Insect-Derived Hybrid Antimicrobial Peptides: Antibiotics for the Next Generation?

by

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Abstract.

There has been recent interest in utilizing natural antimicrobial peptides (AMPs) as novel antibiotics. Hybrid antimicrobial peptides (hAMPs) are synthetic molecules containing active motifs from multiple parental AMPs. This thesis presents *in vitro* activity data of hAMPs designed b y combining binding and killing motifs from different invertebrate and vertebrate AMPs. The goals of hAMP design are to improve antimicrobial efficacy and reduce host cytotoxicity. Activity was further optimized by modifying basic properties of the peptide such as charge, hydrophobicity, peptide length and specific amino acid composition. Overall, hAMPs exhibited higher antimicrobial efficacy than their active motifs alone *in vitro*. Some hAMPs kill bacteria through membrane permeabilization while others likely attack intracellular targets. Synergistic activity was observed when different hAMPs were combined together separately or when combined in the form of a multivalent hAMP. Our results indicate that hAMPs may be suitable for use as novel antimicrobial agents.

Keywords: Antimicrobial Peptides; AMPs; Hybrid Antimicrobial Peptides; hAMPs; Antibiotics

Dedication

To my parents, Karen and Christopher Loh: for all your love and support through this endeavour.

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I will like to express my gratitude toward several individuals who were instrumental in the completion of this project.

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List of Acronyms

Term	Initial components of the term
AMP	Antimicrobial Peptide
Bm	Binding Motif
DMEM	Dulbecco's Modified Eagle Medium
dsRNA	Double stranded RNA
EPEC	Enteropathogenic *E. coli*
FBS	Fetal Bovine Serum
FIC	Fractional Inhibitory Concentration
Gram +	Gram-positive
Gram -	Gram-negative
hAMP	Hybrid Antimicrobial Peptide
HC	Hemolytic Concentration
IIS	Innate Immune System
Km	Killing Motif
LB	Luria Bertani
LPS	Lipopolysaccharide
MHC	Minimum Hemolytic Concentration
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
MVP	Multivalent Peptide
mvAMP	Multivalent Hybrid Antimicrobial Peptide
OD	Optical Density
PAMP	Pattern-associated Molecular Patterns
PBS	Phosphate Buffered Saline
PI	Propidium Iodide
PRR	Pattern Recognition Receptor
RISC	RNA-induced Silencing Complex
ROS	Reactive Oxygen Species
SI	Selectivity Index
SIR	Selectivity Index Range
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth

Glossary

Term	Definition
Fractional Inhibitory Concentration (FIC) Index	A numerical value describing the effects of combining antimicrobial agents
Hybrid Antimicrobial Peptide (hAMP)	A synthetic antimicrobial peptide (AMP) consisting of active motifs from different natural AMPs
Minimum Inhibitory Concentration (MIC)	The lowest concentration of antimicrobial compound at which growth inhibition is observed after an overnight incubation (Andrews, 2001)
Multivalent Peptide	A synthetic molecule consisting of multiple peptide subunits covalently attached onto a single molecular scaffold
Selectivity Index (SI)	A mathematical expression of the therapeutic suitability of our hAMPs; a ratio of host cytotoxicity to antimicrobial efficacy

Chapter 1. Introduction.

1.1. Abstract.

The innate immune system, comprising humoral and cellular components, is the first line of defense against microbial pathogens in all organisms. Antimicrobial peptides (AMPs) play a major role in the innate immune response of all organisms. In invertebrates, AMPs eliminate microbial pathogens in the hemocoel, and regulate the numbers of beneficial and detrimental microbes in the midgut. In vertebrates, besides their antimicrobial role, AMPs also act as immunomodulators and mediators between the innate and adaptive immune systems. Despite their great structural diversity, AMPs share several common features including their cationicity and amphipathicity, both of which enable them to bind to and penetrate prokaryotic membranes. In this chapter I will summarize the current knowledge on AMPs; their properties, mode of action, role in the innate immune response, and potential as novel antibiotics.

1.2. Introduction to Innate Immunity.

The innate immune system (IIS) is the first line of defence against pathogens and parasites. It is nonspecific and does not confer long-lasting protection against pathogens. This is unlike the adaptive immune system in vertebrates, which is highly specific and confers long lasting immunity against subsequent infections. The IIS is the principal means of antimicrobial defense in bacteria, fungi, plants, and invertebrates, including insects.

The IIS (1) acts as a physical and chemical barrier (e.g. skin, cuticle, gastrointestinal lining, stomach acids), (2) destroys foreign particles and microbes within the body via cellular (e.g. phagocytosis) and humoral (e.g. antimicrobial peptides) components, and (3) acts as an immunomodulator (i.e. by recruiting immune cells to sites of infection via cytokines). The complement system of vertebrates acts within the innate immune response and as a bridge between the IIS and the adaptive immune system (Ricklin and Lambris, 2007). Complement consists of proteins, which contribute to opsonisation (tagging of target cells for phagocytosis), chemotaxis, and lysis of pathogens. The complement system also activates the adaptive immune response in several ways. These include (1) augmenting the antibody-mediated (humoral) response, (2) enhancing T-lymphocyte responses, and (3) enhancing immunological memory (Ricklin and Lambris, 2007).

1.3. Innate Immunity in Insects.

Insects are one of the most evolutionarily successful groups of organisms on the planet. They have colonized virtually every continent except Antarctica and occupy a diverse array of habitats with the exception of oceanic and polar environments. There are over 1 million described insect species to date with current estimates of total insect diversity ranging from 2.5-7 million species (Hamilton et al. 2010).

The innate immune system of insects is one of the main contributors to their evolutionary success (Christophides et al. 2004). This first line of defence includes both the integument, which consists of layers of cuticle, and the peritrophic membrane, which

lines the digestive tract of many insects. Once these layers are breached, an array of humoral and cellular responses is activated. These include proteolytic cascades (blood clotting and melanotic encapsulation), phagocytosis, and the expression and mobilization of antimicrobial peptides.

Recognition of non-self is the first essential step to initiate any anti-parasite/pathogen response. Pathogen-associated molecular patterns (PAMPs) are conserved domains located on the surfaces of microbial pathogens such as bacteria and fungi. Examples of PAMPs include the lipopolysaccharide (LPS) layer in Gram-negative (Gram -) bacteria and the peptidoglycan cell wall in Gram-positive (Gram +) bacteria (Nappi et al. 2004). Recognition is first initiated by binding of the specific PAMP to receptors on the cell (hemocyte) surface, known as pattern recognition receptors (PRRs) (Tsakas and Marmaras 2010, Medzhitov and Janeway 1997). PRR-PAMP binding subsequently activates downstream signalling cascades, which upregulate the expression of immunity-associated genes, leading to an immune response (Welchman et al. 2009).

1.3.1. Phagocytosis.

Phagocytosis is a cellular response that involves the intake of large particles into cells via phagosomes. In insects, phagocytosis is performed by designated hemocytes known as plasmatocytes or granulocytes (Rosales, 2011). Phagocytosis in insects serves two functions: (1) to eliminate microbial invaders such as bacteria and (2) to scavenge for dead/dying (apoptotic) cells (Rosales, 2011). This process often is initiated by specific PRR-PAMP binding on the cell surface (Tsakas and Marmaras 2010). Receptor binding results in the activation of signalling pathways, which ultimately activate phagocytosis. Once engulfed, the phagosome fuses with a lysosome to form a phagolysosome, where acidification and destruction of the foreign particle (via hydrolytic enzymes) occurs (Rosales 2011).

1.3.2. Melanotic Encapsulation.

Melanotic encapsulation involves both cellular (encapsulation) and humoral (melanisation) responses. During encapsulation, groups of hemocytes surround and engulf the target (usually a large object) in multiple layers until a capsule is formed (Rosales, 2011). The usual targets of encapsulation are eukaryotic parasites (e.g. protozoa, nematodes) that are too large to phagocytose (Rosales, 2011).

Following encapsulation, melanisation may ensue. This process involves a proteolytic cascade initiated by PAMP recognition followed by the activation of phenoloxidase from prophenoloxidase. This pathway is regulated and catalyzed by serine proteases (Christensen et al. 2005). Melanin biosynthesis begins with the conversion of phenylalanine to tyrosine via phenylalanine hydroxylase. Tyrosine then undergoes a series of enzyme-catalyzed steps to form, in succession, dopaquinone, dopachrome, indolequinone, and eumelanin (Christensen et al. 2005). The encased organism is then killed by reactive oxygen species (ROS), which arise from the melanin biosynthesis pathway.

1.3.3. The Humoral Response: Immune Signaling Pathways.

The humoral response in insects consists mainly of factors secreted by specific tissues. The most prominent of these is the fat body, the equivalent of the vertebrate liver (Tsakas and Marmaras 2010). The most important humoral factors include antimicrobial peptides (AMPs, Section 1.4) and ROS generated from the melanisation pathway. AMP production is regulated by two main immune signalling pathways, Toll and Imd (Tsakas and Marmaras 2010, Welchman et al. 2009). Figure 1.1 illustrates an overview of immune signalling in insects. To initiate the signalling cascade, PRRs on the hemocyte cell surface bind to PAMPs on the pathogen surface (Tsakas and Marmaras 2010, Medzhitov and Janeway 1997). The activation of these immune pathways results in the activation of downstream NF-κB transcription factors and eventually the transcriptional upregulation of AMP genes. Traditionally, we consider that Gram + bacteria and fungi activate the Toll pathway while the Imd pathway is triggered by Gram - bacteria (Welchman et al. 2009). However there is growing evidence that there is

cross-talk between and among immune pathways and that certain molecules function in multiple pathways (Cooper and Mitchell-Foster 2011, Cerenius et al. 2010, Tsakas and Marmaras 2010, Steinert and Levashina 2011).

1.3.4. Immune Responses to Viral Infection.

There are three main pathways that are activated in response to viral infections and other forms of cell stress: (1) JAK-STAT (Tsakas and Marmaras 2010, Welchman et al. 2009, Steinert and Levashina 2011), (2) RNAi (Cooper and Mitchell-Foster 2011, Steinert and Levashina 2011, Tsakas and Marmaras 2010), and (3) apoptosis (Cooper and Mitchell-Foster 2011, Cooper et al. 2009).

In the JAK-STAT pathway, cytokines released from damaged/infected cells bind to the receptor Domeless on the cell surface (Tsakas and Marmaras 2010). This event in turn initiates the phosphorylation of the transcription factor STAT by the kinase JAK. Activated STAT, which is dimerized, migrates to the nucleus and activates anti-viral and repair-associated genes (such as growth factors) (Welchman et al. 2009, Steiner and Levashina 2011).

In the RNAi pathway, silencing of viral mRNAs occurs via the generation of small RNAs. Double-stranded viral RNA (dsRNA) is recognized by an RNase III (Dicer), which is cleaved into small interfering RNAs, or siRNAs (Tsakas and Marmaras 2010). A dsRNA binding protein (R2D2) binds siRNA and separates the strands into a passenger strand and a guide strand. Both of these are loaded onto RISC (RNA-induced silencing complex), where the passenger strand is degraded and the guide strand serves to lead RISC to complementary viral RNA sequences, where further RNA degradation occurs (Steiner and Levashina 2011, Tsakas and Marmaras 2010), preventing expressed mRNAs from being translated into proteins.

Apoptosis, or programmed cell death is a highly conserved mechanism employed by eukaryotic organisms to removed aged, damaged, or abnormal cells (Cooper and Mitchell-Foster 2011, Kaufmann and Hengartner 2001), including cells containing pathogens. In response to a 'death stimulus', a signalling cascade is initiated

by the cell, resulting in the expression of effector cysteine proteases known as caspases (Cooper and Mitchell-Foster 2011, Kornbluth and White 2005, Aravind et al. 2001). Apoptosis is a highly effective strategy in the antiviral response as it can severely limit pathogen production and spread by the self-destruction of virus-infected cells (Cooper and Mitchell-Foster 2011). Apoptotic responses have been documented in insects in response to viral infection (Wu et al. 2013, Ikeda et al. 2011, Cooper and Mitchell-Foster 2011, Bowers et al. 2003), and many insect viruses contain inhibitors of apoptosis genes in their genomes to thwart this anti-viral response (Cooper and Mitchell-Foster 2011, Benedict et al. 2002, Liang et al. 2012).

1.4. Antimicrobial Peptides (AMPs).

Antimicrobial peptides (AMPs) are present in all taxonomic groups of organisms from bacteria to humans. In general, they are short (6-60 amino acids) peptides that adopt an amphipathic conformation when interacting with membranes. Approximately 50% of an AMP consists of hydrophobic residues (Huang et al. 2010). Most AMPs are cationic due to the presence of basic amino acids (Lys and Arg) although anionic AMPs do exist (Brogden, 2005). In insects, AMPs are primarily synthesized by the fat body (Hoffmann 2003, Bulet et al. 1999, Tsakas and Marmaras 2010). The recognition of PAMPs by their corresponding PRRs triggers signalling pathways that result in the transcription, expression, and release of AMPs. In vertebrates, AMPs can have dual roles in killing pathogens directly and modulating the inflammatory response by stimulating the release of chemokines and modulating/activating the cells of the adaptive immune system (Alalwani et al. 2010, Choi and Mookherjee 2012, Cirioni et al. 2006, Niyonsaba et al. 2002, Oppenheim et al. 2003).

AMPs have been described by functional groups, 3D shape, or amino acid composition. α-helical AMPs are the most abundant and widely studied class of AMPs described to date (Amiche and Galanth 2011, Huang et al. 2010) and include cecropins, magainins, buforins, cathelicidins, and pleurocidins (Brogden 2005). The amphipathic helices typically have clusters/faces of hydrophilic and hydrophobic amino acids. These faces often have a repeating motif of a specific sequence of amino acids (Brogden 2005,

Giuliani et al. 2007). In aqueous solution the peptides assume an unstructured conformation but upon interaction with lipid membranes, an α-helical structure is adopted (Texeira et al. 2012). Most α-helical AMPs are thought to damage membranes, inducing microbial cell death by creating holes in outer membranes causing a loss of essential ions and/or lysis (Teixeira et al. 2012). Some AMPs, such as the buforins, however, target nucleic acids without causing extensive damage to the cell membrane (Fleming et al. 2008, Cho et al. 2009, Jang et al. 2012), and certain cecropins have been demonstrated to enter the cell and affect RNA transcription and protein translation (Hong et al. 2003, Wimley, 2010, Lu et al. 2012)

β-sheet AMPs include several important groups of AMPs such as the defensins (Ganz, 2003), thanatin (Fehlbaum et al. 1996), and the protegrins (Steinberg et al. 1997, Ostberg and Kaznessis 2005). These peptides typically contain several cysteine residues that form disulphide bridges, creating cyclic or hairpin-like structures. These disulphide bridges are essential in maintaining both antimicrobial activity and stability in serum (protease resistance) (Ganz 2003, Fehlbaum et al. 1996, Ostberg and Kaznessis 2005). The proposed mechanism of action for many β-sheet AMPs is through membrane permeabilization (Jenssen et al. 2006). There are a few peptides in this category that have intracellular targets. The histatins, peptides belonging to the defensin class, can permeabilise membranes and target mitochondria in fungal cells (Kavanagh and Dowd 2004).

Other AMPs are enriched in one or two specific amino acids. These can form a variety of structures such as extended helices, random coils, and loops (Brogden, 2005). Examples include indolicidin (Trp-rich) (Selsted et al. 1992), abaecin (Pro-rich) (Boman 1995), and bactenecins (Arg & Pro-rich) (Shamova et al. 1999).

Electrostatic attraction and binding has to occur before AMPs can interact with bacterial membranes (Pasupuleti et al. 2012, Epand et al. 2010). The cationic nature of most AMPs allows them to bind to negatively charged elements on the bacterial surface such as anionic phospholipids (e.g. phosphatidylglycerol, disphosphatidylglycerol), the lipopolysaccharide (LPS) layer in Gram - bacteria, and teichoic acids in Gram + bacteria (Epand et al. 2010). Typically, most cationic AMPs

range in charge from +2 to +9 (Jenssen et al. 2006, Giuliani et al. 2007, Fox et al. 2012). This initial binding step results in the clustering of peptides at the lipid-peptide interface, which then leads to further interactions between the peptide and the membrane lipids (Brogden 2005, Pasupuleti et al. 2012).

The accumulation of AMPs at the membrane surface initially results in a conformational change in secondary structure. Upon reaching a threshold concentration, the peptides then insert into the membrane to form pores or translocate into the cytosol (Pasupuleti et al. 2012, Yount and Yeaman 2005, Amiche and Galanth 2011, Fleming et al. 2008). Insertion is mediated by hydrophobic interactions of the amphipathic regions of the peptide with the phospholipid bilayer. Several models have been proposed to describe how insertion/permeabilisation occurs (Figure 1.2). In both the barrel stave and toroidal pore models, distinct pores are formed in the membrane, either lined with peptides only (barrel stave), or with both lipids and peptides (toroidal pore) (Pasupuleti et al. 2012, Teixeira et al. 2012, Brogden 2005). In the carpet model, solubilisation of the membrane occurs via micellization by peptides (Teixeira et al. 2012, Brogden 2005). In the aggregation channel model, peptides form unstructured aggregates with membrane phospholipids, creating channels through which essential ions leak out from the target cell (Li et al. 2012). In the molecular electroporation model, there is an increase in membrane potential due to the accumulation of peptide at the membrane surface. This temporarily increases the permeability to various molecules including the peptides (Teixeira et al. 2012). The consequences of peptide entry include cell death from lysis or loss of essential ions.

Some AMPs, in addition to permeabilizing membranes, also interact with intracellular structures such as nucleic acids and essential proteins involved in processes such as cell wall synthesis, protein synthesis, and enzymatic activity (Brogden 2005, Teixeira et al. 2012). The amphibian-derived buforins are a notable example of such peptides as they bind specifically to nucleic acids following translocation through the membrane (Park et al. 2000, Uyterhoeven et al. 2008, Jang et al. 2012). In the case of buforin II, peptide translocation is mediated by phospholipid composition, with penetration enhanced by negatively-charged phospholipids (phosphatidylglycerol) (Fleming et al. 2008).

In response to infection, organisms typically express more than one AMP (Nguyen et al. 2011, Peschel and Sal 2006). The simultaneous expression of many AMPs may enhance killing efficacy through synergistic interactions between peptides with different modes of action (Nguyen et al. 2011, Mangoni and Shai, 2009, Mor et al. 1994). The probability of resistance mechanisms developing to all AMPs by the target pathogen is also reduced (Nguyen et al. 2011, Peschel and Sahl 2006).

