NOVEL ANTIMICROBIAL PEPTIDES IN ALLIGATOR AND CROCODILE

by

Stephanie Barksdale
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Committee:

_____	Dr. Monique van Hoek, Thesis Director
_____	Dr. Barney Bishop, Committee Member
_____	Dr. Sergei Popov, Committee Member
_____	Dr. James Willett, Director, School of Systems Biology
_____	Dr. Donna M. Fox, Associate Dean, Student Affairs & Special Programs, College of Science
_____	Dr. Peggy Agouris, Dean, College of Science
Date: _____	Fall Semester 2015 George Mason University Fairfax, VA

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Stephanie Barksdale
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Director: Monique van Hoek, Associate Professor
School of Systems Biology

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Fairfax, VA

DEDICATION

This is dedicated to my husband Nick, for his love and support, and to my mother, for instilling in me a love of learning.

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I would like to thank my family, friends, and lab mates for their support. My husband Nick supported me through long hours and hard work. My sisters Jessica and Rachael and my parents have encouraged me to push myself. My PI Dr. van Hoek has mentored me for many years and has helped me grow as a researcher, an employee, and a person. All of my lab mates helped me get through difficult times and difficult problems. And thanks to the Dr. Bishop and his lab for all of the collaboration and advice from the other side of biochemistry.

TABLE OF CONTENTS

Page
List of Tables vi
List of Figures vii
List of Equations viii
List of Abbreviations and/or Symbols ix
Abstract xi
Chapter 1: Introduction 1
Antimicrobial Peptides 1
Order Crocodilia 3
Alligator mississippiensis (American Alligator) 4
Crocodylus siamensis (Siamese Crocodile) 4
Infecting and Commensal Bacteria of Alligators and Crocodiles 5
Innate Immunity of Crocodilians 8
Chapter 2: Hepcidin of the Siamese crocodile 15
Chapter 3: Apolipoprotein-derived peptides of the American Alligator 36
Chapter 4: Cathelicidin of the American Alligator 56
Chapter 5: Conclusion 76
References 80

LIST OF TABLES

Table Page
Table 1 Siamese crocodile hepcidin sequence and properties……...19
Table 2 Reduction conditions and rate equations for antimicrobial assays……...21
Table 3 Amino acid sequence conservation in hepcidins……..24
Table 4 Reported antimicrobial and antifungal activity of various hepcidins……...27
Table 5 Antimicrobial activity of Siamese crocodile hepcidin……..……...28
Table 6 Apo5 and Apo6 sequences and physio-chemical properties……40
Table 7 Reduction conditions and rate equations for antimicrobial assays……...41
Table 8 Percent secondary structure contribution of Apo5 and Apo6……...46
Table 9 Antimicrobial activity of Apo5 and Apo6……....47
Table 10 Genes from the *A. mississippiensis* genome annotated as cathelicidins.....63
Table 11 Identification of alligator cathelicidin genes.....65
Table 12 Known OH-CATH-like antimicrobial peptides.....66
Table 13 Sequence and physico-chemical properties of AM-CATH28.....67
Table 14 Antimicrobial activity of AM-CATH28.....68
Table 15 Percent secondary structure contribution of AM-CATH28.....71

LIST OF FIGURES

Figure Page
Figure 1 Classes of AMP secondary structures....2
Figure 2 Blood cells from *Alligator mississippiensis*....10
Figure 3 Conserved disulfide bond patterns in hepcidins....16
Figure 4 Antimicrobial activity of the Siamese crocodile hepcidin....29
Figure 5 CD spectra of the Siamese crocodile hepcidin....31
Figure 6 Bioinformatical analysis of Apo5 and Apo6....44
Figure 7 CD spectra of Apo5 and Apo6....45
Figure 8 Graph of antimicrobial activity of Apo5 and Apo6....49
Figure 9 Membrane permeabilization activity by Apo5 and Apo6.... 50
Figure 10 Membrane depolarization by Apo5 and Apo6....51
Figure 11 Cytotoxicity of Apo5 and Apo6....52
Figure 12 Helical wheels of AM-CATH28 and AM-CATH....67
Figure 13 Structural prediction of AM-CATH28....70
Figure 14 Membrane permeabilization activity by AM-CATH28....72
Figure 15 Cytotoxicity of AM-CATH28....72

LIST OF EQUATIONS

Equation Page
Equation 1 Growth rate equation for *P. aeruginosa* ATCC 9027.....21
Equation 2 Growth rate equation for *S. aureus* ATCC 25923.....21
Equation 3 Growth rate equation for *E. coli* ATCC 51659.....21
Equation 4 Growth rate equation for *P. aeruginosa* PAO1.....41
Equation 5 Growth rate equation for *S. aureus* ATCC 33592.....41
Equation 6 Growth rate equation for *A. baumannii* ATCC BAA-1794.....41

LIST OF ABBREVIATIONS

Amino Acid.....aa
Cationic Antimicrobial Peptide.....CAMP
Liver Expressed Antimicrobial Peptide-1.....LEAP-1
Lipopolysaccharide.....LPS
Trifluoroethanol.....TFE
Sodium Dodecyl Sulfate.....SDS
Phosphate Buffer.....PB
Phosphate-Buffered Saline.....PBS
Colony-Forming Units.....CFU
Circular Dichroism.....CD
Enterohemorrhagic *E. coli*.....EHEC
Multi-Drug Resistant.....MDR
Reverse Phase High-Performance Liquid Chromatography.....RP-HPLC
Electrospray Ionatization Mass Spectrometry.....ESI-MS
Optical Density.....OD
Centers for Disease Control and Prevention.....CDC
Pathogen-associated molecular pattern.....PAMP
Methicillin-resistant *S. aureus*.....MRSA
Deoxyribonucleic acid.....DNA
Messenger ribonucleic acid.....mRNA
National Center for Biotechnology Information.....NCBI
Expressed sequence tag.....EST
American Type Culture Collection.....ATCC
Sheep Myeloid Antimicrobial Protein-29.....SMAP-29
Basic Local Alignment Search Tool.....BLAST
European Molecular Biology Laboratory.....EMBL
genInfo identifier.....GI
Antimicrobial Peptide Database 2.....APD2
Molecular weight.....MW
Trifluoroacetic acid.....TFA
Weight/volume.....w/v
Volume/volume.....v/v
Confidence interval.....CI
Base pair.....bp
Minimal inhibitory concentration.....MIC
Minimal bactericidal concentration.....MBC

Red blood cell.....RBC
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium.....MTT
3,3’-dipropylthiadicarbocyanine iodide.....DiSC3(5)

ABSTRACT

NOVEL ANTIMICROBIAL PEPTIDES IN ALLIGATOR AND CROCODILE

Stephanie Barksdale, M.S.

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Thesis Director: Dr. Monique van Hoek

Novel antibiotics are needed to fight the rising tide of drug resistance in pathogenic bacteria. One possible source is cationic antimicrobial peptides (CAMPs), small proteins produced by the innate immune system. CAMPs have a range of mechanisms including direct antibacterial action and immunomodulatory effects. Crocodilians are part of an ancient clade, the Archosaurs, and are more closely related to birds and dinosaurs than other living reptiles. Very little is known about the innate immune systems of crocodilians, but research has found that the serum of these species have antimicrobial activity beyond that of human serum. This activity is thought to be partly due to CAMPs, though only a handful of crocodilian CAMPs have been described. In this thesis, four novel CAMPs from members of the order Crocodilia are investigated. A hepcidin from *Crocodylus siamensis,* an iron-regulating peptide with 4 intramolecular disulfide bonds, is found to have weak activity against *Pseudomonas aeruginosa, Escherichia coli,* and *Staphylococcus aureus.* Two fragments of an apolipoprotein found in the blood of

Alligator mississippiensis were found to have strong activity against a range of Gram negative and Gram positive bacteria, including multi-drug resistant bacteria. These fragments were found to be alpha-helical and to depolarize the bacterial membrane. A cathelicidin from *A. mississippiensis* is strongly active against *P. aeruginosa* and multi-drug resistant *Acinetobacter baumannii* and permeabilizes the bacterial membrane. These analyses give us greater understanding of the crocodilian innate immune system. In addition, these CAMPs could be used as a basis for new antimicrobials.

CHAPTER 1: INTRODUCTION

Antimicrobial Peptides

Cationic antimicrobial peptides (CAMPs) are small proteins produced as part of the innate immune system of virtually all higher organisms. CAMPs can have direct antimicrobial activity, have immunomodulatory effects, or be fragments of proteins with other purposes. CAMPs are usually not conserved by amino acid sequence between species, but can be broadly grouped by specific secondary structures or amino acids features. There are four broad types of CAMPs based on secondary structure. First are the α-helical AMPs, such as magainin, a member of the cathelicidin group (**Figure 1A**) (Gesell, Zasloff, & Opella, 1997). Second are CAMPs that are mainly unstructured, such as indolicidin (**Figure 1B**) (Rozek, Friedrich, & Hancock, 2000). Next are CAMPs with mixed structure, such as protegrin-1 from (**Figure 1C**) (Y. Yang, Sanchez, Strub, Brutscher, & Aumelas, 2003). Lastly are CAMPs with β-sheets, often stabilized by disulfide bonds, such as the humanα-defensin-1, which is part of the larger group of defensins (**Figure 1D**) (X. L. Zhang, Selsted, & Pardi, 1992).

AMPs have a range of mechanisms. Some CAMPs associate with and disrupt the bacterial membrane, leading to lysis or membrane depolarization, such as NA-CATH (M. L. Juba et al., 2015). Other CAMPs cross the bacterial membrane and interfere with a variety of processes either specifically or generally. CAMPs can interfere with protein synthesis (Chung, Dean, & van Hoek, 2015), bind DNA and halt transcription or

replication (Xie, Fleming, Chen, & Elmore, 2011), and decrease biofilm formation (Duplantier & van Hoek, 2013). CAMPs can also affect the host by modulating the immune system (G. Wang, 2014).

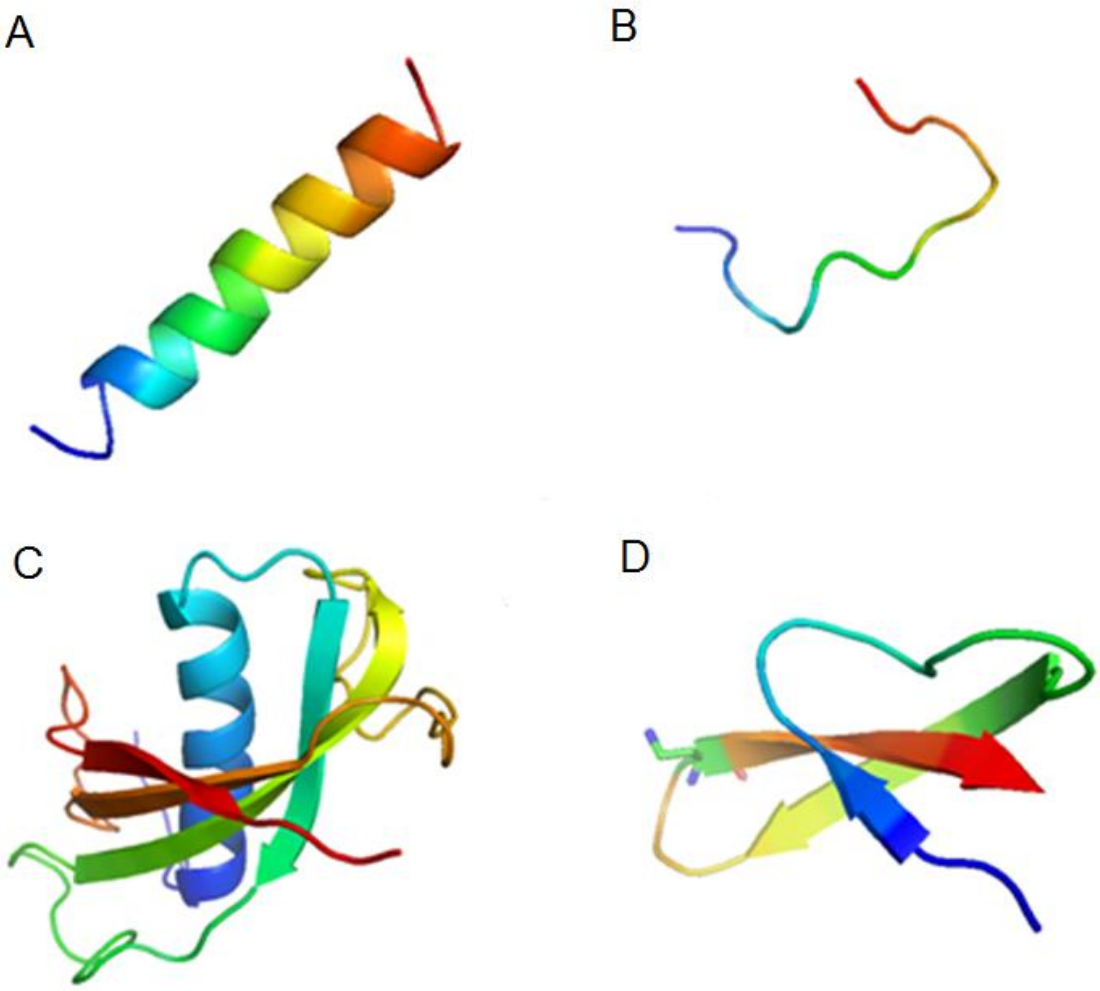

Figure 1. Classes of CAMP secondary structures. Adapted from "Various AMPs" by Ymahn, licensed under CC BY 2.0. **A.** α-helical: magainin (Gesell et al., 1997), **B.** unstructured or random coil: indolicidin (Rozek et al., 2000), **C.** mixed: protegrin-1 (Y. Yang et al., 2003), **D.** β-sheet: human α-defensin-1 (X. L. Zhang et al., 1992).

CAMPs have been studied since 1967, when melittin was first isolated from bee venom (Fennell, Shipman, & Cole, 1967), but interest has grown more recently because of their possible use as novel antibiotics (Nakatsuji & Gallo, 2012). Antibiotic resistance has increased dramatically over the last several decades. In 2013, the CDC reported that there were 2 million infections caused by drug-resistant bacteria, with 23,000 deaths (*Antibiotic Resistance Threats in the United States, 2013*, 2013). Very few antibiotics are in the FDA approval pipeline, and bacteria are already displaying resistance to antibiotics introduced this decade (*Antibiotic Resistance Threats in the United States, 2013*, 2013). CAMPs are attractive bases for new antimicrobials because they tend to be multifunctional and bacteria do not normally acquire genetic resistance to them.

Many organisms could be attractive sources of new CAMPs; for example, organisms that are often exposed to pathogenic bacteria, such as carrion eaters, saprophytes, and those living in environments with high burdens of bacteria such as swamps. Organisms with long lineages that are evolutionarily strong are another; through persistence over millions of years, these organisms have shown to have a strong immune system. Because of these reasons, members from the order Crocodilia, in particular *Alligator mississippiensis* and *Crocodylus siamensis*, were targeted as possible sources of strong CAMPs.

Order Crocodilia

Members of the order Crocodilia, are part of an undiverged lineage more closely related to dinosaurs than are most modern reptiles. Order Crocodilia is part of the clade Archosauria, which also include dinosaurs and birds. This order first emerged more than

80 million years ago. Members of the order are carnivorous ectothermic amniotes, and are normally found in wet tropical environments. The order includes three extant families: Gavialidae, comprised of the gharial and false gharial; Alligatoridae, alligators and caimans; and Crocodylidae, the crocodiles. (Brochu, 2003)

Alligator mississippiensis (American Alligator)

American alligators are found in the continental United States of America, ranging from Texas to Florida to some southern parts of Virginia. They are one of only two crocodilian species commonly found in non-tropical climes, and are thus much more tolerant to cold weather than other crocodilians. They can be found in many fresh bodies of water, including swamps, lakes, and calm rivers. They display sexual dimorphism, with females growing to just under 3 m and males under 5 m. They hibernate during the winter and do not feed at lower temperatures. American alligators are known to feed less as the temperature drops below 27°C and to stop feeding at 23°C. (Britton, 2009a; Pajerski, Schechter, & Street, 2000)

American alligators were previously an endangered animal due to hunting and loss of territory. Conservation programs in the mid-twentieth century helped the population rebound, so this species is now of low concern. Alligator farms now exist in several southern states that produce alligator skin and meat to consumers and maintain a strong wild population. (Britton, 2009a; Pajerski et al., 2000)

Crocodylus siamensis (Siamese Crocodile)

The Siamese crocodile is a smaller crocodilian, with males rarely passing 3 m. Siamese crocodiles were traditionally found across Southeast Asia, including Cambodia,

Indonesia, Malaysia, and Thailand. However, overhunting, loss of habitat, and pollution have greatly decreased the range and population of the Siamese crocodile. This species is now considered critically endangered and is only found wild in Cambodia. Because their skin is considered valuable, the region has made an effort to increasing populations on crocodile farms, especially in Thailand. Little is known about this species because of the decimation of its population. (Britton, 2009b)

Infecting and Commensal Bacteria of Alligators and Crocodiles

Literature about the infecting and commensal bacteria of alligators and crocodiles is limited to mostly case studies of morbidity in alligator and crocodile farms or zoos and surveys of the microbiome of the gastrointestinal tract. Several groups have swabbed the oral and cloacal cavities to survey those microbiomes. In wild *C. acutus* and *C. moreletii* (Morelet's crocodile), *Aeromonas hydrophila* and *Escherichia coli* were most often found in the cloacae, while *A. hydrophila* and *Arcanobacterium pyogenes* were found most often in the oral cavity. *Salmonella arizonae* and *Salmonella typhi* were also found (Charruau, Perez-Flores, Perez-Juarez, Cedeno-Vazquez, & Rosas-Carmona, 2012). A group found that the normal oral flora of *A. mississippiensis* includes *A. hydrophila*, *Pantoea agglomerans, Citrobacter diversus,* and *Clostridium* spp. (Flandry et al., 1989), and another found *Clostridium* in oral swabs (Doering, Iii, Fitts, Rambo, & Bradham, 1971). In South Carolina, a group took cloacal swabs of wild members of *A. mississippiensis* and found a wide variety of species, including *A. hydrophila, Citrobacter braaki, Citrobacter freundii, Edwardsiella tarda, Edwardsiella aerogenes, Enterobacter cloacae, E. coli, Hafnia alvei, Klebsiella pneumoniae, Morganii morganii, Pantoea* spp.,

Pasteurella pneumotropica, Plesiomonas shigelloides, Plesiomonas alvalifacians, Plesiomonas rettgeri, Salmonella spp., *Serratia marcescens, Vibrio cholera*, and *Vibrio fluvialis* (M. A. Johnston, Porter, Scott, Rhodes, & Webster, 2010). *Salmonella* has been found on many African reptiles, particularly captive farmed Nile crocodiles, but also some wild-caught dwarf crocodiles (van der Walt, Huchzermeyer, & Steyn, 1997). Another study found *Salmonella* spp. in a quarter of the cloaca of wild Nile crocodiles in Zimbabwe, but none on the skin of these crocodiles (Madsen, Hangartner, West, & Kelly, 1998). *Salmonella* spp. were also found in the cloaca and feces of farmed crocodiles in Australia (Manolis, Webb, Pinch, Melville, & Hollis, 1991).

