A Modular Approach to the Characterization of Histone H2A-Derived Antimicrobial Peptides

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ABBREVIATIONS

AMP: Antimicrobial Peptide
HDAP: Histone Derived Antimicrobial Peptide
CPP: Cell Penetrating Peptide
RDA: Radial Diffusion Assay
HipC: C-terminus of hipposin
BF2HipC: Buforin 2 + C-terminus (of hipposin)
TSB: Tryptic Soy Broth
CFU: Colony Forming Units
MTT: 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazoliumbromide
TABLES AND FIGURES

Table 1: HDAP primary sequences
Table 2: Mammalian cell lines

Figure 1: AMP mechanisms of action
Figure 2: Schematic of core histone protein
Figure 3: Schematic of radial diffusion assay
Figure 4: Relative strength of H2A-derived AMPs
Figure 5: Determination of MIC in HDAPs
Figure 6: MTT assay schematic
Figure 7: HDAPs are minimally cytotoxic to normal human cells
Figure 8: Composite images of *E. coli* post-HDAP exposure
Figure 9: Buforin 2 translocates bacterial membranes
Figure 10: Parasin permeabilizes bacterial cell membrane
Figure 11: Buforin 1 localizes to the cell membrane
Figure 12: HipC translocates the cell membrane
Figure 13: BF2HipC translocates the cell membrane
Figure 14: Comparison of AMP activity in bacterial and eukaryotic cells
Figure 15: TAT and biocytin as proof of principle
Figure 16: Mechanisms of action of BF1 and BF2 against cancer cells
Figure 17: Ratio of cytotoxicity against normal vs. cancer cells
Figure 18: Trends in anticancer and antibacterial activity
Figure 19: HDAP helical wheel depictions
# Table of Contents

1. Introduction  
   1.1. Antimicrobial peptides  
   1.2. Structure determines antimicrobial peptide function  
      1.2.1. Antibacterial therapeutics  
      1.2.2. Anti-cancer activity  
      1.2.3. Antimicrobial peptides as delivery vectors  
   1.3. Applications of antimicrobial peptide research  
   1.4. Histone-derived antimicrobial peptides  
   1.5. Goals of this thesis  

2. Materials and Methods  
   2.1. Peptides  
   2.2. Mammalian cell culture  
   2.3. Bacterial cytotoxicity assays  
      2.3.1. Radial diffusion (RDA) antimicrobial activity assay  
      2.3.2. Microbroth dilution assay  
   2.4. Cell viability assay  
   2.5. Confocal imaging of AMPs in bacteria  
   2.6. Confocal microscopy in mammalian cell culture  

3. Results and Discussion  
   3.1. Histone H2A-derived Peptides Show Bacterial Selectivity  
   3.2. Histone H2A-derived Peptides Exhibit Modularity in Mechanism  
   3.3. Histone H2A-derived AMPs Demonstrate Activity Against Cancer Cell Lines  
   3.4. Future Directions for the Project  

4. References
ABSTRACT

Previous research with antimicrobial peptides (AMPs) has investigated the effect that individual amino acids have on a peptide’s activity and mechanism. In this study, I am characterizing a family of histone H2A-derived AMPs that share significant sequence similarity based on a more modular approach to understand the relationship between an AMP’s primary structure and its interactions with bacterial and eukaryotic membranes. The antibacterial efficacies of peptides in this family of H2A-derived AMPs was determined using a radial diffusion assay and a microbroth dilution assay. Eukaryotic cytotoxicity assays against non-cancerous fibroblast cells demonstrate that these peptides are selectively cytotoxic for bacterial cells. Peptide mechanisms of action against bacterial cells were investigated using confocal microscopy. Hipposin, a 51-mer AMP derived from the Atlantic halibut, is a cationic histone-derived AMP that has high sequence similarity to a portion of histone subunit H2A. A fairly potent bactericidal agent, it is selectively active against bacterial cells over eukaryotic cells; previous studies in our lab suggest that it acts via a membrane permeabilizing mechanism. The slightly less potent N-terminal region of hipposin, parasin, is a 19-mer AMP that acts via a membrane permeabilizing mechanism. The middle portion of hipposin (buforin 2) and the C-terminal region (HipC) have both been found to translocate into bacterial cells without notable membrane disruption, though only buforin 2 demonstrates bactericidal activity. When a membrane permeabilizing peptide such as parasin is attached to a translocating peptide such as buforin 2, the resulting peptide (buforin 1) is a potent antibacterial agent that does not cross the cell membrane. Similarly, the addition of the HipC translocating fragment to buforin 2 results in a translocating peptide; though the mechanism remains the same, the activity is decreased slightly, due to the addition of an inert peptide constituent. Though minimal anti-cancer activity was observed in this H2A-derived family of AMPs, notable cytotoxicity trends against bacteria and cancerous eukaryotic cells suggest membrane differences that may affect the nature of peptide-membrane interactions.
1. **INTRODUCTION**

The advent of antibacterial drugs at the beginning of the 20th century has allowed for highly successful and effective treatment of bacterial infections and diseases that would previously have been fatal. Recently, however, a host of antibiotic-resistant bacterial strains have emerged both in and out of hospital settings, rendering conventional antibacterial treatments (still chemically very similar to those discovered in the mid-1900s) effectively useless. In response to the increase in antibiotic-resistant bacterial strains, substantial research is now being done in an attempt to identify more effective antibacterial treatments (Yeung et al., 2011), either through the creation of novel drugs or by harnessing the antibacterial properties of naturally occurring immune systems.

1.1. **Antimicrobial peptides**

Antimicrobial peptides (AMPs) are short, amphipathic peptides that are components of many organisms’ innate immune response. Most widely characterized in the skin mucus of fish and amphibians, AMPs are found in nearly all organisms and target bacterial cells and other microbes for destruction, without the specificity of the adaptive immune response. As the name suggests, AMPs show activity against microbes, primarily bacteria, viruses, fungi and other parasitic organisms (Jenssen et al., 2006). For the purposes of this thesis, we will be investigating AMPs in terms of their antibacterial properties.

Naturally occurring in most organisms including plants, invertebrates and vertebrates, AMPs comprise a portion of the nonspecific innate immune system. This ancient defense system is the primary mechanism of preventing invasions by pathogenic organisms; its conservation among species indicates its continued efficacy, despite many
generations of evolution. AMPs themselves are widely distributed among organisms, playing a fundamental role in the defense against bacteria and fungi even in the presence of an active adaptive immune response (Jenssen et al., 2006). Direct antimicrobial activity most often occurs in portions of the organism most consistent with the first line of defense strategy: plant leaves, flowers, tubers, and seeds (Terras et al., 1992), amphibian skin glands (Rinaldi, 2002), and the granules of vertebrate phagocytic cells (Bowdish et al., 2005a; Bowdish et al., 2005b; Scott and Hancock, 2000; Yang et al., 2004; Yang et al., 2002). While some AMPs are expressed constitutively (notably the hemocytes found in shrimp, horseshoe crab and oysters), others rely on a pathogen recognition response to induce expression (Jenssen et al., 2006).

AMPs are charged peptides, usually containing one or more net charges. While some AMPs are anionic, those discussed in this thesis are known as cationic AMPs and are polycationic with a net charge of +2 to +9 (Jenssen et al., 2006). This net cationic charge plays a significant role in the selectivity of cationic AMPs, as bacterial membranes contain more negatively charged membrane components (namely the anionic lipid head group phosphatidylglycerol) than do normal eukaryotic membranes (Janmey and Kinnunen, 2006). Additionally, AMPs are attracted to the outer envelope of Gram-negative bacteria by anionic phosphate groups on lipopolysaccharides (LPS), while teichoic acids on Gram-positive bacteria confer electrostatic attraction between AMP and membrane (Brogden, 2005), rendering both Gram-negative and Gram-positive bacteria susceptible to AMP targeting. This net negative charge is compounded by a negative membrane potential (Janmey and Kinnunen, 2006); these anionic characteristics facilitate
electrostatic interactions between the bacterial membrane and cationic AMP (Brogden, 2005; Jenssen et al., 2006).