1.5. AMPs as Therapeutics.

Within the past 40 years, only three new classes of antibiotics have been developed against Gram + bacteria and none for Gram - bacteria (Oyston et al. 2009). This scarcity of new antibacterial drugs coupled with the increasing incidence of multi-drug resistant pathogens requires that we look for alternative sources of antimicrobial compounds. Consequently, there has been recent interest in the therapeutic potential of AMPs (Brogden and Brogden 2011, Huang et al. 2010, Marshall and Arenas 2003). The attractiveness of AMP-based therapies is due to the selectivity of many AMPs for prokaryotic over eukaryotic membranes. Several studies have noted the decreased susceptibility of AMPs towards microbial resistance (Hancock and Patrzykat, 2002) although others have indicated otherwise (Gruenheid and Moual 2012, Bell and Gouyon 2003, Perron et al. 2006). The evolution of resistance to therapeutic AMPs has been deemed to be of great concern as this may result in the evolution of bacteria resistant to host peptides (Bell and Gouyon 2003, Habets and Brockhurst 2012). Bacteria have developed resistance to therapeutic peptides *in vitro* (Anaya-Lopez et al. 2013). Selection experiments with the therapeutic AMP Pexiganan created resistant strains of *E. coli* and *Pseudomonas* fluorescens within 600-700 generations (Perron et al. 2006, Anaya-Lopez et al. 2013). Bacterial resistance to host AMPs *in vivo* has not been observed (Perron et al. 2006), probably because the expression of multiple AMPs with different modes of action during infection impedes or retards the evolution of resistance (Perron et al. 2006). Some documented mechanisms of bacterial resistance to AMPs include proteolytic degradation (Schmidtchen et al. 2002, Hritonenko and Stathopoulos, 2007, Gutner et al. 2009), shielding of the bacterial surface by ‘decoys’ (Gruenheid and Moual 2012, Llobet et al. 2008, Foschiatti et al. 2009), alteration of net surface charge by

reducing anionicity (Koprivnjak and Peschel 2011, Collins et al. 2002), membrane proteins that export or import AMPs (Gruenheid and Moual 2012, Parra-Lopez et al. 1993, Mason et al. 2005, Shafer et al. 1998), and downregulation of AMP gene expression (Islam et al. 2001, Sperandio et al. 2008, Chakraborty et al. 2008). The disadvantages of AMP-based therapies include the lack of stability in serum due to proteases, possible cytotoxicity toward host cells, and reduced efficacy *in vivo* (Brogden and Brogden 2011).

Several therapeutic AMPs have recently entered clinical development (See Table 1.1) although most have not been commercialized due to efficacy problems encountered during clinical trials. Most of these therapeutic peptides were derived from naturally occurring AMPs with some modifications in amino acid sequence in hopes of improving antimicrobial activity or stability. Pexiganan (MSI-78) is a synthetic 22-amino acid derivative of magainin (Genaera, Plymouth, PA, USA), a naturally occurring AMP from the clawed frog *Xenopus laevis* (Zasloff 1987). It was rejected by the FDA in 1999 despite showing promising results in wound healing during Phase III clinical trials as it did not exhibit any significant improvement in efficacy over current treatments (Marr et al. 2006). Iseganan (Intrabiotics Pharmaceuticals Inc., Mountain View, CA), derived from porcine protegrin, met a similar fate. It was originally developed as a mouth rinse to prevent polymicrobial infection associated with oral mucositis but had efficacy problems during clinical trials (Jenssen et al. 2006, Giles et al. 2004). The company withdrew the product and redeveloped it into an aerosolized spray for treating ventilator-associated pneumonia. This strategy backfired as patients receiving Iseganan treatment had higher rates of pneumonia than the placebos (Jenssen et al. 2006).

Therapeutic peptides that have been commercialized successfully include bacteria-derived polymixin b and gramicidin, both of which are used as topical treatments for infections caused by *Pseudomonas aeruginosa* and *Acinetobacter baumanni* (Marr et al. 2006). Polymixin b is a cyclic lipopeptide (Zavascki et al. 2007) while gramicidin is a linear peptide consisting of alternating L and D amino acids (Burkhart et al. 1999). Both peptides are toxic when administered at clinical doses systemically so the extent of their usage is limited (Marr et al. 2006). Daptomycin is another bacterial-derived lipopeptide that has been approved for systemic treatment of

Gram + bacterial infections such as *Staphyloccocus aureus* and *Streptococcus spp.* (Marr et al. 2006).

Omiganan (Migenix Inc., Vancouver, BC, Canada) is a bovine indolicidin-derived 12-amino acid peptide that is currently undergoing clinical development (Phase III clinical trials). It has demonstrated significant efficacy in reducing catheter infections as well as a topical agent in treating acne. (Marr et al. 2006, Giuliani et al. 2007). It has broad spectrum activity, exhibiting antimicrobial activity against Gram + and Gram - bacteria as well as fungi (Fritsche et al. 2008, Sader et al. 2004).

These examples of therapeutic peptides demonstrate that there are still roadblocks that need to be surmounted before AMPs can be used more widely as antibiotics. To overcome these obstacles, several strategies have been developed. These include:

(1) The incorporation/substitution of D-amino acids; D-substituted AMPs have been found to be significantly less toxic to mammalian cells while maintaining antimicrobial activity (Avrahami et al. 2001, Papo and Shai. 2004, Papo et al. 2002,, Huang et al. 2010) and are also more resistant to proteases in serum (Papo et al. 2002). Bacteria may be less likely to develop resistance to D-substituted AMPs as these peptides are known to be resistant to degradation by bacterial proteases (Gurenheid and Le Moual 2012).

(2) The development of chimeric or 'hybrid' AMPs (hAMPs) that contain active motifs from 2-3 parental peptides. Several studies have found that hAMPs demonstrated improved efficacy in antimicrobial activity as well as decreased host cell cytotoxicity relative to their parental peptides (Fox et al. 2012, Jeong et al. 2011, Hongbiao et al. 2005, Schlamadinger et al. 2012, Shin et al. 1999, Plunkett et al. 2009). The majority of this thesis will deal with the design, synthesis, and evaluation of hAMPs and their incorporation into multivalent/dendrimeric peptides.

(3) The development of 'peptidomimetics'. These molecules are mimics of natural AMPs as they share their basic properties (cationicity, amphipathicity) but may exhibit enhanced activity, improved stability, and/or decreased host cytotoxicity relative

to the natural peptides. Some of these mimics include multivalent/dendrimeric peptides (Liu et al. 2010, Pieters et al. 2009, Young et al. 2011), peptoids (Giuliani and Rinaldi 2011, Chongsiriwatana et al. 2008) ceragenins (Giuliani and Rinaldi 2011, Epand et al. 2007, Savage et al. 2002), carbopeptides and peptidopolysaccharides (Guell et al. 2012, Li et al. 2012), and synthetic lipidated peptides (Giuliani et al. 2008, Makovitzki et al. 2006). Figure 1.4 illustrates some examples of peptidomimetics.

The approach used in this research is to combine motifs from different naturally occurring AMPs into hybrid AMPs (hAMPs). In this approach we first identified regions of AMPs that bound to selected targets; Gram - bacteria, Gram + bacteria, and fungi. We then combined these with peptide motifs from other AMPs that were predicted or reported to kill target organisms. This combination of binding motifs and killing motifs from 2-3 different AMPS from very diverse organisms was used to create over 120 hAMPs that were evaluated for efficacy against target organisms and toxicity toward mammalian cells. The conceptual design of hAMPs is shown in Figure 1.5.

1.6. Thesis Objectives.

The objectives of this thesis are to:

1) Design and synthesize hAMPs that combine putative binding motifs and killing motifs from 2-3 different naturally occuring AMPs (see Figure 1.5 (a-e))

2) Evaluate the efficacy of hAMPs against a range of Gram - and Gram + bacteria.

3) Evaluate the effects of the hAMPs on eukaryotic cells.

4) Modify specific hAMP characteristics such as charge, amphipathicity, hydrophobicity, secondary structure, and D-amino acid substitutions and evaluate these changes on activity against bacteria and eukaryotic cells.

5) Assess the efficacy of hAMPs against bacteria and eukaryotic cells when applied together or synthesized as multivalent hAMPs (mvAMPs) (see Figure 1.5f)

1.7. References.

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1.8. Figures and Tables.

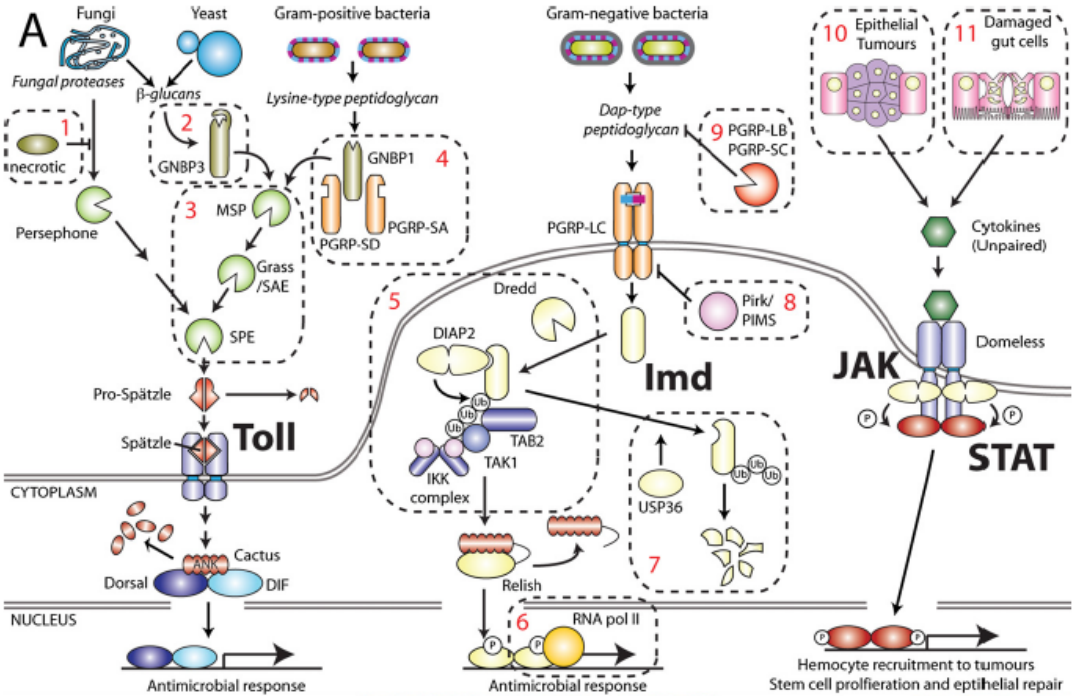

Figure 1.1: Innate immune signaling pathways in insects.

An overview of immune signaling pathways in insects is represented here. Both fungi and Gram + bacteria activate the Toll pathway, which results in the cleavage of Pro-spatzle to form the mature ligand Spatzle. This ligand binds to the Toll receptor on the cell surface, initiating downstream signaling events leading to the transcription of immune (AMP) genes. The Imd pathway responds to the presence of Gram – bacteria. Direct binding of peptidoglycan to PGRP-LC (PGRP= peptidoglycan recognition protein) activates downstream signaling events in the cell where Imd is cleaved by the caspase Dredd and activation of the transcription factor Relish, which then upregulates transcription of immune genes. Cell damage (including viral infection) activates the JAK-STAT pathway via cytokine release- binding of cytokines to the receptor Domeless, resulting in the phosphorylation of the transcription factor STAT by the protein kinase JAK. Dimerized STAT migrates to the nucleus to initiate the transcription of antiviral factors and repair-associated proteins. Note that viral infections also activate other pathways not illustrated here, such as RNAi and apoptosis. Figure from Welchman et al. (2009).

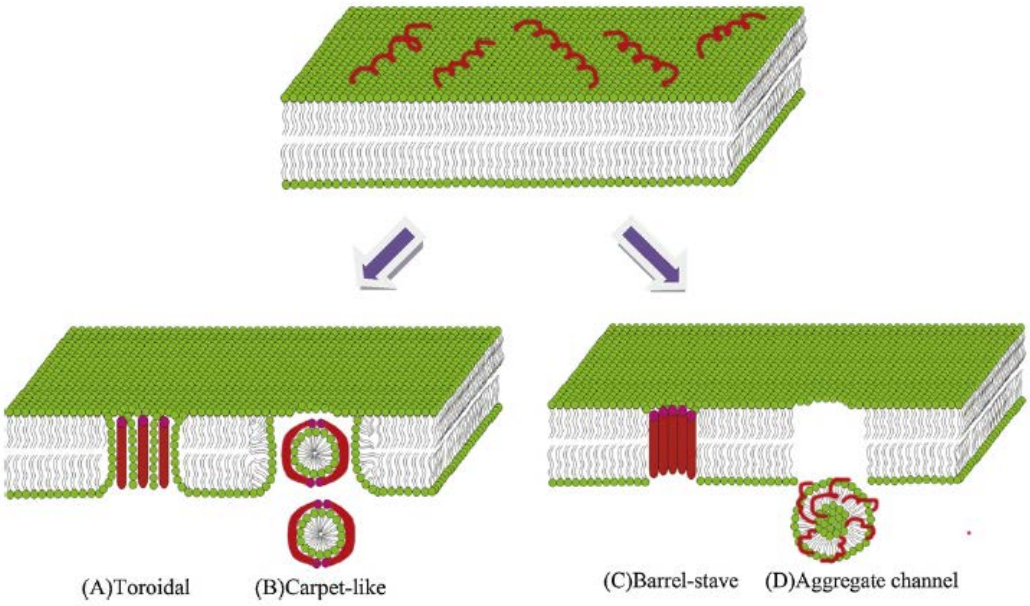

Figure 1.2: Current models of membrane disruption by AMPs.

In the first step (top), an unstructured peptide in solution binds to the membrane surface via electrostatic attraction to the anionic phospholipids. Binding results in a conformation change to the AMP, resulting in a structured peptide. Upon reaching a threshold concentration, pore formation ensues via hydrophobic and hydrophilic interactions with the lipid bilayer. These interactions are mediated by the amphipathic structure of the peptide. In the toroidal pore model (A), the hydrophilic portions of the peptide interact with the phospholipid head groups, forming a pore that is lined with both lipid and peptide. In the carpet model (B), peptides interact with lipids in a detergent-like mechanism, resulting in micelle formation and consequently membrane disruption. In the barrel stave model (C), the peptides span the entire bilayer, forming a peptide-lined pore. The aggregate channel model (D) proposes that peptides interact with the membrane in unstructured clusters/aggregates. Figure from Li et al. (2012).

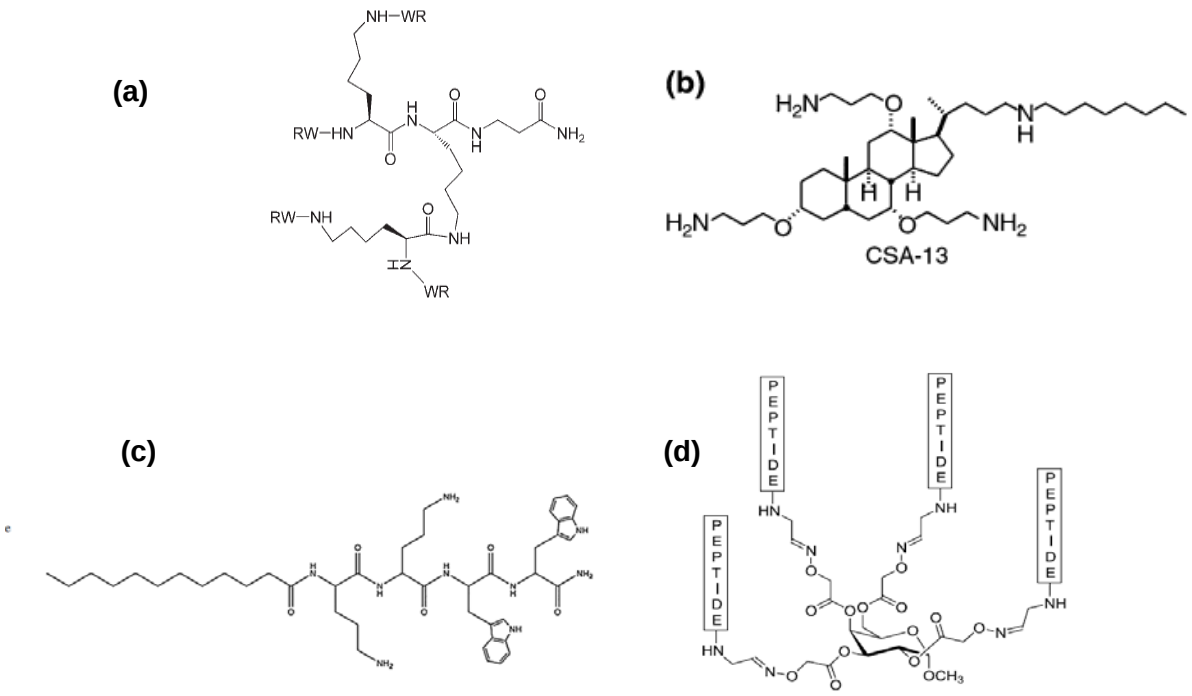

Figure 1.3: Examples of peptidomimetics with peptide subunits attached to different molecular scaffolds. These peptides have increased antimicrobial activity over their individual unattached subunits.