The oral microflora has also be described by identifying bacteria colonizing crocodilian bites. In Africa, separate bites from crocodiles were colonized with *Citrobacter* spp., *Vibrio vulnificus, P. agglomerans, Bacteroides melaninogenicus, A. hydrophila, Serratia fonticola, Clostridium perfringens, Pseudomonas aeruginosa*, and *Proteus vulgaris. Burkholderia pseudomallei* was also found, but it is believed that this bacteria came from environmental exposure of the wound rather than the bite itself. (Wamisho et al., 2009) *E. tarda* was also found in two separate alligator bites (Wallace, White, & Gore, 1966).

Recently, the entire gastrointestinal tract of wild and captive American alligators was surveyed in winter, during wild alligator fasting, and in summer, during wild alligator active feeding (Keenan, Engel, & Elsey, 2013). In wild alligators, GI bacteria in the winter were found to be primarily Proteobacteria and Fusobacteria and some Firmicutes, with Proteobacteria primarily colonizing the tract from tongue to ileum and

Fusobacteria primarily from ileum to colon and in feces. In the spring, bacteria are primarily Firmicutes and Fusobacteria, with the area from tongue to ileum being colonized by Firmicutes and Fusobacteria from ileum to colon and in feces. In captive alligators, which are fed pellets instead of live food, there is little seasonal variation. Actinobacteria and Proteobacteria colonize the tongue and esophagus, Firmicutes from stomach to ileum, and a mixture of bacteria in the colon and feces, including Fusobacteria. (Keenan et al., 2013) The presence of Proteobacteria such as *E. coli, Pantoea* spp., *Clostridium* spp., and *Citrobacter* spp. in the oral cavity of crocodile is unexpected, as these bacteria are often found in the feces of mammalian species. It is possible that bacteria from the fecal material of prey colonize the crocodilian oral cavity when that prey is consumed. (Flandry et al., 1989) This makes sense considering that Proteobacteria are not prevalent in the American alligator oral cavity during the winter fast (Keenan et al., 2013).

There are a few case reports of crocodilian morbidity from bacterial infection in the literature, as well as some studies of colonized skin lesions. In one study, researchers identified colonized microbes in 203 lesions from 180 farmed crocodiles. Twenty-eight percent were infected with *Dermatophilus congolensis*, 32.5% were mixed infections including *D. congoensis*, 15% were infected with fungi, 2.5% with mycobacteria, 3.5% with Poxvirus, and 18.7% with other bacteria (Buenviaje, Ladds, & Martin, 1998). Several farmed captive freshwater crocodiles died of mycobacterial infections during the winter (Ariel, Ladds, & Roberts, 1997). *Mycoplasma alligatoris* is also a particular problem for crocodilians, especially those that are farmed (Brown et al., 2001).

Antibodies to this flesh-eating bacterium have been found in about 5% of tested free-living American alligators in Florida (Brown, Zacher, & Carbonneau, 2005).

Crocodilian morbidity is often associated to commensal bacteria, which, as is the case with humans, can be opportunistic pathogens. A West African dwarf crocodile died of *S. marcescens* septicemia, with *M. morganii* and *Staphylococcus* also infecting wounds and organs (Heard, Jacobson, Clemmons, & Campbell, 1988). A captive zoo Nile crocodile infected with *S. fonticola* died of septicemia and skin lesions (Garcia et al., 2008). *P. rettgeri* caused an outbreak of septicemia and meningoencephalitis is juvenile farmed American alligators (Camus & Hawke, 2002). *A. hydrophila, C. freundii, P. agglomerans, Proteus* spp., *M. morganii, S. marcescens,* and *K. oxytoca* were found to cause septicemia in captive American alligators in another study (Novak & Seigel, 1986). *A. hydrophila* was found in the organs of deceased American alligators, as well as on the skin and in the oral cavity of wild live alligators and on the skin of captive alligators. Researchers posited that bacteria can exist in low levels in organs, and stress from handling or trapping causes immune suppression, while subsequent exposure to high water temperatures can lead to bacterial proliferation. Together this can cause an opportunistic infection from the normal microbiome. (Gorden, Hazen, Esch, & Fuermans, 1979)

Innate Immunity of Crocodilians

The reptilian and mammalian lines split 315 million years ago. The immune systems of reptiles, birds, and mammals contain many similar features of innate and adaptive immunity. Reptiles do not have lymph nodes, but they have several other

lymphoid tissues, such as the thymus, spleen, bone marrow, and a unique organ called the gut-associated lymphoid tissue (GALT) (Zimmerman, Vogel, & Bowden, 2010). Many reptiles hibernate or fast during cooler months. It has been observed that lymphoid tissues undergo species-specific changes during seasons (Zimmerman et al., 2010). During the winter, the white pulp of the spleen becomes depleted and the thymus involuted (Hussein, Badir, El Ridi, & Akef, 1978; Hussein, Badir, El Ridi, & El Deeb, 1979; Ridi, Badir, & Rouby, 1981). As the temperature warms, the spleen redevelops and the thymus once again becomes enlarged and organized (Hussein et al., 1978; Hussein et al., 1979; Ridi et al., 1981). These species have a much less robust immune response during the winter (Hussein et al., 1978; Hussein et al., 1979; Ridi et al., 1981; Zimmerman et al., 2010), and it has been seen that some species are more susceptible to infection during the colder months (Ariel et al., 1997; Ladds, Bradley, & Hirst, 1996).

Blood cells in the reptile are also somewhat different than in the mammal. Reptiles have nucleated red blood cells (**Figure 2A**); it has not yet been shown if these red blood cells participate in active division or protein synthesis. Crocodilians have three granulocytes, similar to mammals: the eosinophil (**Figure 2B**), the basophil (**Figure 2C**) and the heterophil (**Figure 2D**). The heterophil is similar to the mammalian neutrophil in that it is normally the most abundant circulating white blood cell. Monocytes can sometimes be observed (**Figure 2E**). Reptiles also have the same variety of lymphocytes (**Figure 2F**). Reptilian B cells are phagocytic, however (Zimmerman et al., 2010). The adaptive humoral immune response is much less robust than in mammals. The initial

response to immunogens is slower, and subsequent exposures do not see a great increase in antibody titer or any increase in antibody binding affinity. (Zimmerman et al., 2010)

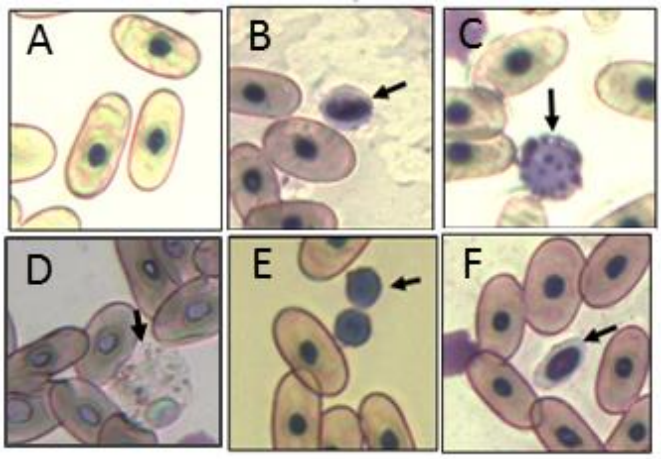

Figure 2. Blood cells from *Alligator mississippiensis.* Visualized by Wright stain and imaged at 40x. Cell types are **A.** nucleated red blood cell, **B.** eosinophil, **C.** basophil, **D.** heterophil, **E.** monocyte, and **F.** lymphocyte.

However, the ecosystems that these inhabit have an abundance and diverse range of pathogenic bacteria, and these species must be highly resistant to bacterial infections. (Ikenaga, Guevara, Dean, Pisani, & Boyer, 2010). This suggests that crocodiles have a robust and well-adapted immune system outside of the humoral adaptive immune system. One suggestion has been that reptiles rely more heavily on natural antibodies produced by B cells (Zimmerman et al., 2010). It has also been suggested that there are several extremely effective components of the crocodilian innate immune system.

There has not been much research published about the efficacy of the crocodilian innate immune system. Crocodilians have been shown to have several of the same innate immune tactics as mammalians. When *A. mississippiensis* is injected with bacterial LPS,

there is a dose-dependent increase in the number of heterophils and lymphocytes after one and two days (M. Merchant et al., 2006). *A. mississippiensis* also develops a fever (M. Merchant, Williams, Trosclair, Elsey, & Mills, 2007) and lowers plasma iron concentration (Mark Merchant, Paige Sanders, Jessica Dronette, Kaili Mills, & Jennifer Berken, 2007) in response to LPS injection. These are tactics also seen in mammalians; the febrile response drives invading bacteria into heat shock and denatures proteins, while lowering plasma iron concentrations "starves" bacteria of iron (Ong, Ho, Ho, & Ding, 2006).

Various organs and tissues have been shown to have antimicrobial activity *in vitro.* Lung and adrenal tissue from *Crocodylus niloticus* (Nile crocodile) had clearing activity against *Micrococcus luteus* (Shaharabany et al., 1999). Oil made from the fat of crocodiles has been in use as a folk treatment for various infections, and has been shown to have antimicrobial activity *in vitro* against *Staphylococcus aureus, K. pneumoniae,* and *Candida albicans* (Buthelezi, Southway, Govinden, Bodenstein, & du Toit, 2012).

The serum and plasma of many crocodilian species have antimicrobial activity. Serum and plasma from *Crocodylus siamensis* (Siamese crocodile) has been shown to have antibacterial activity against *E. coli, Vibrio cholera, P. aeruginosa, S. typhi, K. pneumoniae,* and *Staphylococcus epidermidis* (Kommanee et al., 2012; Leelawongtawon, Siruntawineti, Chaeychomsri, & Sattaponpan, 2010; Preecharram et al., 2010). Serum from *Caiman latirostris* (broad-snouted caiman) has shown a synergistic effect on *E. coli* when tested in combination with ciprofloxacin (Siroski, Russi, Ortega, & Formentini, 2015). *A. mississippiensis* serum has been tested against a wide range of organisms. Its

serum had activity against *K. oxytoca, Providencia stuartii, E. coli, Proteus mirabilis, Enterobacter aerogenes, Salmonella typhimurium, P. aeruginosa, C. freundii, Shigella sonnei, Shigella dysenterae, Salmonella poona, Yersinia enterocolitica, S. pyogenes, Streptococcus epidermidis, S. aureus,* and *E. faecalis,* while human serum was only effective against *P. stuartii, P. mirabilis, E. aerogenes, P. aeruginosa, S. sonnei,* and *S. poona* (M. E. Merchant, Roche, Elsey, & Prudhomme, 2003). Serum from *A. mississippiensis* (American alligator) also had antiviral activity against HIV-1, West Nile Virus, and HSV-1 (M. E. Merchant, Pallansch, et al., 2005), as well as amoebacidal activity against several *Naegleria* and *Acanthamoeba* spp. (Mark Merchant, Damon, Loubser, & Elsey, 2004) Much of this activity was lost when the serum was treated with heat, EDTA, or proteases, which suggests much of this activity is due to complement (Mark Merchant et al., 2004; M. E. Merchant, Pallansch, et al., 2005; M. E. Merchant et al., 2003). This is supported by the identification of an alternative pathway for complement in *A. mississippiensis* that was inhibited by the addition of human C3 antibodies *in vitro* (M. E. Merchant, Roche, Thibodeaux, & Elsey, 2005) and the discovery that the serum of *C. siamensis* increases phagocytosis by murine macrophages of bacteria *in vitro* (Aree, Siruntawineti, & Chaeychomsri, 2011). Complement activity has also been found from the serum of *C. porosus* (saltwater crocodile), *C. johnstoni* (freshwater crocodile) (M. Merchant & Britton, 2006), and *C. acutus* (American crocodile) (M. Merchant, McFatter, Mead, McAdon, & Wasilewski, 2010), indicating this is a conserved immune tactic.

Several non-complement proteins in plasma and produced by leukocytes have been identified and also have been shown to have antimicrobial activity. A lysozyme-like was found in *C. siamensis* in leukocyte extracts; this protein was active against *P. aeruginosa* and *V. cholera* (Pata, Daduang, Svasti, & Thammasirirak, 2007). Fragments of hemoglobin from *C. siamensis* have shown antimicrobial activity *in vitro*, similar to mammals (Srihongthong et al., 2012). Secreted phospholipase a(2) was found in the serum of *C. niloticus*, *Mecistops cataphractus* (slender-snouted crocodile), and *Osteolaemus tetraspis* (African dwarf crocodile) with high phospholipase activity against *E. cloacae, K. oxytoca,* and *E. faecalis* (M. Merchant et al., 2011). Low molecular weight extracts from the leukocytes of *A. mississippiensis* had activity against a range of microbes, including several *Candida* spp., HIV-1, HSV-1, *E. coli, Shigella flexneri, E. cloacae, K. oxytoca, C. freundii, Salmonella enterica, P. aeruginosa, Streptococcus pyogenes,* and *E. faecalis.* Proteases inhibited much of this activity, and the authors suggested the effect may be due to antimicrobial peptides. (M. E. Merchant et al., 2006) A separate group fractionated the blood of *C. siamensis* using RP-HPLC and found the first fraction had antimicrobial activity through membrane penetration; this group also suggested this activity was due to antimicrobial peptides (Kommanee et al., 2012; Leelawongtawon et al., 2010). Another group found four antimicrobial peptides from the leukocyte extracts of *C. siamensis* using RP-HPLC, which they named Leucrocins I-IV. These peptides are short (7-11 aa) and not highly charged, with Leucrocin I and II having no charge and +1 charge respectively. All four showed strong activity against *V. cholerae*. Leucrocins II and IV were strongly active against *S. epidermidis,* and

Leucrocin II was additionally strongly active against *S. typhi.* This group found that Leucrocins I and II caused blebbing in *S. epidermidis*, while Leucrocins II and IV permeabilized both membranes of Gram negative bacteria. (Pata et al., 2011) Another antimicrobial peptide called crocosin was also isolated by RP-HPLC from the blood of *C. siamensis*. This peptide was reported to have activity against *S. typhi* and *S. aureus* and to cause blebbing of the bacteria membrane (Preecharram et al., 2010).

Hypothesis and Aims

In this work, we hypothesize that there are a wide variety of CAMPs to be found in crocodilians, many of which may be homologous to those found in mammals. First, we analyze a CAMP from the Siamese crocodile that is homologous to the hepcidin found in mammals and fish. We examine its physio-chemical properties, perform CD, and test its activity against pathogenic bacteria. Next, we examine two CAMPs fragmented from an apolipoprotein found in the blood of the American alligator. We test these peptides against multiple bacteria, including those with multi-drug resistance, examine secondary structure, and perform experiments to identify mechanism of action. Lastly, we test a cathelicidin from the American alligator against pathogenic bacteria, as well as consider its secondary structure and possible mechanisms of action. In this, we hope to gather more understanding about the innate immune system of crocodilians because of their value in the economy and as part of the ecosystem. In addition, because of the strong antimicrobial activity of crocodilian serum, we aim to discover novel CAMPs with strong activity against human pathogens.

CHAPTER 2: HEPCIDIN OF THE SIAMESE CROCODILE

In the quest to discover novel antimicrobial peptides, we have identified a new peptide in *Crocodylus siamensis* through bioinformatics analysis that is a potential homolog of human hepcidin. This 25 amino acid peptide was synthesized and tested for antimicrobial activity. *De novo* sequencing mass spectrometry confirmed the sequence of the peptide. The hepcidin-like CAMP has 8 cysteine residues, predicted to form 4 intramolecular disulfide bonds. Both the reduced form and oxidized form exhibit significant broad-spectrum antimicrobial activity against *Staphylococcus aureus* (EC50=43.4 μg/ml and 37.5 μg/ml), *Escherichia coli* (11.9 μg/ml and 31.2 μg/ml), and *Pseudomonas aeruginosa* (11.3 μg/ml and 56.6 μg/ml). CD spectroscopy was performed and confirmed that the mainly unfolded structure of the reduced form is markedly different than that of the oxidized form, which contains multiple disulfide bonds. The Siamese crocodile hepcidin is similar to a recently published 26 amino acid peptide from *C. siamensis,* but the active hepcidin peptide has not yet been purified from Siamese crocodiles *in vivo* due to their status as critically endangered animals. However, in this work we were able to examine the antimicrobial activity of a purified linear and folded Siamese crocodile hepcidin peptide side-by-side.

Introduction

Hepcidins are a class of vertebrate CAMPs with 8 cysteine residues (Hocquellet, le Senechal, & Garbay, 2012; C. H. Park, E. V. Valore, A. J. Waring, & T. Ganz, 2001) and 4 disulfide bonds (A. Krause et al., 2000). The positioning of cysteines and disulfide bonds has been found to be extremely conserved among vertebrates, including primates, pigs, dogs, and fishes (Clark et al., 2011), shown in **Figure 3**.

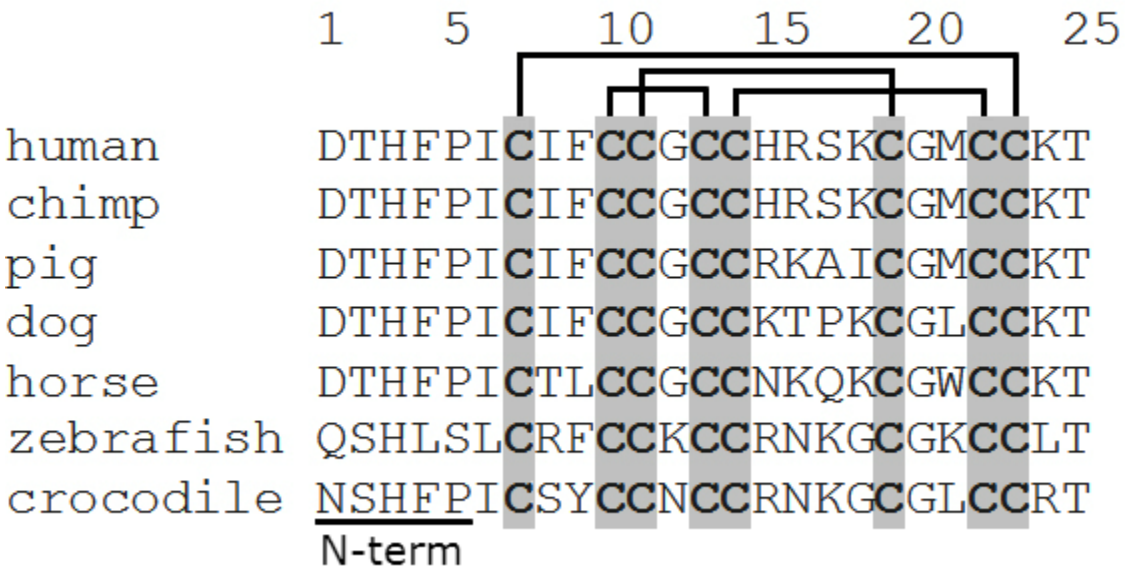

Figure 3. **Conserved disulfide bond patterns in hepcidins.** (Adapted from Clark et al (Clark et al., 2011)) Conserved cysteine placement indicates the conserved disulfide bond formation of Cys7-Cy23, Cys10-Cys13, Cys11-Cys22, and Cys14-Cys19 (Clark et al., 2011).