Additional bacterial selectivity is conferred by the high levels of cholesterol present in eukaryotic cell membranes. Studies indicate that cholesterol, which is found in eukaryotic but not prokaryotic cell membranes, inhibits antimicrobial activity of magainins, a class of AMPs derived from *Xenopus laevis*, by inhibiting AMP-induced disordering of lipid bilayers (Matsuzaki, 1999). It is not yet clear how specific these effects are to magainin-induced cell death.

In addition to their initial role in an organism’s innate immune system, some mammalian AMPs have been implicated as a chemotactic signal for monocytes (Territo et al., 1989) and T cells (Chertov et al., 1996), signaling the transition from innate to adaptive immunity. Defensins NP1 and NP2 are AMPs that are found within human neutrophil granules and released into the medium and neutrophil’s phagocytic vesicle by the neutrophil at concentrations up to 3 – 5 picograms when activated by IL-8 (Chertov et al., 1996; Ganz et al., 1986). There, they may deliver a T-lymphocyte activating signal in addition to destroying microbes via cytotoxic mechanisms (Chertov et al., 1996). The stimulation of a host organism’s immune response is also a quality important to the development of various peptide-based therapeutic strategies (Bowdish et al., 2005a; Bowdish et al., 2005b; Lee et al., 2004), though not the focus of this current body of work.

1.2. **Structure determines antimicrobial peptide function**

As are all proteins, AMPs are composed of a primary structure of amino acids, and a secondary structure wherein these amino acid chains interact to form simple three-
dimensional structures. The most common secondary structures observed in AMPs are alpha helices, beta sheets, and random coils. Biological dogma states that a protein’s structure determines its function, or in the case of antimicrobial peptides, its mechanism of action. While the level of correlation between AMP structure and mechanism is not clear, certain mechanisms seem to be more effective at selectively killing bacterial cells. Cationic AMPs have several distinct mechanisms of bactericidal activity, with the majority falling into one of three categories. The first two common mechanisms of action rely on disruption of membrane barrier function. The first of these occurs through general destruction of membrane integrity (Figure 1A) similar to the effects of a detergent, and is known as the “carpet model” of membrane destruction (Brogden, 2005). Peptides align parallel to the cell membrane (Jenssen et al., 2006; Pouny et al., 1992) and cause local disturbances in membrane stability, resulting in unstructured holes in the membrane.

Figure 1. Antimicrobial peptides interact with a lipid membrane in one of three ways. Peptides may A. disrupt the membrane structure, resulting in a loss of membrane integrity, form a pore in the membrane by B. rearranging phospholipid head groups to be in contact with AMPs (toroidal pore) or C. allowing peptides to come in contact with hydrophilic and hydrophobic lipid components, allowing outside matter to enter the cell, or D. translocate across the membrane and into the cell without disrupting the membrane structure or composition.

Peptides may also interact with each other, forming distinct pores across the lipid bilayer in a more controlled form of membrane permeabilization. Two types of pores may form: the “barrel-stave” model (Brogden, 2005; Matsuzaki et al., 1996) involves no
lipid rearrangement, so peptides come in contact with both the hydrophilic phospholipid heads and the hydrophobic tails of the lipid membrane (Figure 1C); the “toroidal pore” model (Baumann and Mueller, 1974) requires the movement of lipids in the membrane such that only the phospholipid head groups come in contact with the AMP pore (Figure 1B). Through either of these membrane permeabilizing mechanisms, the resulting ion imbalance and general loss of protective barrier function causes cell death.

The third category of AMPs are those known as membrane translocating peptides (Figure 1D). These peptides are capable of crossing the plasma membrane without damaging the membrane integrity. Once inside a cell, they interfere with DNA and RNA synthesis (Patrzykat et al., 2001; Subbalakshmi and Sitaram, 1998; Uyterhoeven et al., 2008) protein synthesis (pleurocidin and indolicidin; (Boman et al., 1993; Friedrich et al., 2000; Patrzykat et al., 2001; Subbalakshmi and Sitaram, 1998), or cellular enzymatic activity (Kragol et al., 2001), resulting in cell death at their minimum inhibitory concentration (MIC).

AMP mechanism of action is determined using various in vitro and in vivo assays (Steinberg and Lehrer, 1997). While membrane permeabilization can be measured in vivo in a bacterial system using the propidium iodide uptake assay (Steinberg and Lehrer, 1997), membrane translocation is most commonly determined using lipid-based vesicle assays. Because they need not be maintained at physiological conditions, these synthetic lipid bilayers allow for maximal manipulation of the system and the environment, while mimicking a biological plasma membrane. However, as a purely biochemical assay, these techniques cannot provide specific information about the interactions between an AMP and a live cell. To that end, a more biological method (both bacterial and
eukaryotic) must be employed that will allow for the monitoring of peptides as they interact with a biological membrane. Microscopy allows researchers to visualize the effects of AMP-membrane interactions and distinguish between mechanisms of action. Scanning and transmission electron microscopy can be used to examine ultra-structure damage following exposure to peptides (Hancock and Sahl, 2006). As demonstrated in this thesis, confocal laser-scanning microscopy can determine mechanism of action by localizing a fluorescently tagged peptide to either the cell surface or cytoplasm.

1.3. Applications of antimicrobial peptide research

Antibacterial therapeutics

From a basic science perspective, the characterization of histone-derived AMP structure and mechanism is interesting as a way to better understand the structure-function relationships of other AMPs and proteins in general. A more thorough understanding of the effects of a particular primary or secondary structure on the mechanism, or function, of an AMP will allow researchers to predict the nature of similar AMPs. Additionally, the design and development of novel designed AMPs with specific properties is highly dependent upon a basic understanding of the relationship between peptide structure and function.

Additionally, AMPs may serve a more applied role as potential therapeutic treatments in a clinical setting. While evidence of bacterial resistance to AMPs has been found, their continued efficacy as part of the innate immune response indicates that they remain potent antimicrobial agents and are minimally susceptible to antibacterial resistance. This is likely due, at least in part, to the electrostatic interactions that recruit cationic AMPs such as those described here to the bacterial membrane (Hancock and
Sahl, 2006). Because the presence of negatively-charged phospholipids and teichoic acids are inherent components of a bacterium’s outer envelope, it would be non-trivial for the bacterial cell to change the makeup of its membrane such that no net negative charge is present. That AMPs target such a general and fundamental structural characteristic also renders them potentially toxic to a wide range of organisms (Jenssen et al., 2006), particularly when compared with many modern antibiotics, which often have very specific molecular targets. Despite the high barrier to developing AMP resistance, some bacteria have proven quite adept at rendering the antimicrobial response ineffective. Many Gram-negative species have adapted mechanisms to shield their cell surface from attack by cationic AMPs through the use of “decoy” macromolecules such as capsule polysaccharides and the exopolysaccharides involved in the formation of biofilms (Gruenheid and Le Moual, 2012). These decoys bind AMPs, reducing the number of peptides able to reach the bacterial cell surface (Gruenheid and Le Moual, 2012). Other bacteria utilize bacterial transporters such as the resistance-nodulation-division efflux exporter pump to pump AMPs from the intracellular environment to the extracellular matrix (Aires and Nikaido, 2005), or the ABC importer family which transports AMPs from the periplasm into the cytosol where they are proteolytically degraded (Parra-Lopez et al., 1993). Still other bacterial species degrade AMPs prior to peptide-membrane interaction using proteases that are either localized to the bacterial outer membrane, or secreted into the extracellular matrix (Gruenheid and Le Moual, 2012). Although there is a wide range in the mechanisms of developing AMP resistance, it is not yet a widespread phenomenon and several studies in which bacteria was serially passaged in sub-MIC
peptide concentrations substantiate the claim that it is difficult for AMP resistance to develop (Ge et al., 1999; Steinberg et al., 1997; Zhang et al., 2005).