(a) A tetrameric peptide designed on a lysine scaffold containing 4 copies of the peptide RW that was found to be protease resistant and highly active against multi-drug resistant bacteria compared to individual RW molecules (Liu et al. 2007). (b) CSA-13, an example of a ceragenin that attaches peptides to a central sterol backbone (Epand et al. 2007). (c) An antimicrobial synthetic lipopeptide that contains a short peptide sequence/peptidyl backbone (pink) attached to a fatty acid chain at the N-terminus (green) that is responsible for antimicrobial activity (Giuliani and Rinaldi 2011). (d) A tetrameric carbopeptide that attaches peptide subunits to a modified carbohydrate scaffold, α-D-galactopyranoside (Gueell et al. 2012)

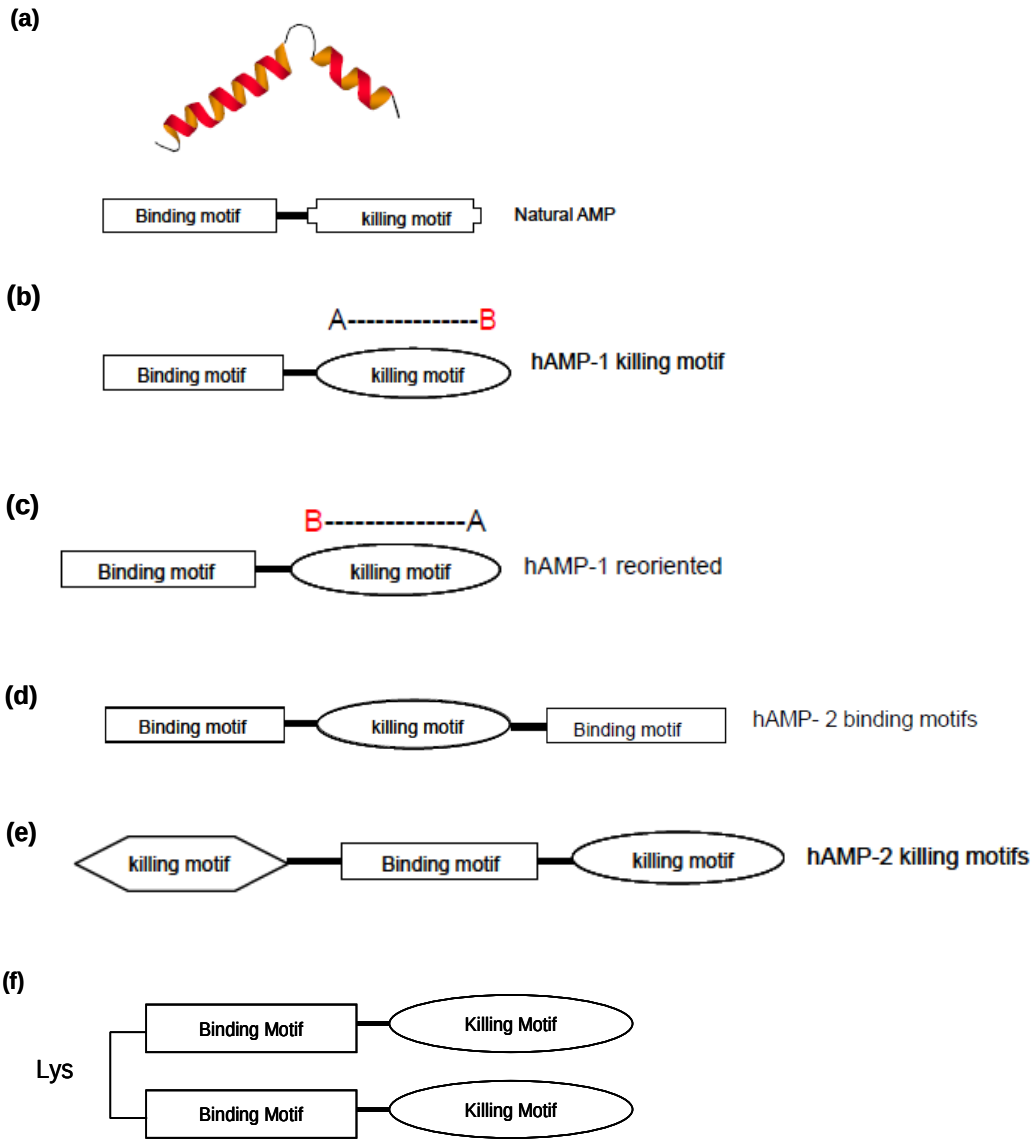

Figure 1.4: Generalized schematic of hAMP combinations designed and screened in this study.

Schematic of different combinations of hAMPs designed by combining active motifs from different peptides into a single "hybrid" protein and tested in the study. (a) A generalized schematic of a natural alpha-helical AMP, with an N-terminal binding motif and C-terminal killing motif separated by a hinge. (b) A hAMP designed as a natural AMP mimic with single killing motif oriented in natural N-C (A-B) conformation. (c) Same hAMP design as (b) but with killing motif oriented in reverse (B-A) conformation. (d) hAMP with 2 binding motifs flanking a central killing motif. (e) hAMP with 2 killing motifs and a central binding motif. (f) A multivalent construct with 2 hAMPs attached to a lysine scaffold.

Table 1.1: Examples of Synthetic AMPs in Commercial Development.

	Product	Company	Trials Outcome and Applications	Planned Trials
Available on the Market	Polymixin b	RX Generic Drugs	G(-) skin infections	None
	Daptomycin (Cubicin®)	Cubist Pharmaceuticals	G(+) skin infections	None
Late Clinical Development (Phase III & later)	Pexiganan	Genaera	Failed FDA approval	None
	Iseganan	Intrabiotics Pharmaceuticals	Failed phase III trials for both oral mucositis and pneumonia	Phase IIa as drug for cystic fibrosis patients
	Omiganan (MX594AN)	Migenix & Cutanea Life Sciences Inc.	Failed Phase III for catheter-related bloodstream infections Successfully completed Phase IIb trials for topical acne treatment, currently in Phase III trials as a topical antimicrobial against rosacea & acne	Not announced
Early Clinical Development (preclinical, Phase I-II)	SB006	Spider Biotech	G(-) infections Preclinical	Not announced
	Neuprex	Xoma	Failed Phase III trials for pediatric meningococcemia	None
	XMP-629	Xoma	Failed Phase II trials as an acne treatment	None
	Mersacidin	Novacta Biosystems	Systemic G(+) infections incl. MRSA Preclinical	Not announced
	Plectasin	Novozymes (partnered with Sanofi Aventis)	G(+) infections Preclinical	Not announced

Note: adapted from Giuliani et al. 2007.

Connecting Statement 1.

In Chapter 1, I presented background information on antimicrobial peptides (AMPs). AMPs are an essential component of the innate immune response of most organisms. Some AMPs are expressed as zymogens whereas many are expressed through signalling cascades after the presence of the pathogen is recognized by PRR-PAMP interactions. Despite the diversity in size and 3D structure, all AMPs share a similar mode of action: electrostatic binding onto the surface of the pathogen followed by killing via membrane disruption and/or damage to intracellular structures. AMP activity is mediated by a combination of factors such as charge, amphipathicity, secondary structure, peptide length and amino acid composition. There is recent interest in utilizing AMPs as novel antibiotics primarily due to their selectivity for bacterial membranes. Hybrid AMPs (hAMPs) are synthetic peptides combining binding and killing motifs from different natural AMPs with the aim of improving antimicrobial efficacy and lowering host cytotoxicity. In the next chapter we present *in vitro* data on the design, screening, efficacy, and hemolytic activities of selected hAMPs.

Chapter 2. Design and *In vitro* Assessment of Six Hybrid Antimicrobial Peptides.

2.1. Abstract.

There has been recent interest in the therapeutic potential of antimicrobial peptides (AMPs) to treat bacterial infections. In simplest terms, an AMP consists of a binding motif to facilitate electrostatic attachment to the bacterial membrane surface, and a killing motif that causes membrane disruption via hydrophobic interactions with the lipid bilayer. One strategy to improve AMP efficacy is to design hybrid antimicrobial peptides (hAMPs) that contain active motifs from 2-3 different parental peptides. Here, we present *in vitro* activity data of selected hAMPs comprised of binding and killing motifs of AMPs from different organisms. Antimicrobial activity was evaluated against a range of Gram-positive (Gram +) and Gram-negative (Gram -) bacteria and eukaryotic host cell toxicity was assessed against erythrocytes and two immortalized human cell lines. Toxicity to bacteria was improved by altering various parameters such as charge, hydrophobicity, amphipathicity, peptide length, and D-amino acid substitutions. The data indicate that a balance among hydrophobicity, amphipathicity, and charge is required to achieve optimal antimicrobial activity while minimising hemolytic activity. We calculated a universal selectivity index range for each hAMP against all Gram + and Gram - bacteria tested and also a selectivity index for each hAMP against each bacterium tested. Our hAMPs varied in their activities against different bacteria, ranging from broad-spectrum activity to greater toxicity towards either Gram + or Gram – bacteria, or to a specific bacterium. This study highlights important parameters that contribute to the efficacy and toxicity of hAMPs and demonstrates the potential of hAMPs as broad spectrum, or tailor-made antibiotics to treat a particular pathogen or a group of pathogens.

2.2. Introduction.

Antimicrobial peptides (AMPs) are an essential component of the innate immune system of all organisms. Over 2,300 AMPs have been characterized as of 2014 (Antimicrobial Peptide Database: http://aps.unmc.edu/AP/main.php). AMPs are typically short (6-60 amino acids) amphipathic peptides with diverse secondary structures. AMPs range from simple linear cationic alpha-helical peptides to peptides with complex beta-sheet structures and disulfide bridges (Bulet et al. 1999, Haney et al., 2009). AMPs target a broad spectrum of microbial pathogens including viruses, bacteria, fungi and parasites (Jenssen et al. 2006, Marshall and Arenas, 2003).

The mode of action of many AMPs is not well understood. Electrostatic binding by the cationic AMPs to the anionic bacterial membrane must first occur before killing can proceed (Teixeira et al. 2012, Hancock and Rozek 2006). Killing may be the result of membrane permeabilisation via pore formation, causing either cell lysis or loss of essential ions (Teixeira et al. 2012). Some AMPs also interact with intracellular targets (Brogden 2005, Hancock and Rozek 2006, Teixeira et al. 2012) while others act as immunomodulators (Huang and Chen, 2010), controlling the release of cytokines or acting as anti-inflammatory agents during infection (Alalwani et al. 2010, Choi and Mookherjee, 2012).

There is interest in developing AMPs as novel therapeutic agents due to the lack of new conventional antibiotics being developed and the emergence of multi-drug resistant pathogens (Huang et al. 2010, Marshall and Arenas, 2003, Brogden and Brogden 2011). MRSA (methicillin-resistant *Staphylococcus aureus*) is an increasing problem in hospitals, nursing homes, and community settings worldwide (Appelbaum 2006), and multidrug resistant strains of *Acinetobacter baumannii*, have developed resistance to all known antibiotics (Peleg et al. 2008). Epidemics of multi-drug resistant tuberculosis are becoming an increasing threat on a global scale especially when coupled with the ongoing HIV epidemic (Barnard et al. 2008). Equally worrisome is the reduced research emphasis into the development of new antibiotics (Oyston et al. 2009, Williams et al. 2009). The reasons for this stem, in part, from the high cost of drug development and the shift in the interests of biopharmaceutical companies towards more

lucrative avenues of research into therapies for cancer and other chronic/age-related disorders (Williams et al. 2009). There are currently only three classes of antibiotics regularly used. AMPs would constitute a new class of antibiotics if they can be developed to be efficacious, demonstrate low cytotoxicity toward host cells (Hancock and Patryzkat 2002, Maher & McClean, 2006, Maher & McClean 2008), and are stable in biological fluids (e.g. serum) (Papo et al., 2002, Jung et al., 2007).

Hybrid antimicrobial peptides (hAMPs) are synthetic AMPs comprised of two to three active binding and killing motifs from different naturally occurring parental peptides arranged in non-normal conformations. hAMPs ideally would have enhanced antimicrobial properties with minimal toxicity toward host cells (Fox et al. 2012, Brogden & Brogden, 2011, Huang et al. 2010). Melittin, an AMP from the honey bee, kills both prokaryotic and eukaryotic cells (Habermann 1972, Dempsey 1990, Raghuraman and Chattopadhyay 2007) whereas cecropin-melittin hybrids display comparable, if not greater, antimicrobial activity than either parental peptide while having reduced, but still significant host cell toxicity (Shin et al. 1997, Shin et al. 1999). hAMP design must consider the effects of charge, hydrophobicity, amphipathicity, peptide length, sequence composition, and secondary structure (Yin et al., 2012, Rekdal et al., 2012, Hoernke et al. 2012, Jiang et al., 2008, Wiradharma et al., 2011., Dathe et al. 2001) on efficacy and non-target effects.

The objectives of this study were to design, screen, and assess hAMPs created by combining binding and killing motifs from different naturally occurring AMPs. We generated and screened ~120 hAMPs putatively designed to kill Gram +, Gram -, or both and report here *in vitro* activity data of 6 hAMPs against bacteria and cytotoxicity data against eukaryotic cells. The effect of these hAMPs against extracellular and intracellular bacterial pathogens and their host cells was visualized *in vitro* using fluorescent nucleic acid stains (Live/Dead staining).

2.3. Materials and Methods.

2.3.1. Bacterial Cultures.

Bacterial cultures of *Escherichia coli* ATCC 11303*, Enterobacter aerogenes* ATCC 13408*, Bacillus subtilis* ATCC 6051*, Staphylococcus epidermidis* ATCC 14990*, Salmonella enterica* serovar Typhimurium=*Salmonella* Typhimurium ATCC SL1344*,* enteropathogenic *E. coli* (EPEC) ATCC 43887 and *Listeria monocytogenes* (EGD BUG 600) were grown in Luria Bertani (LB) broth and on agar plates using standard microbiological techniques. *Streptococcus pyogenes* ATCC 700294 cultures were grown on blood agar (Tryptic soy agar (TSA) + 5% defibrinated sheep's blood) and in Todd Hewitt broth (THB). For all *in vitro* assays, 3-4 ml of liquid media was inoculated with bacteria from a streak plate. The cultures were grown overnight in an incubator with shaking (~250 rpm) at 37°C for 15-18 hours. Subsequently, 5-10 µl of each culture was inoculated into 4 ml of fresh growth medium and grown to log phase (Optical density at 620 nm (OD_{620}) 0.4-0.8) for use in radial diffusion and MIC assays.

2.3.2. Peptide Design and Synthesis.

All hAMPs were synthesized commercially (LifeTein LLC, South Plainfield, NJ, USA, or Genscript USA Inc. Piscataway, NJ, USA). hAMPs were designed with regions of parental molecules known or predicted to bind to Gram - bacteria, Gram + bacteria, or fungi (Degraaf et al. unpublished, MacLean et al. unpublished) linked by a hinge region to a putative killing motif from characterized or unpublished AMPs. Alternatively the binding motif (Bm) and the killing motif (Km) were reversed in orientation, or a single Bm was flanked on both sides by the same or different Kms in forward or reversed orientations. A generalized schematic of our designed constructs can be seen in Figure 2.1. To aid in the identification and design of Kms and Bms, we used a secondary structure prediction program (SIMPA96:http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_simpa96.html) to locate potential amphipathic helical regions, where membrane interactions were most likely to occur. Subsequently, in selected hAMPS, we substituted amino acids in both the Bm and Km to alter the charge, hydrophobicity, and amphipathicity, or substituted L-amino acids with D-amino acids. To

evaluate the effects of charge, we substituted/added lysine or arginine residues to either or both C and N termini of the peptides. Hydrophobicity was altered by substitution with tryptophan residues in the amphipathic helices and/or the N-termini. D-amino acid substitutions were made with D-leucines, particularly within the hydrophobic faces of predicted amphipathic helices in both Bms and Kms. The names, molecular weights, and charges of hAMPs discussed in this chapter are listed in Table A1 in Appendix 1.

2.3.3. In vitro Screening Procedures for Antimicrobial Activity.

Radial diffusion assays were used as a qualitative assessment of the antimicrobial efficacy of hAMPs. Log-phase bacteria suspensions (30-40 µl) were spread on agar plates to create a lawn. 10 µl of a 10 µg/µl solution (100 µg of peptide) of each peptide was applied to a sterile filter paper disc which was then placed directly on the agar surface. For each plate, a negative control (disc applied with 10 µl nuclease-free water) and positive control (disc applied with 10 µl of a 5 mg/ml solution of carbenicillin) were included. Plates were then incubated at 37°C for 15-18 hours and examined the following morning for growth inhibition.

hAMPs that showed zones of inhibition from the radial diffusion assays (See Figure A1 in Appendix 1) were assayed to determine their minimum inhibitory concentration (MIC) values following the protocol described by Wiegand et al. (2008). The MIC of an antimicrobial compound is the lowest concentration of compound at which growth inhibition is observed after an overnight incubation (Andrews, 2001). Briefly, subcultured bacteria in mid-log phase (OD_{620} 0.4-0.8) were diluted to OD_{620} 0.1 before being further diluted 100-fold in 10 ml LB media to achieve a working concentration of 2 x 10^6 cfu (colony forming units)/ml. Bacteria (100 ul) were aliquoted into wells of a 96-well plate (Greiner Bio-One, Germany). Stock hAMP concentrations (10000 µg/ml) were prepared followed by 1:10 serial dilutions such that 10 fold serial dilutions of peptides were added to adjoining wells (final peptide concentration ranges: 1000 µg/ml- 0.1 µg/ml). For each plate, a row of wells containing bacterial suspension (100 µl each) with no peptides was included as a positive growth control and a column of wells containing 100 µl of LB or nuclease-free water were used as sterile controls. Plates were sealed with optically clear sealing tape (Sarstedt Inc., Montreal, QC) and incubated at 37°C

(25°C for *M. luteus* and *P. fluorescens*) with gentle shaking (20 rpm) for 14-20 hours. The OD_{620} was then measured in an ASYS Expert Plus 96-well plate reader (Montreal Biotech Inc., Montreal, Quebec) (See Figure A2 in Appendix 1). For each peptide, an "MIC range" was calculated by determining the lowest peptide concentration at which there was no visible bacterial growth and the proceeding peptide concentration at which growth was not eliminated. The true MICs of the hAMPs are somewhere between these 2 concentrations.

2.3.4. Hemolysis Assays and Selectivity Index Calculations.

Hemolysis assays were conducted as previously described (Plunkett et al. 2009, Park et al. 2007) to determine the degree of toxicity of selected hAMPs towards eukaryotic cells (erythrocytes). Briefly, hemolytic activity was determined by measuring the amount of heme released from lysed sheep erythrocytes after a designated incubation period with hAMPs. Defibrinated sheep's blood (Cedarlane Laboratories, Burlington, ONT, Canada) was diluted to 1% in 1X phosphate-buffered saline (PBS). Cells were centrifuged (1000 rpm, 5 min, 4°C) and washed twice with PBS, with centrifugation steps in between washes (1000 rpm, 5 min, 4°C), resuspended in PBS and aliquoted into 0.6 ml polypropylene Eppendorf tubes. Serial dilutions of peptides were added to the cell suspension (final concentration range: 1000 µg/ml- 0.1 µg/ml, final volume: 200 µl). Positive controls (cells incubated with 0.2% Triton-X) and negative controls (cells incubated with 1X PBS) were also included. After incubation for 1 h at 37°C, cells were centrifuged for 5 min (1000 rpm, 4°C) and the supernatants (100 µl each) transferred into sterile polypropylene 96-well plates. The amount of heme released via cell lysis was determined by measuring the absorbance at 405 nm (A_{405}) with an ASYS Expert Plus 96-well plate reader (Montreal Biotech Inc., Montreal, Quebec). The percent hemolysis was calculated using the following formula:

% hemolysis= [(A_{405} Experimental)-(A_{405} PBS)/(A_{405} Triton X)-($A4_{05}$ PBS)] x 100%

We then determined hemolytic concentrations (HCs) resulting in 1 (MHC), 5 (HC_5), and 10% (HC_{10}) hemolysis. The MHC, or minimal hemolytic concentration is defined as the highest peptide concentration at which no detectable lysis (≤1%

hemoglobin release) (Chen et al. 2005) is observed. From these data, we calculated a selectivity index (SI), defined as the ratio of the amount of therapeutic agent (hAMP concentration) resulting in a toxic effect (hemolysis) to the concentration that causes a therapeutic effect (MIC) (Muller and Miller 2012):

SI= HC/MIC

Using this ratio, we calculated 2 sets of values:

1) a universal selectivity index range (SIR) for each peptide by comparing its MHC, HC_5 and HC_{10} to the geometric mean of all observed MIC ranges (HC/MIC range) versus Gram - or Gram + bacteria.