This peptide is most strongly expressed in the liver, where it is secreted by hepatocytes (C. H. Park et al., 2001). The active form of human hepcidin, LEAP-1 (Liver Expressed Hepcidin Peptide-1), is a 25 amino acid (aa) peptide found in humans, also called Hep25c

(Hunter, Fulton, Ganz, & Vogel, 2002; A. Krause et al., 2000). The LEAP-1 hepcidin has some antimicrobial activity against multiple pathogens (Hocquellet et al., 2012), though it is not as potent as other CAMPs such as LL-37 and NA-CATH. LEAP-1 is also central to iron regulation in humans (Ganz & Nemeth, 2012); by binding ferroportin, which transports iron from the cell into the blood, and degrading this protein, LEAP-1 can effectively lower iron levels in the blood, which may control the growth of pathogens (De Domenico et al., 2007; Jones et al., 2012). The ferroportin interacting domain is predicted to be in the N-terminal 7-9 amino acids of the active hepcidin peptide (Preza et al., 2011).

Our group sought to identify potential hepcidins from the crocodilian order. From an mRNA available on the NCBI database for the Siamese crocodile (*Crocodylus siamensis*), we predicted and synthesized a 25 aa peptide, which is very homologous to the active Hepc25 human hepcidin peptide, LEAP-1.

The purpose of this study was to identify, synthesize, and characterize a crocodilian hepcidin - we chose the hepcidin from *C. siamensis* for further study. The sequence of this peptide was produced both in folded form and in linear form. In these experiments, we determined the antimicrobial activity of both the folded and linear 25aa Siamese crocodile hepcidin against several pathogenic bacteria. By CD spectroscopy in its unfolded form, this hepcidin exhibits mixed random coil characteristics. When folded with four intramolecular disulfide bonds, this peptide displays mixed β-sheet and random coil characteristics. This report demonstrates that crocodilians likely have a 25 aa hepcidin peptide, homologous to the active hepcidin-25 peptides identified in many other vertebrates, including humans.

Methods and Materials

Bacterial Strains and Media

P. aeruginosa strain ATCC 9027, *Staphylococcus aureus* ATCC 25923, and *Escherichia coli* ATCC 51659 were obtained from American Type Culture Collection (Manassas, VA). Bacteria were grown overnight in Nutrient Broth (Difco 234000) in a 37°C shaking incubator. Stocks were aliquoted, suspended in 20% glycerol, and kept frozen at -80°C. All stocks were enumerated prior to antimicrobial assays on Nutrient Agar (NA).

Bioinformatics Analysis

NCBI databases and programs including BLAST and Gene were used to search for the crocodile hepcidin based on homology to known hepcidin peptides. At the time of our searching, an mRNA was identified that may encode a hepcidin, *C. siamensis* (Taxonomy ID 68455) hepcidin mRNA, EMBL Accession: FJ968771.1 (http://www.ebi.ac.uk/ena/data/view/FJ968771), NCBI GI: 283106144. Uniprot E8ZAD0_CROSI predicted the full-length preproprotein from that mRNA. Homology searching and alignments with known hepcidins (**Figure 3**) led to the prediction of Siamese crocodile hepcidin. NCBI databases and programs including BLAST and Gene were used and known hepcidin peptides such as Hep25C were used in alignments and searching approaches. Antimicrobial peptide database APD2 was used to predict physicochemical properties of the proposed Siamese crocodile hepcidin peptide (G. Wang, Li, & Wang, 2009). The CAMP databases AntiBP and AntiBP2 (Lata, Mishra, & Raghava) were used to obtain computed scores of probable antimicrobial activity of the proposed peptide.

Table 1: Siamese crocodile hepcidin sequence and properties. Analysis of the amino acid sequence of crocodile hepcidin. The predicted and computed properties of this peptide are listed.

Property:	**Details**
Predicted Properties:	APD2 [(G. Wang et al., 2009)]
Sequence	NSHFPICSYCCNCCRNKGCGLCCRT
Total net charge	+ 3
Total hydrophobic ratio	44 %
Predicted molecular weight (kDa)	2780.329
Protein-binding Potential (Boman index)	1.78 kcal/mol
Cysteines	8 (4 possible disulfides)
Hydrophobic amino acids	I: 1, V: 0, L: 1, F: 1, C: 8, M: 0, A: 0, W: 0
G and P amino acids	G: 2, P: 1
Negatively charged amino acids	E: 0, D: 0
Positively charged amino acids	K: 1, R: 2, H: 1
Other amino acids	T: 1, S: 2, Y: 1, Q: 0, N: 3
Predicted Antibacterial activity Score	
AntiBP Method 1 (Lata, Sharma, & Raghava, 2007): QM	Score: 1.771 YES Threshold -0.2 Default: C-terminus= 0; NC-terminus= -0.6.
AntiBP2 Method 2 (Lata et al.): SVM	Score: 1.007 YES Threshold 0.

Chemical Synthesis

The 25 aa Siamese crocodile hepcidin linear peptide (2786.27 predicted MW) was synthesized by China Peptide Co, Ltd. RP-HPLC analysis was performed using a Kromasil 100-5C18 column (4.6 mm x 250 mm, 5 micron) with Buffer A= 0.1% trifluoroacetic acid (TFA) in water and Buffer B=0.1% TFA in acetonitrile, a flow rate of 1 ml/min and a gradient of 10-50% Buffer B in 14 minutes. 10 µl of sample was injected, and the readout was performed at 220 nm. A peak with one shoulder was identified

(Peak1 at 11.021 min, Peak2 at 11.221 minutes) with approx. 1500 mV height, representing >99% purity. ESI-MS was performed with Buffer A=0.1% formic acid in water, and Buffer B=0.1% formic acid in acetonitrile. A 1-minute run at 0.2 ml/min revealed 4 peaks with masses of (in order of intensity [cps], highest to lowest): 929.7, 1394.1, 1115.1 and 1124.9 m/z Da. Folded Siamese crocodile hepcidin was synthesized by Peptides International, using Fmoc chemistry and an S-4,4'dimethylsulfinylbenzhydryl protection chemistry recently described (Dekan et al., 2014). Briefly, the peptide was synthesized using Fmoc chemistry. Cys 1 and 8 were protected with 4-methylbenzyl, Cys 3 and 6 with acetamidomethyl, and Cys 5 and 7 with Msbh. The Cys2-Cys4 bond was formed using DMSO. The bond between Cys3 and Cys6 were then deprotected oxidatively in a TFA solution, and the disulfide bond formed with I2 and ascorbic acid. Deprotection of Cys1 and Cys8 was achieved using HF and p-cresol, and the disulfide bond created using I2 and ascorbic acid. Deprotection with simultaneous bond formation was produced with neat TFA, followed by the addition of DMS solution, NH4I, and ascorbic acid. Peptide was purified using RP-HPLC to 90% purity. Both peptides were synthesized terminal amino and carboxy-free.

Antimicrobial Assays

The antimicrobial activity of Siamese crocodile hepcidin was determined in 10 mM phosphate buffer as previously described, with minor modification (Amer, Bishop, & van Hoek, 2010; de Latour, Amer, Papanstasiou, Bishop, & van Hoek, 2010; Dean, Bishop, & van Hoek, 2011a; Han, Bishop, & van Hoek, 2008). 10 mM phosphate buffer, pH 7.4 is prepared as a 1:10 dilution of 100 mM phosphate buffer (47.5 ml of 200 mM monobasic phosphate buffer ($NaH_2PO_4 \cdot H_2O$), 202.5 ml dibasic phosphate buffer

($Na_2HPO_4 \cdot 2\ H_2O$) plus 250 ml H_2O). The sequences and net charges of Siamese crocodile hepcidin is shown in **Table 1**. In a 96 well plate, 10^5 bacteria were incubated in 10 mM phosphate buffer (6.12 mM sodium monohydrogen phosphate heptahydrate, 3.92 mM monosodium phosphate, pH 7.4) with various dilutions of peptide (3 h, 37°C). Cation-adjusted Mueller Hinton Broth with resazurin sodium salt was then added to each well, and resazurin reduction measured kinetically on a Tecan Safire^2 fluorospectrometer overnight at 37°C. Percent survival was calculated based on previously determined reduction rate growth curves. Reduction conditions and growth curve equations are listed in **Table 2**. Each experiment was repeated at least twice, and a representative experiment is shown. 95% confidence intervals (CI) are reported to indicate the error of each EC50 determination.

Table 2: Reduction conditions and rate equations for antimicrobial assays.

Bacteria	Mueller Hinton Broth (w/v) %	Resazurin (µM)	Reduction rate equation for determining initial CFU
S. aureus	2.2	109	$\log(CFU) = \frac{(time\ onset_{15000RFU} - 78260)}{-40094}$
P. aeruginosa	2.2	54.5	$\log(CFU) = \frac{(time\ onset_{20000RFU} - 79784)}{-9165.7}$
E. coli	2.2	109	$\log(CFU) = \frac{(time\ onset_{15000RFU} - 37673)}{-4292.9}$

CD Spectroscopy

Circular dichroism (CD) was performed using a Jasco J-1500 spectropolarimeter. Peptides were analyzed in 10 mM sodium phosphate (pH 7.4), and 50% (v/v)

trifluoroethanol (TFE) in 10 mM sodium phosphate buffer (Lee et al., 2003), and 100 µg/ml peptide was used in each experiment. Samples were allowed to equilibrate for 3 min prior to data collection at 25°C in a 1-mm path length cuvette. Spectra were collected from 190 to 260 nm at 0.2-nm intervals, data integration time of 4 s, and a 1 nm bandwidth; four scans per sample were averaged. Data smoothing was performed using a moving average.

Statistical Analysis

Antimicrobial assays were performed in triplicate, and repeated at least once, with the results analyzed using GraphPad Prism 5. 95% confidence intervals (CI) are stated for each EC50 calculation, which reflects the significance ($p=0.05$) of the calculated EC50 values. EC50 that do not overlap in the CI are predicted to be different, $p<0.05$.

Results

Peptide Design

In searching for potential antimicrobial peptides in the crocodilian genomic sequences, we predicted a 25 aa peptide, the Siamese crocodile hepcidin (**Table 1**), within the predicted full-length protein sequence of *C. siamensis* hepcidin (Uniprot E8ZAD0_CROSI) based on our analysis of LEAP-1 and other hepcidins (See **Figure 3**). Our analysis led to the prediction of Siamese crocodile hepcidin peptide with the sequence NSHFPICSYCCNCCRNKGCGLCCRT. We had Siamese crocodile hepcidin chemically synthesized both oxidized (folded) and reduced for testing. In the intervening time, another group published a 26 aa peptide based on their prediction of the enzymatic processing site of the predicted propeptide from the 426 bp mRNA from *C. siamensis* that had been deposited into the NCBI database (Accession: FJ968771.1 GI: 283106144),

leading to a different starting aa to the peptide, with the sequence FNSHFPICSYCCNCCRNKGCGLCCRT (J. Hao, Y. W. Li, M. Q. Xie, & A. X. Li, 2012). In addition, Hao et al proposes a highly unusual vicinal disulfide bond formation as part of the folding of this molecule, which is not predicted for the 25 aa form we identified as Siamese crocodile hepcidin (Clark et al., 2011).

The proposed Siamese crocodile hepcidin peptide is highly similar to amino acids 60-84 of the human hepcidin (LEAP-1) (P81172, pdb 2KEF-A, GENE ID: 57817 HAMP/LEAP-1) (**Figure 3**). Siamese crocodile hepcidin and LEAP-1 have fourteen out of 25 aa are identical and twenty out of 25 are similar. Siamese crocodile hepcidin and LEAP-1 have a BLAST score of 52.8 bits (117), and the expected value is 5e-07. By BLAST analysis, there are 16/25 identities, 19/25 positives with 2 gaps (Altschul et al., 1997).

Interestingly, the Siamese crocodile hepcidin nucleotide sequence is rather less similar to many other hepcidin genes. Indeed, the full-length mRNA identified is very poorly conserved, with the highest homology to various species of fish. In addition, if the analysis is done with the full-length translated protein sequence, it is also not highly homologous to hepcidin peptides from many other taxa except in the 25 aa C-terminal region described above (**Table 3**). This highlights the concept that CAMPs are often highly conserved in their protein structure (e.g. β-hairpins) and their peptide properties (such as hepcidin disulfide bonding patterns), but can be poorly conserved by genomic sequence (van Hoek, 2014). Due to the lack of annotated, complete genomes in the NCBI database, no significant homologues can be found within other members of the order

Crocodilia (taxid: 8493) or monitor lizards (van Hoek, 2014), but have been found in the anole (*Anolis carolinensis*) (Hilton & Lambert, 2008) and the turtle.

Table 3. Amino acid sequence conservation in hepcidins. Data adapted from NCBI BLAST. Though cysteine sequence and placement is highly conserved (of 25 aa, ~32% of the peptide), the remaining residues are poorly conserved between the predicted active hepcidin peptide of *C. siamensis* and the other species listed (see also **Figure 3**).

Accession	Species Source	Protein Names	E value	Max identity
ADA68357.1	*C. siamensis*	Hepcidin	8e-53	100%
XP_006269766.1	*A. mississippiensis*	Hypothetical	5e-31	85%
ABU75214.1	*Trachypithecus obscurus*	Hepcidin	2e-07	48%
XP_003937406.1	*Saimiri boliviensis boliviensis*	Predicted hepcidin isoform 1	2e-07	44%
EHB14005.1	*Heterocephalus glaber*	Hepcidin	2e-07	41%
XP_002762058.1	*Callithrix jacchus*	Predicted hepcidin	2e-07	46%
XP_001094273.1	*Macaca mulatta, M. fasicularis, Papio papio*	Predicted hepcidin isoform 1	2e-07	48%
ABU75220.1	*Callithrix jacchus*	Hepcidin	2e-07	46%
ABU75217.1	*M. fuscata*	Hepcidin	2e-07	48%
ABU75221.1	*Chlorocebus aethiops*	Hepcidin	3e-07	48%
XP_003915389.1	*P. anubis*	Predicted hepcidin isoform 1	3e-07	48%
NP_001103163.1	*Pan troglodytes, P. peniscus*	Hepcidin precursor	5e-07	46%
ABU75222.1	*Ateles fusciceps*	Hepcidin	1e-06	46%
AAK14912.1	*Homo sapiens*	Putative liver tumor regressor	1e-06	44%
XP_004060563.1	*Gorilla gorilla*	Predicted hepcidin isoform 1	1e-06	46%
NP_066998.1	*H. sapiens*	Hepcidin pre-proprotein	1e-06	44%

As is common with many antimicrobial peptides, proteolytic processing is required for the liberation of the smaller hepcidin peptide from the full-length propeptide, although the *in vivo* relevant protease in not known in the crocodile (J Hao, Y. W. Li, M. Q. Xie, & A. X. Li, 2012). Independently of Hao et al, our group identified this mRNA as potentially encoding crocodile hepcidin and proceeded to synthesize a 25aa form of the peptide (without the Hao group's leading Phe residue). We predicted the sequence of Siamese crocodile hepcidin based on our analysis of the fully processed active forms of

hepcidin in human (Hepc25, for example), mouse, and other animals which corresponds to the last 25 aa (e.g. LEAP-1, FTId=PRO_0000013379), and this region displays high homology with our predicted crocodile hepcidin (**Figure 3**), including the highly conserved 8 cysteine residues, and good alignment of the N-term domain. Since the *in vivo* processing enzymes and cleavage sites that are responsible for hepcidin processing in the crocodile are unknown but are likely conserved with other higher vertebrates, we predicted that the active crocodile hepcidin peptide will actually be a 25 amino-acid peptide, similar to the hepcidins of several other higher order animals. Thus, we predicted and tested a Siamese crocodile hepcidin peptide composed of 25 aa with the sequence NSHFPICSYCCNCCRNKGCGLCCRT. The N-terminal sequence of NSHFPICST is analogous to the mammalian N-terminal sequence, DTHFPICTF. Because the N-terminus of mammalian hepcidins is known to be the ferroportin binding domain, it is possible this difference in the N-terminal sequence reflect differences between the human and crocodilian ferroportin.

Based on analysis of Siamese crocodile hepcidin sequence, this 2.78 kDa peptide has a net charge of +3 (**Table 1**). It is predicted to be an antimicrobial peptide by the APD2 CAMP prediction program (G. Wang et al., 2009) with a hydrophobic ratio of 44%, and a Boman index of 1.78 kcal/mol. The Boman index is the sum of the free energies of the respective side chains for transfer from cyclohexane to water, divided by the total number of the residues of the proposed antimicrobial peptide (Boman, 2003). Thus, the Boman index is an estimate of the potential of peptides to bind to other proteins. For this index, a low index value (<1) should indicate that the peptide has

mostly antibacterial activity without many other effects, whereas a high index value (2.5-3) is reported to indicate that the peptide is multifunctional, may bind other proteins, or may exhibit hormone-like activities (Boman, 2003). For hepcidin peptides, this could reflect their interaction with ferroportin (Hunter et al., 2002; A. Krause et al., 2000), likely through the N-terminal sequence, which is highly divergent. Our predicted Siamese crocodile hepcidin peptide sequence was highly homologous to the LEAP-1 sequence, and the critical cysteine residues aligned exactly (**Figure 3**), suggesting that this may be the Siamese crocodile hepcidin.

Antibacterial Activity

Both reduced and oxidized Siamese crocodile hepcidin have antimicrobial activity against multiple pathogens, though this peptide is less effective than other, more potent antimicrobial peptides such as LL-37 or maginin-2. Reported antimicrobial activities of LEAP-1, buffalo hepcidin and the hepcidins of other species against other pathogens including yeast are reviewed in **Table 4** and range from low to minimally active.

When reduced and oxidized Siamese crocodile hepcidin were tested against bacteria in traditional minimal inhibitory concentration determination conditions, it was found that neither peptide had antimicrobial activity nor delayed the log growth of bacteria, similar to LL-37 (Amer et al., 2010; Dean et al., 2011a; Dean, Bishop, & van Hoek, 2011b) (**Table 5**). The published antimicrobial activity of many LEAP-1-homologous hepcidins is based on the particular disulfide bond formation previously determined as Cys7-Cys23, Cys11-Cys19, Cys10-Cys13, and Cys14-Cys22

Table 4. Reported antimicrobial and antifungal activity of various hepcidins.