As a result of many bacteria species’ inability to form effective resistance strategies, AMPs are prime candidates for antibacterial therapeutics. Several AMPs have advanced through the drug development pipeline to phase 3 clinical trials: the magainin derivative MSI-78 to treat diabetic foot ulcers, the protegrin derivative IB-367 to treat oral mucosaitis, and the indolicidin variant CP-226 for catheter-associated infections are among those tested (Hancock and Sahl, 2006). Despite these clinical trial successes, getting AMPs into use in the clinic has been much more difficult, due in large part to their complex toxicity mechanisms. While AMPs are largely selective for bacteria and have fewer interactions with eukaryotic cells, such an interaction remains a possibility, particularly when AMPs are present in high concentrations. Additionally, AMPs are highly susceptible to proteolytic break down, potentially causing unexpected and undesirable pharmacokinetics (Hancock and Sahl, 2006). Therefore, designing a peptide with minimal eukaryotic cytotoxicity is of utmost importance if AMPs are to be considered for clinical antibacterial uses.

Anti-cancer activity

In addition to the aforementioned antibacterial uses, cationic AMPs may also have a use as potential anti-cancer treatments. Buforin IIb, a synthetic analog of buforin II, has been found to have significantly higher cytotoxic properties towards cancerous cells than normal eukaryotic cells (Lee et al., 2008). Similar findings have been described in other cationic AMPs, such as melittin (Sharma, 1992), cecropin (Moore et al., 1994), magainin
(Cruciani et al., 1991), LL-37 (Henzler-Wildman et al., 2003), and alloferons 1 and 2 (Chernysh et al., 2002).

Differences in membrane composition of cancerous and non-cancerous cells may help to explain the cancer-selective toxicity observed in some cationic AMPs such as buforin IIb. While normal mammalian cell membranes are composed largely of zwitterionic phospholipids (Hoskin and Ramamoorthy, 2008), cancer cell membranes contain a number of anionic molecules such as phosphatidylserine (Dobrzynska et al., 2005; Utsgui et al., 1991), O-glycosylated mucins (Burdick et al., 1997), heparin sulfates (Kleeff et al., 1998), and sialilated gangliosides (Lee et al., 2008). As with bacterial membranes, these molecules combine with a net negative membrane potential (Hoskin and Ramamoorthy, 2008; Schweizer, 2009) to lend the cancer cell a net negative charge, suggesting that cancer-selectivity may be due to a similar electrostatic interaction between the cationic peptide and anionic cell membrane that is seen in bacterial cells.

Additionally, tumorigenic cells have a comparatively high number of microvilli on their surface when compared to normal cells, providing an increased surface area with which an AMP could interact (Chan et al., 1998; Zwaal and Schroit, 1997). Current research suggests that AMPs act through both membrane permeabilizing and non-permeabilizing mechanisms when interacting with cancer cells (Schweizer, 2009).

Investigations of AMPs as potential anti-cancer agents are fundamentally interesting on several levels. Not only might AMPs provide a novel form of anti-cancer therapeutic, the apparent selectivity of AMPs for cancerous cells over normal eukaryotic cells may further elucidate characteristics of peptide-lipid interactions in a eukaryotic system. Much of the research in the field is conducted in bacterial systems, and
comparatively little is known about peptide mechanisms or selectivity for and against various eukaryotic cell membranes.

Much of modern cancer treatment today relies on chemical treatments such as alkylating agents and antimetabolites to attack malignant cells. While highly effective, these treatments are not specific to cancerous cells but instead are damaging to all cells of the body, including those cells that display a normal phenotype.

Antimicrobial peptides as delivery vectors

In addition to direct therapeutic uses of AMPs, the selectivity and unique mechanism of membrane translocating peptides offer promising potential for future drug delivery vectors. These membrane translocating peptides, also known as cell-penetrating peptides (CPPs), have been widely studied as potential delivery vectors due to their ability to be electrostatically or covalently bound to molecules that could then be transported across the cell membrane (Lindgren and Langel, 2011). Recent trials have been successful with the introduction of small molecule pharmaceuticals (Torchilin, 2008), siRNA (Haas, 2011), and other proteins into a cell using targeted CPPs. One of the most well known CPPs is Tat, the protein coded for by the Trans-Activator of Transcription (Tat) gene of human immunodeficiency virus (HIV). An arginine-rich alpha helix, Tat interacts with heparin sulfates on the cell membrane, and is able to translocate into the cell without significantly comprising membrane integrity, likely via an endocytotic mechanism (Vivès et al., 2003). As with most AMPs, the exact primary sequence that confers translocating ability of most CPPs is unknown, preventing informed engineering of a CPP with a predicted mechanism of action (Lindgren and
Langel, 2011). With a better understanding of the structural requirements of the translocating mechanism, we may one day be able to design highly specific vectors for the delivery of compounds into a cell.

1.4. Histone-derived antimicrobial peptides

The most thoroughly characterized translocating AMP is the 21-mer buforin II (Kobayashi et al., 2004). Derived from the Asiatic toad, *Bufo bufo gargarizans*, this peptide displays antibacterial activity against both gram-positive and gram-negative bacteria (Park et al., 1996). Interestingly, buforin II shares a high sequence similarity to a DNA-binding portion of the H2A histone subunit. Buforin II also has high affinity to DNA, and its antimicrobial activity has been found to be dependent upon this binding ability (Uyterhoeven et al., 2008).

Whole histones themselves display some antimicrobial properties (Rosenbluh et al., 2005; Rosenbluh et al., 2004) and have been observed to be cell penetrating, independent of endocytotic pathways in HeLa and Colo-205 cultured cells (Hariton-Gazal et al., 2003), as well as synthetic large unilamellar vesicles and multilamellar vesicles (Rosenbluh et al., 2005). Several AMPs with sequence homology to portions of histone subunits have been derived from natural sources such as the Atlantic halibut (Birkemo et al., 2003), catfish (Park et al., 1998) and rainbow trout (Fernandes et al., 2004).

Studies suggest that histone-derived AMPs such as buforin I is produced by proteolytic breakdown of histone H2A in the toad stomach (Cho et al., 2009). Histone H2A is overproduced in the toad’s gastric mucosal cell, creating an excess of what is
required for DNA packaging purposes (Kim et al., 2000). These excess histone subunits remain unacetylated and are stored in cytoplasmic secretory granules until secretion into the gastric lumen, where pepsin C isozymes process the H2A subunit and produce buforin I (Kim et al., 2000). A similar process is involved in parasin synthesis, with cathepsin D taking the place of pepsin C as the proteolytic enzyme responsible for histone H2A cleavage (Park et al., 1998).

