

Future Directions

To further examine the modular nature of this family of peptides, different combinations of peptides within the histone H2A family should be examined. For example, PARA and CTERM could be added together to reinforce the hypothesis that the new peptide will have the membrane permeabilizing ability of the more permeabilizing parent, in this case PARA. Additionally, repeating peptides, such as BF2+BF2, would be helpful to determine if the relationship between the number of translocating domains and the translocating ability is linear. The same could be done with PARA+PARA and membrane permeabilization. It would also be useful test the effects of domains such as PARA on peptides outside the histone H2A family. PARA could be added to MAG to see if membrane permeabilization increased. It could also be added to non-membrane permeabilizing peptides such as DesHDAP1, to see if addition of a lytic domain could induce greater membrane permeabilizing ability.

But, to be able to meaningfully compare these peptides, assays with more exact reproducibility must be developed. In order to be able to distinguish between highly membrane permeabilizing peptides, data analysis of the β-galactosidase assay will continue to be developed. Other members of the Elmore lab are also developing a high-throughput version of the propidium iodide assay. Thus, one could conceivably have enough replicates to determine statistical significance between peptides more easily despite variation between individual assays.

For translocation, further replications of the trypsin digestion assay are necessary to make significant conclusions about the relationship between the translocating abilities of BF2, CTERM, and BF2+CTERM. For the other peptides, investigation is required concerning why this assay may produce a false positive in some cases, especially since it appears to give reliable results for some permeabilizing peptides, but not others.

Ideally, there would be an assay that could measure membrane permeabilizing ability and translocating ability simultaneously, which would determine if peptides such as BF1 and HIP operated via dual mechanisms. Every translocating and membrane permeabilizing assay mentioned in this thesis relies (at least temporarily) on separation of the contents inside the membrane from the solvent outside the membrane. However, it is an inherent characteristic of membrane permeabilizing peptides to destroy the integrity of a lipid membrane, which can make it difficult for any differential between the inside and the outside of the membrane to be maintained over the course of the assay. Thus any possible solutions would likely be even further removed from *in vivo* conditions.

Literature Cited

1. Diseases, N. I. o. A. a. I., *Methicillin-Resistant Staphylococcus aureus (MRSA) History*. 2008.
2. Neu, H. C., THE CRISIS IN ANTIBIOTIC-RESISTANCE. *Science* **1992,** *257* (5073), 1064-1073.
3. Blumberg, H. M.; Rimland, D.; Carroll, D. J.; Terry, P.; Wachsmuth, I. K., RAPID DEVELOPMENT OF CIPROFLOXACIN RESISTANCE IN METHICILLIN-SUSCEPTIBLE AND METHICILLIN-RESISTANT STAPHYLOCOCCUS-AUREUS. *J. Infect. Dis.* **1991,** *163* (6), 1279-1285.
4. Centers for Disease Control and Prevention, F. a. D. A., National Institues of Health *2011 Progress towards the Implementation of: A Public Health Action Plan to Combat Antimicrobial Resistance*; 2011; pp 3-5.
5. McManus, M. C., Mechanisms of bacterial resistance to antimicrobial agents. *Am. J. Health-Syst. Pharm.* **1997,** *54* (12), 1420-1433.
6. Drlica, K.; Zhao, X. L., DNA gyrase, topoisomerase IV, and the 4-quinolones. *Microbiol. Mol. Biol. Rev.* **1997,** *61* (3), 377-+.
7. Tenover, F. C., Mechanisms of antimicrobial resistance in bacteria. *Am. J. Infect. Control* **2006,** *34* (5), S3-S10.
8. Segura, A.; Moreno, M.; Molina, A.; Garcia-Olmedo, F., Novel defensin subfamily from spinach (Spinacia oleracea). *FEBS Lett.* **1998,** *435* (2-3), 159-162.
9. Hultmark, D.; Engstrom, A.; Bennich, H.; Kapur, R.; Boman, H. G., INSECT IMMUNITY - ISOLATION AND STRUCTURE OF CECROPIN-D AND 4 MINOR ANTIBACTERIAL COMPONENTS FROM CECROPIA PUPAE. *Eur. J. Biochem.* **1982,** *127* (1), 207-217.
10. Taguchi, S.; Maeno, M.; Momose, H., EXTRACELLULAR PRODUCTION SYSTEM OF HETEROLOGOUS PEPTIDE DRIVEN BY A SECRETORY PROTEASE INHIBITOR OF STREPTOMYCES. *Appl. Microbiol. Biotechnol.* **1992,** *36* (6), 749-753.
11. Ganz, T.; Selsted, M. E.; Szklarek, D.; Harwig, S. S. L.; Daher, K.; Bainton, D. F.; Lehrer, R. I., DEFENSINS - NATURAL PEPTIDE ANTIBIOTICS OF HUMAN-NEUTROPHILS. *J. Clin. Invest.* **1985,** *76* (4), 1427-1435.
12. Gudmundsson, G. H.; Agerberth, B.; Odeberg, J.; Bergman, T.; Olsson, B.; Salcedo, R., The human gene FALL39 and processing of the cathelin precursor to the antibacterial peptide LL-37 in granulocytes. *Eur. J. Biochem.* **1996,** *238* (2), 325-332.
13. Ohsaki, Y.; Gazdar, A. F.; Chen, H. C.; Johnson, B. E., ANTITUMOR-ACTIVITY OF MAGAININ ANALOGS AGAINST HUMAN LUNG-CANCER CELL-LINES. *Cancer Res.* **1992,** *52* (13), 3534-3538.
14. Microbiology, U. D. o. P., Antimicrobial Peptide Database. 2013.
15. Sato, H.; Felix, J. B., Peptide-membrane interactions and mechanisms of membrane destruction by amphipathic alpha-helical antimicrobial peptides. *Biochim. Biophys. Acta-Biomembr.* **2006,** *1758* (9), 1245-1256.
16. Storm, D. R.; Rosenthal, K. S.; Swanson, P. E., POLYMYXIN AND RELATED PEPTIDE ANTIBIOTICS. *Annu. Rev. Biochem.* **1977,** *46*, 723-763.
17. Jiang, Z. Q.; Vasil, A. I.; Hale, J. D.; Hancock, R. E. W.; Vasil, M. L.; Hodges, R. S., Effects of net charge and the number of positively charged residues on the biological activity of amphipathic alpha-helical cationic antimicrobial peptides. *Biopolymers* **2008,** *90* (3), 369-383.
18. Geisler, I.; Chmielewski, J., Cationic Amphiphilic Polyproline Helices: Side-Chain Variations and Cell-Specific Internalization. *Chem. Biol. Drug Des.* **2009,** *73* (1), 39-45.
19. Chen, Y. X.; Vasil, A. I.; Rehaume, L.; Mant, C. T.; Burns, J. L.; Vasil, M. L.; Hancock, R. E. W.; Hodges, R. S., Comparison of biophysical and biologic properties of alpha-helical enantiomeric antimicrobial peptides. *Chem. Biol. Drug Des.* **2006,** *67* (2), 162-173.