Peptide	Form	Microbe	MIC/MBC (μM)	Reference
LEAP-1	Folded (vicinal bond)	*Bacillus subtilis*	14.4 (IC50)	(Alexander Krause et al., 2000)
Hepcidin 2 (*Danio rario*)	Folded	*Escherichia coli*	18	(Lin, Liu, Hu, & Zhang, 2014)
		Vibrio anguillarum	15	
		Staphylococcus aureus	13	
		B. subtilis	9	
LEAP-1	Folded	*E. coli*	50	(Hocquellet et al., 2012)
		B. megaterium	50	
		B. subtilis	100	
		Micrococcus luteus	100	
Hepcidin (*Oryzias melastigmus*)	Linear	*Corynebacterium glutamicum*	12	(Lin et al., 2014)
		S. aureus	12	
		E. coli	24	
		Aeromonas hydrophila	12	
		Pseudomonas stutzeri	12	
Hepcidin (*Morone* hybrid)	Folded (vicinal bond)	*S. aureus*	>44	(Lauth et al., 2005)
		E. coli	11	
		P. aeruginosa	>44	
Hepcidin (*Pseudosciaena crocea*)	Linear	*A. hydrophila*	3-6	(K. J. Wang et al., 2009)
		E. coli	12-24	
		M. luteus	1.5-3	
		S. aureus	3-6	
		B. subtilis	3-6	
Hep-JF2 (*Paralichthys olivaceus*)	Linear	*E. coli*	25	(Hirono et al., 2005)
		S. aureus	50	
TH1-5 (*Oreochromis mossambicus*)	Linear	*S. aureus*	21	(P.-H. Huang, J.-Y. Chen, & C.-M. Kuo, 2007)
HepcD (*Camelus dromedaries*)	Folded	*S. aureus*	15	(Boumaiza, Ezzine, Jaouen, Sari, & Marzouki, 2014)

(Hocquellet et al., 2012; Jordan et al., 2009). Hocquellet et al has demonstrated that these bonds are critical to the antimicrobial abilities of these hepcidins (Hocquellet et al., 2012). However, many of the published hepcidin papers report the activity of unfolded hepcidin peptide (Hirono et al., 2005; P.-H. Huang et al., 2007; Lin et al., 2014; K. J. Wang et al., 2009). In our experiments, unfolded Siamese crocodile hepcidin was shown to retain good and sometimes better antimicrobial activity in its unfolded state depending on the organism, similar to human β-defensin-1 (HBD-1) (Schroeder et al., 2011).

Table 5. Antimicrobial activity of Siamese crocodile hepcidin.

Bacteria	**EC50 Folded (μg/ml)**	**EC50 Unfolded (μg/ml)**	**MIC Folded (μg/ml)**	**MIC Unfolded (μg/ml)**
P. aeruginosa ATCC 9027	56.6	11.8	>100	>100
E. coli ATCC 51659	31.2	11.9	>100	>100
S. aureus ATCC 25923	37.5	43.4	>100	>100

In 10 mM sodium phosphate buffer, the EC50 of reduced Siamese crocodile hepcidin against *P. aeruginosa* ATCC 9027 was 11.8 μg/ml, 43.4 μg/ml against *S. aureus* ATCC 25923, and 11.9 μg/ml against *E. coli* ATCC 51659. The oxidized form, containing four intramolecular disulfide bonds, is much more difficult to produce *in vitro*, though this form could be found *in vivo*. The oxidized form of the peptide was statistically less effective against both *P. aeruginosa* (EC50=56.6 μg/ml) and *E. coli* (EC50=31.2 μg/ml), and had similar efficacy against *S. aureus* (EC50=37.5 μg/ml). The antimicrobial assay results including the confidence intervals are shown in **Figure 4**.

These EC50 values are similar to linear human LEAP-1 against *S. aureus* and *P. aeruginosa*.

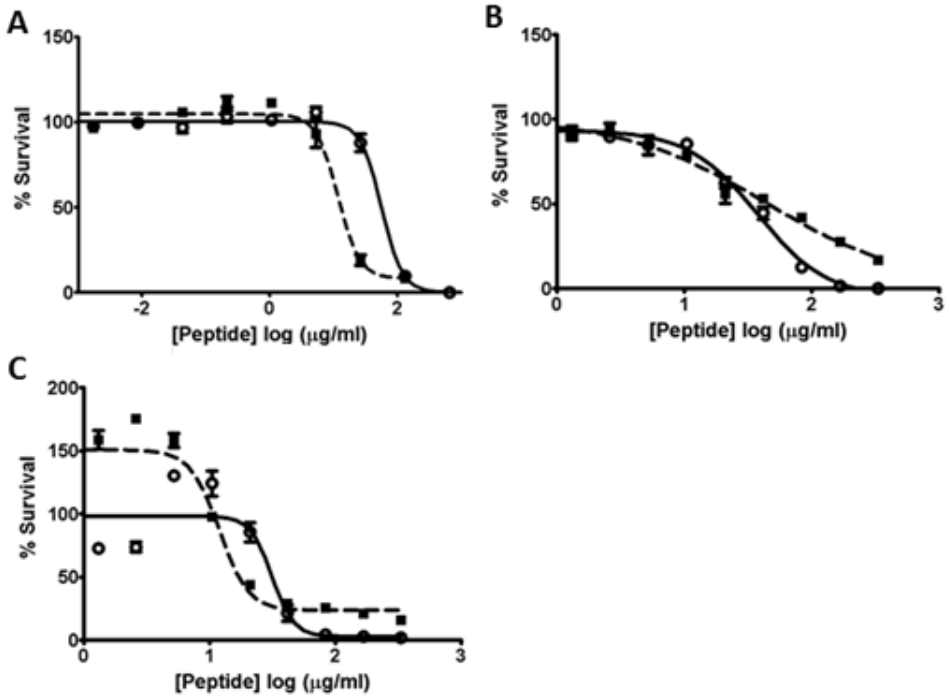

Figure 4. Antimicrobial activity of the Siamese crocodile hepcidin. Siamese crocodile hepcidin antimicrobial activity in 10 mM sodium phosphate buffer against (**A**) *P. aeruginosa*: reduced crocodile hepcidin (○) EC50=11.8 μg/ml (4.24 μM),, 95% CI=8.64 to 16.0 μg/ml (3.11 to 5.76 μM); oxidized crocodile hepcidin (■) EC50=56.6 μg/ml (20.4 μM), 95% CI=45.1 to 71.0 μg/ml (16.3 to 25.6 μM); (**B**) *S. aureus*: reduced crocodile hepcidin (○) EC50=43.4 μg/ml (15.6 μM), 95% CI=19.6 to 95.8 μg/ml (7.05 to 34.5 μM); oxidized crocodile hepcidin (■) EC50=37.5 μg/ml (13.5 μM), 95% CI=30.6 to 46.0 μg/ml (11.0 to 16.6 μM); and (**C**) *E. coli*: reduced crocodile hepcidin (○) EC50=11.9 μg/ml (4.28 μM), 95% CI=9.66 to 14.7 μg/ml (3.47 to 5.29 μM); oxidized crocodile hepcidin (■) EC50=31.2 μg/ml (11.3 μM), 95% CI=22.9 to 42.7 μg/ml.

CD Spectra

The resolved structure of the human LEAP-1 with intra-molecular disulfide bonding displays a mixed structure with some β-sheet portions, with a peak around 200

nm (Luo et al., 2012; Nemeth et al., 2006). CD spectroscopy was performed to determine if the oxidized Siamese crocodile hepcidin displayed similar structural characteristics as predicted, and how the lack of disulfide bonds may change the reduced Siamese crocodile hepcidin. As shown in **Figure 5**, the reduced Siamese crocodile hepcidin has some α-helical characteristics when CD is performed in phosphate buffer and 50% TFE, as indicted by peaks at 208 nm and 222 nm. However, α-helical structures often display more intense spectra than other secondary structures, especially when considering peaks at these wavelengths (Whitmore & Wallace, 2008). Instead, the secondary structure contribution method described by Raussens et al. (Raussens, Ruysschaert, & Goormaghtigh, 2003) was used in order to provide a more accurate description of the secondary structure. Using this method, it was determined that in 50% TFE, the reduced Siamese crocodile hepcidin had primarily random coil (36.9%) and β-sheet (27.8%) characteristics, with some contributions from the α-helix (12.4%) and turn (12.5%). Oxidized Siamese crocodile hepcidin had more strong β-sheet (50.1%) and random coil (43.1%) characteristics, with little influence from turn (11.9%) and virtually no α-helix (1.3%). Quantitatively, the structure of oxidized folded Siamese crocodile hepcidin is very similar to published spectra of LEAP-1, with the same peak around 200 nm, suggesting it has the same bonding pattern (Luo et al., 2012; Nemeth et al., 2006; Christina H. Park, Erika V. Valore, Alan J. Waring, & Tomas Ganz, 2001).

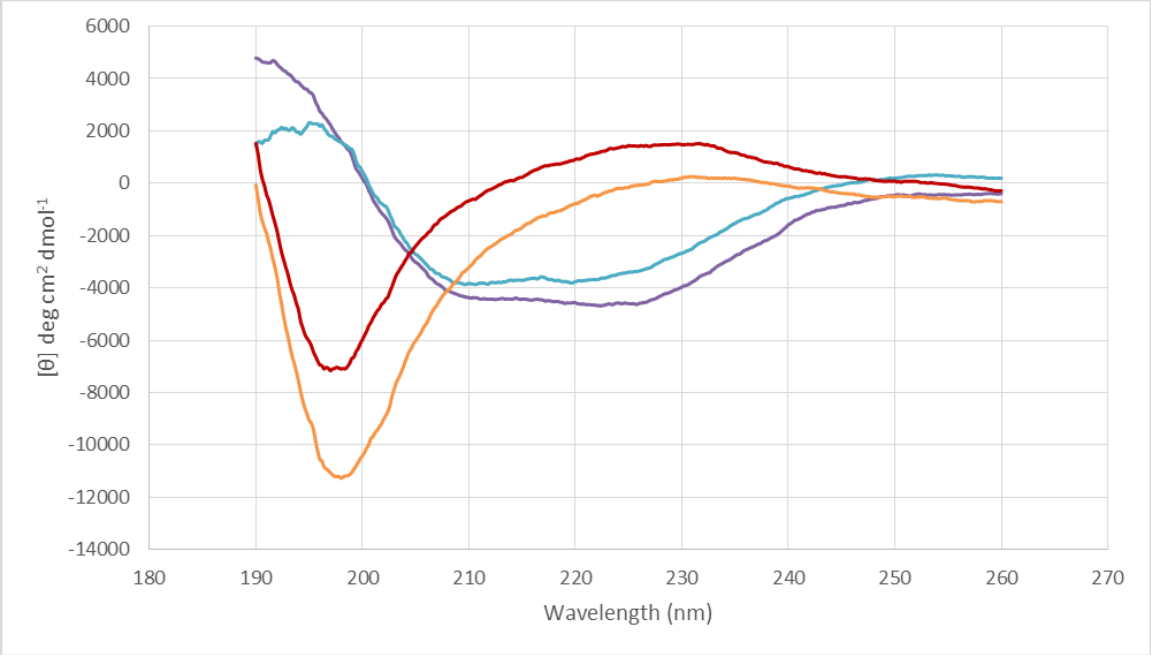

Figure 5. CD spectra of Siamese crocodile hepcidin. CD was performed on linear Siamese crocodile hepcidin in 10 mM sodium phosphate (----) and 50% TFE (----) and on folded hepcidin in 10 mM sodium phosphate (----) and 50% TFE (----).

Discussion

In this work, we sought to identify a new crocodilian antimicrobial peptide, as so few are known (van Hoek, 2014). Through data-mining and bioinformatics analysis, we predicted a 25aa hepcidin-like peptide from *C. siamensis* mRNA deposited in the NCBI database and synthesized this peptide, which we have shown has antimicrobial activity. This peptide differs from that published by Hao et al, who predicted a 26 aa peptide based on trypsin cleavage sites (J Hao et al., 2012). In addition, Hao et al predicted that the crocodile hepcidin would have a vicinal disulfide bond between Cys3 and Cys4. This bonding pattern was recently determined to not be the correct bonding pattern for the human hepcidin, and thus it is likely not the correct bonding pattern for the crocodile

hepcidin. Instead, the bonding pattern was found to follow the format Cys1-Cys8, Cys3-Cys6, Cys2-Cys4, Cys5-Cys7 (Hocquellet et al., 2012). The folding process used to produce our oxidized Siamese crocodile hepcidin produces a hepcidin with this pattern. No form of the peptide has been confirmed to be present *in vivo* in crocodile tissue or serum to date due to the critically endangered status of the Siamese crocodile (van Hoek, 2014).

The presence of this mRNA and the resultant antimicrobial activity found in the synthesized Siamese crocodile hepcidin peptide support the hypothesis that though many antimicrobial peptides, even hepcidins, are poorly conserved by amino acid sequence (**Table 3**), they are often remarkably conserved in structure (especially through the cysteine-based folding pattern and resulting 3D structure) and overall peptide properties. Known hepcidins function in the innate immunity of mammals, avians, and fishes by acting primarily as an iron regulation peptide that removes iron from the outside of the cell in response to pattern-associated molecular patterns such as LPS (Ganz, 2002; Hunter et al., 2002), and secondarily as an antimicrobial peptide that directly takes action against the pathogen, similar to other antimicrobial peptides that can show poor performance *in vitro* in some situations (Amer et al., 2010; Schittek, Paulmann, Senyurek, & Steffen, 2008). A hepcidin peptide has not been isolated from a crocodilian, and the direct binding of a crocodilian ferroportin to a crocodilian hepcidin and a decrease in iron serum levels in crocodilians after an injection of crocodilian hepcidin have not been observed. However, it has been found that American alligators withhold iron in response to LPS stimulation (M. Merchant, P. Sanders, J. Dronette, K. Mills, & J.

Berken, 2007), and a likely ferroportin can be found in NCBI protein databases (XP_006264814.1). It is very likely that crocodilian hepcidin regulates iron in response to PAMPs in a similar manner to fishes, mammals, and avians.

The published antimicrobial activity of many LEAP-1-homologous hepcidins is based on the particular disulfide bond formation previously determined as Cys7-Cys23, Cys11-Cys19, Cys10-Cys13, and Cys14-Cys22 (Hocquellet et al., 2012). Hocquellet et al. has demonstrated that these bonds are critical to the antimicrobial abilities of these hepcidins (Hocquellet et al., 2012). Many of the published hepcidin papers report the activity of reduced linear hepcidins, several of which tend to be more active than oxidized folded hepcidins. When the MICs of both reduced and oxidized hepcidins are determined, efficacy seems to vary widely. As an extreme example, synthesized linear hepcidins originally found in *Epinephelus coioides* displayed MIC values so high as to be undeterminable, even though the MIC values of the folded version was measured from 50-100 μM against *E. coli* and *S. aureus* (J. G. Zhou et al., 2011). Linear hepcidins from *Oreochromis mossambicus* were shown to have an MIC of around 21.5 μM against *S. aureus* (P. H. Huang, J. Y. Chen, & C. M. Kuo, 2007), and those from *Paralichthys olivaceus* cleared *S. aureus* at 50 μM (Hirono et al., 2005). The antimicrobial activities of a variety of hepcidins with and without disulfide bonds, and some with the incorrect vicinal bond are listed in **Table 4**. Researchers testing the antimicrobial activity of linear hepcidins have remarked that versions with intramolecular bonds would likely have stronger activity. However, when the antimicrobial activities of hepcidins in different forms were compared by us, it does not seem that folded hepcidins have stronger activity.

In fact, in the case of another heavily disulfide-bonded peptide, human β-defensin-1, antimicrobial activity was found to greatly increase when the peptide was reduced (Schroeder et al., 2011).

In our experiments, linear Siamese crocodile hepcidin was shown to have comparable activity to these other published hepcidins and to be more strongly antimicrobial than Siamese crocodile hepcidin with intramolecular bonds, except in the case of *S. aureus*, against which there was no significant change in efficacy. In general, these EC50 values are comparable to MIC and EC50 values reported for other hepcidins. Very few reptile hepcidins have been tested for their antimicrobial activity, and it is not clear if in all cases that the reduced hepcidin would be stronger than the folded oxidized version. It is also possible that the reduced Siamese crocodile hepcidin could aggregate or form disulfide bonds between reduced Cys residues. However, multiple spectra were obtained using CD with linear Siamese crocodile hepcidin that had been stored for different lengths of time, lyophilized and in solution, and it was found that the reduced spectra were identical with each scan.

The mode of antibacterial action for hepcidins as a family of peptides is not yet known. Previous research has shown that LEAP-1 does not permanently disrupt the bacterial membrane like LL-37 or SMAP-29 (Hocquellet et al., 2012; Shin et al., 2001; Vandamme, Landuyt, Luyten, & Schoofs, 2012). Our experiments with ethidium bromide uptake assays suggest that neither version of Siamese crocodile hepcidin causes permeabilization of the bacterial membrane (data not shown). Research continues on crocodilian hepcidins in our lab. In the future, we will characterize the American alligator

hepcidin, and we will work to isolate the native hepcidin peptide from American alligator liver and to observe the levels of hepcidin and iron in LPS-stimulated alligators.

CHAPTER 3: APOLIPOPROTEIN-DERIVED PEPTIDES OF THE AMERICAN ALLIGATOR

Our group has developed a new process for isolating and identifying novel cationic antimicrobial peptides from small amounts of biological samples, in this case the plasma of *Alligator mississippiensis*. In this chapter, two of the novel peptides discovered using this process are characterized: Apo5 and Apo6. These peptides were found to have *in vitro* antimicrobial activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus.* We also examined their activity against multiple multi-drug resistant strains and clinical isolates of common human pathogens. We also characterized their structural characteristics using circular dichroism and membrane permeabilization studies, and tested for other properties such as DNA binding. These peptides were found to have strong *in vitro* activity against multi-drug resistant *S. aureus, E. coli,* and *A. baumannii*, as well as a clinical isolate of *P. aeruginosa*. Apo5 and Apo6, peptides derived from alligator apolipoprotein C-1, depolarized the bacterial membrane. Performing circular dichroism analysis, Apo5 and Apo6 were found to be predominantly helical in SDS and TFE buffer. Neither of these peptides were found to be hemolytic to human cells *in vivo*, but were found to be cytotoxic after 24 h at 300 μg/ml. Overall, we suggest that Apo5 and Apo6 make promising candidates for the treatment of drug resistant bacteria.

Introduction

Traditionally, new CAMPs are identified through either bioinformatics or fractionation. CAMPS are a diverse group of proteins by nucleic acid, amino acid, and secondary structure, which can make locating new CAMPs difficult. Fractionation through HPLC or other methods typically requires large amounts of biological sample, typically a liter or more (Preecharram et al., 2010), which can be problematic when working with small or endangered animals. Fractionation and testing is typically insensitive due to dilution of samples, as CAMPs can be present in very small amounts.