![Figure 2. Schematic of the core histone protein. Histone cores are octamers comprised of two each of four subunits: H2A, H2B, H3, and H4.](image)

Previous work in our lab has focused on the cell penetrating ability of buforin II as well as further characterization of the 51-mer cationic AMP hipposin, derived from the skin mucus of the Atlantic halibut (Birkemo et al., 2003). Both these peptides share sequence homology with the same portion of histone subunit H2A (Table 1) – buforin II is homologous to the middle portion of hipposin - yet display drastically different characteristics. While buforin II is a well-characterized membrane translocating peptide and does not disrupt the lipid bilayer (Kobayashi et al., 2004), hipposin has been found to be extremely damaging to the cell membrane (Fischer, 2013). Additionally, the N-terminal portion of hipposin, parasin, is a highly membrane-permeabilizing peptide (Fischer, 2013; Koo et al., 2008). Such disparate mechanisms among peptides with shared derivation and similar sequence suggest that portions of a peptide may confer
certain characteristics and mechanisms even when attached to another peptide fragment as could be observed in a parasin-buforin 2 chimeric peptide.

In an effort to determine the cause for the mechanistic differences between these otherwise similar histone H2A-derived AMPs, the current study will investigate a number of peptides in the histone H2A family (Table 1), some naturally occurring (names italicized) and some found only as chemically synthesized samples for the purposes of this study.

Table 1. Amino acid sequences of histone H2A-derived antimicrobial peptides of interest. Names in italics indicate that the peptide is naturally occurring. Text color reflects color-coding throughout this document. All peptides synthesized for laboratory use.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino Acid Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hippocin</td>
<td>KGRGKQGGKVRASKRSSRAGLQWPVGRVHRLLRKGNYAHRVGAAPVWL</td>
</tr>
<tr>
<td>Buforin I</td>
<td>KGRGKQGGKVRASKRSSRAGLQWPVGRVHRLLRK</td>
</tr>
<tr>
<td>Buforin II</td>
<td>TRSSRAGLQWPVGRVHRLLR</td>
</tr>
<tr>
<td>Parasin</td>
<td>KGRGKQGGKVRASKRSS</td>
</tr>
<tr>
<td>Hippocin C-terminus</td>
<td></td>
</tr>
<tr>
<td>Buforin II + HipC</td>
<td>TRSSRAGLQWPVGRVHRLLRKGNYAHRVGAAPVWL</td>
</tr>
</tbody>
</table>

1.5. Goals of this thesis

The global goal of this thesis project is to better understand the relationship between an antimicrobial peptide’s primary structure, mechanism of action and subsequent efficacy in bacterial and eukaryotic cytotoxicity. Additionally, in keeping with long-term research interests in our lab, my project investigates differences in
peptide-lipid interactions in a bacterial and eukaryotic system. Further understanding of these two key components of the biochemistry of antimicrobial peptides will allow conclusions to be drawn about the usefulness and viability of a similar system as potential antibacterial or anticancer therapeutic systems.

The modular approach that we have taken to these questions of structure-function relationships also allows us to investigate the extent to which AMPs can act as modules, forming chimeric peptides with specific mechanistic properties as conferred by their individual pieces. If histone-derived AMPs such as those characterized in this thesis can be mixed-and-matched into an effective chimeric system, novel peptides can be designed and developed with very specific desired properties or characteristics.
2. MATERIALS AND METHODS

2.1. Peptides

Buforin 2 and hipposin (wild type) were synthesized and purified by GenScript (Piscataway, NJ) to > 95% purity. Hipposin (S1K, T6Q, A10V; TrpHip), buforin 1, parasin, hipposin C-terminus (HipC), and buforin 2 + hipposin C-terminus (BF2HipC) were synthesized and purified by NeoBioSci (Cambridge, MA) to > 95% purity (Table 1). All peptides were dissolved in NanoPure water and stored at -20°C. Prepared peptide concentrations were determined from the absorbance of endogenous tyrosine or tryptophan residues using both the BioRad Smart-Spec Plus spectrophotometer (Hercules, CA) and Thermo Scientific NanoDrop 2000 (Wilmington, DE). TrpHip, buforin 1, buforin 2, parasin, HipC, and BF2HipC were also purchased biotinylated by NeoBioSci (Cambridge, MA) for use in confocal microscopy.

2.2. Mammalian cell culture

Pancreatic adenocarcinoma cell lines “Capan-2” (a sub-clone of the ATCC Capan-2 cell line) and BxPC-3, and prostate carcinoma cell line DU-145 were obtained frozen (-80°C) from the Webb lab (Wellesley College, Wellesley, MA) (Table 2.1). Fetal fibroblast cell line WI-38 was obtained frozen from ATCC (Manassas, VA) (Table 2.1). Cells were grown to confluency in RPMI 1640 (Sigma Aldrich R6504) medium containing 10% fetal bovine serum (HyClone SH30070), 0.2% sodium bicarbonate solution (Sigma Aldrich S8761), and a penicillin-streptomycin-amphotericin antibiotic solution (Sigma Aldrich A5955). Cell culture conditions were maintained at 37°C, 95% humidity, and 5% CO₂. Cells were passaged at confluency, generally 5-10 days.
Table 2. Cell lines used in this study.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>ATCC Number</th>
<th>Source</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>WI-38</td>
<td>CCL-75</td>
<td>Human fibroblast; lung isolation</td>
<td>None</td>
</tr>
<tr>
<td>DU-145</td>
<td>HTB-81</td>
<td>Human prostate; isolated from brain metastasis</td>
<td>Carcinoma</td>
</tr>
<tr>
<td>“Capan-2”</td>
<td>Subclone derived from HTB-80</td>
<td>Human pancreas</td>
<td>Adenocarcinoma</td>
</tr>
<tr>
<td>BxPC-3</td>
<td>CRL-1687</td>
<td>Human pancreas</td>
<td>Adenocarcinoma</td>
</tr>
</tbody>
</table>

2.3. **Bacterial cytotoxicity assays**

Top10 *E. coli* (Invitrogen) was transfected with the ampicillin-resistance plasmid pET45b (Novagen) and stored frozen at -80°C. Thawed stocks were maintained on Luria Bertoni (20% LB w/v, 10% Bacto™ agar w/v, Sigma, St. Louis, MO) nutrient agar at 4°C. Individual colonies from LB nutrient agar plates were isolated and grown overnight at 37°C with shaking in Bacto™ tryptic soy broth soybean-casein digest medium (30% w/v TSB) (BD Biosciences 211825) with 25 µL/mL ampicillin to log phase. Bacteria were then diluted 1:100 in TSB and allowed to grow until mid-log phase at 37°C with shaking. Cells were pelleted by centrifugation for 10 minutes at 800 g and 4°C and resuspended to relevant concentrations in sterile 10 mM sodium phosphate buffer. Concentration of colony forming units (CFU) was calculated to be 2.5 x 10^8 CFU/ mL based on the OD_{600} as determined by Agilent 8453 UV-Vis Diode Array spectrophotometer (Santa Clara, CA).

2.3.1. **Radial diffusion (RDA) antimicrobial activity assay**

Relative antibacterial activity was determined based on the radial diffusion assay described by Steinberg and Lehrer (Steinberg and Lehrer, 1997). Culture of Top10 *E.
coli (pET45b) was prepared as described above. 10 mL of melted TSB agarose underlay gel (10 mM Na₃PO₄, 1% TSB v/v, 1% agarose w/v, pH 7.4) was inoculated with 4 x 10⁶ CFU of Top10 E. coli (pET45b) in mid-log phase growth and cooled in a petri dish. 1 mm wells were created in the agar and filled with 2 µL of 1 x 10⁻⁴ M peptide solutions. Bacteria were incubated with peptide solutions for 3 hours at 37°C. Underlay gel was covered by 10 mL of melted TSB overlay gel (30% w/v TSB, 1% w/v agarose) and incubated overnight for approximately 16 hours at 37°C. The diameter of bacterial clearing was measured optically at 7X magnification.