2) a bacterium-specific SI for each hAMP. For this calculation, we divided the HC_5 by the lowest MIC value for each tested bacterial species.

2.3.5. Monolayer Experiments.

In these experiments, we examined the effects of hAMPs against an extracellular bacterial pathogen, enteropathogenic *E. coli* (EPEC) and an intracellular intestinal pathogen, *Salmonella* Typhimurium. Both pathogens were permitted to infect their host cells, grown as a monolayer. With experiments involving *S.* Typhimurium, the effects of hAMPs were evaluated both before and after the bacteria had time to enter the host cells.

2.3.5.1. Maintenance of Host Cell Lines and Bacterial Cultures.

HeLa (cervical cancer cell line derived from Henrietta Lacks) cells were grown and maintained (37°C) in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 100 µg/ml gentamicin. Cells were passaged every 3-5 days: cells were washed three times with phosphate buffered saline (PBS), trypsinized (3 ml, 3 minutes at 37°C), and centrifuged (1000 rpm, 25°C). Pelleted cells were resuspended in DMEM and 2-3 ml of this suspension was added to a flask of fresh medium. To seed, 0.5-1 ml of cell suspension was added to each culture dish containing

2 ml of DMEM (+ 10% FBS). Seeded culture dishes were allowed to grow to 60-80% confluency at 37°C for infection experiments (1-2 days).

Cultures of EPEC and *S.* Typhimurium were maintained on LB agar plates. Prior to each infection experiment, a liquid culture was set up by inoculating a bacterial colony from a freshly streaked plate in 2-3 ml liquid LB and grown for 14-18 hours to stationary phase with shaking (220 rpm, 37°C).

2.3.5.2. Infection Experiments.

EPEC: During infection experiments with EPEC, cells were grown in DMEM supplemented with 10% FBS and 5 µg/ml of gentamicin. Cells were washed 6X with DMEM (+10% FBS) to eliminate the antibiotic. Each culture dish was infected with 2 µl of overnight EPEC culture and incubated for 3 hours at 37°C. After this initial incubation, growth media was replaced and hAMPs were added to the experimental dishes (at 10 µg/ml & 1 µg/ml concentrations), PBS to the negative control, and a conventional antibiotic (gentamicin- 5 µg/ml) to the positive control. Dishes were then incubated for an additional hour.

S. Typhimurium*:* We added 60 µl of an overnight culture of *S.* Typhimurium to 2 ml of fresh LB and allowed the culture to grow for 3 hours with shaking (37°C, 220 rpm). 1 ml of the subculture was centrifuged (4 g, 3 minutes, room temperature) and re-suspended in 1 ml of serum-free DMEM. Growth media from each seeded culture dish was removed and replaced with 2 ml of serum-free DMEM after repeated washes with the same medium. Experiments were performed pre-invasion and post-invasion to assess the ability of the peptides to disrupt bacterial membranes before and after they have entered host cells. In pre-invasion experiments, peptides were added prior to a 1 hour incubation period of bacteria and host cells. We added 7.5-10 µl of the resuspended *S.* Typhimurium subculture to the cells immediately followed by the addition of peptides (to achieve a final peptide concentration of either 10 or 1 µg/ml). Dishes were then incubated for 1 hour at 37°C. In post-invasion experiments, bacteria and host cells were first incubated without peptides to allow for internalization of the bacteria by host cells to occur. Cells were then washed, growth media was replaced, and peptides were added (10 or 1 µg/ml) and left to incubate for an additional 30-60 minutes

at 37°C. Cells were incubated with a similar volume of PBS (negative control) or carbenicillin (150 µg/ml) (positive control).

2.3.5.3. Post-infection Procedures (Live/Dead Staining) and Imaging.

After the incubation period with peptides, growth media was removed from each culture dish and cells were washed 3X with PBS and incubated with 0.25 µl of Baclight™ Live/Dead Stain (Life Technologies Inc., Burlington, ONT, Canada) in 2 ml PBS in the dark for 15 minutes at room temperature. The stain used in these experiments was a 1:1 mixture of nucleic acid stains SYTO 9 (green) and propidium iodide (PI, red). SYTO 9 stains both intact (live) and permeabilized (dead) cells while PI stains permeabilized cells only. Following incubation, cells were washed again once in PBS and visualized on a Leica DMI4000B inverted fluorescence microscope equipped with a Hamamatsu Orca R2 CCD camera (Hamamatsu, Japan) and Metamorph Imaging System software (Molecular Devices, Sunnyvale, CA, USA).

2.3.6. Cytotoxicity Towards other Human Cell Lines.

Because some cytotoxicity was seen in HeLa cells, we evaluated hAMP toxicity against other commonly used human cell lines: T84 (colon epithelial cell line) and A549 (alveolar basal epithelial cell line). T84 cells were grown and maintained (37ºC) in DME/F-12 (Dulbecco's Modified Eagle Medium and Ham's F-12 Nutrient Mixture) supplemented with 5% FBS. A549 cells were grown and maintained in F12K Nutrient Mixture (Kaigh's Modification, supplemented with L-Glutamine). Seeding and passaging procedures for T84 and A549 cell lines were similar to that for HeLa.

Prior to the start of experiments, cells grown overnight were washed repeatedly with fresh growth media (2X) before peptides were added. Cells were incubated with peptides for 1 hour at 37ºC. Negative (PBS) and positive (Triton-X) controls were included as well. Post-incubation treatment with Live/Dead stain and live cell imaging procedures followed those used with HeLa cells.

2.4. Results.

2.4.1. Antimicrobial Activity Data.

We screened over 120 hAMPs in radial diffusion and MIC assays. The radial diffusion data of selected hAMPs are listed in Table 2.1 and corresponding MIC data are listed in Table 2.2. Data from the monolayer experiments are summarized in Figures 2.2-2.5 and Table 2.3.

hAMP-83 and hAMP-100 exhibited the strongest broad spectrum activity against Gram + and Gram - bacteria . Both molecules were designed with the same putative binding motif. hAMP-54, a short (14-amino acid) peptide previously determined to exhibit moderate broad spectrum activity (data not shown) was incorporated as a killing motif in hAMP-83. hAMP-100 had slightly higher overall efficacy against Gram - than Gram + bacteria but was highly active against the Gram + bacteria *S. pyogenes* (MIC range: 2.59-0.26 μM) and *B. subtilis* (MIC ≤0.026 μM).

hAMP-95 and hAMP-90 were more toxic towards Gram + bacteria with exceptions (Tables 2.2, 2.7). The leucines in the predicted amphipathic regions of these molecules were D-substituted. The L-analogs of these hAMPs were more toxic towards eukaryotic cells (See Table A2 in Appendix). Interestingly, activity against Gram - bacteria was diminished in hAMP-95 when compared with the L-analog while activity against Gram + bacteria was retained. hAMP-90 displayed marked improvement in antimicrobial activity over the L analog against both Gram - and Gram + bacteria. Activity, however, was more pronounced (lower MICs) against Gram + bacteria (See Table A3 in the Appendix)

hAMP-80 and hAMP-66 were more lethal towards Gram - bacteria (Tables 2.2, 2.7). These are nearly identical molecules but differ in that hAMP-66 contains a disulfide bridge linking the 2 cysteine residues in the C-terminal region. MIC ranges for *E. aerogenes* and *B. subtilis* were 10-fold lower when treated with hAMP-66 compared with the hAMP-80 (Table 2.2). Three of these peptides (hAMP-83, hAMP-100, hAMP-95) were further evaluated for toxicity against selected Level 2 pathogens (EPEC and *S.*

Typhimurium) and their eukaryotic host cells (in monolayers). These effects were visualized via Live/Dead staining. Imaging data for both hAMP-100 and hAMP-83 are presented in figures 2.2-2.5. From these experiments, hAMP-83 displayed permeabilizing activity at both high (10 µg/ml) and low (1 µg/ml) peptide concentrations against EPEC and extracellular *S.* Typhimurium (Figures 2.2-2.4A-C). However, it was unable to target internalized *S.* Typhimurium at 1 µg/ml (Figures 2.4D-F). Unfortunately, this high efficacy was accompanied by visible host cytotoxicity (Figures 2.2B & E, 2.3B, 2.4B & E), which was more extensive at 10 µg/ml. hAMP-100 was able to permeabilize extracellular EPEC and *S.* Typhimurium (pre-invasion experiments) with no toxic effects against host cells (Figure 2.5 (EPEC), Table 2.3). However, it was unable to attack internalized *S.* Typhimurium in the post-invasion experiments (Table 2.3). hAMP-95 had low permeabilizing activity against EPEC at both test concentrations as relatively few bacteria stained for PI.

2.4.2. Host Cytotoxicity Data.

Hemolysis data for 6 selected peptides are listed in Table 2.4. MHC, HC_5, and HC_{10} values (in µM) are included. hAMP-100 was the least toxic peptide with hemolysis values below 5% at the highest tested concentration (259 µM). Interestingly hAMP-66 was less toxic than its analog without the disulfide bridge (10 fold higher MHC than hAMP-80). hAMP-83 was the most toxic with a HC_5 of 2.28 µM (compared with >259 µM for hAMP-100). Both listed D-peptides (hAMP-95 and hAMP-90) were significantly less toxic than their L-analogs (See Table A2 in the Appendix).

Toxicity data of 3 peptides (hAMP-100, hAMP-83, and hAMP-95) against monolayers of 2 human cell lines (T84 colon and A549 lung cells) are presented in Table 2.5. All 3 peptides were nontoxic (or displayed slight toxicity) to T84 cells at 10 and 1 µg/ml. 2 out of the 3 tested peptides displayed negligible to no toxicity to A549 cells. Only hAMP-83 exhibited detectable toxicity at 10 µg/ml, with about 15-20% permeabilization (positive PI stain) detected out of all the cells observed. No permeabilization was detected at 1 µg/ml.

2.4.3. Selectivity Indices.

The universal SIRs based on MHC and HC_5 values are listed in Table 2.6. The SIR is a measure of overall therapeutic potential and takes into account both cytotoxicity (hemolysis) and antimicrobial activity (MIC). SIR values greater than 1 indicate acceptable therapeutic potential. Based on these calculations, hAMP-100 displayed the highest SIR (therefore the best peptide, SIR: 13.3-74 at MHC, 133.5-740 at HC_5) while hAMP-83 (SIR: 0.013-0.13 at MHC, 1.29-12.81 at HC_5) had the lowest.

Besides a universal SIR, which incorporates the geometric mean of MIC ranges against all bacteria tested, individual hAMP SIs based on the ratio of the calculated HC_5 value to the lowest MIC value were calculated against each bacterium (Table 2.7). From these data, the activity and selectivity of each peptide can be seen. Both hAMP-100 and hAMP-83 were broad spectrum due to similar SI values for most Gram - and Gram + bacteria. hAMP-95 and hAMP-90 exhibited higher SIs against the tested Gram + bacteria compared to Gram - bacteria. hAMP-80 and hAMP-66 displayed comparatively higher SIs against Gram - bacteria than Gram + bacteria.

2.5. Discussion.

2.5.1. Parameters Affecting Antimicrobial Activity and Host Cytotoxicity.

Charge, hydrophobicity, amphipathicity and D-amino acid substitutions all had noticeable effects on antimicrobial activity and host cytotoxicity. It appears that the overall activity of a peptide is dependent on the interplay of these 4 factors rather than the result of a single parameter. Increases in charge and hydrophobicity index correlated to improvements in antimicrobial activity to a limited extent. There appears to be a window where these 2 parameters can be optimized to ensure both high antimicrobial efficacy and low host cytotoxicity.

An increase in charge from +6 to +14 between hAMP-54 and hAMP-83 resulted in a 10-100 fold improvement in MIC against most bacteria tested accompanied

by a 10 fold increase in hemolytic activity (data not shown). In comparison, hAMP-100, the hAMP with the highest selectivity index in our study, was moderately cationic (charge of +9) relative to hAMP-54 and hAMP-83. This peptide demonstrated both high overall antimicrobial efficacy and low host cytotoxicity. Previous studies (Jiang et al. 2008, Dathe et al. 2001) noted increases in hemolytic activities of AMPs when charge was increased beyond a certain threshold while MICs remained unchanged. It has been proposed that the excessive positive charge promotes pore formation in eukaryotic cell membranes by increasing the hydrophilicity of the pore, allowing favorable association with the surfaces of the lipid bilayer (Jiang et al. 2008).

hAMP-80, (charge +12) had a high efficacy against most Gram - bacteria tested while displaying low hemolytic activity. The addition of a disulfide bridge between the cysteine residues of the C-terminal killing motif of hAMP-66 increased activity against some bacteria (*B. subtilis* and *E. aerogenes*) and decreased hemolytic activity 10-fold compared with hAMP-80. The cationic cysteine loop created by the disulfide bridge appeared to enhance antimicrobial activity. The retention of activity in the linear molecule, hAMP-80 may be the result of compensation by the N-terminal binding motifs, both of which have predicted alpha-helical regions. The activity of these 2 hAMPs may be affected by their molecular size that can influence the ease of passage of AMPs across the bacterial cell wall (Papo and Shai, 2004). In our study, both hAMPs are relatively large peptides and may have difficulty getting past the thick peptidoglycan cell walls of many Gram + bacteria. The inclusion of a disulfide bridge may further increase the "bulk" of the molecule and could explain the 100-fold increase in MIC range in *L. monocytogenes* for the disulfide bridge analog compared to the linear hAMP.

Increases in hydrophobicity correlated with improved antimicrobial activity within a narrow window, beyond which antimicrobial activity decreased and/or hemolytic activity increased. The L-analog of hAMP-95, hAMP-41 was more hemolytic, having a 10 fold higher HC_5 (See Table A4 in Appendix). The peptide had a relatively high hydrophobicity index (predicted Hi of -0.15) due to the tryptophan residue in the binding motif. The solubility of the D-analog was improved although overall antimicrobial activity was similar to the L analog. Previous studies with synthetic AMPs (Chen et al. 2007, Jiang et al. 2008, Gopal et al. 2013) confirmed a window of optimum hydrophobicity. The

decreased antimicrobial activity of highly hydrophobic peptides may be due to their increased tendencies to self-associate in solution that may prevent their passage through the bacterial cell wall while having no effect on their entry into eukaryotic cells (Chen et al. 2007).

Amphipathicity/helicity played a role in the activity of the L analog of hAMP-90, hAMP-29. This peptide contained 3 predicted alpha-helical repeats of the putative killing motif compared with 2 in hAMP 41. Although both peptides displayed similar hydrophobicity indices, hAMP 29 had substantially higher hemolytic activity and lower antimicrobial efficacy (data not shown). The substitution of D-leucines in the D-analog greatly reduced hemolytic activity and increased antimicrobial activity, with greater toxicity towards Gram + bacteria. Highly helical AMPs have been reported to be toxic towards eukaryotic cells (Chen et al. 2005). The inclusion of D-amino acids within the amphipathic helices disrupts secondary structure and reduces hydrophobicity (Chen et al. 2002, Song et al. 2004, Papo et al. 2002, Lee et al. 2004). These changes could interfere with pore formation, resulting in lowered toxicity. The observed increase in antimicrobial activity could indicate reduced self-aggregation of the peptide due to improved solubility/reduced hydrophobicity and/or a different mode of action employed by the D-analog to enter bacterial cells.

2.5.2. Membrane Permeabilization as a Possible Mode of Action.

Our initial *in vitro* screens (radial diffusion & MIC assays) assessed the inhibitory effects of our hAMPs on bacterial growth. Efficacy was evaluated by determining MICs, and 6 hAMPs were evaluated in monolayer studies for their ability to enter cells. Indirect killing effects such as cytokine release and other immunomodulatory triggers were not evaluated with these techniques.

Many cells incubated with hAMPs stained positively with propidium iodide (PI), suggesting that they act via membrane disruption/permeabilization. The differences in concentration of hAMPs required to enter the cells may be a function of different concentration thresholds required by each hAMP. hAMP-83 and hAMP-100 were the

only hAMPs that were clearly active at their lowest test concentrations (1 µg/ml), against EPEC.

hAMP-95 exhibited a relatively low MIC range (4.69-0.47 µM- see Table 2.2) against EPEC but displayed weak permeabilizing activity in the monolayer experiments involving the same bacteria (Table 2.3). The possibility of unfavorable interactions with serum in the growth medium can be excluded as the activities of other tested peptides were not affected by the same growth medium. D-peptides have also been found to be resistant to proteases in serum (Papo et al. 2002). An alternative explanation may be a different mode of action employed by the peptide. The limitation of our experimental technique is that only permeabilized cells can be visualized since PI only enters membrane-compromised cells. Bacterial cells killed by other means, such as intracellular interactions with nucleic acids and essential proteins will not be detected by the stain as long as the membrane stays intact. Buforins, for example, can translocate through bacterial membranes without disruption in order to bind to nucleic acids (Cho et al. 2009, Fleming et al. 2008, Jang et al. 2012, Uyterhoeven et al. 2008). Our data may be a conservative estimate of the actual number of cells affected by our hAMPs. The use of assays that measure cellular metabolic activity (e.g. MTT, ATP detection assays) will determine the extent of killing by hAMPs more accurately.

2.5.3: Cytotoxicity differences between various host cell types.

Three peptides (hAMP-100, hAMP-83, hAMP-95) were tested further for cytotoxicity against two immortalized human cell lines: T84 (colon) and A549 (lung). Both hAMP-100 and hAMP-95, that were non-toxic to erythrocytes and HeLa cells, were also non-toxic towards both cell lines. Only hAMP-83 was inconsistent, being non-toxic towards T84 cells while displaying detectable toxicity towards A549 cells. Interestingly the peptide was observed to be more toxic towards HeLa than A549 cells (est. 15-20% A549 cells stained for PI versus 70-80% HeLa cells stained).