Our group recently developed a novel and powerful process, the peptidome Bioprospecter process, for discovering and identifying new and potentially useful CAMPs (Bishop et al., 2015). We have successfully employed this process to discover a large number of new peptides, several of which show antibacterial activity against Gram-positive and/or Gram-negative bacteria, from only 100 µl of plasma from the American alligator, *Alligator mississippiensis*. Our CAMP discovery process is sample agnostic and requires very small samples for analysis. Moreover, it is unique in its approach to CAMP identification in that it directly mines the native antimicrobial peptidome. This bioprospecting approach provides us access to the CAMP peptidomes of some of the world's most remarkable species, to dramatically expand the current CAMP library and potentially unlock the key to overcoming antibiotic resistance via the discovery of new antimicrobial peptides.

Using this tool, we discovered 2 novel peptides (Apo5 and Apo6) with activity against various bacteria including important bacterial pathogens (Bishop et al., 2015). Apo5 and Apo6 are highly related peptides, both fragments of a purported apolipoprotein.

Apolipoproteins are known to have antimicrobial activity against a variety of pathogenic bacteria (Biedzka-Sarek et al., 2011; L. D. Johnston et al., 2008; Pridgeon & Klesius, 2013; Sigel et al., 2012; Villarroel, Bastías, Casado, Amthauer, & Concha, 2007; C. Q. Wang et al., 2013)**.** In this work, we characterize the activity of these peptides. In addition, we determined the secondary structure of these peptides in order to understand the mechanism by which they exert their antibacterial activity, and to further analyze antimicrobial activity against relevant pathogens resistant against multiple antibiotics, as well as clinical isolates, to the aim of developing new antimicrobial platforms.

Materials and Methods

Bacteria.

Staphylococcus aureus ATCC 25923 and 33592, *Escherichia coli* strain O157:H7 ATCC 51659, *Pseudomonas aeruginosa* ATCC 9027, and *Acinetobacter baumannii* ATCC BAA-1794 were purchased from the American Type Culture Collection (Manassas, VA). *Pseudomonas aeruginosa* strain PAO1 was generously provided by Karin Sauer from Binghamton University (Binghamton, NY). Bacteria were grown in Nutrient Broth (Difco 234000) overnight in a shaking incubator (37˚C). Bacteria were aliquoted and frozen at -80 ˚C and enumerated via serial dilution and plating prior to experimentation.

Peptides

All peptides were synthesized by ChinaPeptides, Inc (Shanghai, China) using Fmoc chemistry. Peptides were provided at >95% purity, and purity and structure were confirmed with RP-HPLC and ESI-MS.

Bioinformatics

Physico-chemical properties were calculated using the Antimicrobial Peptide Database (APD2) (G. Wang et al., 2009). The percent hydrophobicity is defined as the ratio of hydrophobic residues to total residues. The full-length sequence for the apolipoprotein C-1 containing Apo5 and Apo6 (Accession XP_006276575.1) was found on the BLAST NCBI database (Altschul, Gish, Miller, Myers, & Lipman, 1990). Ribbon models displaying the full proteins were created using SWISS MODEL. The *A. mississippiensis* apolipoprotein C-1 was modeled on the human apolipoprotein C-1 (SMTL id: 1ioj.1.A, Sequence identity = 43.40%) (Arnold, Bordoli, Kopp, & Schwede, 2006; Biasini et al., 2014; Bordoli et al., 2008; Guex, Peitsch, & Schwede, 2009; Kiefer, Arnold, Kunzli, Bordoli, & Schwede, 2009; Kopp & Schwede, 2006). Helical wheel projections and hydrophobic moment was calculated using HeliQuest (Gautier, Douguet, Antonny, & Drin, 2008).

Circular dichroism spectroscopy

CD was performed using a Jasco J-1500 spectropolarimeter. 100 µg/ml of peptide was used in each experiment. Samples were allowed to equilibrate for 3 min prior to data collection at 25°C in a 1 mm path length cuvette. Spectra were collected from 190 to 260 nm at 0.2 nm intervals, with a data integration time of 4 s and a 1 nm bandwidth. Data presented is an average of four spectra. Peptides were analyzed in 10 mM sodium phosphate buffer (6.12 mM sodium monohydrogen phosphate heptahydrate; 3.92 mM monosodium phosphate anhydrous; pH 7.4), 50% (v/v) trifluoroethanol (TFE) in phosphate buffer, or 60 mM sodium dodecyl sulfate (SDS) in phosphate buffer. Percent contribution to secondary structure was measured using methods determined by Raussens

et al (Raussens et al., 2003).

Antimicrobial assays.

The antimicrobial MIC activity of the peptides was first determined in cation-adjusted Mueller Hinton Broth (BD 212322) in a polystyrene tissue-culture treated 96 well plate. Enumerated bacteria were diluted in broth and 10^5 CFU was added to each well with varying dilutions of peptide. The plate was incubated for 24 h at 37˚C and then read on a spectrophotometer at OD 600nm.

Table 6. Apo5 and Apo6 sequences and physico-chemical properties.

Peptide name	Sequence	Molecular Weight (kDa)	Charge	Hydro-phobicity
Apo5	FSTKTRNWFSEHFKKVKEKLKDTFA	3103.57	+4	32%
Apo6	KTRNWFSEHFKKVKEKLKDTFA	2766.49	+4	31%
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	4493.33	+6	35%

The EC50 antibacterial activity of the peptides was then determined in 10 mM phosphate buffer using resazurin as an indicator of CFU as previously described (Bishop et al., 2015). This method was validated by Bishop et al (Bishop et al., 2015). The sequences and net charges of the peptides tested are shown in **Table 6**. In a black polypropylene 96 well plate, 10^5 bacteria were incubated in 10 mM phosphate buffer with various dilutions of peptide (3 h; 37 °C) (1:5). Cation-adjusted Mueller Hinton Broth (BD 212322, final concentration 2.2% unless otherwise notes) and resazurin (final concentration 109 µM unless otherwise notes) dissolved in PBS were added to each well, and the plate was read kinetically overnight on a Tecan Safire^2 spectrofluorometer (37 ˚C; excitation=540 nm; emission=590 nm). Percent survival after treatment was then

calculated with previously determined growth curves (**Tables 2 and 7**). GraphPad Prism 6.0 was used to calculate EC50 using log concentration vs. percent survival. Each experiment was repeated at least twice, and a representative experiment is shown. 95% confidence intervals (CI) are reported to indicate the error of each EC50 determination.

Table 7. Reduction rate equations for antimicrobial assays.

Bacterial strain	**Reduction rate equation**
P. aeruginosa PAO1	$\log(CFU_{P.aeruginosa}) = (T_{20000} - 60948) / -5823.3$
E. coli ATCC 51659	$\log(CFU_{E.\ coli}) = (T_{20000} - 37673) / -4292.9$
S. aureus ATCC 33592	$\log(CFU_{S.\ aureus}) = (T_{20000} - 59514) / -7912.2$
A. baumannii ATCC BAA-1794	$\log(CFU_{A.\ baumannii}) = (T_{20000} - 34042) / -4398.1$

Ethidium bromide uptake assay.

The ethidium bromide uptake assay was performed as previously detailed (Y. Li et al., 2012; Murata, Tseng, Guina, Miller, & Nikaido, 2007) with some modifications. *E. coli* was grown overnight in caMHB in a shaking incubator (37°C). Bacteria was centrifuged, washed with PBS, and then adjusted to an OD 600 nm of 0.1 in 10 mM phosphate buffer. 180 µl of bacteria was added to 10 µl ethidium bromide (10 mM final concentration) and 10 µl peptide in various concentrations. The plate was read in a Tecan Safire^2 spectrofluorometer every 2 min for 30 min (37°C; excitation = 540 nm; emission = 590 nm). Peak RFU at 50 µg/ml was used in **Figure 9.**

Membrane depolarization study.

Membrane depolarization was studied using $DiSC_3(5)$ as has previously been

reported (Gupta, Singh, & van Hoek, 2015; Rodriguez, Papanastasiou, Juba, & Bishop, 2014). Depolarization of a membrane can be visualized by a drop in fluorescence. Enumerated frozen bacteria were pelleted and washed twice in 10 mM phosphate buffer and then resuspended to $4x10^7$ CFU/ml in 10 mM phosphate buffer containing 50 µg/ml $DiSC_3(5)$. 100 µl of this suspension was added to wells of a black 96 well plate. Plate was incubated in a Tecan Safire^2 spectrofluorometer and monitored until fluorescence leveled off. 100 µl various concentrations of peptide in 10 mM phosphate buffer was added to each well. Bacteria without peptide and peptide without bacteria served as controls. Plate was immediately returned to the spectrofluorometer. Readings were taken every 15 s for 5 min (excitation = 622 nm; emission = 670 nm). Peak RFU at each concentration was used in **Figure 10**.

Hemolysis Assay

To measure the hemolytic activity of peptides, 2% sheep red blood cells were added to various dilutions of peptide reconstituted in PBS in a sterile U-bottom 96 well plate. 2% RBCs with PBS alone served as the negative control, and 2% RBCs in water as the positive control. The plate was incubated for 1 h at 37°C and then centrifuged at 1000 rpm for 2 min. The supernatant was transferred to a fresh plate and read at OD 540 nm (Dean et al., 2011a).

Cytotoxicity Assay

Cytotoxicity assays were performed using the Vybrant MTT Cell Proliferation Assay Kit (Life Technologies) according to manufacturer's instructions (Kehn-Hall et al., 2011). Assays were performed using human lung epithelial lung carcinoma line A549

(ATCC CCL-185), which were maintained at a low passage in Dulbecco's Minimal Essential Media (Life Technologies 11995073) with 10% heat-inactivated fetal bovine serum and 18 U/ml penicillin-streptomycin. 300 µg/ml of peptide was used for each experimental well. Each experiment was performed in triplicate three times. A representative experiment is shown.

Statistics

Statistical analyses were performed using GraphPad Prism 6.0. To determine statistical significance, the one-way ANOVA with Tukey's multiple comparisons was performed in all instances.

Results:

Bioinformatics.

The native, plasma-derived peptides were analyzed using APD2 and studying the secondary structure and placement within the parent proteins. Apo5 and Apo6 are both part of the C-terminus of an apolipoprotein, apolipoprotein C-1. Apo5 is comprised of aa 64-86, while Apo6 is the smaller fragment (aa 67-86, shown in **Figure 6A**). Both peptides have a +4 charge and a hydrophobic ratio of just over 30%. Apo5 is cleaved at a Glu-Phe site and Apo6 at a Thr-Lys site. On the full apolipoprotein, both cleavages sites are placed in a disordered hinge proceeding a C-terminal alpha helix as seen in **Figure 6B**. The helical wheels are shown in **Figures 6C** and **6D**, illustrating the distribution of charge on the amphipathic face. Because Apo6 is missing 3 aa on the N-terminus, it has a slightly stronger hydrophobic moment (0.484 vs 0.436).

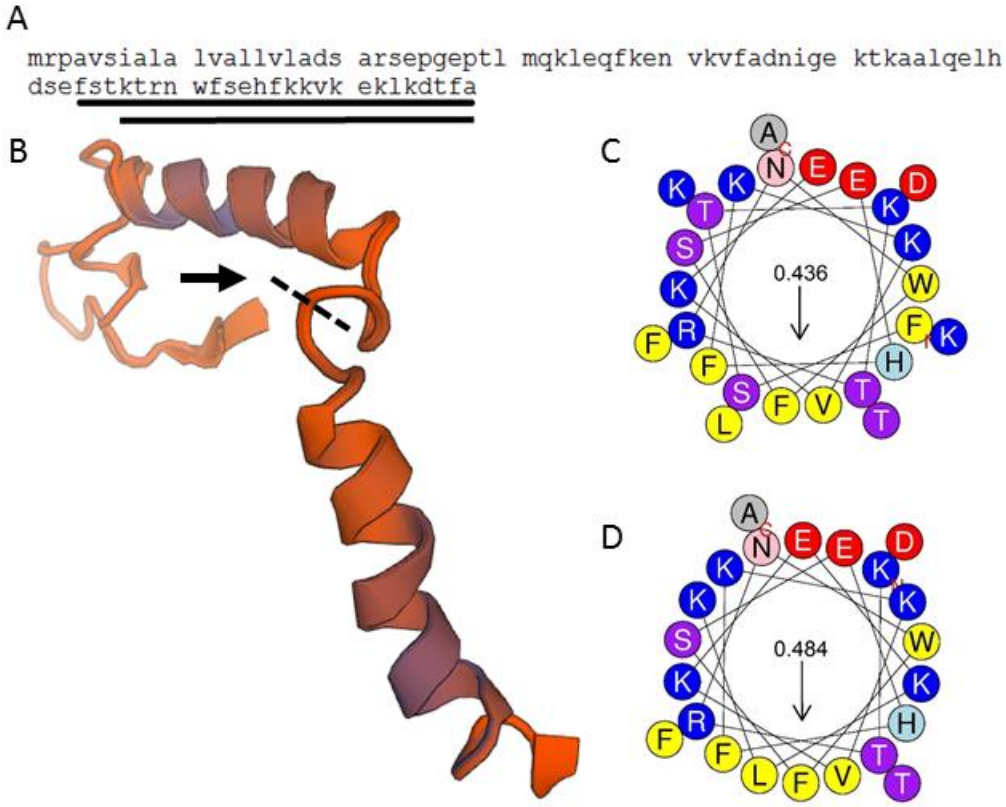

Figure 6. Bioinformatical analysis of Apo5 and Apo6. **A.** Amino acid sequence of *A. mississippiensis* apolipoprotein C-1; Apo5 is underlined; Apo6 is double underlined. **B.** Ribbon model of apolipoprotein C-1 showing cleavage site of Apo5. **C.** Helical wheel of Apo5 with hydrophobic moment. **D.** Helical wheel of Apo6 with hydrophobic moment.

Secondary structure determination.

To determine general secondary structure of Apo5 and Apo6, CD spectroscopy was used. CD was performed in 10 mM phosphate buffer, 60 mM SDS, and 50% TFE in phosphate buffer. Helical CAMPs such as LL-37 maintain a random or disordered structure until associated with a membrane or micelle. SDS mimics the bacterial membrane, forcing the CAMP into a more ordered conformation.

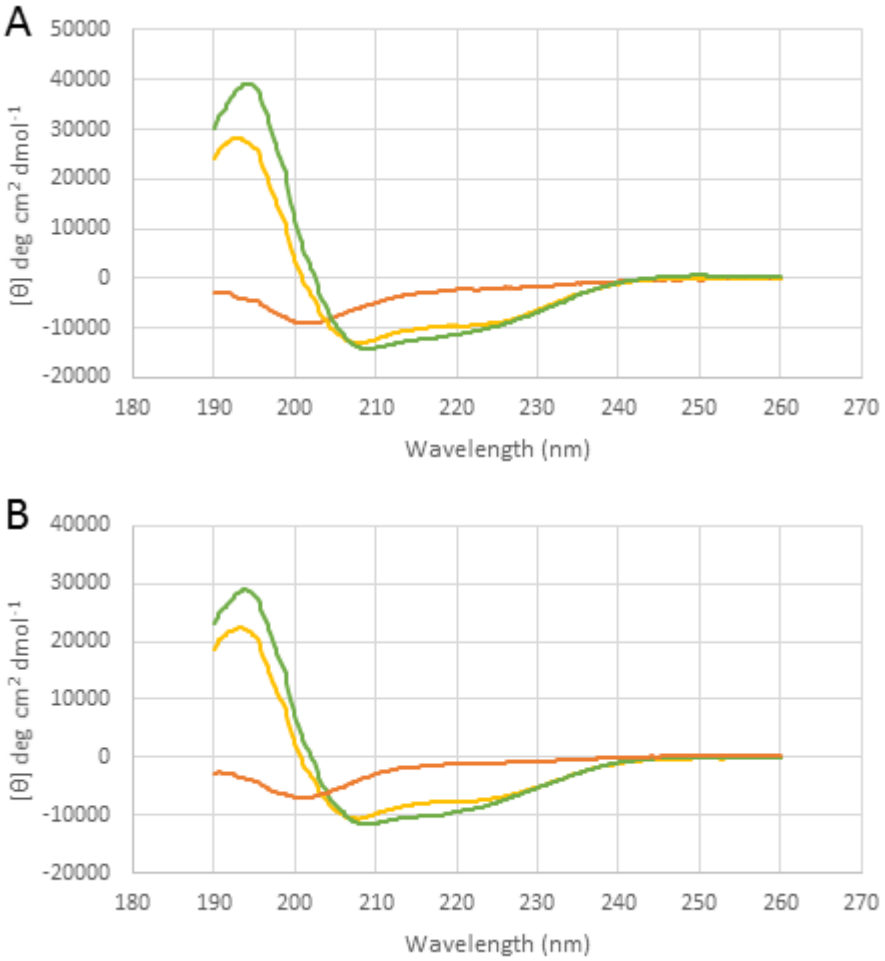

Figure 7. CD spectra of peptides to determine secondary structure. **A**. Apo5 and **B**. Apo6. All spectra were taken with peptide concentrations of 100 µg/ml in a 1 mm pathway cuvette. Spectra were read in 10 mM phosphate buffer (----), 50% TFE in 10 mM phosphate buffer (----), and 60 mM SDS in 10 mM phosphate buffer (----).

As expected, Apo5 (**Figure 7A**) and Apo6 (**Figure 7B**) had nearly identical spectra. Both peptides have random coil and β-sheet characteristics in 10 mM phosphate buffer, and are primarily α-helical when CD is measured in buffers with SDS and TFE. Interestingly, Apo5, the longer of the two peptides, is calculated to be more α-helical than Apo6 in both SDS (63.3% vs. 57.0%) and TFE (51.0% vs. 50.3%), shown in **Table 8**.

When evaluating α-helical properties of these two peptides by simple intensity at 208 nm and 222 nm, it is notable that the peaks at these wavelengths are more intense for Apo5 than Apo6, though the two Apo peptides maintain 12.5% turn no matter the buffer used. Apo5 and Apo6 likely have a primarily α-helical structure with some random coil portions. Based on helical wheel projections, it appears that both Apo5 and Apo6 have great amphipathic character, with several hydrophobic residues on one face and several basic amino acids on the other. This is consistent with the predicted structure of the c-terminal portion of the parental protein, apolipoprotein C-1 (**Figure 6B**).

Table 8. Percent secondary structure contribution of Apo5 and Apo6. As calculated by a method described by Raussens et al (Raussens et al., 2003).

	α-helical	**β-sheet**	**Turn**	**Random**	**Sum**
Apo5					
PB	8.9	30.3	12.5	40.6	92.3
TFE	51	4.6	12.5	29.3	97.4
SDS	63.3	-4.8	12.5	25.1	96.1
Apo6					
PB	9.4	30.5	12.5	41	93.4
TFE	50.3	4.8	12.5	29.7	97.3
SDS	57	0.7	12.5	26.9	97.1

Antimicrobial activity.