2.3.2 Microbroth dilution assay for bactericidal activity

Minimum inhibitory concentrations (MICs) of HDAPs of interest were determined using a micro-dilution protocol modified from Steinberg and Lehrer (Steinberg and Lehrer, 1997). Culture of Top10 E. coli (pET45b) was prepared as described above. Wells of a 96 well plate were filled with 100 µL of bacterial solutions at a concentration of 1 x 10³ CFU/mL and treated with peptide solutions of the appropriate concentration (final concentrations tested ranged from 0.005 – 100 µg/mL. Plates were then covered tightly with parafilm to prevent evaporation of liquid, and incubated for 3 hours at 37°C with shaking. Post-incubation, 100 µL of 2X TSB (60% w/v TSB) was added to each well, the plate was covered again and incubated with shaking for 16 hours at 37°C. Antimicrobial activity was determined by visual analysis based on a binary growth/no growth condition.

2.4. Cell viability assay
Cytotoxicity was determined by the MTT assay as described by Wu et al. (Wu, 2009). Cells were seeded in 96-well plates at $3 \times 10^3$ cells/well and incubated overnight at 37°C and 5% CO$_2$. Cells were then incubated with 1 µM peptide solutions for 48 hours. Each peptide solution was tested in quadruplicate. Cell viability was measured by introducing the 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazoliumbromide (MTT) indicator (0.5 mg/mL) (Invitrogen, Carlsbad, CA) into treated cell solutions for 4 hours. MTT is reduced to a purple formazan salt by metabolically active cells; formazan crystals were dissolved after 4 hours by addition of 5% sodium dodecyl sulfate (SDS) (Sigma Aldrich L4390), and mixtures were incubated overnight. Absorbance was read at 570 nm on a Molecular Devices SpectraMax M3 microtiter plate reader (Sunnyvale, CA). Percent cell viability was calculated by taking the ratio of the $A_{570nm}$ of cells treated with peptide solutions over the $A_{570nm}$ of cells treated with water. Staurosporine, a known cytotoxic agent, was used as a comparison for negative growth.

2.5. **Confocal imaging of AMPs in bacteria**

Peptide translocation into Top10 *E. coli* (pET45b) was visualized as described by Park et al. (Park et al., 2000). Overnight cultures from frozen bacterial stocks were diluted 1:100 in TSB liquid media and allowed to grow to mid-logarithmic phase. Bacteria were then pelleted by centrifugation at approximately 880 g and resuspended in sterile 10 mM sodium phosphate buffer (pH 7.4). Bacterial cells at a concentration of $10^7$ CFU/mL were exposed to 4 µg/mL biotinylated peptide solutions for 30 minutes at 37°C. Cell/peptide solutions were then placed on a poly-L-lysine coated glass slide and treated with 0.066% Triton-X for 1 – 2 minutes. Biotinylated peptides were rendered visible by
the addition of streptavidin-AlexaFluor 488 (Invitrogen) tags at a final concentration of 5 µg/mL. Cells were visualized with a Leica TCS SP5 laser scanning confocal microscope with excitation at 488 nm by an argon laser at 20% output. All 8-bit 512 x 512 images represent the average of six scans at 63X magnification (Leica Plan-Apochromat objective; numerical aperture 1.40). Composite images were produced by Leica LAS AF software (Buffalo Grove, IL). Z-stacks of 0.07 – 0.09 µm thickness were analyzed using ImageJ image analysis software (NIH) in conjunction with the LOCI BioFormats plug-in (LOCI, Madison, WI). Unlabeled images were evaluated by outside individuals for location of peptide fluorescence within the cell to prevent bias in the reading of the data.

2.6. Confocal microscopy in mammalian cell culture

Peptide translocation into mammalian cell culture was visualized as described by Hilchie et al. (Hilchie et al., 2011). Cells were grown in RPMI 1640 medium (Sigma Aldrich) as described above (Section 2.2). 5 x 10³ cells were seeded into each well of a sterile 8-well chamber slide and allowed to incubate overnight at 37°C in RMPI 1640 without phenol red. Cells were then exposed to 10 µM biotinylated peptide solutions for an hour at 37°C, fixed with 4% paraformaldehyde and stained with DAPI nuclear stain and streptavidin-AlexaFluor 488 fluorescent conjugates (Invitrogen). Cells were visualized with a Leica TCS SP5 laser scanning confocal microscope with excitation at 408 nm and 488 nm by an argon laser at 20% output. 8-bit 512 x 512 images were obtained at 20X and 40X optical magnification with a 2X zoom in z-stack slices of 0.5 – 1.0 µm thickness. All images represent the average of six scans. Z-stacks were analyzed
using ImageJ image analysis software (NIH) in conjunction with the LOCI BioFormats plug-in (LOCI, Madison, WI).
3. **RESULTS AND DISCUSSION**

Previous work in the lab has focused on the role of specific amino acids in determining a peptide’s mechanism of action. This study seeks to characterize the relationship between an antimicrobial peptide’s (AMP) primary structure and its activity and mechanism in a more modular sense. That is, can we predict the efficacy and mechanism of action of a particular peptide based on the characteristics of the peptide fragments it contains? This characterization of a family of histone subunit H2A-derived AMPs occurs in three parts: first, a determination of the bactericidal activity and specificity of each fragment of the hipposin “parent” (Table 1); second, a mechanistic study that investigate potential correlations between activity and peptide mechanism of action; third, studies on AMP activity against mammalian cells that elucidate differences between how peptides interact with eukaryotic and bacterial membranes and the effects those differences have on AMP activity.

3.1. **Histone H2A-derived Peptides Show Bacterial Selectivity**

In order to determine the bacterial selectivity exhibited by the H2A-derived peptides of interest, antibacterial activity was compared with eukaryotic cytotoxicity induced by peptide solutions. Relative antibacterial potency was determined by allowing the diffusion of peptide solutions in solid media inoculated with bacteria (Top10 *E. coli*) to create a peptide concentration gradient and measuring the diameter of bacterial clearing after an overnight incubation. More potent antibacterials result in a larger diameter of bacterial clearing, indicating that they are still active against bacteria even at low concentrations (Figure 3).
Figure 3. Schematic of radial diffusion assay to test bactericidal activity of AMPs (left). Peptide diffuses out from well (shown in gray), creating a concentration gradient in a bacterial lawn. Diffusion rates among peptides remain relatively consistent among cationic AMPs (Steinberg and Lehrer, 1997), therefore the diameter of bacterial clearing is directly proportional to the potency of the peptide against bacteria. In this example, the purple line indicates the diameter of bacterial clearing of a weak peptide; the red line indicates the diameter of bacterial clearing of a stronger peptide. Inactive peptides do not display any bacterial clearing, but instead are measured as the diameter of the well. Example of diameters of clearing in a Top10 *E. coli* bacterial plate (right). Most potent peptides are found on the bottom row, while least active peptides are in the middle. Image of plate courtesy of Katie Pavia (Kathryn Pavia, personal communication).

Buforin 2 and Buforin 1 displayed the most significant bactericidal activity, resulting in diameters of clearing of 9.1 ± 0.2 and 8.7 ± 0.14 mm, respectively (uncertainty reported as standard error) (Figure 4). In contrast, the C-terminal region of hipposin (HipC) had a diameter of clearing that was not significantly greater than water, indicating that HipC is an inert peptide (Figure 4). Peptides with significant bacterial clearing were active against both gram-positive (*Bacillus subtilis*) and gram-negative (*E. coli*) bacteria (Fischer, 2013).
Figure 4. **Relative strength of H2A-derived AMPs.** Larger diameters of bacterial clearing indicate more potent antibacterials. Water shown as a negative control for antibacterial activity. N = 15 trials (peptides tested in triplicate on five separate days); error bars indicate standard error. Adapted from (Fischer, 2013).