These results indicated that different cell types exhibited a range of sensitivities towards our hAMPs. This variation can be attributed to differences in membrane lipid composition among cell types, which will ultimately affect properties

such as membrane permeability and fluidity (Spector and Yorek 1985). These differences may include the types of phospholipids present, cholesterol content, and fatty acid composition of the phospholipids (Spector and Yorek 1985). HeLa cells have been reported to be less robust than other cell lines used in tissue culture (J. Guttman, pers. comm.), a feature that could explain their susceptibility to many of our hAMPs. Our findings also raise important considerations in the evaluation of therapeutic suitability. In most studies hemolytic activity is used to assess host cytotoxicity *in vitro* as it is quantitative and easy to perform. Because different cell types exhibit varying levels of robustness to antibiotics, a toxicity assessment using erythrocytes alone would be limited in scope and thus would provide an incomplete picture of the full therapeutic potential of a candidate drug. Toxicity data on cell types other than erythrocytes will more effectively assess suitability of the drug in treating infections in specific organs. A peptide that exhibits negligible toxicity toward lung cells may be a potential candidate in treating bacterial infections that occur in the respiratory system (e.g. tuberculosis).

2.5.4. Practical Applications: Therapeutic Suitability.

The objective of this study was to evaluate hAMPs as a potentially new source of antibiotics. The suitability of a candidate molecule as a therapeutic is dependent on 2 factors: antimicrobial efficacy, measured as an 'MIC' range, or minimum inhibitory concentration range, and host cytotoxicity, represented as a hemolytic concentration (HC). The ratio of HC to MIC, known as a selectivity index, therefore represents the overall suitability of a potential drug as a therapeutic. We calculated 2 values based on the data we gathered: a universal selectivity index range (SIR), which incorporates the geometric mean of observed MIC ranges against all tested bacteria and an individual SI for each bacterial species (Table 2.7). Although a universal SIR provides a sound indicator of the overall therapeutic suitability of a hAMP, it does not reveal information on its selectivity. With the individual SI values, we were able to determine hAMPs that were broad spectrum, or those that were more toxic to Gram + or Gram - bacteria. We were also able to determine hAMPs that were effective against a specified bacterial species or strain. Both broad spectrum and narrow spectrum antibiotics would be extremely useful tools to treat infections. Broad spectrum antibiotics are useful in treating general or multiple infections (e.g. sepsis) or where there are drug-resistant bacteria. The non-

selective nature of broad-spectrum drugs, however, can result in the loss of beneficial host microflora (Newton et al. 2013, Bartosch et al. 2004). If the infection is known or suspected (i.e. EPEC), a narrow spectrum antibiotic that targets very few bacteria, or has a much lower MIC against the target organism, could eliminate the target pathogen with minimal effect on beneficial bacteria. This assumes that we know the symptoms are caused by a single, identified pathogen. Our data suggest that hAMPs can be designed as general or group selective antibiotics.

2.6. References.

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2.7. Figures and Tables.

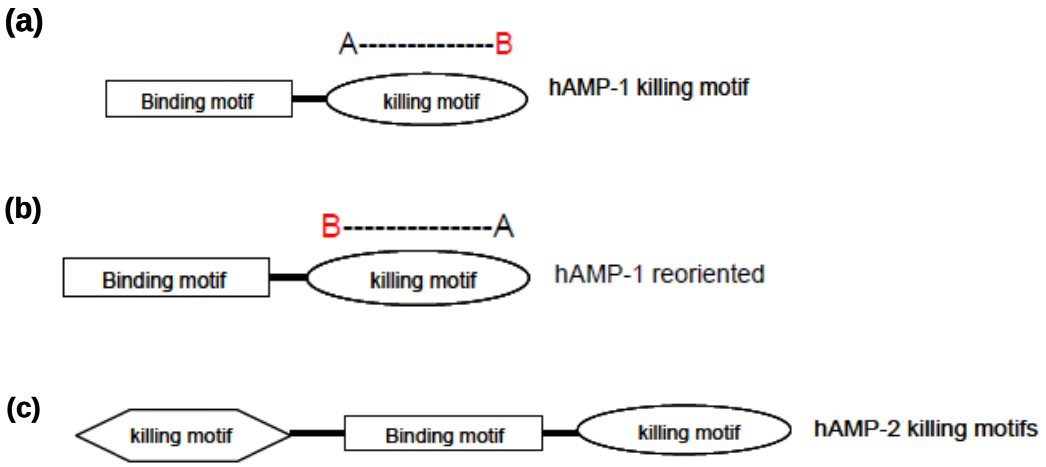


Figure 2.1: Generalized schematic of hAMP combinations designed and tested in this study.

(a) A hAMP designed as a natural AMP mimic with single killing motif oriented in natural N-C (A-B) conformation. (b) Same hAMP design as (a) but with killing motif oriented in reverse (B-A) conformation. (c) hAMP designed with a central binding motif flanked by 2 killing motifs.

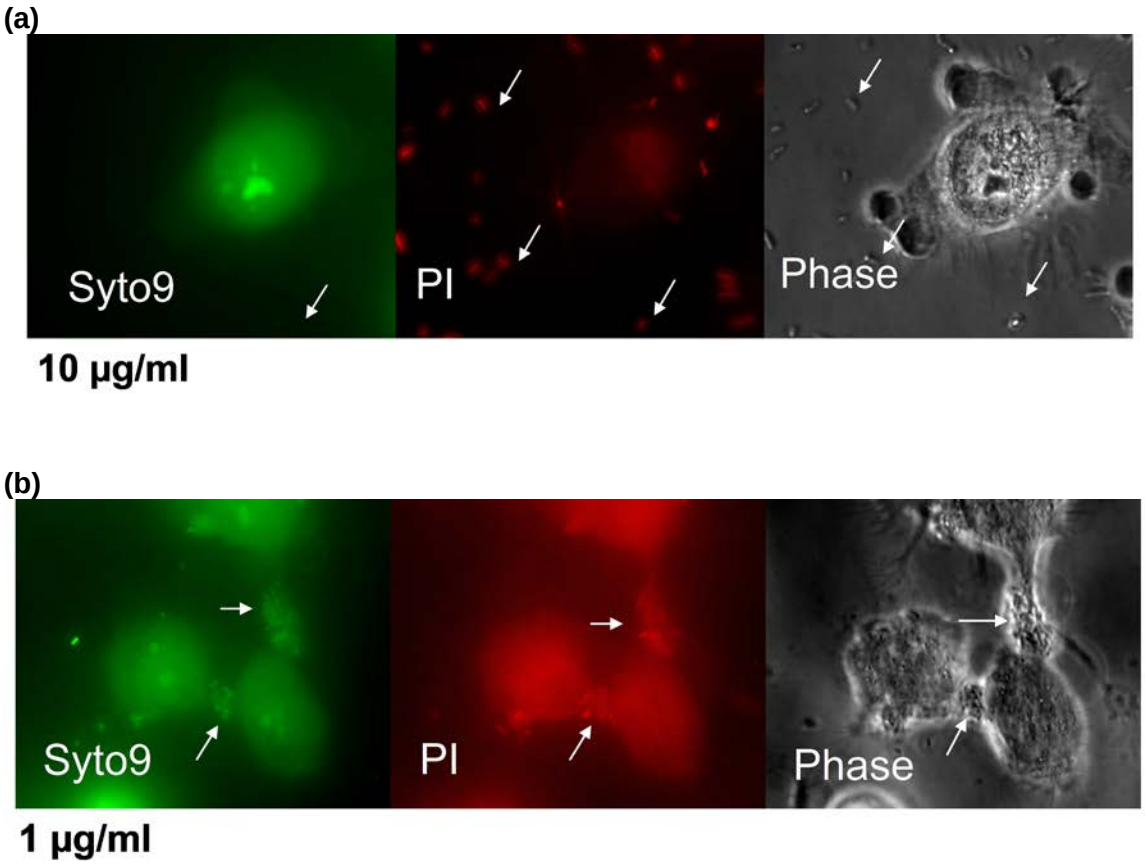


Figure 2.2: Live cell imaging data showing the effects of hAMP-83 on enteropathogenic *E. coli* (EPEC) and their host cells (HeLa).

HeLa cells and bacteria were treated with either 10 µg/ml (a) or 1 µg/ml (b) peptide and later stained with live/dead stain. Syto9 (green) stain (left panels) enters both intact and compromised cells, propidium iodide (red) stain (center panels) enters only membrane-compromised cells. Right panels represent corresponding Phase contrast images. White arrows in the images indicate individual bacterial cells. From the images, the peptide appears to be toxic towards both the bacteria and host cells.

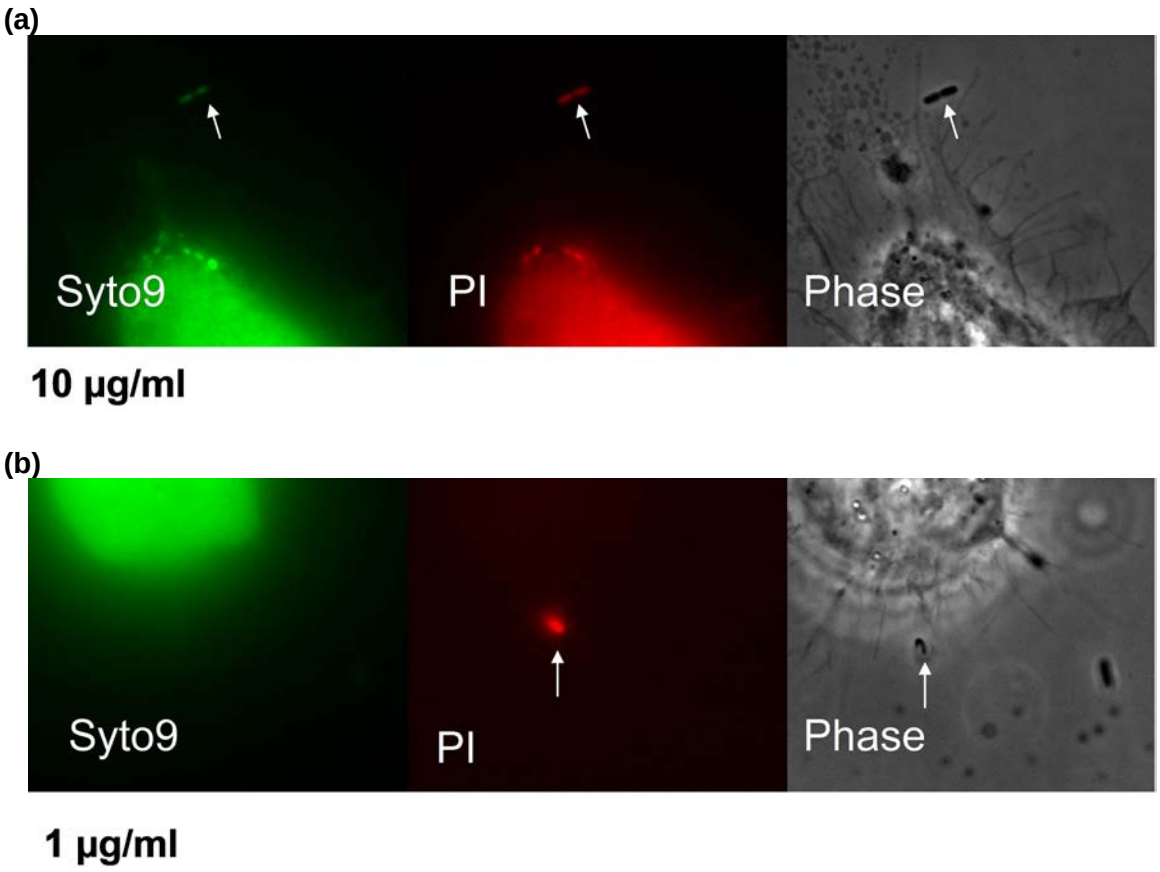


Figure 2.3: Live cell imaging data showing the effects of hAMP-83 on *Salmonella* Typhimurium and their host cells (HeLa) in a pre-invasion experiment.

In pre-invasion experiments HeLa cells and bacteria were treated with either 10 µg/ml (a) or 1 µg/ml (b) peptide prior to the time bacteria parasitize their host cells and later stained with live/dead stain. Syto9 (green) stain (left panels) enters both intact and compromised cells while propidium iodide (red) stain (center panels) enters only membrane-compromised cells. Right panels represent corresponding Phase contrast images. White arrows in the images indicate individual bacterial cells. The peptide was determined to be toxic to bacteria at both tested concentrations and to host cells at 10 µg/ml.

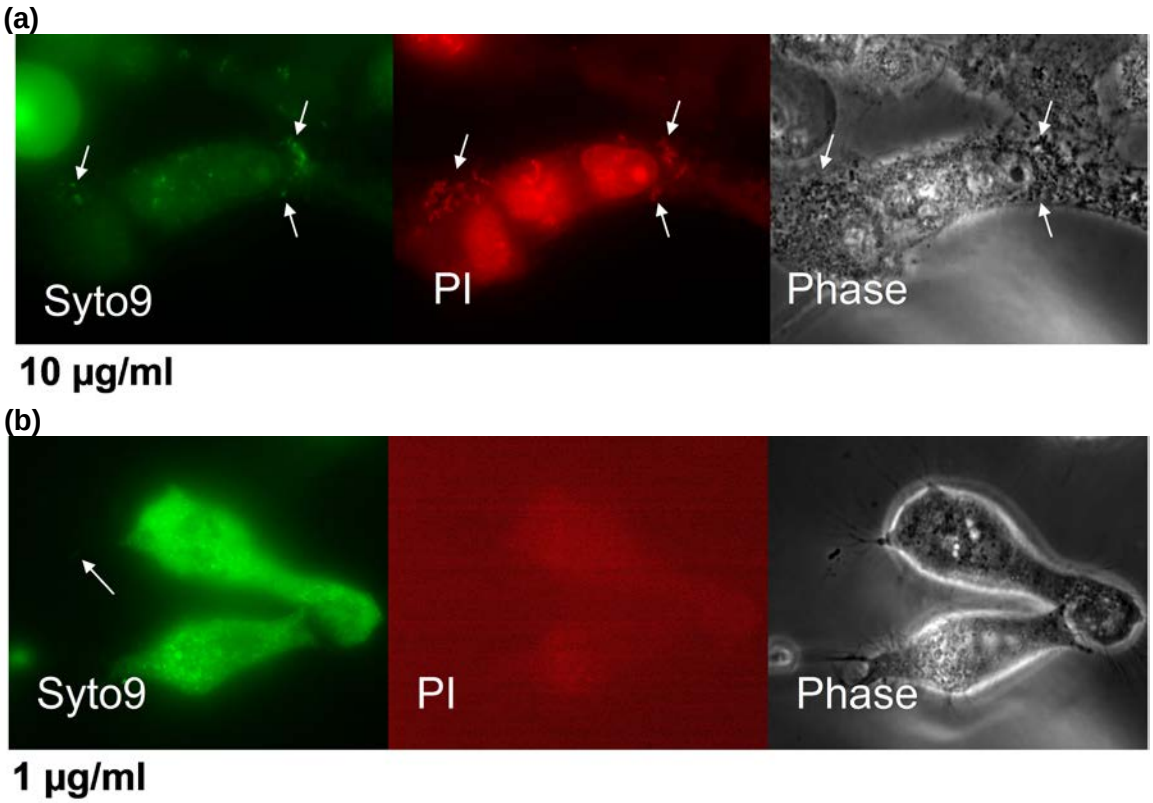
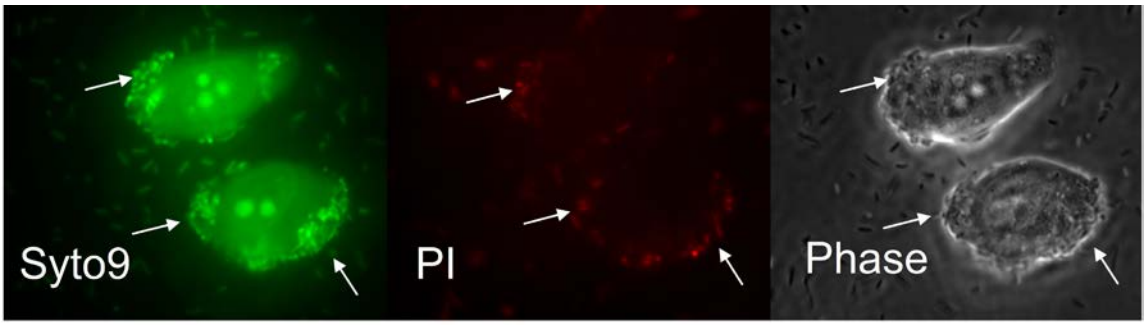


Figure 2.4: Live cell imaging data showing the effects of hAMP-83 on *Salmonella* Typhimurium and their host cells (HeLa) in a post-invasion experiment.

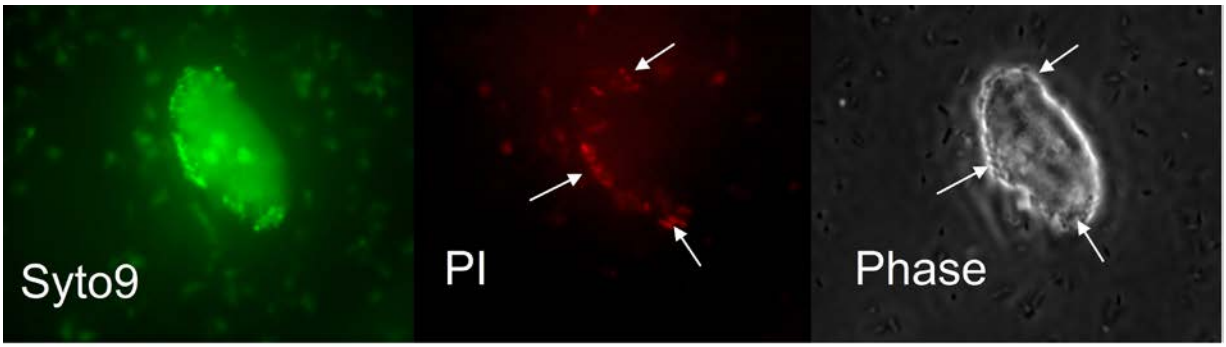
HeLa cells and bacteria were treated with either 10 µg/ml (a) or 1 µg/ml (b) peptide after bacteria were permitted to enter their host cells and later stained with live/dead stain. Syto9 (green) stain (left panels) enters both intact and compromised cells while propidium iodide (red) stain (center panels) enters only membrane compromised cells. Right panels represent corresponding Phase contrast images. White arrows in the images indicate individual bacterial cells. From the images, the peptide was found to be toxic to both host cells and their internalized bacteria at 10 µg/ml but had no effect at 1 µg/ml.

(a)



10 µg/ml

(b)



1 µg/ml

Figure 2.5: Live cell imaging data showing the effects of hAMP-100 on enteropathogenic *E. coli* (EPEC) and their host cells (HeLa).