20. Nizet, V., Antimicrobial peptide resistance mechanisms of human bacterial pathogens. *Curr. Issues Mol. Biol.* **2006,** *8*, 11-26.
21. Brogden, K. A., Antimicrobial peptides: Pore formers or metabolic inhibitors in bacteria? *Nat. Rev. Microbiol.* **2005,** *3* (3), 238-250.
22. Yang, L.; Harroun, T. A.; Weiss, T. M.; Ding, L.; Huang, H. W., Barrel-stave model or toroidal model? A case study on melittin pores. *Biophys. J.* **2001,** *81* (3), 1475-1485.
23. Saint, N.; Marri, L.; Marchini, D.; Molle, G., The antibacterial peptide ceratotoxin A displays alamethicin-like behavior in lipid bilayers. *Peptides* **2003,** *24* (11), 1779-1784.
24. Marion, D.; Zasloff, M.; Bax, A., A TWO-DIMENSIONAL NMR-STUDY OF THE ANTIMICROBIAL PEPTIDE MAGAININ-2. *FEBS Lett.* **1988,** *227* (1), 21-26.
25. Ludtke, S. J.; He, K.; Heller, W. T.; Harroun, T. A.; Yang, L.; Huang, H. W., Membrane Pores Induced by Magainin†. *Biochemistry* **1996,** *35* (43), 13723-13728.
26. Park, C. B.; Kim, M. S.; Kim, S. C., Novel antimicrobial peptide from Bufo bufo gargarizans. *Biochem. Biophys. Res. Commun.* **1996,** *218* (1), 408-413.
27. Boman, H. G.; Agerberth, B.; Boman, A., MECHANISMS OF ACTION ON ESCHERICHIA-COLI OF CECROPIN-P1 AND PR-39, 2 ANTIBACTERIAL PEPTIDES FROM PIG INTESTINE. *Infect. Immun.* **1993,** *61* (7), 2978-2984.
28. Park, C. B.; Kim, H. S.; Kim, S. C., Mechanism of action of the antimicrobial peptide buforin II: Buforin II kills microorganisms by penetrating the cell membrane and inhibiting cellular functions. *Biochem. Biophys. Res. Commun.* **1998,** *244* (1), 253-257.
29. Shin, S. Y.; Kang, J. H.; Jang, S. Y.; Kim, Y.; Kim, K. L.; Hahm, K. S., Effects of the hinge region of cecropin A(1-8)-magainin 2(1-12), a synthetic antimicrobial peptide, on liposomes, bacterial and tumor cells. *Biochim. Biophys. Acta-Biomembr.* **2000,** *1463* (2), 209-218.
30. Park, C. B.; Yi, K. S.; Matsuzaki, K.; Kim, M. S.; Kim, S. C., Structure-activity analysis of buforin II, a histone H2A-derived antimicrobial peptide: The proline hinge is responsible for the cell-penetrating ability of buforin II. *Proc. Natl. Acad. Sci. U. S. A.* **2000,** *97* (15), 8245-8250.
31. Xie, Y.; Fleming, E.; Chen, J. L.; Elmore, D. E., Effect of proline position on the antimicrobial mechanism of buforin II. *Peptides* **2011,** *32* (4), 677-682.
32. Dathe, M.; Schumann, M.; Wieprecht, T.; Winkler, A.; Beyermann, M.; Krause, E.; Matsuzaki, K.; Murase, O.; Bienert, M., Peptide helicity and membrane surface charge modulate the balance of electrostatic and hydrophobic interactions with lipid bilayers and biological membranes. *Biochemistry* **1996,** *35* (38), 12612-12622.
33. Chen, Y. X.; Guarnieri, M. T.; Vasil, A. I.; Vasil, M. L.; Mant, C. T.; Hodges, R. S., Role of peptide hydrophobicity in the mechanism of action of alpha-helical antimicrobial peptides. *Antimicrob. Agents Chemother.* **2007,** *51* (4), 1398-1406.
34. Wu, Y.; Huang, H. W.; Olah, G. A., METHOD OF ORIENTED CIRCULAR-DICHROISM. *Biophys. J.* **1990,** *57* (4), 797-806.
35. Ladokhin, A. S.; Wimley, W. C.; White, S. H., Leakage of membrane vesicle contents: Determination of mechanism using fluorescence requenching. *Biophys. J.* **1995,** *69* (5), 1964-1971.
36. Rausch, J. M.; Wimley, W. C., A high-throughput screen for identifying transmembrane pore-forming peptides. *Anal. Biochem.* **2001,** *293* (2), 258-263.
37. Goodson, B.; Ehrhardt, A.; Ng, S.; Nuss, J.; Johnson, K.; Giedlin, M.; Yamamoto, R.; Moos, W. H.; Krebber, A.; Ladner, M.; Giacona, M. B.; Vitt, C.; Winter, J., Characterization of novel antimicrobial peptoids. *Antimicrob. Agents Chemother.* **1999,** *43* (6), 1429-1434.
38. van Kraaij, C.; Breukink, E.; Noordermeer, M. A.; Demel, R. A.; Siezen, R. J.; Kuipers, O. P.; de Kruijff, B., Pore formation by nisin involves translocation of its C-terminal part across the membrane. *Biochemistry* **1998,** *37* (46), 16033-16040.