Because the exact peptide concentration at a specific point in the peptide gradient is unknown, the radial diffusion assay provides information about the relative activity of each peptide, but does not illustrate exactly how much peptide is required for bactericidal activity. It is also not very specifically known how consistent peptide diffusion remains through the nutrient agar plate. Additionally, the overlay nutrient agar added in RDA renders it an anaerobic environment for the bacterial lawn between the two agar layers. Because the standard bacterial strains measured are not anaerobic strains, this may affect their growth in ways that would be unexpected were they to be grown in a more physiologically relevant manner. To this end, measuring AMP-induced bacterial clearing
with RDA is a fair relative measure of peptide efficacy, but may not provide physiologically accurate cytotoxicity measurements.

The minimum inhibitory concentration (MIC) can be determined using a microbroth dilution assay. Bacteria are plated in a 96-well plate at a density of $1 \times 10^3$ CFU/mL, exposed to peptides in a serial dilution and allowed to incubate overnight. Bacterial growth is determined on a growth-no growth scale after visual examination; the MIC represents the lowest concentration of peptide required for bactericidal activity to be observed. Though bacterial growth prior to peptide exposure was consistent, notable inconsistencies in reported MIC were observed both on a day-to-day basis, and within trials each day (Figure 5). Due to the aforementioned challenges with consistency in the protocol, antibacterial activity was determined in this way only for buforin 1, buforin 2, and parasin, presented here (Figure 5). Kanamycin was used as a positive antibacterial control.
Figure 5. **Overnight growth of Top10 E. coli after treatment with the indicated concentrations of peptide.** Shaded boxes represent significant bacterial growth after 16-hour incubation at 37°C. Trials were performed in quadruplicate on four separate days; plates shown here represent results from one single day.

Of the peptides for which data were collected, buforin 1 is the most potent antibacterial, with a MIC around 0.5 µg/mL peptide (Figure 5). Buforin 2 is observed to have a MIC...
of approximately 1 µg/mL, twice as potent as parasin with a MIC of 2 µg/mL (Figure 5). While the relationship between buforin 2- and parasin-induced antibacterial activity corresponds with that found with radial diffusion analysis (Figure 4), microbroth dilution suggests that buforin 1 is twice as potent as buforin 2, a relationship that is not observed in relative antibacterial activity studies (Figure 4). Literature values cite buforin 2’s MIC at 2 – 4 µg/mL (Kobayashi et al., 2000); the values described here suggest similar MIC values. Similarly, published values for the MIC of parasin against E. coli (ATCC 27325) are 1 µg/mL, similar to those reported here in Top10 E. coli (Park et al., 1998). Interestingly, the same study (Park et al., 1998) published a MIC for buforin 1 against E. coli of 8 µg/mL, highlighting a discrepancy between MIC determinations using similar methods.

A number of factors may help to elucidate the cause for recurring discrepancies in MIC determinations using this turbidity-based microbroth dilution assay. Peptide age and storage conditions play a significant role in the quality and potency of antimicrobial peptides; it is possible that individual labs may treat their peptide stocks differently, thus changing peptide potency even when concentrations remain the same. Additionally, some variation of MIC values may be due to variation in laboratory strain susceptibility. Though bacterial strains are most often acquired commercially from ATCC, culture and growth conditions may vary by lab and may affect bacterial susceptibility to AMP treatments. Careful consideration of exact bacterial growth media used is also critical when comparing MIC values between studies as variance in bacterial growth will also affect reported MIC values of peptides tested.
Certain patterns become apparent when examining the antibacterial activities of modules of the H2A-derived AMP family. Generally speaking, chimeric peptides displayed antimicrobial activity similar to that observed in the most potent “parent” peptide, rather than an additive effect. The addition of an inert fragment such as HipC appears to decrease peptide efficacy as is seen in both BF2HipC and hipposin, peptides whose other constituent fragments have notably higher bacterial toxicity (Figure 4).

![Diagram of MTT assay](image)

**Figure 6. MTT assay used to determine cell viability of eukaryotic cells.** Yellow tetrazolium salts are reduced by active cell metabolism to an insoluble purple formazan crystal. Sodium dodecyl sulfate is added to aid in formazan solubilization. Absorbance of purple color is measured using a SpectraMax plate reader at 570 nm.

In order for AMPs to be effective as clinical antibacterial therapeutics, they must be selective for bacteria and minimally cytotoxic against normal mammalian cells. Cell viability of a human fibroblast cell line after exposure to histone H2A AMPs was determined by a standard MTT cytotoxicity assay to examine their cytotoxicity in normal mammalian systems. MTT (3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazoliumbromide) is a chemical indicator that is reduced to a purple formazan crystal when exposed to active cell metabolisms (Figure 6). Data are presented as percent viability as determined by crystal formation in AMP-treated cells and cells treated with water. No significant decrease in cell viability was observed in cells treated with 1 µM of all six peptides tested (Figure 7).
Though cellular viability calculations lead to reported percent viabilities greater than 100% (Figure 7), such viabilities do not have physiologic meaning. We have understood these numbers to mean (as mentioned above) that the H2A-derived HDAP family does not induce significant decreases in cell viability of populations of normal eukaryotic cell lines. Due in part to cell clumping and imprecise cell counts, cell number varies slightly in each well. Therefore, individual trials of the MTT cytotoxicity assay may result in these seemingly nonsensical viabilities. One can thus consider viabilities higher than 100% to be 100% ± observed viability as a result of variance in the number of cells (and thus observed viability) per well. Additionally, such variance is preserved when large data sets are combined; because percent viabilities or cell number may vary across separate trials, viabilities greater than 100% are more likely to be found in multi-trial, compiled data sets. Minimization of cell clumping to ensure accurate cell counts, and a more stringent mathematical standardization of initial cell viability numbers may reduce the occurrence of these non-physiological viabilities.
Figure 7. **Histone H2A-derived AMPs are minimally cytotoxic to normal human fibroblast cells.** Viability of the WI-38 human fetal fibroblast cell line after exposure to 1 µM peptide solution was determined using a standard MTT cytotoxicity assay. All values reported are the average of at least twelve wells over three separate days and are standardized to a set of control wells treated with water in place of peptide solution. Error bars indicate standard error.

3.2. **Histone H2A-derived Peptides Exhibit Modularity In Mechanism**

AMP mechanism of action was observed using confocal microscopy of biotinylated peptides in a Top10 *E. coli* bacterial system (Figure 8). A streptavidin-AlexaFluor 488 conjugate allowed for visualization of peptides when excited by an argon 488 nm laser. Because *E. coli* bacteria are so small, z-stack imaging allowed for increased resolution, as well as noting specific localization of the peptide within the bacterial cell.
Figure 8. Composite images of Top10 *E. coli* after exposure to A) buforin 2, B) buforin 1, C) parasin, D) BF2HipC, and E) HipC. Biotinylated peptides were conjugated to a streptavidin-AlexaFluor 488 tag and visualized using a Leica SP5 LSCM fitted with an argon laser at 488 nm. Composite images created by overlaying individual z-stack slices taken at 0.07 – 0.1 µm with a six frame-average. Scale bars indicate 2 µm.