HeLa cells and bacteria were treated with either 10 µg/ml (a) or 1 µg/ml (b) peptide and later stained with live/dead stain. Syto9 (green) stain (left panels) enters both intact and compromised cells while propidium iodide (red) stain (center panels) enters only membrane compromised cells. Right panels represent corresponding Phase contrast images. White arrows in the images indicate individual bacterial cells. From the images, the hAMP was selectively toxic towards EPEC but had no visible effects on host cells.

Table 2.1: Radial diffusion data of six hAMPs against four Gram-negative (Gram -) and four Gram-positive (Gram +) bacteria.

Peptide	*E. coli* (Gram -)	EPEC (Gram -)	*E. aer.* (Gram -)	*S.* Typ. (Gram -)	*B. sub.* (Gram +)	*S. epi.* (Gram +)	*S. pyo.* (Gram +)	*L. mon.* (Gram +)
hAMP-66	++	+	++	+	+	+	NT	0
hAMP-80	+	+	+	+	+	+	NT	+
hAMP-83	++	+	++	+	+++	+	++	++
hAMP-90	+	++	+	++	++	++	++	++
hAMP-95	++	++	+	++	++	++	++	+
hAMP-100	++	++	++	++	++	++	++	NT

Legend: '+' indicates a zone of inhibition of at least 0.5 mm width around the disc, '++', at least 1.5 mm, '+++', at least 4 mm; NT: not tested; 0: no zone of inhibition detected; Gram -: Gram-negative, Gram +: Gram-positive; EPEC= enteropathogenic *E. coli*, *E. aer.= Enterobacter aerogenes*, S. Typ.= *Salmonella* Typhimurium*, B. sub.= Bacillus subtilis*, *S. epi= Staphylococcus epidermidis*, *S. pyo.= Streptococcus pyogenes*, *L. mon*.= *Listeria monocytogenes*

Table 2.2: MIC ranges (μM) of six hAMPs against four Gram-negative (Gram -) and four Gram-positive (Gram +) bacteria.

Peptide	*E. coli* (Gram -)	EPEC (Gram -)	*E. aer.* (Gram -)	*S.* Typ. (Gram -)	*B. sub.* (Gram +)	*S. epi.* (Gram +)	*S. pyo.* (Gram +)	*L. mon.* (Gram +)
hAMP-66	1.84-0.18	1.84-0.18	0.18-0.018	1.84-0.18	1.84-0.18	184-18.4	NT	184-18.4
hAMP-80	1.81-0.18	1.81-0.18	1.81-0.18	1.81-0.18	18.1-1.81	181-18.1	NT	1.81-0.18
hAMP-83	2.28-0.23	0.23-0.023	2.28-0.23	2.28-0.23	0.23-0.023	2.28-0.23	2.28-0.23	0.23-0.023
hAMP-90	41.4-4.14	4.14-0.41	41.4-4.14	41.4-4.14	4.14-0.41	4.14-0.41	≤0.041	41.4-4.14
hAMP-95	46.9-4.69	4.69-0.47	469-46.9	4.69-0.47	46.9-4.69	4.69-0.47	0.47-0.047	46.9-4.69
hAMP-100	0.26-0.026	0.26-0.026	2.59-0.26	2.59-0.26	≤0.026	25.9-2.59	2.59-0.26	NT

MIC (Minimal inhibitory concentration) ranges are expressed in μM; an MIC range includes the lowest tested peptide concentration where there was observed growth inhibition and the highest tested peptide concentration where there is visible microbial growth; note that the experimentally undetermined MIC is located within this range of values; Gram -: Gram-negative, Gram +: Gram-positive, NT: not tested; EPEC= enteropathogenic *E. coli*, *E. aer.= Enterobacter aerogenes*, S. Typ.= *Salmonella* Typhimurium*, B. sub.= Bacillus subtilis*, *S. epi= Staphylococcus epidermidis*, *S. pyo.= Streptococcus pyogenes*, *L. mon.= Listeria monocytogenes*

Table 2.3: Permeabilizing activity of three hAMPs at 10 and 1 µg/ml against enteropathogenic *E. coli* (EPEC) and *Salmonella* Typhimurium with HeLa cells.

Bacteria	EPEC		*S.* Typhimurium (Pre-Invasion)		*S.* Typhimurium (Post-Invasion)	
	PI Stain (Y/N)					
Peptide	10 µg/ml Peptide	1 µg/ml Peptide	10 µg/ml Peptide	1 µg/ml Peptide	10 µg/ml Peptide	1 µg/ml Peptide
hAMP-83	Y*	Y*	Y*	Y	Y*	N
hAMP-95	Y -weak	Y -weak	NT	NT	NT	NT
hAMP-100	Y	Y	Y	N	N	N

PI: propidium iodide stain, PI stain only enters permeabilized cells; Y= PI stain (permeabilization) detected, N= not detected; weak- fewer than 3 cells were stained; NT: not tested; * host cell (HeLa) toxicity detected

Table 2.4: Host cytotoxicity data of six hAMPs: hAMP concentrations resulting in 1 (MHC), 5 (HC_5), and 10% (HC_{10}) heme release from red blood cells (hemolysis).

hAMP	MHC	HC_5	HC_{10}
hAMP-66	1.84	18.4	184
hAMP-80	0.18	18.1	181
hAMP-83	0.023	2.28	22.8
hAMP-90	4.14	41.4	414
hAMP-95	4.69	46.9	>469
hAMP-100	25.9	>259	>259

The MHC refers to the minimal peptide concentration resulting in ≤1% hemolysis; concentrations are in μM

Table 2.5: Permeabilizing activity of three hAMPs at 10 and 1 µg/ml against two human cell lines grown as monolayers.

Cell Line Type	T84		A549	
	PI Stain (Y/N)			
Peptide	10 µg/ml Peptide	1 µg/ml Peptide	10 µg/ml Peptide	1 µg/ml Peptide
hAMP-83	N	N	Y**	N
hAMP-95	N*	N	N	N
hAMP-100	N	N	N	N

PI: propidium iodide stain, Y= stain (permeabilization) detected, N: stain not detected
* Slight toxicity (<2% stained for PI) detected
** Toxicity detected- ~15-20% of visualized cells were permeabilized

Table 2.6: Universal selectivity index ranges of six hAMPs based on overall MIC ranges per peptide and 1% (MHC) and 5% hemolytic concentrations (HC_5).

Peptide	Selectivity Index Range*	
	MHC	HC_5
hAMP-66	0.37-3.79	3.74-37.9
hAMP-80	0.037-0.37	3.72-37.4
hAMP-83	0.024-0.24	2.37-23.5
hAMP-90	0.56-4.22	5.63-42.2
hAMP-95	0.32-3.17	3.17-31.7
hAMP-100	26.7-199.2	267-1992

* Selectivity index ranges for each peptide were calculated by dividing the hemolytic concentration (causing either 1 or 5% hemolysis) by the overall MIC range (the geometric mean of the MIC ranges determined for all tested bacteria); both MHC and HC_5 values were used in the calculations

Table 2.7: Selectivity indices of six hAMPs based on specific MICs and 5% hemolytic concentrations (HC_5)*.

Peptide	*E. coli* (Gram -)	EPEC (Gram -)	*E. aer.* (Gram -)	*S.* Typ. (Gram -)	*B. sub.* (Gram +)	*S. epi.* (Gram +)	*S. pyo.* (Gram +)	*L. mon.* (Gram +)
hAMP-66	102	102	1022	102	102	1	NT	1
hAMP-80	100	101	101	101	10	1	NT	100
hAMP-83	9.9	99	9.9	9.9	99	9.9	9.9	9.9
hAMP-90	10	101	10	10	101	101	1010	10
hAMP-95	10	100	1	100	100	10	100	10
hAMP-100	9961	9961	996	996	9961	100	996	NT

* Selectivity indices for each bacteria were calculated by dividing the 5% cut-off value (HC_5) by the low end of the previously determined MIC range for each peptide per bacteria; Gram -: Gram-negative, Gram +: Gram-positive; NT: not tested; EPEC= enteropathogenic *E. coli*, *E. aer.= Enterobacter aerogenes*, S. Typ.= *Salmonella* Typhimurium, *B. sub.= Bacillus subtilis*, *S. epi= Staphylococcus epidermidis*, *S. pyo.= Streptococcus pyogenes*, *L. mon*.= *Listeria monocytogenes*

Connecting Statement 2

In Chapter 2, we screened hAMPs designed from the killing and binding motifs of various natural alpha-helical AMPs for antimicrobial activity and host cytotoxicity. We explored the effects of modifying charge, amphipathicity, amino acid composition, peptide length, and helicity on antimicrobial activity and toxicity towards eukaryotic cells. Some hAMPs exhibited broad spectrum antibacterial activity while others were more toxic to a group of microbes or even to a limited number of species.

These data raised the question whether combining different hAMPs together would further increase toxicity towards bacteria. In the next chapter we explore two strategies to increase hAMP efficacy: (1) determining synergistic activity between various hAMPs, and (2) designing and assessing multivalent hAMPs (mvAMPs) designed by attaching pairs of linear hAMP subunits onto a single molecular scaffold. The results indicate that the mvAMPs were more lethal towards bacteria, but also damaged eukaryotic cells.

Chapter 3. In vitro Activity of Three Multivalent Hybrid Antimicrobial Peptides (hAMPs).

3.1. Abstract.

Multivalent hybrid antimicrobial peptides (mvAMPs) were designed by attaching copies of a single or different hAMP onto a lysine scaffold. Three divalent molecules were designed: a heterodimer (mvAMP 3) consisting of two different peptide constituents hAMP-83 and hAMP-97, and two homodimers (mvAMP 1 and mvAMP 2) each consisting of a pair of each hAMP. hAMPs were selected on the basis of previous *in vitro* screening data (Chapter 2) and combinatorial studies that assessed synergy between peptides. Both hAMP-83 and hAMP-97 displayed the highest synergistic activity against both Gram-negative (Gram -) and Gram-positive (Gram +) bacteria and were thus selected for design of the heterodimer. All constructs displayed broad-spectrum activity against Gram - and Gram + bacteria with the highest antimicrobial activity observed in the heterodimer. This increased efficacy, however, was accompanied by a significant increase in hemolytic activity, particularly with the heterodimer. These results suggest that hAMP selectivity is reduced with multivalency and therefore has significant implications on the design and therapeutic use of mvAMPs.

3.2. Introduction.

Because of the problems associated with drug resistant strains of bacteria (Chapter 1), there has been growing interest in the therapeutic potential of antimicrobial peptides (AMPs) (Brogden and Brogden 2011, Huang et al. 2010, Marshall and Arenas 2003). AMPs are a large and diverse group of small (6-60 amino acids long), amphipathic, mostly cationic peptides found in all classes of organisms (Bulet et al. 1999, Zanetti 2004). Although the killing mechanism of many cationic AMPs is poorly understood, it is proposed that they bind electrostatically to the anionic bacterial membrane and create pores via hydrophobic interactions with the lipid bilayer of the membrane. Several models have been proposed to explain the mechanism of pore formation (See Chapter 1). Cell death eventually results from cell lysis and/or loss of essential ions from the cell. Some AMPs can translocate through the membrane with minimal disruption and disrupt DNA replication and protein synthesis (Cho et al. 2009, Kavanagh and Dowd 2004).

AMPs are more active against prokaryotic than eukaryotic cells. However, several obstacles must be surmounted before they can be used as therapeutics including cytotoxicity towards host cells, stability in serum, and susceptibility to protease activity. Strategies that address these issues include hybrid AMPs (hAMPs- covered in Chapter 2), substitutions of specific amino acids (covered in Chapter 2), and peptidomimetics, which will be the subject of this study.

Peptidomimetics are synthetic or semi-synthetic molecules that share (or mimic) the properties and mode of action of natural AMPs (Giuliani and Rinaldi 2011). Some groups of peptidomimetics include dendrimeric/multivalent peptides, the subject of this chapter (Liu et al. 2010, Pieters et al. 2009, Young et al. 2011, Pini et al. 2008) peptoids (Giuliani and Rinaldi 2011, Chongsiriwatana et al. 2008), carbohydrate-based peptides (Guell et al. 2012, Li et al. 2012), synthetic lipidated peptides (Giuliani et al. 2008, Makovitzki et al. 2006), and ceragenins, which are based on a steroid backbone (Giuliani and Rinaldi 2011, Epand et al. 2007, Savage et al. 2002). (Figure 3.1)

Multivalent peptides (MVPs) are molecules that consist of a central polymeric core/scaffold where peptides are attached. MVPs were designed originally as peptide immunogens (Tam, 1988). More recently, there has been interest in developing MVPs as novel antibiotics. MVPs have increased resistance to proteases (Falciani et al. 2007, Liu et al. 2007), higher stability in high salt concentrations (Tam et al. 2002), and improved antimicrobial activity (Tam et al. 2002, Pini et al. 2005). This enhanced antimicrobial activity is thought to be the result of increased local concentration of active peptide units (Giuliani and Rinaldi 2011). Scaffolds used in MVP design include α-amino acids (e.g. lysine) (Tam et al. 2002, Sadler and Tam 2002, Bai et al. 2012), small organic molecules (e.g. benzoic acid) (Arnusch et al. 2007), and reactive polymers (e.g. polymaleic anhydride, carbohydrates) (Liu et al. 2006, Guell et al. 2012, Li et al. 2012). The number of attached bioactive units varies with the type of scaffold used. Dimers, tetramers, and octamers have been designed with both lysine and small organic molecule cores (Liu et al. 2007, Arnusch et al., 2007, Bai et al. 2012). With reactive polymers, tens to hundreds of AMPs can be attached (Sal-Man et al. 2002, Liu et al. 2006).

In this study we report *in vitro* activity data of multivalent hAMPs (mvAMPs) designed with hAMPs previously designed and evaluated (See Chapter 2). Individual hAMPs were selected based on activity data against Gram + and Gram - bacteria. These were evaluated in combinatorial studies to determine pairs that generated a synergistic antimicrobial effect. Selected pairs were synthesized as mvAMP homodimers and heterodimers attached to a lysine scaffold (Figure 3.2).

3.3. Materials and Methods.

3.3.1. Bacterial Cultures.

In this study we studied the synergistic effects of hAMPs against several species of bacteria: *Escherichia coli* ATCC 11303, *Enterobacter aerogenes* ATCC 13408, *Alicaligenes faecalis* ATCC 8750, *Citrobacter freundii* ATCC 13316, *Pseudomonas fluorescens* ATCC 13525*, Bacillus subtilis* ATCC 6051, *Staphylococcus epidermidis* ATCC 14990, *Staphylococcus aureus* ATCC 12600*, Salmonella enterica* serovar

Typhimurium=*S.* Typhimurium ATCC SL1344 and enteropathogenic *E. coli* (EPEC) ATCC 43887. Bacterial cultures were grown in Luria Bertani (LB) broth and agar plates using standard microbiological techniques.

For all *in vitro* assays, 3-4 ml of liquid media were inoculated with bacteria from a streak plate. The cultures were grown overnight in an incubator with shaking (~250 rpm) at 37°C for 15-18 hours. Subsequently, 5-10 µl of each overnight culture were inoculated into 2-4 ml of fresh LB medium and grown to log phase (Optical density at 620 nm (OD_{620}) 0.4-0.8) for use in disk and MIC assays.

3.3.2. Combinatorial Studies with Selected Individual hAMPs.

Combinatorial studies to assess synergy between different hAMPs followed the "checkerboard" approach described previously (Anantharaman et al. 2010, Chongsiriwatana et al. 2011). Individual hAMPs were selected based on *in vitro* activity (Minimum Inhibitory Concentration (MIC) assays) reported previously (see Chapter 2 and Table 2.3). Table 3.1 lists the names and sequences of hAMPs tested in our combinatorial experiments.

Combinations of peptides were added to wells of a 96-well plate and were serially diluted 10 fold /well in a row. Bacterial suspensions were prepared as for MIC assays. Briefly, subcultured bacteria in mid-log phase (OD_{620} 0.4-0.8) were diluted to OD_{620} 0.1 before being further diluted 100-fold in 10 ml LB media to achieve a working concentration of 2×10^6 cfu (colony forming units)/ml. This diluted suspension was then aliquoted into wells of a 96-well plate. The first well of each combination of peptides corresponded to concentrations 10 times the upper end of the MIC range of each hAMP for each bacteria strain tested, and 10 fold serial dilutions extended to 1/100 of each individual MIC. As a comparison, single peptides (monomers) were tested at the same concentration range alongside the combinations. Plates were sealed with optically clear sealing tape (Sarstedt Inc., Montreal, QC) and incubated at 37°C with gentle shaking for 15-20 hours. Growth inhibition was measured by determining the OD_{620} of the suspension in an ASYS Expert Plus 96-well plate reader (Montreal Biotech Inc.,

Montreal, Quebec). MICs were calculated as described for individual peptides (Chapter 2).

Synergy was measured using Fractional Inhibitory Concentration (FIC) Indices. FIC indices were calculated using the following formula (Rabel et al. 2004, Meletiadis et al. 2010, Anantharaman et al. 2010):

MIC(A+B)/ MIC A + MIC(A+B)/ MIC B where MIC(A+B) represents the MIC of components A and B in combination and MIC A and MIC B are the MICs of the components alone. Synergy was determined using the following FIC index values: less than 1; possible synergism, 1; an additive effect, 2; no effect, greater than 2; an antagonistic effect. We calculated 2 FIC values based on the lower and upper values of the MIC range of each tested hAMP combination. We therefore expressed this range of values as an 'FIC index range'.

3.3.3. mvAMP Design and Synthesis.

The peptide pair that displayed the best synergistic activity from the combinatorial experiments was used to design the multivalent constructs (Figure 3.2). A lysine scaffold was used in the design of the dimers, with peptides attached to the N-terminal and R amide groups. Two homodimers and one heterodimer were designed (Figure 3.2). All AMPs and mvAMPs were synthesized commercially (LifeTein LLC, South Plainfield, NJ, USA).

3.3.4. Radial Diffusion Assays.

Radial diffusion assays were used as a qualitative assessment of our designed mvAMPs. Log-phase bacteria suspensions (30-40 µl) were spread on LB agar plates to create a lawn. 10 µl of a 10 µg/µl solution (100 µg of peptide) of each peptide was applied onto a sterile filter paper disc that was then placed directly on the agar surface. On each plate, a negative control (disc applied with 10 µl nuclease-free water) and positive control (disc applied with 10 µl of a 5 mg/ml solution of carbenicillin) were included. Plates were incubated at 37°C for 15-18 hours (25°C for *P. fluorescens*) and

examined the following morning for growth inhibition, defined as a zone of no visible growth around each disc.