39. Yeaman, M. R.; Yount, N. Y.; Waring, A. J.; Gank, K. D.; Kupferwasser, D.; Wiese, R.; Bayer, A. S.; Welch, W. H., Modular determinants of antimicrobial activity in platelet factor-4 family kinocidins. *Biochim. Biophys. Acta-Biomembr.* **2007,** *1768* (3), 609-619.
40. Uyterhoeven, E. T.; Butler, C. H.; Ko, D.; Elmore, D. E., Investigating the nucleic acid interactions and antimicrobial mechanism of buforin II. *FEBS Lett.* **2008,** *582* (12), 1715-1718.
41. Birkemo, G. A.; Luders, T.; Andersen, O.; Nes, I. F.; Nissen-Meyer, J., Hipposin, a histone-derived antimicrobial peptide in Atlantic halibut (Hippoglossus hippoglossus L.). *BBA-Proteins Proteomics* **2003,** *1646* (1-2), 207-215.
42. Park, I. Y.; Park, C. B.; Kim, M. S.; Kim, S. C., Parasin I, an antimicrobial peptide derived from histone H2A in the catfish, Parasilurus asotus. *FEBS Lett.* **1998,** *437* (3), 258-262.
43. Pavia, K. E., *Studying Antimicrobial Peptide Mechanisms: Analysis of Novel HDAPS and Development of High-Throughput Techniques*. Wellesley College Thesis: Wellesley 2012; p 69.
44. Koo, Y. S.; Kim, J. M.; Park, I. Y.; Yu, B. J.; Jang, S. A.; Kim, K. S.; Park, C. B.; Cho, J. H.; Kim, S. C., Structure-activity relations of parasin I, a histone H2A-derived antimicrobial peptide. *Peptides* **2008,** *29* (7), 1102-1108.
45. Matsuzaki, K.; Murase, O.; Fujii, N.; Miyajima, K., TRANSLOCATION OF A CHANNEL-FORMING ANTIMICROBIAL PEPTIDE, MAGAININ-2, ACROSS LIPID BILAYERS BY FORMING A PORE. *Biochemistry* **1995,** *34* (19), 6521-6526.
46. Pavia, K. E.; Spinella, S. A.; Elmore, D. E., Novel histone-derived antimicrobial peptides use different antimicrobial mechanisms. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **2012,** *1818* (3), 869-876.
47. Turner, J.; Cho, Y.; Dinh, N. N.; Waring, A. J.; Lehrer, R. I., Activities of LL-37, a cathelin-associated antimicrobial peptide of human neutrophils. *Antimicrob. Agents Chemother.* **1998,** *42* (9), 2206-2214.
48. Bustillo, M., *A Modular Approach to the Histone H2A Family of Antimicrobial Peptides*. Wellesley College Thesis: Wellesley, 2013.