Microscopy images of buforin 2 corroborate the previously characterized mechanism (Kobayashi et al., 2000) of peptide translocation across the cell membrane and into the cell as observed by uniform fluorescence through the entire cell (Figure 9). The rod shape demonstrated in these images is characteristic of *E. coli* and its presence is clearly indicative of an intact membrane.
Figure 9. Buforin 2 translocates the bacterial cell membrane and enters the cell. Z-stack montage of *E. coli* (10^7 CFU/mL) after exposure to 4 µg/mL biotinylated buforin 2. Each z-step represents a 0.085 µm slice on the z-axis. Peptides were visualized using a streptavidin-AlexaFluor 488 conjugate to bind to biotin tag on peptide N-terminal region and visualized on a Leica SP5 LSCM confocal microscope with excitation at 488 nm by an argon laser.

In contrast, images of parasin and Top10 *E. coli* show no rod shaped bacteria, but rather, extensive cell debris indicative of cell lysis (Figure 10). This phenomenon is observed throughout the entire depth of the cell, further suggesting that parasin is active via a detergent-like, lytic mechanism (Figure 10). These data are verified by biochemical assays with non-biotinylated peptides to measure membrane permeabilization, such as the propidium iodide uptake assay (Fischer, 2013). Propidium iodide uptake suggests that parasin is as strong a permeabilizing agent as magainin, a known permeabilizing agent (Fischer, 2013).
Figure 10. Parasin permeabilizes the cell membrane, causing cell lysis. Z-stack montage of \textit{E. coli} (10^7 CFU/mL) after exposure to 4 µg/mL biotinylated parasin. Each z-step represents a 0.09 µm slice on the z-axis. Peptides were visualized using a streptavidin-AlexaFluor 488 conjugate to bind to biotin tag on peptide N-terminal region and visualized on a Leica SP5 LSCM confocal microscope with excitation at 488 nm by an argon laser.

Bioimaging studies with non-biotinylated fluorescent peptides demonstrate similar results (Takeshima et al., 2003) to biotinylated streptavidin conjugates, suggesting that there is not always a notable change in AMP mechanism after the addition of a biotin tag, rendering microscopy a valuable quantitative analytic for determining the mechanism of AMP activity. Additionally, our biochemical data corroborate the bioimaging studies presented here, further confirming the validity of the mechanistic conclusions drawn from these data.
Figure 11. Buforin 1 localizes to the cell membrane but does not translocate into the cell. Z-stack montage of *E. coli* (10^7 CFU/mL) after exposure to 4 µg/mL biotinylated buforin 1. Each z-step represents a 0.07 µm slice through the z-plane. Peptides were visualized using a streptavidin-AlexaFluor 488 conjugate to bind to biotin tag on peptide N-terminal region and visualized on a Leica SP5 LSCM confocal microscope with excitation at 488 nm by an argon laser.

The propidium iodide uptake assay, a biochemical assay for membrane permeabilization, suggests that buforin 1 acts as a potent permeabilizing agent (Fischer, 2013), similar to parasin. Microscopic evidence suggests that buforin 1 is not a substantially lytic peptide (that is, does not lead to widespread cell lysis) such as parasin (Figure 9), but rather localizes to the bacterial cell membrane (Figure 10). Together these data indicate that buforin 1 may demonstrate bactericidal activity via a pore-forming mechanism, thus compromising membrane integrity while remaining embedded in an at least somewhat intact lipid bilayer. It is also possible that buforin 1 acts in a similar manner as parasin, but at a slower rate so by the time it is imaged, the membrane is still
visible. However, while long-term microscopic studies have not been conducted, cell lysis has not been observed in buforin 1-treated bacterial cells even after three hours exposure. Because biochemical studies at similar peptide concentrations result in measurable permeabilization at this time point, it is unlikely that the apparent mechanistic differences observed in these imaging studies are due solely to differences in the time required for a peptide to be an efficient bactericidal agent.

It is interesting to note that the addition of parasin, a membrane permeabilizing peptide, to buforin 2, a membrane translocating peptide, results in a peptide with membrane permeabilizing ability (Figure 10), albeit one with a non-lytic mechanism. Were this trend to continue, it would suggest that the addition of a permeabilizing fragment to a more translocating peptide could be predicted to display mechanistic characteristics more similar to the more permeabilizing constituent. Although mechanism of action appears to have favored the more permeabilizing fragment (Figure 10), buforin 1’s antibacterial activity is more similar to its translocating constitution, buforin 2 (Figures 4 & 5). This pattern suggests that peptide mechanism alone is not sufficient to predict the efficacy of a peptide as an antimicrobial agent, though certain mechanistic characteristics may be conserved.

Though the C-terminal portion of hipposin (HipC) is not naturally occurring, for the purposes of investigating the modularity of histone-derived peptides, we investigated the mechanism of a synthesized quantity of this peptide. As indicated by radial diffusion analysis of the relative strength of our peptides of interest, this C-terminal portion does not exhibit substantial antibacterial activity (Figure 4). Such lack of bactericidal activity might suggest an inability to target the bacterial membrane, a less potent interaction
between peptide and membrane, or an interaction that does not result in cellular toxicity. Microscopic analysis demonstrates that HipC is a translocating peptide (Figure 12), indicating that it is the latter of the three options outlined above that holds true.

![Confocal microscopy of the C-terminal region of hipposin. Z-stack montage of E. coli (10⁷ CFU/mL) after exposure to 4 µg/mL biotinylated HipC. Each z-step represents a 0.07 µm slice through the z-plane. Peptides were visualized using a streptavidin-AlexaFluor 488 conjugate to bind to biotin tag on peptide N-terminal region and visualized on a Leica SP5 LSCM confocal microscope with excitation at 488 nm by an argon laser.](image)

The hybrid AMP of buforin 2 and the C-terminal portion of hipposin (BF2HipC), in contrast, exhibits significant bactericidal activity that is similar to that of hipposin (Figure 4). Mechanistically, BF2HipC appears to cross the membrane (Figure 13) without causing significant membrane damage (compare to parasin, Figure 8C).
Figure 13. Confocal microscopy analysis of bufferin 2 + C-terminus (BF2HipC). Z-stack montage of *E. coli* (10⁷ CFU/mL) after exposure to 4 µg/mL biotinylated BF2HipC. Each z-step represents a 0.07 µm slice through the z-plane. Peptides were visualized using a streptavidin-AlexaFluor 488 conjugate to bind to biotin tag on peptide N-terminal region and visualized on a Leica SP5 LSCM confocal microscope with excitation at 488 nm by an argon laser.

The notable discrepancy in antibacterial activity between BF2HipC and HipC can be explained by HipC’s lack of DNA-binding sequences. Buforin 2 is known to contain DNA binding sequences of the histone H2A subunits (Cho et al., 2009); this DNA binding is likely necessary for the antibacterial activity observed in both BF2HipC and buforin 2. Because both BF2HipC and buforin 2 are antibacterially active yet share a translocating mechanism with the inactive HipC, it follows then that the reason for HipC’s lack of antibacterial activity is likely related to the lack of the buforin 2 sequence that is common to both BF2HipC and buforin 2. Therefore, despite a translocating mechanism being observed in HipC, its probable lack of DNA binding region limits its effectiveness as an antibacterial agent.

3.3. Histone H2A-derived AMPs Demonstrate Activity Against Cancer Cell Lines
Research implicates buforin 2b, an analogue of buforin 2, in anti-cancer activity as well as activity against bacterial cells (Lee et al., 2008). In order to determine whether our peptides also exhibited anti-cancer properties, results from an MTT cytotoxicity assay in “Capan 2” pancreatic adenocarcinoma (Figure 14) were compared to those from non-carcinogenic cell lines exposed to the same AMP treatment (Figure 17).