Apo5 and Apo6 were first tested against drug-sensitive strains of *S. aureus* and *P. aeruginosa.* It was found that they had no activity in broth up to 250 µg/ml. It has been found that antimicrobial assays performed in broth do not show the full extent of the activity of CAMPs (Chromek, Arvidsson, & Karpman, 2012; Dean et al., 2011a, 2011b), so Apo5 and Apo6 were subsequently tested in 10 mM sodium phosphate buffer. Apo5

Table 9. Antimicrobial activity of Apo5 and Apo6.

Peptide	Bacteria	MIC (µg/ml)	EC50 (µg/ml)	95% CI (µg/ml)	EC50 (µM)	95% CI (µM)
Apo5	*S. aureus* ATCC 25923	>80	30.0	23.9 to 37.9	9.66	7.69 to 12.2
	S. aureus ATCC 33592	>80	0.0680	0.0401 to 0.115	0.0219	0.0401 to 0.115
	P. aeruginosa ATCC 9027	>80	4.38	2.81 to 6.92	1.41	0.906 to 2.23
	P. aeruginosa PAO1	>80	0.0878	0.0326 to 0.237	0.0283	0.0105 to 0.764
	E. coli ATCC 51659	>80	13.9	10.7 to 18.0	4.48	3.45 to 5.80
	A. baumannii ATCC BAA-1794	>80	0.2344	0.122 to 0.449	0.0755	0.0394 to 0.145
Apo6	*S. aureus* ATCC 25923	>80	19.6	14.9 to 25.7	7.08	5.39 to 9.29
	S. aureus ATCC 33592	>80	0.882	0.5250 to 1.48	0.319	0.190 to 0.536
	P. aeruginosa ATCC 9027	>80	2.62	19.5 to 3.51	0.948	0.706 to 1.27
	P. aeruginosa PAO1	>80	1.19	0.867 to 1.63	0.429	0.313 to 0.587
	E. coli ATCC 51659	>80	9.07	7.11 to 11.6	3.28	2.57 to 4.19
	A. baumannii ATCC BAA-1794	>80	0.348	0.249 to 0.487	0.126	0.0899 to 0.176
LL-37	*S. aureus* ATCC 25923	>80	2.48	1.72 to 3.58	0.552	0.383 to 0.797
	S. aureus ATCC 33592	>80	0.208	0.139 to 0.312	0.0462	0.0308 to 0.694
	P. aeruginosa ATCC 9027	>80	2.36	2.00 to 2.76	0.525	0.446 to .615
	P. aeruginosa PAO1	>80	0.647	0.0598 to 6.92	0.144	0.0133 to 1.55
	E. coli ATCC 51659	>80	0.298	0.208 to 0.428	0.0663	0.0463 to 0.0953
	A. baumannii ATCC BAA-1794	>80	0.803	0.367 to 1.75	0.179	0.0823 to 0.389

and Apo6 were found to be very effective against susceptible *P. aeruginosa* ATCC 9027 with EC50 values of 4.38 and 2.62 µg/ml, but were only moderately effective against susceptible *S. aureus* ATCC 25923, with EC50 values of 30.0 and 19.6 µg/ml, shown in **Table 9** and represented graphically in **Figure 8**.

Antibiotic resistance has been increasing steadily for the past several decades, and CAMPs are considered a possible basis for novel antimicrobials. Because of this, the novel peptides were also tested against multi-drug resistant strains of *S. aureus*, *A. baumannii*, and *E. coli*, as well as clinical isolate *P. aeruginosa* strain PAO1. Sharing a salt-sensitive phenotype with LL-37, the CAMPs had no effect in MIC experiments as high as 250 µg/ml, so experiments were performed to determine the EC50 in 10 mM sodium phosphate buffer.

In general, it was found that the EC_{50} values of Apo5 and Apo6 were statistically similar. Full numerical and statistical data shown in µg/ml and µM can be found in **Table 9**. Both apolipoprotein-derived peptides had very strong activity against methicillin-resistant *S. aureus* (Apo5 EC50 = 0.0755 µM; Apo6 EC50 = 0.126 µM), a broadly antibiotic-resistant strain of *A. baumannii* (Apo5 EC50 = 0.0755 µM; Apo6 EC50 = 0.126 µM), as well as strong activity against *P. aeruginosa* clinical isolate strain PAO1 (Apo5 EC50 =0.0283 µM; Apo6 EC50 = 0.429 µM). These peptides also had good but weaker activity against MDR EHEC *E. coli* (Apo5 EC50 = 4.48 µM; Apo6 EC50 = 3.28 µM).

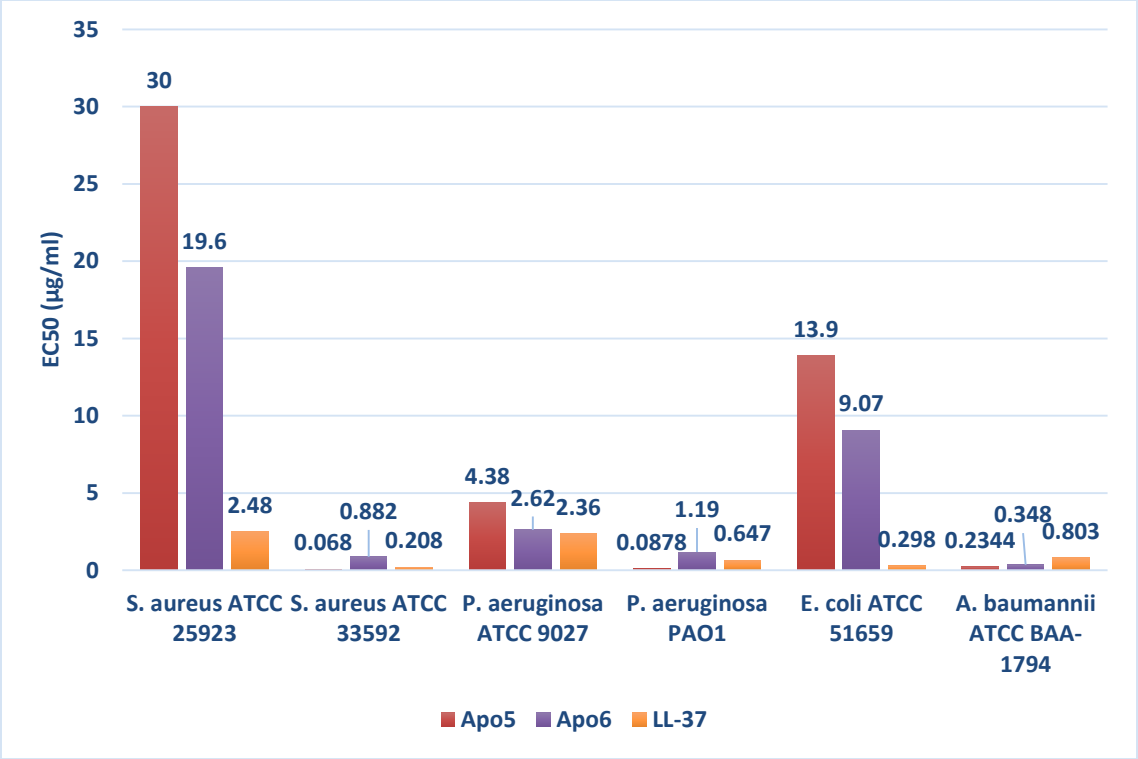

Figure 8. Graph of antimicrobial activity of Apo5 and Apo6.

Membrane permeabilization and depolarization by peptides.

To determine whether Apo5 and Apo6 interacted with the bacterial membrane, each peptide's ability to permeabilize the membrane was measured by the ethidium bromide uptake assay, while membrane depolarization was measured with the fluorescent chemical DiSC3(5), which is sensitive to the polarization of membranes.

When the ethidium bromide uptake assay was performed (**Figure 9A**), it was found that Apo5 and Apo6 permeabilized the *E. coli* membrane at concentrations at 50 µg/ml quickly, comparable to control peptide LL-37 ($p<0.001$). Neither Apo peptide permeabilized membranes at lower concentration.

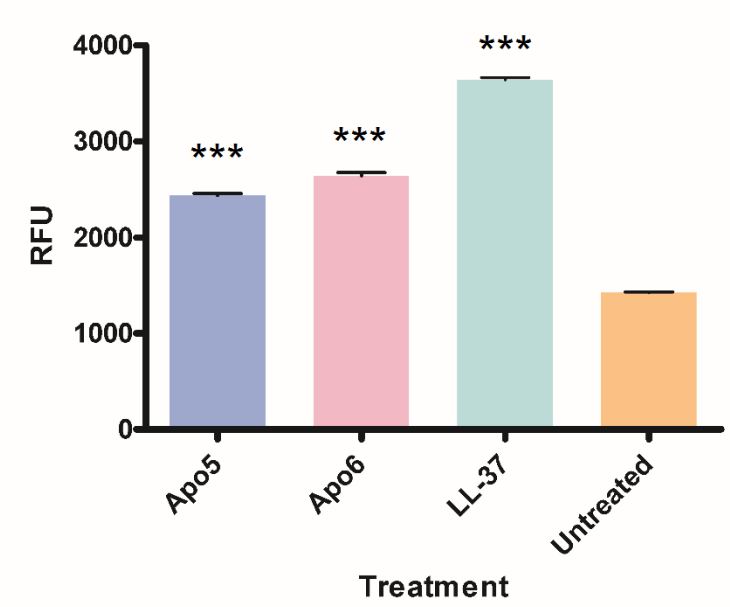

Figure 9. Membrane permeabilization activity by Apo5 and Apo6. An increase in fluorescence demonstrates greater binding of DNA by ethidium bromide, which indicates the permeabilization of the bacterial membrane. Shown is permeabilization of membrane after 20 min at 50 µg/ml.

Depolarization of a bacterial membrane indicates very small, transient pore formation only large enough to leak ions, which damages the proton motive force and other gradients that store chemical energy. As shown in **Figure 10**, within 1 min it was found that Apo5 and Apo6 depolarized bacterial membranes at concentrations as low as 0.5 µg/ml ($p<0.001$), showing a clear dose-dependent response to peptide concentration. Depolarization signals at 0.5 µg/ml were well below more EC50s found for Apo5, Apo6, and LL-37 ($p<0.001$).

These results indicate that Apo5 and Apo6 depolarize the bacteria membrane quickly, suggesting this is the mechanism by which these peptides kill bacteria.

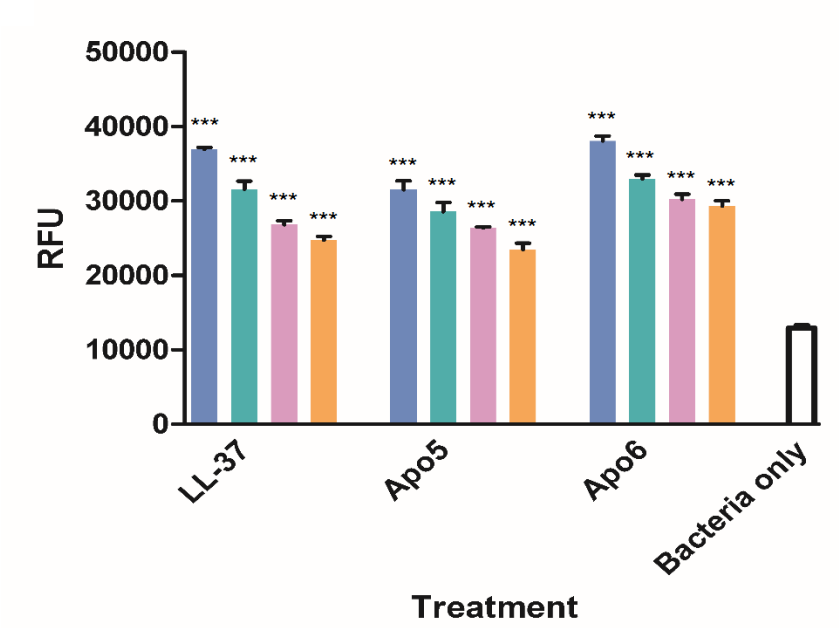

Figure 10. Membrane depolarization activity by Apo5 and Apo6. Depolarization determined using DiSC3(5) for each peptide at 50 µg/ml (■), 25 µg/ml (■), 5 µg/ml (■), 0.5 µg/ml (■), and no treatment (□).

Cytotoxicity.

The physiochemical properties of CAMPs preclude cytotoxicity; however, some CAMPs have been found to cause damage to eukaryotic cells, such as SMAP-29. Potential cytotoxicity against red blood cells and A549 lung epithelial cells was measured. For red blood cells, a spectrophotometric assay that measures free heme was used, while an MTT assay was used for A549 cells. In **Figure 11A**, hemolytic activity is shown as percent hemolysis. At 300 µg/ml, all four peptides showed hemolysis of less than 1% of RBCs, comparable to the control peptide LL-37. It was found there was no statistically significant difference between the untreated control and all peptides, indicating that Apo5 and Apo6 are not hemolytic.

Using the MTT assay, we tested cytotoxicity of the peptides at 1 log higher than the highest EC50 calculated (30 µg/ml against *S. aureus* ATCC 25923). It was shown in **Figure 11B** that at 300 µg/ml, both peptides were found to be statistically cytotoxic ($p<0.0001$) against A549 epithelial cells after 24 h exposure, at the same level as LL-37 (**Figure 11C**).

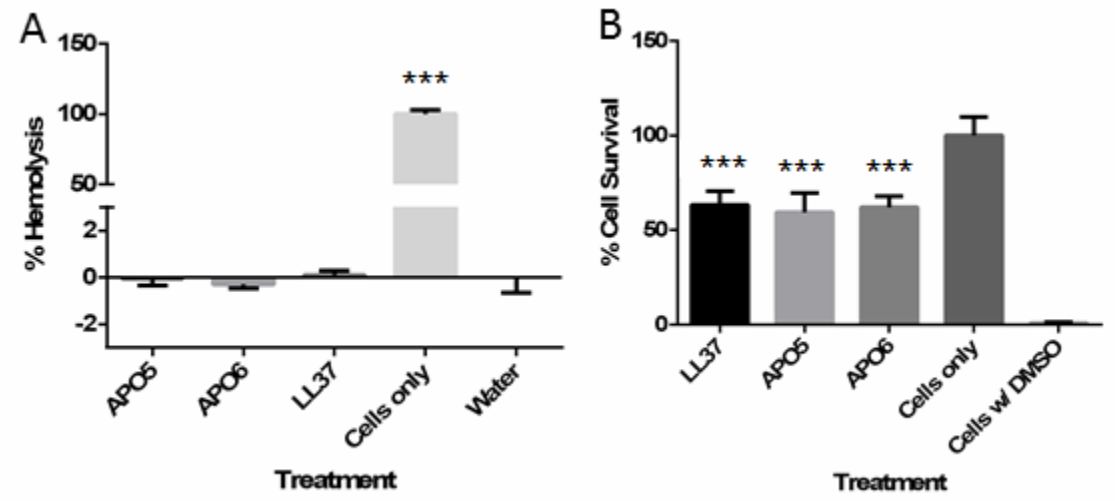

Figure 11. Cytotoxicity of Apo5 and Apo6. Cytotoxicity was tested against **A.** sheep red blood cells measured using a photometric assay after exposure to 300 µg/ml for 1 h and **B.** A549 human lung epithelial cells measured using the MTT assay after exposure 300 µg/ml for 24 h.

Discussion

Using our CAMP discovery process and only 100 µl of alligator plasma, we have successfully identified several novel peptides from *A. mississippiensis* that exhibit antibacterial activity (Bishop et al., 2015). Our bioprospecting-based process provides a unique access to the antimicrobial peptidome, and is a significant advance in the effort to identify novel antimicrobial peptides in nature. In this study we present detailed

characterization of the structure and function of two alligator plasma-derived peptides: Apo5 and Apo6. Apo5 and Apo6 are potent antimicrobial peptides effective against multi-drug resistant and clinically relevant pathogens.

Apo5 and Apo6 are both derived from a predicted apolipoprotein C-1 in *A. mississippiensis*. Apolipoproteins bind lipids; apolipoprotein C-1 in particular is known to bind phospholipids and is a marker of apoptosis (Beck et al., 2013; L. D. Johnston et al., 2008; Navab et al., 2002; Villarroel et al., 2007). Apo6 is a smaller derivative of Apo5; Apo5 contains residues 64-88 of predicted apolipoprotein C-1, while Apo6 contains residues 67-88. These peptides were very active against Gram-negative bacteria. We discovered that Apo5 and Apo6, two highly related peptides derived from the alligator protein apolipoprotein C1, are predominantly helical peptides. Their mode of antibacterial activity appears to be primarily through membrane interaction. At low concentrations (approximately the EC_{50} for all bacteria), Apo5 and Apo6 depolarized the membrane of *E. coli*. At high concentrations, well above the EC_{50} for all bacteria tested, these peptides caused greater disruption and permeabilization of the bacterial membrane, allowing for the escape of much larger cellular milieu. Based on these observations, it seems that the primary mode of action of these apolipoprotein-derived peptides is membrane perturbation and depolarization.

Apolipoprotein-derived antimicrobial peptides have been identified in other organisms, although they are more typically derived from apolipoprotein E or A (Biedzka-Sarek et al., 2011; L. D. Johnston et al., 2008; Kelly et al., 2007; Pridgeon & Klesius, 2013; Villarroel et al., 2007; C. Q. Wang et al., 2013). Many whole

apolipoproteins have been found to have antimicrobial activity against a variety of bacteria, as well as some viruses. Apolipoprotein A-1 from various fishes have been shown to have antimicrobial activity against *E. coli* (Concha, Molina, Oyarzún, Villanueva, & Amthauer, 2003; L. D. Johnston et al., 2008; Villarroel et al., 2007), *Streptococcus* spp. (L. D. Johnston et al., 2008) and fish pathogens such as *Mycobacterium marinum (L. D. Johnston et al., 2008)* and *Yersinia ruckeri* (Villarroel et al., 2007). The antimicrobial activity of human apolipoprotein A-1 has also be examined, and several groups have found that this protein is effective against *E. coli, K. pneumoniae,* and *Yersinia enterocolitica* (Beck et al., 2013; Biedzka-Sarek et al., 2011). The primary mode of action of apolipoprotein A-1 seems to be as a detergent; it has been shown to bind lipids including LPS and to dissolve micelles (Beck et al., 2013; Ulevitch, Johnston, & Weinstein, 1979; Villarroel et al., 2007). The LPS-binding activity in particular has been shown to be reliant on the N-terminus of the protein (Beck et al., 2013). The mechanism of action and antimicrobial activity of apolipoprotein C-1 has not yet been explored, but it likely binds lipids as some part of its mechanism. Apo5 and Apo6 were not found to bind LPS as part of its mechanism (data not shown); considering these two peptides are part of the C-terminus, this make sense if the properties and domains of apolipoprotein C-1 are similar to those of apolipoprotein A-1. This characterization of the broad antimicrobial activity of apolipoprotein C-1-derived peptides adds to this family of CAMPs.