3.3.5. Minimum Inhibitory Concentration (MIC) Assays.

mvAMPs were assayed to determine their minimum inhibitory concentration (MIC) values following the protocol described by Wiegand et al. (2008). Briefly, subcultured bacteria in mid-log phase (OD_{620} 0.4-0.8) were diluted to OD_{620} 0.1 before being further diluted 100-fold in 10 ml LB media to achieve a working concentration of 2 x 10^6 cfu (colony forming units)/ml and 100 µl of this bacterial growth was aliquoted into wells of a 96-well plate (Greiner Bio-One, Germany). Stock mvAMP concentrations (10000 µg/ml) were prepared followed by 1:10 serial dilutions (down to 0.1 µg/ml). 11 µl of each peptide solution were aliquoted into wells containing bacterial suspension, resulting in final peptide concentration ranges of 1000 µg/ml- 0.1 µg/ml. For each plate, a row of wells containing bacterial suspension (100 µl each) with no peptides was included as a positive growth control and a column of wells containing 100 µl of LB or nuclease-free water were used a sterility controls (100 µl per well). Plates were sealed with optically clear sealing tape (Sarstedt Inc., Montreal, QC) and incubated at 37°C with gentle shaking (20 rpm) for 14-20 hours. The OD_{620} was then measured in an ASYS Expert Plus 96-well plate reader (Montreal Biotech Inc., Montreal, Quebec). MIC ranges were calculated by determining the lowest peptide concentration where growth inhibition is observed and the proceeding dilution where significant growth resumed.

3.3.6. Hemolysis Assays and Selectivity Index Range Calculations.

Hemolysis assays were conducted as previously described (Plunkett et al. 2009, Park et al. 2007) to determine the degree of toxicity of mvAMPs towards eukaryotic cells (erythrocytes). Hemolytic activity was determined by measuring the amount of heme released from sheep erythrocytes after incubation with mvAMPs. Defibrinated sheep's blood (Cedarlane Laboratories, Burlington, ONT, Canada) was diluted to a concentration of 1% in 1X phosphate-buffered saline (PBS). Cells were then centrifuged (1000 rpm, 5 min, 4°C) and washed twice with PBS, with centrifugation steps in between washes (1000 rpm, 5 min, 4°C), before being resuspended in PBS and

aliquoted into 0.6 ml polypropylene Eppendorf tubes. Serial dilutions of peptides were added to the cell suspension (final concentration range: 1000-0.1 µg/ml, final volume: 200 µl). Positive controls (cells incubated with 0.2% Triton-X) and negative controls (cells incubated with 1X PBS) were also included. After an incubation step (37°C, 1 hour), mixtures of peptides and cells were centrifuged for 5 min (1000 rpm, 4°C) and 100 µl of each supernatant were transferred to sterile polypropylene 96-well plates. The amount of heme released via cell lysis was determined by measuring the absorbance at 405 nm (A_{405}) with an ASYS Expert Plus 96-well plate reader (Montreal Biotech Inc., Montreal, Quebec). The percent hemolysis was calculated using the following formula:

$$\% \text{ hemolysis} = [(A_{405} \text{ Experimental}) - (A_{405} \text{ PBS}) / (A_{405} \text{ Triton X}) - (A_{405} \text{ PBS})] \times 100\%$$

For each peptide we calculated a selectivity index (SI), expressed as a ratio of its hemolytic concentration (HC) to its MIC (Chen et al. 2005):

$$SI = HC / MIC$$

In our study we determined (1) a universal selectivity index range (SIR) for each peptide against all bacteria tested, (2) SIRs against either Gram + or Gram - bacteria, or (3) an individual SI for each hAMP against each bacterium tested. For (1) and (2), we calculated the SIR by comparing the hemolytic concentration (HC) resulting in 1 (MHC), 5 (HC_5), and 10% (HC_{10}) hemolysis (hemoglobin release) to the geometric mean of MIC ranges (HC/MIC range) for all bacteria tested (1) and tested Gram + or Gram - bacteria collectively (2). The MHC (minimum hemolytic concentration) is defined as the highest peptide concentration where there is no detectable hemolysis (% hemolysis= <1%) (Chen et al. 2005). For (3) we calculated the SI of each hAMP against each bacterial species tested because a broad spectrum SIR does not reveal target selectivity. In this calculation, we determined the ratio of the HC_5 to the lowest MIC value for each bacteria species.

3.4. Results.

3.4.1. Combinatorial Studies.

Data from the combinatorial studies are presented in Table 3.2. A total of 13 different peptide combinations were tested against *S. epidermidis* (Gram +) and *E. coli* (Gram -). 11 combinations displayed synergism (FIC ≤0.2) towards at least one bacterial species. Of these, the peptides hAMP-97 and hAMP-83 displayed the highest synergistic activity against both *E. coli* and *S. epidermidis* and were selected as candidates for the design of our multivalent molecules. The constructs included two homodimers, mvAMP-1 (hAMP-97) and mvAMP-2 (hAMP-83), and one heterodimer incorporating both hAMP 97 and hAMP-83, mvAMP-3 (Table 3.3).

3.4.2. In vitro Antibacterial Assays of mvAMPs.

In vitro antibacterial data (radial diffusion and MIC) of the three mvAMPs are presented in Tables 3.4 and 3.5. Overall all mvAMPs displayed broad spectrum activity against Gram - and Gram + bacteria. mvAMP-1 had ~10 fold lower MICs than mvAMP-2 against most bacteria, the exceptions being *E. coli* and *C. freundii,* where there was no observed difference in MIC between the peptides. The heterodimer, mvAMP-3, was highly active with MIC ranges for *E. coli* (Gram -) and *S. epidermidis* (Gram +) at 2-0.2 µg/ml and <0.2 µg/ml respectively. mvAMP-3 also displayed significant activity against enteropathogenic *E. coli* (EPEC) and *S.* Typhimurium (both Gram -), with MIC range values of 2-0.2 µg/ml for both species. The least sensitive bacteria were *P. fluorescens* (Gram -, MIC range: 200-20 µg/ml) and *S. aureus* (Gram +, MIC range: 200-20 µg/ml). With the exception of EPEC, these values were significantly lower than the MICs of either individual hAMP units or the homodimers (Table 3.7).

3.4.3. Hemolysis Assays and Selectivity Index Ranges.

Data from hemolysis assays are presented in Table 3.6. Both mvAMP-1 and mvAMP-2 had MHC values of ~10 µg/ml. The heterodimer mvAMP-3 was the most

hemolytic, even at low concentrations (80.4% at 1000 µg/ml, 42% at 1 µg/ml, MHC, HC_5, and HC_{10} of ≤0.2 µg/ml).

SIRs/SIs for the mvAMPs and their corresponding monomeric units are listed in Tables 3.7 and 3.8. Three sets of SIR/SI values were calculated for each peptide: 1) against all bacteria tested, 2) against all Gram + bacteria and against all Gram - bacteria, and 3) against each bacterial species tested. There was no apparent selectivity for either Gram + or Gram - bacteria; i.e. broad spectrum activity was observed by all mvAMPs. Both homodimers displayed significantly higher SIRs than the heterodimer, due to lower hemolytic activity and moderate antimicrobial efficacy. mvAMP-1 had the best overall SIR (0.0512-4.07), scoring higher than its corresponding monomer. This was largely attributed to its relatively low hemolytic activity relative to the other 2 constructs.

The specific SIs for 8 bacteria (5 Gram - and 3 Gram +) are summarized in Table 3.8. Both mvAMP-3 and mvAMP-1 displayed low SIs (≤5). mvAMP-2 displayed comparatively higher SIs (≥10) against most of the Gram - and Gram + bacteria, with the exceptions being *E. aerogenes* (SI= 1) and *S. aureus* (SI= 0.5).

3.5. Discussion.

Synergism between AMPs and other antimicrobial agents (including other AMPs) has been well documented (Westerhoff et al. 1995, Yan and Hancock 2001, Haney et al. 2009, Anantharaman et al. 2010, Niu et al. 2013, Myers et al. 2012, Cirioni et al. 2010, Chongsiriwatana et al. 2011). In pharmacological terms, synergy between two antibiotics implies that the combined antimicrobial effect of the two drugs is substantially greater than the sum of the effects exerted by each drug alone. Many organisms produce a diverse array of AMPs in response to pathogens, most likely to ensure a more complete clearance of the infection through synergistic interactions between AMPs and other immunity-associated proteins (Haney et al. 2009, Yan and Hancock 2001). Synergy also has been reported between host AMPs and administered peptide and non-peptide antibiotics (Anantharaman et al. 2010, Chongsiriwatana et al. 2011, Niu et al. 2013). This interaction is desired if it improves efficacy, decreases host cytotoxicity (due to reduced dosage needed for optimal antimicrobial activity), and

reduces the likelihood of resistance development in the pathogen (Haney et al. 2009). In this study, we evaluated changes in antimicrobial efficacy when combining selected individual hAMPs (combinatorial studies) and using these data to generate a multivalent heterodimeric construct that incorporated hAMPs exhibiting synergistic activity. We used a multivalent design approach to increase the proximity and thus, the local concentration of bioactive peptide units, in the hopes of enhancing antimicrobial efficacy.

In this study, hAMP-97 and hAMP-83 demonstrated high synergy against most bacteria tested when both combined individually and attached as a single heterodimeric unit. Previous screening data on these two hAMPs indicated that on their own, both had broad spectrum activity (See Chapter 2 for hAMP-83). hAMP-83 was also more hemolytic and was able to disrupt the membranes of internalized *S.* Typhimurium within its host eukaryotic cells at low concentrations (Chapter 2). hAMP-97 in contrast, was relatively less cytotoxic and only permeabilized intracellular *S.* Typhimurium at higher concentrations (data not shown). The synergy observed could be due to enhanced pore formation. Both peptides have a similar secondary structure (linear alpha helical active motifs separated by a proline hinge), which suggests that they act on membranes in a similar fashion (as pore formers). Westerhoff et al. (1995) reported synergistic interactions between magainin 2 and PGLa, both natural AMPs derived from *Xenopus laevis*, in the form of enhanced membrane permeabilization. It was suggested that these findings were the result of multimeric transmembrane pore formation from the combined actions of both peptides. Alternatively, these peptides may exhibit different modes of action and the actions of one peptide may enhance the activity of the other. hAMP-83 has been found to permeabilize membranes (Chapter 2). If hAMP-97 primarily interacts with intracellular structures without causing significant membrane damage, the membrane permeabilizing activity of hAMP-83 may allow for easier access of hAMP-97 into the interior of the cell, resulting in higher killing efficacy.

Despite exhibiting potent broad-spectrum activity against bacteria, the heterodimer was cytotoxic, even at low concentrations, towards eukaryotic cells. In comparison, both homodimers were significantly less active against both bacteria and red blood cells. The loss of selectivity for prokaryotic cells in the heterodimer may be attributed to enhanced pore formation from peptide "preassembly". Since both individual

units of the heterodimer displayed synergism in the combinatorial studies against bacteria, linking them together in a multivalent conformation would increase proximity between the units, increasing the local concentration of peptides and thus enhancing pore formation. Sal-man et al. (2002) compared antimicrobial and hemolytic activities between monomeric cationic diastereomic AMPs and their "bundled" versions. These bundles were groups of five individual AMPs attached to the leucine residues of a peptide scaffold. Both the monomer and corresponding bundle displayed similar antimicrobial activity. However, the bundle was significantly more hemolytic and had a greater affinity for zwitterionic vesicles than the monomeric form. This observation was interpreted as the result of the bundle being more "preassembled" as the bioactive units were in closer proximity to each other and thus were able to form more organized amphipathic structures and consequently enhanced pore formation.

Our heterodimer had MICs 10-100 fold lower than the homodimers. The homodimer with the shorter subunits, mvAMP-1, had higher antimicrobial activity than mvAMP-2. The lower MICs, however, were accompanied by greater hemolytic activity. The HC_5 of mvAMP-1 was 10-100 fold lower than mvAMP-2. Consequently, the individual SIs per bacteria (Table 3.8) were lower for mvAMP-2 than mvAMP-1 based on HC_5 values. The differences in activity observed between the 2 homodimers may be attributed to the overall size of each construct and the relative ease in crossing the bacterial cell wall or the eukaryotic cell membrane. Molecular size is one of the parameters that determine the ability of an AMP to penetrate the bacterial cytoplasmic membrane. Larger peptides have more difficulty crossing the cell wall (Papo and Shai, 2003). Both the relatively small size and increased local concentration of hAMP subunits in mvAMP-1 may contribute to the higher antimicrobial and hemolytic activity observed in the dimer. The larger mvAMP- 2 may have difficulty in traversing the bacterial cell wall/eukaryotic cell membrane, reducing its efficacy relative to the corresponding monomer (See Table 3.7).

A problem encountered in this study was the issue of peptide purity. Due to the difficulty of synthesizing complex multivalent molecules, we obtained our constructs at 35-45% purity, about half of the usual purity range we obtained previously for linear hAMPs (≥90%). The composition of the impure fraction was thought to be a mixture of

unattached peptide fragments, amino acids, and unreacted reagents used in the synthesis process (Lifetein LLC, pers. comm.). The purity issue had potential implications on the results of this study. As each construct contained two attached hAMP constituents, we estimated that the number of individual hAMP units in a 45% pure solution of mvAMPs would be equivalent to a 90% pure solution of the same linear peptides. This assumption may hold true for the homodimers, which led us to carry out the *in vitro* assays with mvAMP concentrations identical to assays carried out with linear peptides (from 1000 µg/ml to 0.1 µg/ml). The heterodimer mvAMP-3 contained two different peptide constituents. Using the same assumption, a 45% pure solution of heterodimer would contain about half A and half B compared with an equivalent 90% pure solution of either A or B. This led us to double the test concentrations of heterodimer in the *in vitro* assays to equal the relative number of molecules we get with a 90% pure solution of linear hAMPs, hence the corrected test concentrations for purity. Concentration adjustments improved the MICs 10-100 fold but as the same time increased hemolytic activity. MIC assays may have also been affected by impurities in the mvAMP solutions. The presence of unattached peptide fragments and amino acids could trigger undesired interactions with bacterial and eukaryotic membranes in our assays, negatively affecting the results (e.g. low MICs, high hemolytic activity). If heterodimer concentrations were doubled in our assays, the amount of impurities would also be doubled. This raises the question of the relative contributions of mvAMP and impurity fractions to the observed toxicity. The full effects of these unwanted constituents on our results remain unknown but have to be taken into consideration when evaluating the data.

3.6. Conclusions.

This study indicated that (1) mvAMPs increased overall antimicrobial activity compared with their monomers, most likely the result of increased local concentration of peptides, (2) synergistic antimicrobial activity observed between different hAMPs in the combination studies was also observed in the heterodimer, (3) the high toxicity of the heterodimer towards eukaryotic cells may be due to enhanced pore formation from peptide pre-assembly, and (4) size of designed molecules may be a contributing

determinant of antimicrobial activity. Future studies on mvAMPs will need to focus on the mechanism of synergistic interaction and pore formation between the two peptide units of the heterodimer in both prokaryotic and eukaryotic membranes. Knowledge of these areas may help develop constructs with greater selectivity towards bacteria and with minimal toxicity towards host eukaryotic cells. Further insight into the parameters that affect mvAMP activity will also contribute to improvements in design.

3.7. References.

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3.8. Figures and Tables.

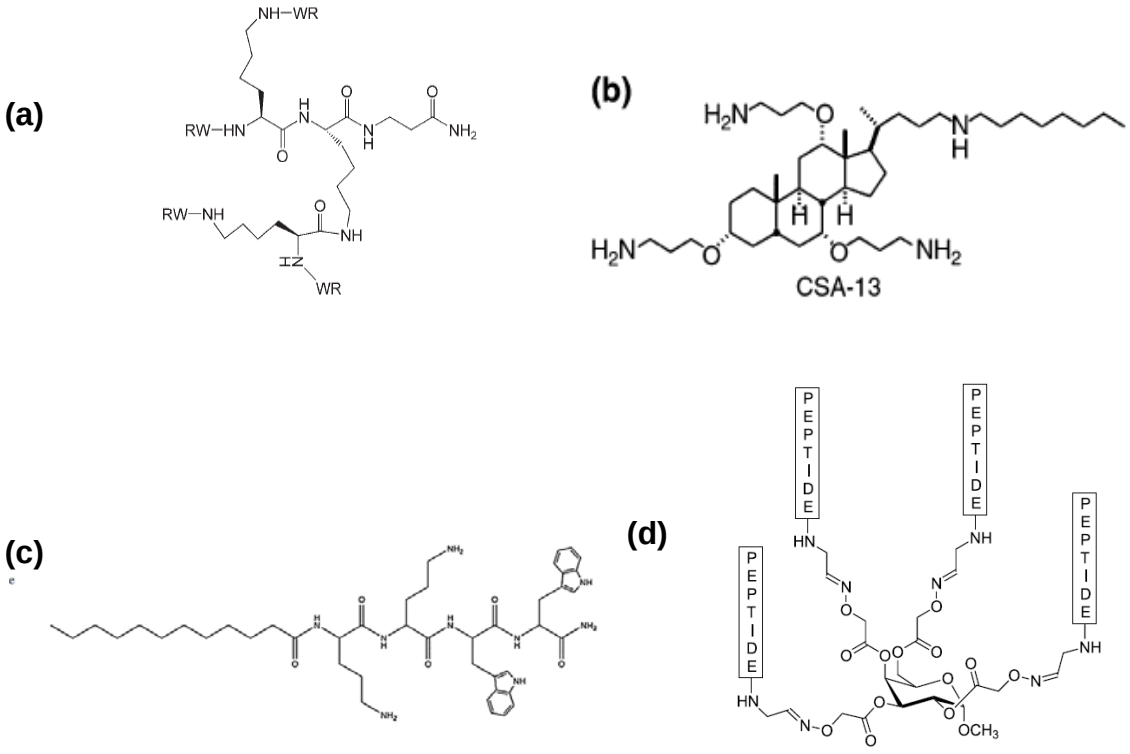

Figure 3.1: Examples of peptidomimetics with peptide subunits attached to different molecular scaffolds. These peptides have increased antimicrobial activity over their individual unattached subunits.

(a) A tetrameric peptide designed on a lysine scaffold containing 4 copies of the peptide RW that was found to be protease resistant and highly active against multi-drug resistant bacteria compared with individual RW molecules (Liu et al. 2007). (b) CSA-13, an example of a ceragenin that attaches peptides to a central sterol backbone (Epand et al. 2007). (c) An antimicrobial synthetic lipopeptide that contains a short peptide sequence/peptidyl backbone (pink) attached to a fatty acid chain at the N-terminus (green) that is responsible for antimicrobial activity (Giuliani and Rinaldi 2011). (d) A tetrameric carbopeptide that attaches peptide subunits to a modified carbohydrate scaffold, α-D-galactopyranoside (Gueell et al. 2012).