![Figure 14. Comparison of trends in AMP activity against cancerous and bacterial cells.](image)

To better understand the nature of the differences in peptide-membrane interactions between bacterial and eukaryotic membranes, pancreatic adenocarcinoma cells were imaged following exposure to biotinylated peptides. As shown in Figure 15,
images of biocytin and TAT provided proof of principle that interactions can be visualized, and mechanisms of peptide’s action differentiated (Figure 15).

![Biocytin and TAT as negative and positive controls, respectively, for translocation into “Capan-2” pancreatic adenocarcinoma cells.](image)

**Figure 15.** Biocytin and TAT as negative and positive controls, respectively, for translocation into “Capan-2” pancreatic adenocarcinoma cells. Peptides were visualized using a streptavidin-AlexaFluor 488 conjugate to bind to biotin tag on peptide N-terminal region and visualized at 40X on a Leica SP5 LSCM confocal microscope with excitation at 488 nm by an argon laser. Images were sectioned along the z-axis in 0.5 – 1 \( \mu \text{m} \) z-slices; images shown here are taken from one z-position and are representative of all the samples imaged. Blue, DAPI; green, AlexaFluor 488-conjugated peptide. Scale bars indicate 20 \( \mu \text{m} \).

Confocal microscopy of “Capan-2” cells exposed to buforin 1 and buforin 2 suggest that the mechanisms of action of these HDAPs against eukaryotic cells may be similar to those observed in bacterial cells (Figure 16). Buforin 1, determined by both the propidium iodide uptake assay and bacterial imaging studies to be membrane permeabilizing in some manner, is seen here to localize around the plasma membrane.
indicating a peptide-membrane interaction but minimal translocation across the membrane when compared to buforin 2 (Figure 16). Buforin 2 appears to maintain its translocating ability even in eukaryotic systems as is evidenced by the presence of green fluorescence in the same locations stained by DAPI (indicating the presence of a cell nucleus) (Figure 16). Most clearly seen as turquoise fluorescence in the merged image, buforin 2’s translocating ability is also noted by the lack of staining around the cell membrane as is observed in samples treated with biocytin (Figure 15) and buforin 1 (Figure 16).
Figure 16. Buforin 1 and buforin 2 exhibit differing interactions with mammalian cell membranes ("Capan-2" cells). Peptides were visualized using a streptavidin-AlexaFluor 488 conjugate to bind to biotin tag on peptide N-terminal region and visualized at 20X (buforin 2) and 40X (buforin 1) with 2X digital zoom on a Leica SP5 LSCM confocal microscope with excitation at 488 nm by an argon laser. Images were sectioned along the z-axis in 0.5 – 1 µm z-slices; images shown here are taken from one z-position and are representative of all the samples imaged. Blue, DAPI; green, AlexaFluor 488-conjugated peptide. Scale bars indicate 20 µm.

Comparison of cytotoxicity data in pancreatic adenocarcinoma cells with normal eukaryotic fibroblasts (Figure 17) suggest that there is variability in anti-cancer activity within the H2A-derived HDAP family of interest. This variability can be compared with relative antibacterial activity to determine whether trends in eukaryotic cytotoxicity are also seen in bacterial studies.
Figure 17. Ratio of viabilities of normal eukaryotic cells to cancerous cells after treatment with 1µM HDAPs. Percent viabilities were determined by the MTT assay as described above and a ratio was taken of the amount of cellular growth observed in normal, fibroblast cells compared with a pancreatic adenocarcinoma cell line (“Capan-2”). Larger bars suggest higher rates of cancer-selective cell death.

Perhaps most notable among the anti-cancer data presented here is the lack of correlation between antibacterial and anticancer activity. As seen in Figure 18, increased anticancer activity does not always correlate with increased antibacterial activity, indicating that membrane differences between prokaryotes and eukaryotes may have a significant effect on the ability of HDAPs to interact with the membrane, as well as the nature of those interactions. Additionally, certain peptides may play a critical role in the correlation of interactions between bacterial and eukaryotic membranes. For example, peptides whose cytotoxic activity is dependent upon the translocating ability of buforin 2 (buforin 2 and BF2HipC) do not correlate as well as peptides whose activity is dependent upon parasin-induced permeabilizing mechanisms. This trend further supports the
hypothesis that membrane composition is critical for translocating ability to be conferred, while permeabilizing ability may depend less upon individual membrane compositions. Though HipC is a translocating peptide, it appears universally inert (Figures 4 & 14) and is therefore not relevant to this claim.

![Figure 18. Trends in anti-cancer and anti-bacterial activity among HDAPs. Red line indicates anticancer activity; green line indicates antibacterial activity. Anticancer activity against “Capan-2” cells was determined by the MTT cytotoxicity assay (Figure 6). Relative antibacterial activity of HDAPs against ampicillin-resistant E. coli was determined by the RDA assay (Figure 3).](image)

3.4. Future Directions for the Project

This thesis begins to answer several questions about the mechanics of antimicrobial peptides, namely the level of modularity that is conferred on AMP mechanism, and on the differences in interactions between eukaryotic cells and bacterial cells.
Further studies will help to elucidate the extent to which predictions can be made on AMP mechanism of action and activity based on the subunits of which each peptide is made. Preliminary studies in our lab have investigated the activity and mechanism of a parasin + DesHDAP1 chimeric peptide. As reported here, parasin is a highly membrane permeabilizing peptide; DesHDAP1 is a novel design peptide designed by our lab that has been found to act by a translocating mechanism. Thus far, preliminary data indicate that the parasin + DesHDAP1 chimera (P+D) is selectively active against bacterial cells. Additionally, confocal microscopy suggests that P+D acts via a translocating mechanism (Maria LaBouyer, personal communication). These results suggest that constituent mechanism alone may not be sufficient to characterize the mechanisms of the resulting chimeric peptide. Investigating the nature of individual amino acids may provide a clue as to the mechanism of efficacy of the resulting peptide. As shown below, many of these AMPs are amphipathic (Figure 19), a trait that has often been implicated in increased antibacterial activity due to an improved ability to maneuver within the plasma membrane.
Figure 19. HDAP helical wheel predictions generated by Helixator (Transporter Classification Database, Saier Lab Bioinformatics Group). Hydrophobic residues are shown in blue.

Additionally, future studies on AMP activity against cancer cell lines may help to further elucidate trends in peptide-membrane interactions. To this end, a more robust microscopy protocol would be helpful. Current studies involve fixed cells on slides, allowing for the reuse of individual slides, as well as a more time-sensitive “image” being captured. Though this method has been used in AMP studies published by other labs (Hilchie et al., 2011; Takeshima et al., 2003), there is cause for concern if fixation with
paraformaldehyde allows for cell entry by the peptides of interest. This issue was of particular interest to the field of cell penetrating peptides (CPP) a number of years ago when it was discovered that the cell fixation used in slide preparation for imaging studies led to artifactual redistribution of CPPs to the nucleus (Richard et al., 2003; Vivès et al., 2003). At the time of writing, non-fixed control experiments have not yet been run, but the minimal cell entry observed in both biocytin- and buforin 1-treated cells (Figures 15 and 16) suggests that this method of study may still provide valuable information about the interactions between AMP and eukaryotic plasma membrane.

Further investigations are required to confirm the ability to characterize and categorize AMPs based on their modular makeup. The study presented here represents the first time to our knowledge that the characterization of AMPs has been approached in such a manner; this perspective may allow for faster, more efficient peptide design for antibacterial therapeutics as well as potential drug delivery systems.