The novel CAMPs characterized here are among the first discovered using our hydrogel bioprospecting technology, illustrating the power of this technology to capture

naturally occurring small peptides with net cationic charge and antimicrobial activity (Bishop et al., 2015). Considering the strong and broad-spectrum activity of these CAMPs, it seems likely that there is a greater role for protein fragments in peptide-based innate immunity than previously thought. In addition, these CAMPs are more effective against drug-resistant strains than test strains, indicating these CAMPs may be valuable bases for treatments against multi-drug resistant bacteria. In future work on this project, we will continue to test Apo5 and Apo6 on MDR bacterial strains. Considering the strength of these two CAMPs on MDR *A. baumannii* in particular, these could be strong candidates for *in vivo* testing. In addition, we will also examine apolipoprotein gene expression and protein/peptide abundance in LPS-stimulated alligators to further our understanding of the role of this protein and its fragmentation in the innate immune response.

CHAPTER 4: CATHELICIDIN OF THE AMERICAN ALLIGATOR

Alligator mississippiensis (American alligator), as a member of order Crocodilia, is more closely related to dinosaurs than other modern reptiles. This species lives in bacteria-laden environments, but are not often known to succumb to bacterial infections. Their serum has been shown to have antibacterial activity beyond that of human serum, and it is believed that this activity is partially due to CAMPs. CAMPs are small proteins produced by many organisms as part of the innate immune system. CAMPs are attractive possible platforms against multi-drug resistant bacteria, such as those found in biofilm-infected war wounds, because they seldom cause genetic resistance in bacteria and are effective against antibiotic resistant bacteria. In this work, we located, synthesized, and characterized a cathelicidin from the American alligator (*Alligator mississippiensis*) named AM-CATH. We discovered this CAMP using BLAST alignment and compared *A. mississippiensis* ESTs with propeptide cathelicidins of other reptiles. We analyzed the structure using bioinformatics tools and circular dichroism and found that AM-CATH has a mixed structure, with an N-terminal α-helix and a center Pro hinge. In MIC assays, it was determined that AM-CATH has strong activity against Gram negative bacteria, including multi-drug resistant (MDR) *A. baumannii*. Using the ethidium bromide uptake assay, it was found that AM-CATH permeabilizes the bacterial membrane and is less sensitive to salt than many other CAMPs. AM-CATH was not found to be hemolytic

against sheep red blood cells at 300 μg/ml, and was not found to be significantly cytotoxic against A549 human lung epithelial cells after 24 h exposure in MTT assays. AM-CATH has activity similar to other CAMPs from reptiles such as NA-CATH. It is possible that AM-CATH plays an important role in the innate immune of *A. mississippiensis*, similar to LL-37 in humans. In addition, due to its activity against MDR bacteria and lack of cytotoxicity, AM-CATH could be an attractive platform for a clinical product.

Introduction

Cathelicidins are a class of CAMPs conserved in the propeptide sequence. Cathelicidin CAMPs form a critical part of vertebrate innate immunity and host defense. These CAMPs are widely expressed throughout the body (Dürr, Sudheendra, & Ramamoorthy, 2006), and are highly expressed and packaged in secondary granules in neutrophils or heterophils (Borregaard & Cowland, 1997), from which they are released in response to encounters with pathogens (Gullberg et al., 1999). The cathelicidins have been found to have a wide range of roles within the innate immune system. Not only have they been found to be directly affect invading pathogens by killing the bacteria and reducing biofilm formation (Amer et al., 2010; de Latour et al., 2010; Dean et al., 2011a, 2011b), cathelicidins are known to have an immunomodulatory effect by increasing neutrophil and eosinophil movement (Kahlenberg & Kaplan, 2013) and moderating the release of inflammatory molecules from neutrophils (Alalwani et al., 2010) and other immune cells (Kahlenberg & Kaplan, 2013).

One of the most interesting features of cathelicidin peptides is that the sequence of the active antimicrobial peptide is extremely diverse among vertebrates, with almost no homology between classes or orders (for example, when snake cathelicidin is compared to human cathelicidin, they are 12% homologous) (Zelezetsky et al., 2006). These genes are most commonly identified by homology within the N-terminal cathelin domain in the propeptide sequence. The entire translated propeptide contains four exons, the last of which typically encodes the active cathelicidin peptide (Chin-I Chang, Pleguezuelos, Zhang, Zou, & Secombes, 2005; Chin- I. Chang, Zhang, Zou, Nie, & Secombes, 2006; G. H. Gudmundsson et al., 1996; G H Gudmundsson et al., 1995; Maier, Dorn, Gudmundsdottir, & Gudmundsson, 2008; Scocchi, Wang, Gennaro, & Zanetti, 1998; C. Zhao, Ganz, & Lehrer, 1995).

Cathelicidin peptides have been identified and characterized in many mammals, including wallabies (J. Wang et al., 2011), dogs (Sang et al., 2007), sheep (Huttner, Lambeth, Burkin, Burkin, & Broad, 1998), pigs, cattle (Scocchi, Wang, & Zanetti, 1997), mice, horses, and humans (Larrick et al., 1996), as well as birds (G. Zhang & Sunkara, 2014) and toads (Sun, Zhan, & Gao, 2015), but very few have been studied in reptiles. Four cathelicidins have been found to be produced by *Anolis carolinensis*, which are released in response to wounding or infection in a similar manner to the human cathelicidin peptide (Alibardi, 2014; Dalla Valle, Benato, Paccanaro, & Alibardi, 2013). A group of elapid snakes, including *Ophiphagus hannah, Bungarus fasciatus,* and *Naja atra,* encode for very similar cathelicidin peptides, called OH-CATH-like (Y. Wang et al., 2008; H. Zhao et al., 2008). This includes the cathelicidin from *N. atra*, NA-CATH,

which, along with smaller derivatives, is a very highly active antimicrobial peptide against multiple human pathogens, including *P. aeruginosa, S. aureus,* and *Francisella tularensis* (Amer et al., 2010; de Latour et al., 2010; Dean et al., 2011a, 2011b).

We sought to identify an active cathelicidin in *Alligator mississippiensis* and to test the predicted peptide for its activity. While no active cathelicidins have been identified or demonstrated yet in the alligator, the partially annotated genome of *A. mississippiensis* has recently been released, enabling us predict, synthesize, and test potential cathelicidins. From this genomic data and using techniques that allowed for the discovery of chicken cathelicidins (Yanjing Xiao et al., 2006), we predicted both a 36 aa peptide (AM-CATH), and a 28 aa antimicrobial peptide with sequence: KIKKGFKKIFKRLPPIGVGVSIPLAGKR. We named this peptide AM-CATH28, keeping the OH-, BF-, NA-CATH naming convention. This peptide is highly cationic (+9), predicted to be helical, and amphipathic. We had this peptide synthesized, determined its activity *in vitro* against multiple bacteria, and tested its mechanism of action. In conclusion, we propose that AM-CATH28 is a part of cathelicidin-like peptide of *A. mississippiensis* and may play an important role in the innate immune response of *A. mississippiensis* to bacteria.

Methods and Materials

Bacterial strains

S. aureus ATCC 33592 and ATCC 25923, *P. aeruginosa* ATCC BAA-2110 and ATCC 9027, *A. baumannii* ATCC BAA-1795, and *Burkholderia thailandensis* ATCC 700388 were purchased from the American Type Culture Collection (Manassas, VA).

Bacteria were grown in N) overnight in a shaking incubator (37˚C). Bacteria were aliquoted and frozen at -80˚C and enumerated via serial dilution and plating prior to experimentation.

Peptides

AM-CATH28 and NA-CATH was synthesized by ChinaPeptides, Inc (Shanghai, China) using Fmoc chemistry. Peptide was provided at >95% purity, which was confirmed with RP-HPLC and ESI-MS.

Bioinformatics

Sequence analysis and searching was primarily performed using the NCBI website. Specific databases used are indicated in the results section below.

Antimicrobial assays.

The MIC of AM-CATH28 was determined according to CLSI guidelines with some modification (Ahmad, Hunter, Qin, Mann, & van Hoek, 2010; Ulrey, Barksdale, Zhou, & van Hoek, 2014). The MIC assay was performed concurrently in caMHB and NB. Each experiment was performed in triplicate three times.

Ethidium bromide uptake assay.

E. coli ATCC 51659 was grown overnight in cation-adjusted Mueller Hinton Broth in a shaking incubator (37°C). Bacteria was centrifuged, washed with PBS, and then adjusted to an absorbance of 0.1 OD600 nm in 10 mM sodium phosphate buffer or PBS. 180 µl of bacteria was added to 10 µl ethidium bromide (10 mM) and 10 µl peptide in various concentrations. The plate was read in a Tecan Safire^2 fluorimeter every 2 min for 30 min (37°C; excitation=540nm; emission=590nm).

Circular dichroism.

CD was performed using a Jasco J-1500 spectropolarimeter. 100 µg/ml of peptide was used in each experiment. Samples were allowed to equilibrate for 5 min prior to data collection at 25°C in a 1 mm path length cuvette. Spectra were collected from 190 to 260 nm at 0.2-nm intervals, with a data integration time of 4 s and a 1 nm bandwidth. Data presented is an average of four spectra. AM-CATH was analyzed in 10 mM sodium phosphate buffer, 50% (v/v) TFE in 10 mM phosphate buffer, or 60 mM SDS in 10 mM phosphate buffer. 100 µg/ml of peptide was used for each experiment. Spectral values were analyzed used method developed by Raussens et al (Raussens et al., 2003) to determine percent secondary structure contribution.

Cytotoxicity assays.

Cytotoxicity assays were performed using the Vybrant MTT Cell Proliferation Assay Kit (Life Technologies) according to manufacturer's instructions. Assays were performed using the human lung epithelial lung carcinoma line A549 (ATCC CCL-185), which was maintained at a low passage number in Dulbecco's Minimal Essential Media with 10% fetal bovine serum and 18 U/ml penicillin-streptomycin. 300 µg/ml of peptide was used for each experimental well and incubated 24 h before addition of MTT. 1% Triton-X is used as a positive control. Each experiment was performed in triplicate three times. A representative experiment is shown.

Results

Bioinformatics analysis

We sought to identify an active cathelicidin peptide *A. mississippiensis*. The partially annotated genome of *A. mississippiensis* has recently been released, and three

candidate genes have already been annotated as potential cathelicidins (**Table 10**), including two OH-CATH-like genes. However, none of these seemed to encode for the NA-CATH or OH-CATH analogs in the C-terminal predicted peptide sequence, despite their annotations (shown in **Table 10**). In a previous work (van Hoek, 2014), an examination of the full-length sequence of snake cathelicidin-like peptide precursors was undertaken. At least within this group, the active cathelicidin always represented the terminal 34 amino acids, preceded by a sequence containing a valine followed by K/R. The sequence upstream of the active peptide included the typical 4 cysteines of cathelin domains and a valine, followed by K/R, then the active cathelicidin peptide sequence.

Our approach was to begin with the sequence of the *Naja atra* cathelicidin (NA-CATH) and look for similar sequences in alligator genomic sequences. We identified a protein in *A. siamensis* (XP_006037273.1) that had a very similar sequence to the C-terminal end to NA-CATH. This gene is annotated as a cathelicidin-OH-like antimicrobial peptide in *A. siamensis*. The upstream sequence of this preproprotein contains the typical cathelin domain with four cysteines and a "VRR" sequence. Following this comes a 34 aa sequence "GLFKKLRRKIKKGFKKIFKRLPPVGVGVSIPLAGRR", which has the typical features of a cathelicidin in that it is highly charged as well as hydrophobic.

However, we only have access to biological samples from *A. mississippiensis* and not *A. siamensis*, so we sought to identify the same sequence in the American alligator whole genome shotgun sequences using BLAST. Following the approach used to identify fowlicidins in whole genome shotgun sequences (Y. Xiao et al., 2006), we searched *A.*

mississippiensis whole genome shotgun sequences (St John et al., 2012) and found a very similar nucleotide sequence gi|397225485|gb|AKHW01084514.1| (**Table 11**). Translation of this fragment of whole genome shotgun sequence through the ExPASy Translate Tool

Table 10. Genes from *A. mississippiensis* genome annotated as cathelicidins (van Hoek, 2014), and their predicted C-terminal peptides. Highlighted in grey is the possible C-terminal peptide sequence, either following the fourth CYs or the C-terminal 34 aa.

Gene Identifier	**Predicted protein sequence**	**Predicted peptide following the cathelin domain.**	**Net charge**	**Similarity to OH-CATH**
XM_006262429.1 cathelicidin-2-like	XP_006262491.1 MKSCWALVLLVGCMASAATAQSQLNFNEAVSLAVDFYNRGLAVNNTFQLLRTAPSGDVVSSPSEFRRLNFTIMETTCPVGSQPTQEXCQFKENGLVRACVGFFSTQQVAPLIVVTCEEAPSEPVRVTRWLWLLRGGLKAAGWGIRAHLNRNQ	APSEPVRVTRWLWLLRGGLKAAGWGIRAHLNRNQ (terminal 34 aa)	+5	No
XM_006262430.1 cathelicidin-OH antimicrobial peptide-like	XP_006262492.1 METCLCLLLLLGVATAVATAQAQAQAQTQSQSQAGYEDAVTTAVDIFNQESGLPQAYRLLEAEPQSEWNPSSQAAQPLKFSVKETVCPIAQKGNLKQCDFKENGLVKDCSGLFTAGKKPPVTAVKCEDTSQEGGRGQNSS ESPGLTLSQ	EDTSQEGGRGQNSSESPGLTLSQ (terminal aa after the last “c”)	-3	No
XM_006262431.1 cathelicidin-OH antimicrobial peptide-like	XP_006262493.1 MPKGTHGCFRLRETGTEAPALSSYQEALAAAVNTYNQESGLPQAYRLLEAEPQPQWDPSSQPAQPLKFSIKETECLVSEKRDVSQCPFKDK	EDAGQEPQLVKRVNWPKVGRTVLRFLPYIXGG (for charge calculations and alignment, replaced x	+2	No

	GLVKDCKGLYAEEKE PPVITAKCEDAGQEPQ LVKRVNWPKVGRTV LRFLPYIXGG	with a, terminal aa after the last "c").		

predicted a possible cathelicidin propeptide sequence (**Table 11**). From this propeptide sequence, and through homology to the *A. siamensis* cathelicidin sequences, we predicted a 36 aa cathelicidin peptide to begin "GFKKLRR…" (underlined in **Table 11**), which we call AM-CATH, keeping the OH-, BF-. NA-CATH naming convention. When the fully annotated genome of *A. mississippiensis* is published, the full gene sequence will hopefully be clarified. AM-CATH is very similar in length to the ~34-37aa cathelicidins of other higher vertebrates such as rabbits, cows, and pigs (**Table 12**). Since the enzyme that processes cathelicidin peptides in reptiles is unknown at this time, we are not able to confirm the *in vivo* expressed natural peptide.

We chose to synthesize a smaller fragment, AM-CATH28, as it is likely to represent the antimicrobial activity of both the longer (AM-CATH) and shorter (AM-CATH28) form, following the examples of NA-CATH, OH-CATH and BF-CATH. The location of AM-CATH28 in the full propeptide sequence can be seen in bold in **Table 11**. Based on the two Arg residues immediately prior to the start of this sequence, which are likely trypsin cleavage sites, AM-CATH28 could be to be a tryptic fragment of full AM-CATH, similar to fragmentation seen other cathelicidins such as LL-37 (Murakami, Lopez-Garcia, Braff, Dorschner, & Gallo, 2004).

However, we only have access to biological samples from *A. mississippiensis* and not *A. siamensis*, so we sought to identify the same sequence in the American alligator whole genome shotgun sequences using BLAST. Following the approach used to identify fowlicidins in whole genome shotgun sequences (Y. Xiao et al., 2006), we searched *A. mississippiensis* whole genome shotgun sequences (St John et al., 2012) and found a very similar nucleotide sequence gi|397225485|gb|AKHW01084514.1| (**Table 11**). Translation of this fragment of whole genome shotgun sequence through the ExPASy Translate Tool

Table 11. Identification of alligator cathelicidin genes. AS-CATH propeptide in *A. sinensis* and AM-CATH propeptide in *A. mississippiensis* whole genome shotgun sequence.

Gene	**Identifiers**	**Predicted protein sequence of cathelicidin propeptide.**
AS-CATH	XP_006037273.1 GI: 557328700 GeneID: 102379244	MQTCWVILLLPLLGAASTELPTPGTDPPQLTPTYAQALATAVDVY NQGPGVDFAFRLLEAESRDDWDASTDPLRQLEFTLKETE**C**PVGE DQPLDQ**C**DFKDGGAVLD**C**TGTFS**C**SEASLMVLVT**C**QPAEPLPDR **VRR**GLFKKLRR**KIKKGFKKIFKRLPPVGVGVSIPLAGRR**
AM-CATH	gi|561049244|ref|NW_006226102.1| gi|397285537|gb|AKHW01024462.1| gi|397225485|gb|AKHW01084514.1|	MGASLLQPDR**VRRGLFKKLRRKIKKGFKKIFKRLPPXGVGVSIPLAGKR** (x=v or i)

Using Heliquest program and the sequence listed in **Table 13**, a helical wheel projection of AM-CATH28, a smaller predicted cathelicidin-like peptide from *A. mississippiensis* was obtained (**Figure 12A**). From this analysis, it is predicted that this peptide has a hydrophobicity index (H) of 0.34, and hydrophobic moment (μH) of 0.341,

and a net charge of +9 (shown in **Table 13**). Overall, half of the amino acids are polar and half are non-polar. A hydrophobic face is predicted, composed of residues IVLFLG, agreeing with the prediction of amphipathicity for cathelicidin peptides. This peptide has three proline residues, which is relatively high for cathelicidin peptides.

Table 12. Known OH-CATH-like antimicrobial peptides. Identified in APD2 (G. Wang et al., 2009) and other databases.