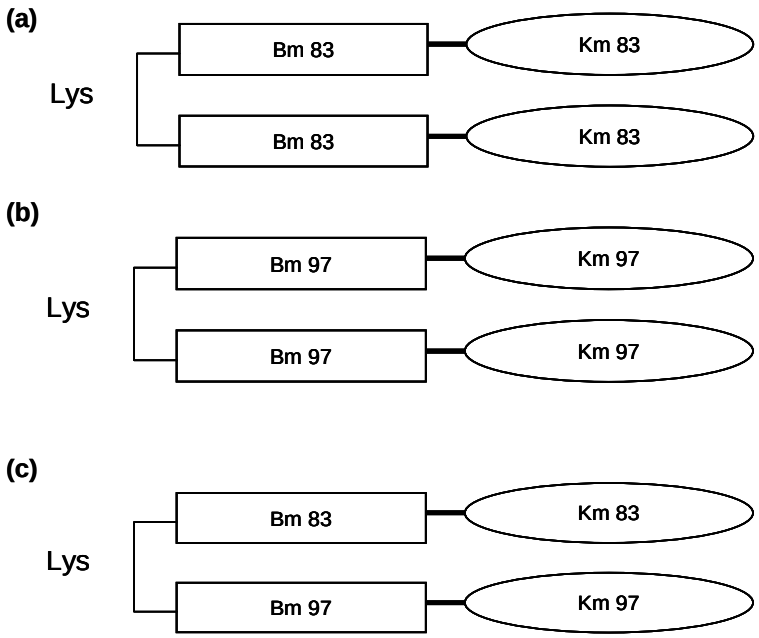

Figure 3.2: Schematic of multivalent hAMPs (mvAMPs) designed and tested in this study.

Schematic of our mvAMP design; peptides were attached onto a lysine scaffold (Lys). Both (a) and (b) represent homodimer constructs, containing either monomer (hAMP) 83 or 97. (c) represents a heterodimer, with both monomers 83 and 97 attached to the lysine scaffold. Lys= lysine scaffold, Bm= binding motif, Km= killing motif.

Table 3.1: Names and previously determined target selectivities of linear hAMPs assayed for synergistic antimicrobial activity in combinatorial studies.

Peptide	Selectivity*
hAMP-91	Gram +
hAMP-92	Gram +
hAMP-93	Gram +
hAMP-102	Gram +
hAMP-83	Broad Spectrum
hAMP-97	Broad Spectrum
hAMP-101	Broad Spectrum
hAMP-82	Gram -
hAMP-100	Gram -

* based on in vitro assay (MIC) data from screening (see Chapter 2)
Bold leucine residues (*L*) indicate D-substituted leucines
Gram +: Gram-positive, Gram -: Gram-negative

Table 3.2: The degree of synergistic activity* of paired hAMP combinations against Gram - (E. coli) and/or Gram + (S. epidermidis) bacteria.

hAMP Combination	FIC Index Range* vs. Gram + bacteria	FIC Index Range* vs. Gram - bacteria
93+91	≤0.02	X**
93+92	≤0.02	X
91+92	0.2-0.02	X
93+102	0.2-0.02	X
91+102	0.2-0.02	X
92+102	0.2-0.02	X
102+83	1-0.2	X
102+101	1-0.2	X
92+83	≤0.02	X
93+83	0.2-0.02	X
101+92	1-0.2	X
101+93	1-0.2	X
83+100	X	1-0.2
100+101	X	1-0.2
83+101	1-0.2	1-0.2
83+97	0.2-0.02	≤0.2

* The degree of synergy was expressed as a fractional inhibitory concentration (FIC) index range, calculated using the formula: MIC (A+B)/MIC A +MIC (A+B)/MIC B where MIC (A+B) are the MIC ranges of the components in combination and MIC A and MIC B are the previously determined MICs of each component alone; an FIC index of ≤0.2 indicates synergy between the 2 components.

**An 'X' indicates that the components of the particular hAMP combination were previously found to be inactive against the target group of bacteria (Gram + or Gram -) and thus was not tested.

Table 3.3: Names and hAMP units of lysine scaffold (K)-based multivalent constructs.

Construct Name	hAMP Units
mvAMP 1	(hAMP 97) X 2
mvAMP 2	(hAMP 83) X 2
mvAMP 3	hAMP-83 + hAMP 97

Table 3.4: Radial diffusion assay data of multivalent hAMP constructs against ten bacterial species; '+' Indicates a zone of inhibition of at least 0.5 mm around each disc; 0 indicates no activity.

Bacteria	Multivalent Construct		
	mvAMP 1	mvAMP 2	mvAMP 3
E.coli	+	+	+
A. faecalis	+	+	+
C. freundii	+	+	+
E. aerogenes	+	0	+
P. fluorescens	0	0	+
EPEC	+	+	+
S. Typhimurium	+	+	+
B. subtilis	+	+	+
S. epidermidis	+	+	+
S. aureus	+	+	+

Table 3.5: MIC data (µg/ml): Comparison of antimicrobial activity between single linear hAMPs and their multivalent combinations.

Bacteria	hAMP/Multivalent Construct				
	hAMP-83	hAMP-97	mvAMP 1	mvAMP 2	mvAMP 3
E.coli	10-1	100-10	10-1	10-1	2-0.2*
A. faecalis	100-10	100-10	100-10	1000-100	2-0.2*
C. freundii	10-1	100-10	10-1	10-1	≤0.2*
E. aerogenes	10-1	1000-100	100-10	1000-100	2-0.2*
P. fluorescens	1000-100	1000-100	1000-100	≥1000	200-20*
EPEC	1-0.1	1-0.1	≤0.2*	20-2*	2-0.2*
S. Typhimurium	10-1	1-0.1	2-0.2*	20-2*	2-0.2*
B. subtilis	1-0.1	10-1	10-1	10-1	≤0.2*
S. epidermidis	10-1	10-1	10-1	100-10	≤0.2*
S. aureus	100-10	1000-100	200-20*	2000-200*	200-20*

*values represent corrected tested concentrations for purity.

Table 3.6: Host cytotoxicity data of multivalent hAMPS: Hemolytic concentrations (HCs) resulting in 1 (MHC), 5 (HC_5) , and 10% (HC_{10}) hemolysis.

Construct	MHC*	HC_5	HC_{10}
mvAMP 1	10-1 μg/ml	10-1 μg/ml	10-1 μg/ml
mvAMP 2	10-1 μg/ml	100 μg/ml	1000-100 μg/ml
mvAMP 3	<0.2 μg/ml	<0.2 μg/ml	<0.2 μg/ml

*MHC: Minimal hemolytic concentration- the highest peptide concentration at which there is no detectable release of hemoglobin (% hemolysis of ≤1).

Table 3.7: Comparison of selectivity index ranges* between single linear hAMPs and their multivalent combinations against Gram-positive (Gram +) bacteria only, Gram-negative (Gram -) bacteria only, and both Gram + and Gram - bacteria combined.

Peptide/Construct	Selectivity Index Range		
	Gram +	Gram -	Gram + & Gram -
hAMP-83	0.001-0.1	0.0052-0.037	0.0063-0.063
hAMP-97	0.021-0.21	0.019-0.19	0.02-0.2
mvAMP 1	0.037-3.68	0.059-4.25	0.0512-4.07
mvAMP 2	0.0079-0.79	0.011-0.82	0.010-0.81
mvAMP 3	0.1-0.21	0.072-0.52	0.079-0.398

*Selectivity index ranges were calculated by dividing the MHC by the geometric mean of determined MIC ranges; the higher the index the greater the therapeutic potential and vice versa.

Table 3.8: Therapeutic indices of multivalent hAMPs based on specific MIC per bacteria and 5% hemolytic concentration (HC_5) values*.

Multivalent Construct	Bacteria							
	E. coli (Gram -)	EPEC (Gram -)	*E. aer.* (Gram -)	*C. fre.* (Gram -)	*S.* Typh. (Gram -)	*B. sub.* (Gram +)	*S. epi.* (Gram +)	*S. aur.* (Gram +)
mvAMP 1	1	1	0.1	1	5	1	1	0.05
mvAMP 2	103	51	1	103	51	103	10	0.50
mvAMP 3	1	1	1	1	1	1	1	0.01

*Therapeutic indices for each bacteria were calculated by dividing the HC_5 value by the low end of the previously determined MIC range for each peptide per bacteria; Gram -: Gram-negative bacteria, Gram +: Gram-positive bacteria; EPEC= enteropathogenic *E. coli*, *E. aer.= Enterobacter aerogenes*, *C. freu.= Citrobacter freundii,* S. Typ.= *Salmonella* Typhimurium*, B. sub.= Bacillus subtilis*, *S. epi= Staphylococcus epidermidis*,*S. aur.= Staphylococcus aureus*.

Chapter 4. Overall Conclusions.

Within the past 40 years, only three new classes of antibiotics have been developed against Gram + bacteria and none for Gram - bacteria (Oyston et al. 2009). This scarcity of new antibacterial drugs coupled with the increasing incidence of multi-drug resistant pathogens requires that we look for alternative sources of antimicrobial compounds.

Because of recent interest in developing AMPs as new antibiotics (Brogden and Brogden 2011, Huang et al. 2010, Marshall and Arenas 2003), we began this study with the objective of evaluating and developing naturally occurring (AMPs) and creating hybrid antimicrobial peptides (hAMPs) as new potential antibiotics. Because some AMPs have recently entered clinical development (See Table 1.1) we thought we could improve on the basic design by incorporating novel motifs or charge or characteristics that would enhance overall antimicrobial activity.

Over the course of this study we:

1) synthesized ~ 120 hAMPs,
2) evaluated hAMPs in disc assays,
3) determined Minimum Inhibitory Concentration (MIC) assays on all hAMPs,
4) determined the effects of selected hAMPs on eukaryotic cells (hemolysis assays),
5) evaluated the ability of hAMPs to kill bacteria attached to, or within, cells grown in monolayers,
6) evaluated combinations of hAMPs to identify those that could work synergistically to increase overall activity, and
7) synthesized multivalent AMPs (mvAMPs) to create the perfect antibiotic.

We were successful in synthesizing many hAMPs; some that worked as planned, others that were rejected outright. Some hAMPs acted equally against Gram - and Gram + bacteria, while others were more lethal against one group of bacteria, or even against a

particular species of bacteria. Through the course of these studies we determined many factors (length, charge, orientation, D- amino acids, hydrophobicity) that contributed to antimicrobial activity.

The research described in this thesis should not be the end point, but should be the impetus for future studies. And these future studies should:

1) further modify these molecules to enhance desired characteristics,
2) begin *in vivo* studies with bacterial diseases in animal models,
3) use bioinformatics to design “rational” hAMPs with higher probability of success, and
4) understanding modes of action (membrane disruption, intracellular targets, immunomodulation).

As a species, we are exposed to, and suffer from, numerous microbes. Some make us very sick, some are lethal. There will always be a need for cheap, efficacious drugs to eliminate infections. Expanding on the studies described in this thesis might be the start of a logical design of new antibiotics.

References

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Huang Y, Huang J, Chen Y. 2010. Alpha-helical cationic antimicrobial peptides: Relationships of structure and function. Protein & Cell 1:143-52.

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Oyston PCF, Fox MA, Richards SJ, Clark GC. 2009. Novel peptide therapeutics for treatment of infections. J Med Microbiol 58:977-87.

Appendix 1.

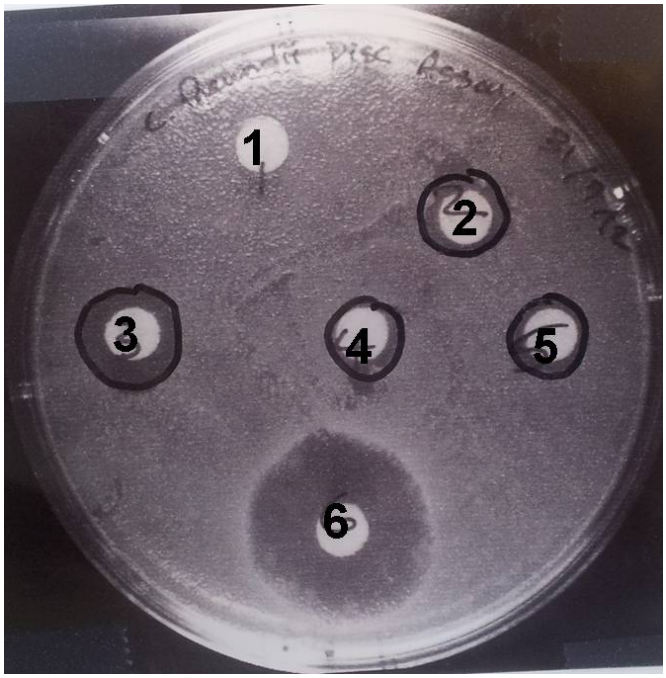
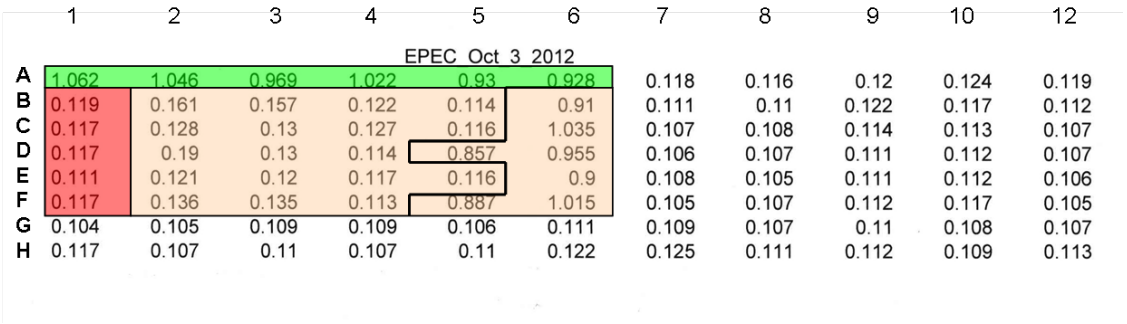


Figure A1: Example of a disc assay with hAMPs.

In this example, sterile filter paper discs applied with hAMP solutions (discs 2-11), water as a negative control (disc 1) and a commercial antibiotic (disc 6, carbenicillin) were placed onto an LB agar plate spread with log phase bacteria (*C. freundii* in this example). After an incubation period overnight (15-20 hours) at 37ºC, the discs were examined for zones of inhibition which are halos of zero visible growth of bacteria around each disc. The diameters of the zones of inhibition were qualitatively assessed- in this example, the hAMP applied to disc 3 displayed the widest zone of inhibition of the four tested here.



	1	2	3	4	5	6	7	8	9	10	12
					EPEC Oct 3 2012						
A	1.062	1.046	0.969	1.022	0.93	0.928	0.118	0.116	0.12	0.124	0.119
B	0.119	0.161	0.157	0.122	0.114	0.91	0.111	0.11	0.122	0.117	0.112
C	0.117	0.128	0.13	0.127	0.116	1.035	0.107	0.108	0.114	0.113	0.107
D	0.117	0.19	0.13	0.114	0.857	0.955	0.106	0.107	0.111	0.112	0.107
E	0.111	0.121	0.12	0.117	0.116	0.9	0.108	0.105	0.111	0.112	0.106
F	0.117	0.136	0.135	0.113	0.887	1.015	0.105	0.107	0.112	0.117	0.105
G	0.104	0.105	0.109	0.109	0.106	0.111	0.109	0.107	0.11	0.108	0.107
H	0.117	0.107	0.11	0.107	0.11	0.122	0.125	0.111	0.112	0.109	0.113

Figure A2: A 96-well plate readout of an MIC assay with five hAMPs.

An example of a plate readout of an MIC assay. Numbers indicate turbidity measurements (OD_{620}) in each of the 96 wells. OD_{620} readings enclosed in the green box represent wells aliquoted with only media with bacterial culture as positive growth controls (Wells A1-A6). Wells enclosed in the red box (Column B1-F1) contain growth media (LB) alone with no bacteria and represent negative growth controls. Measurements in the orange box (Rows B2-6 to F2-6) represent experimental wells containing bacteria and hAMPs in 10 fold serial dilutions between wells (From 1000 µg/ml in column 2 to 0.1 µg/ml in column 6). Each row of experimental wells represents an individual hAMP. The line drawn through the shaded box separates the experimental wells where no growth is detected (to the left of the line) from the wells where growth resumes (to the right of the line). For each hAMP, the concentrations corresponding to these wells represent its calculated 'MIC range'.

Table A1: Names, molecular weights, and predicted charges of hAMPs discussed in Chapter 2.

Peptide	MW (Da)	Charge
hAMP-29	2413	+7
hAMP-41	2131.67	+6
hAMP-54	1737.13	+6
hAMP-66	5639.8	+12
hAMP-80	5513.63	+12
hAMP-83	4383.48	+14
hAMP-90	2413	+7
hAMP-95	2131.67	+6
hAMP-100	3858.8	+9

MW: molecular weight (in daltons)

Table A2: Comparison of hemolytic concentrations (MHC and HC_5) between L and D hAMP analogs discussed in Chapter 2.

Peptide	MHC	HC_5
hAMP-29 (L)	0.0041>	0.041
hAMP-90 (D)	4.14	41.4
hAMP-41 (L)	4.69	4.69
hAMP-95 (D)	4.69	46.9

The MHC, or minimum hemolytic concentration, refers to the peptide concentration resulting in 1% observed hemolysis; the HC_5 refers to the peptide concentration that causes 5% observed hemolysis; concentrations are expressed in μM.

Table A3: Comparison of MIC range data against six bacterial species between L and D hAMP analogs discussed in Chapter 2.

Bacteria	*E. coli* (Gram -)	*E. aer.* (Gram -)	EPEC (Gram -)	*S. epi.* (Gram +)	*B. sub.* (Gram +)	*L. mon.* (Gram +)
Peptide	MIC Range (μM)					
hAMP-29 (L)	414-41.4	41.4-4.14	4.14-0.41	41.4-4.14	414-41.4	4.14-0.41
hAMP-90 (D)	41.4-4.14	41.4-4.14	4.14-0.41	4.14-0.41	4.14-0.41	41.4-4.14
hAMP-41 (L)	4.69-0.47	46.9-4.69	4.69-0.47	4.69-0.47	4.69-0.47	4.69-0.47
hAMP-95 (D)	46.9-4.69	469-46.9	4.69-0.47	46.9-4.69	4.69-0.47	46.9-4.69

hAMP pairs 29 & 90 and 41 & 95 each represent L and D analogs; EPEC= enteropathogenic *E. coli*, *E. aer.= Enterobacter aerogenes*, *B. sub.= Bacillus subtilis*, *S. epi= Staphylococcus epidermidis*, *L. mon.= Listeria monocytogenes*