Peptide name	**Sequence**	**Source Organism**	**APD Identifier**
OH-CATH (34 aa)	KRFKKFFKKLKNSVKKRAKKFFKKPRVIGVSIPF	*O. hannah* (Li, Lee, & Zhang, 2012; H. Zhao et al., 2008)	AP00895
Derivative: OH-CATH30 (30 aa)	KFFKKLKNSVKKRAKKFFKKPRVIGVSIPF		
BF-CATH (34 aa)	KRFKKFFKKLKKSVKKRAKKFFKKPRVIGVSIPF	*B. fasciatus* (Y. Wang et al., 2008)	AP00896
Derivative: BF-30 (30 aa)	KFFRKLKKSVKKRAKEFFKKPRVIGVSIPF		AP01239
NA-CATH (34 aa)	KRFKKFFKKLKNSVKKRAKKFFKKPKVIGVTFPF	*N. atra* (H. Zhao et al., 2008)	AP00897
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	*Homo sapiens*, *Pan troglodytes*	AP00310
PMAP-36	GRFRRLRKKTRKRLKKIGKVLKWIPPIVGSIPLGCG	*Sus scrofa*	AP00370
BMAP-27 Derivatives: BMAP-18 BMAP-15	GRFKRFRKKFKKLFKKLSPVIPLLHLG	*Bos taurus*	AP00366
SMAP-29	RGLRRLGRKIAHGVKKYGPTVLRIIRIAG	*Ovis aries* (Tack et al., 2002)	AP00155
Fowlicidin-1	RVKRVWPLVIRTVIAGYNLYRAIKKK	*Gallus gallus*	
Fowlicidin-2	LVQRGRFGRFLRKIRRFRPKVTITIQGSARF	*Gallus gallus*	
Fowlicidin-3	KRFWPLVPVAINTVAAGINLYKAIRRK	*Gallus gallus*	
HFIAP-1	GFFKKAWRKVKHAGRRVLDTAKGVGRHYVNNWLNRYR	Hagfish	AP00691
WAM1	KRGFGKKLRKRLKKFRNSIKKRLKNFNVVIPIPLPG	*Macropus eugenii*	AP01799
CAP18 (106-142)	GLRKRLRKFRNKIKEKLKKIGQKIQGFVPKLAPRTDY	*Oryctolagus cuniculus*	AP00418

AM-CATH36	GLFKKLRRKIKKGFKKIFKRLPPIGVGVSIPLAGKR	*A. mississippiensis*	
AM-CATH28	KIKKGFKKIFKRLPPIGVGVSIPLAGKR	*A. mississippiensis*	
AS-CATH36	GLFKKLRRKIKKGFKKIFKRLPPVGVGVSIPLAG	*A. sinensis*	

The helical wheel projection of the longer AM-CATH 36 aa peptide was also predicted. This peptide has a net charge <z> of +13, a very similar hydrophobic face and a similar balance of polar and non-polar residues. The helical wheel for this longer cathelicidin-like AM-CATH is shown in **Figure 12B**. The hydrophobic moment for AM-CATH28 is slightly higher than for AM-CATH (0.341 vs 0.313), indicating that AM-CATH28 has a stronger hydrophobic face and greater amphipathic character.

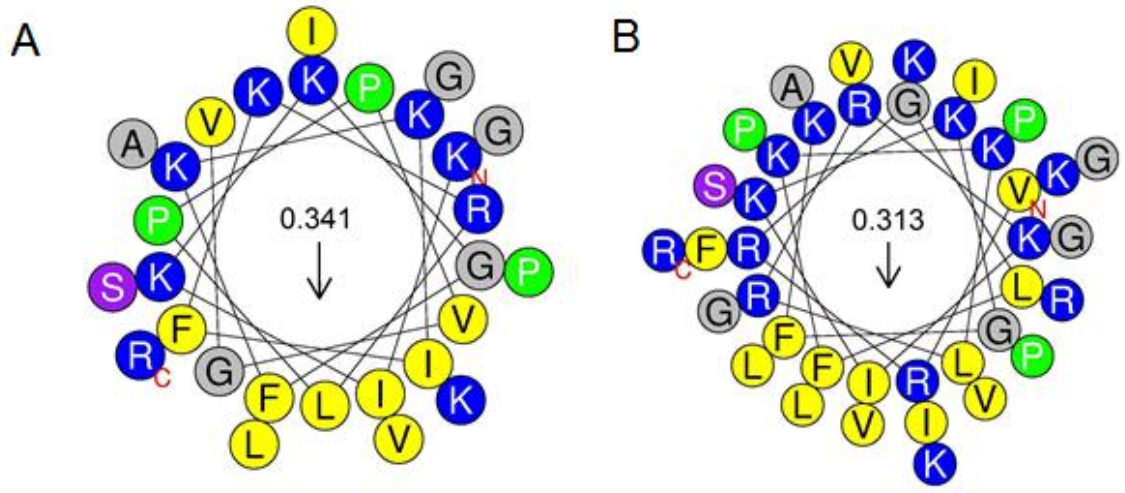

Figure 12. Helical wheels of A. AM-CATH28 and B. AM-CATH. Wheels produced by Heliquest (Gautier et al., 2008) and altered to display hydrophobic moment.

Table 13. Sequence and physico-chemical properties of AM-CATH28.

Peptide Name	Sequence	Molecular Weight (Da)	Hydrophobicity (H)	Charge
AM-CATH28	KIKKGFKKIFKRLPPIGVGVSIPLAGKR	3076.9	0.34	+9
NA-CATH	KRFKKFFKKLKNSVKKRAKKFFKKPKVIGVTFPF	4175.26	0.181	+15

Antimicrobial activity

AM-CATH28 was tested against drug sensitive and drug resistant bacteria. MICs were determined in cation-adjusted Mueller Hinton Broth (caMHB) and NB (**Table 14**). In general, AM-CATH28 had stronger activity in NB than caMHB. In NB, AM-CATH28 was strongly active against *P. aeruginosa* ATCC 9027 and BAA-2110 with MICs of 1.7 µM and 3.4 µM respectively, while in caMHB, the MIC against *P. aeruginosa* ATCC 9027 was 6.8 µM and higher than concentrations tested (>14 µM) against *P. aeruginosa* ATCC BAA-2110. AM-CATH28 was also strongly active against *A. baumannii* ATCC BAA-1794 in both NB (MIC=0.43 µM) and caMHB (3.4 µM). AM-CATH28 was not active in either broth at concentrations tested against *S. aureus* or *B. thailandensis.*

Table 14. Antimicrobial activity of AM-CATH28.

Bacterial strain	Peptide	MIC MHB (µg/ml)	MIC MHB (µM)	MIC NB (µg/ml)	MIC NB (µM)
A. baumannii ATCC BAA-1794	AM-CATH-28	10.5	3.4	1.3	0.43
	NA-CATH	10.5	2.5	5.1	1.2
P. aeruginosa ATCC 9027	AM-CATH-28	21	6.8	5.1	1.7
	NA-CATH	5.1	1.2	5.1	1.2

P. aeruginosa ATCC BAA-2110	AM-CATH-28	>40	>13	10.5	3.4
	NA-CATH	5.1	1.2	5.1	1.2
S. aureus ATCC 25923	AM-CATH-28	>40	>13	>40	>13
	NA-CATH	21	5.0	>40	>9.9
S. aureus ATCC 33592	AM-CATH-28	>40	> 13	>40	>13
	NA-CATH	10.5	2.5	>40	>9.9
B. thailandensis ATCC 70388	AM-CATH-28	>40	>13	>40	>13
	NA-CATH	>40	>9.9	>40	>9.9

Structural studies

The structure of AM-CATH28 was studied using circular dichroism. Many cathelicidins are helical, but some are random coil or beta sheet. The CD spectra of AM-CATH28 is shown in **Figure 13A**. This spectra shows a mixed structure. Though there are peaks around 208 and 222 nm, as is expected in an alpha-helical peptide, the signal at 222 nm is much weaker in both SDS and TFE. The 222:208 ratios is very low, around 0.5, indicating that there are also some random coil aspects to the structure. It seems likely that the two Pro residues at the 14^{th} and 15^{th} position interrupt the helicity of the peptide. When spectral values are analyzed by the technique developed by Raussens et al (Raussens et al., 2003), AM-CATH28 has mixed characteristics in both TFE and SDS. Shown in **Table 15**, α-helical characteristics are stronger in TFE (22.6% in TFE vs 11.9% in SDS), while β-sheet characteristics are stronger in SDS (20.2% in TFE vs. 24.1% in SDS), as is random coil (37.3 in TFE vs 40.3% in SDS).

To further predict the secondary structure of AM-CATH28, the amino acid sequence was entered into I-TASSER. I-TASSER provides images of likely structures, as

well as confidence scores as to how likely an amino acid is in a particular structure type. I-TASSER predicts that the aa 2-12 form an alpha helix with reasonably high confidence, while aa 19-24 form a beta sheet with much lower confidence. It is predicted that aa 13-18 are random coil, and it is likely that the two Pro residues at the midpoint create a hairpin turn. When only the aa predicted to be in the helix by I-TASSER are placed on a helical wheel, shown in **Figure 13B**, the helical portion of AM-CATH28 shows a strong amphipathic character and hydrophobic moment (0.802 μH). The ribbon model produced by I-TASSER, shown in **Figure 13C**, illustrates a probable structure of AM-CATH28 with a mixed structure, consistent with our CD results.

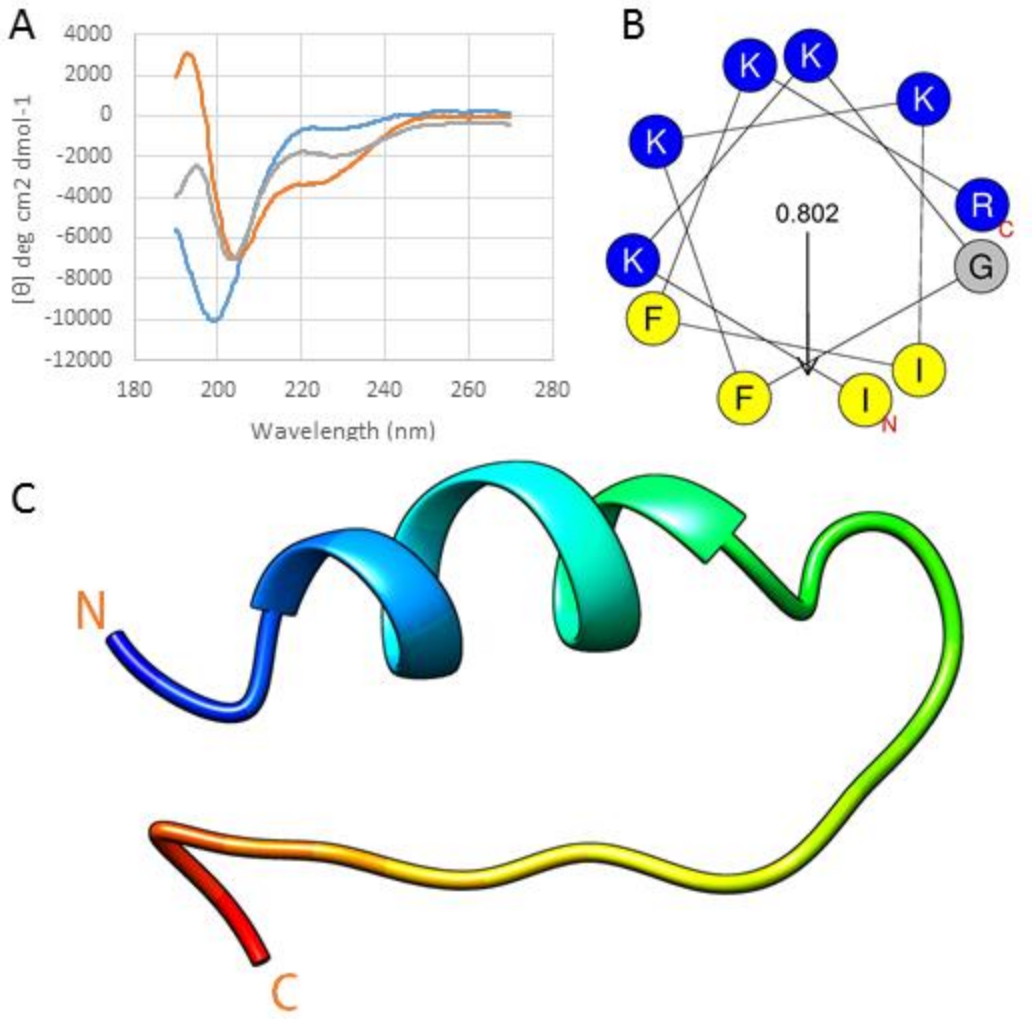

Figure 13. Structural prediction of AM-CATH28. A. CD spectra of AM-CATH28 in 10 mM phosphate buffer (----), 50% TFE (----), and 60 mM SDS (----). **B.** Helical wheel of aa 2-12 adapted from Heliquest (Gautier et al., 2008) with hydrophobic moment added. **C.** Structure predicted by iTASSER (J. Yang et al., 2015) and visualized by Chimera (Pettersen et al., 2004)

Table 15. Percent secondary structure contribution of AM-CATH28. Calculations are based on method by Raussens et al (Raussens et al., 2003).

	10 mM PB	**50% TFE**	**60 mM SDS**
α-helix	4.2%	22.6%	11.9%
β-sheet	34.8%	20.2%	24.1%

Turn	12.5%	12.5%	12.5%
Random	42.0%	37.3%	40.3%
Total	93.4%	92.7%	88.9%

Membrane permeabilization

To explore the mechanism of action of AM-CATH28, the ethidium bromide uptake assay was performed. If a peptide permeabilizes the bacterial membrane, ethidium bromide will enter the cell and bind DNA, causing an increase in fluorescence. Shown in **Figure 14**, when *E. coli* was exposed to 50 µg/ml AM-CATH28 for 20 min, fluorescence increased significantly ($p<0.001$), comparable to control NA-CATH, which has been shown to greatly permeabilize the membrane (M. Juba, Porter, Dean, Gillmor, & Bishop, 2013; M. L. Juba et al., 2015).

Cytotoxicity of AM-CATH28

To determine whether AM-CATH28 may be cytotoxic to mammalian cells, an MTT assay was performed using A549 human lung cells. After 24 h exposure to 300 µg/ml of AM-CATH28, cell survival levels were not significantly different from the untreated control (**Figure 15**).

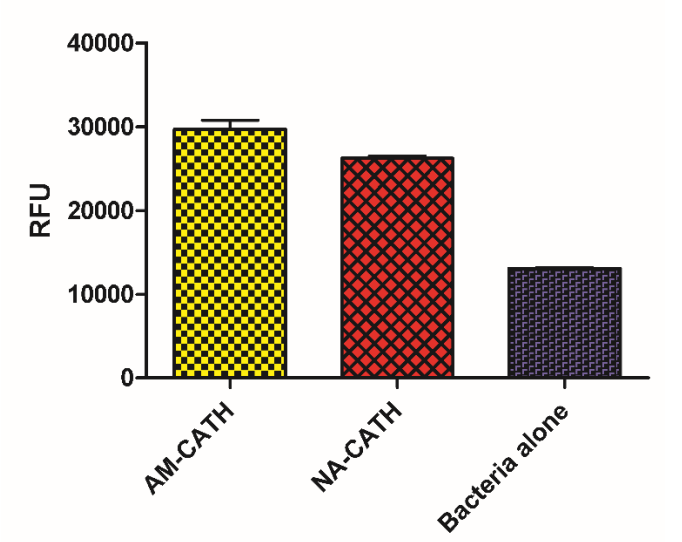

Figure 14. Membrane permeabilization activity of AM-CATH28. Assay was performed in 10 mM phosphate buffer on *E. coli* ATCC 51659. Bacteria were exposed to 50 µg/ml peptide for 20 min.

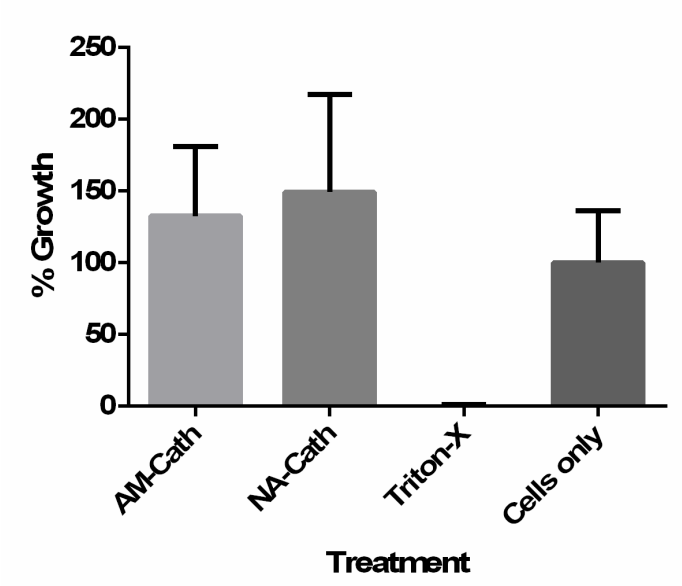

Figure 15. Cytotoxicity of AM-CATH28. A549 human lung epithelial cells were exposed to 300 µg/ml of peptide for 24 h and the MTT assay was then performed.

Discussion

Despite the close evolutionary linkage between avians and crocodilians, the identified alligator cathelicidin AM-CATH36 is not very similar to the identified chicken cathelicidin (fowlicidin) peptides. The most closely related peptide is the predicted OH-like cathelicidin in *A. sinensis*. Following that, the next most similar peptide is the Rabbit CAP18 peptide. This is very consistent with the observation that the active cathelicidin peptides are extremely sequence diverse, but structurally and biophysically homologous.

The C-terminal peptide of 28aa is highly charged (+9) and predicted to be helical, four properties consistent with the properties of known cathelicidins. This shorter version of the peptide is very similar to the OH-CATH30 (B. Y. Zhang, Li, Gao, & Shen, 2013; Y. Zhang et al., 2010) and BF-30 (Chen et al., 2011; H. Wang et al., 2013; H. Zhou et al., 2011).

This peptide is highly active against multi-drug resistant pathogens including MDR *A. baumannii* and MDR *P. aeruginosa*. A very interesting property of this peptide is its ability to demonstrate antibacterial activity in broth, indicating a resistance to high concentrations of salt. This property differentiates AM-CATH28 from the human cathelicidin peptide LL-37, for example, whose activity is highly salt-sensitive (Amer et al., 2010). AM-CATH28 showed greater activity in NB than caMHB. It is possible that this peptide is sensitive to the divalent cations that are added to caMHB, a trait seen in other CAMPs such as thanatin, an insect peptide (Wu et al., 2008). Further testing is needed to understand this effect.

AM-CATH28 was shown to permeabilize the membrane of *E. coli*. Several CAMPs, particularly in the cathelicidin class, lyse bacteria by interacting with the

membrane (M. Juba et al., 2013; M. L. Juba et al., 2015; Skerlavaj, Benincasa, Risso, Zanetti, & Gennaro, 1999). There have been two models proposed to explain this mechanism: the barrel-stave model, in which CAMPs aggregate or assemble near the bacterium and then become inserted into the membrane, creating a channel; and the carpet model, in which hydrophobic surfaces of CAMPs associate with the bacteria membrane and thin the membrane until pores are formed or the membrane is totally disrupted (Shai, 2002). Based on results produced in this work, neither model is more likely. SEM and peptide NMR would to be performed to gather more information.

In conclusion, AM-CATH28, mined from *A. mississippiensis* ESTs, is a possible cathelicidin in the American alligator. It has strong activity against Gram-negative bacteria, and is not highly cytotoxic towards mammalians cells. Due to this, AM-CATH28 could be a strong candidate for further improvements and *in vivo* testing, particularly against MDR *A. baumannii*, a pathogen deemed of urgent concern by the CDC (*Antibiotic Resistance Threats in the United States, 2013*, 2013). It is possible this CAMP plays an important role in the innate immune system of the American alligator, just as LL-37 does in humans. To determine this, we will continue work to analyze gene expression of AM-CATH in stimulated blood and live alligators and to isolate the native cathelicidin peptide.

CHAPTER 5: CONCLUSION

In this thesis, four novel CAMPs from the Siamese crocodile and American alligator were characterized. Little is known about the innate immune system of crocodilians, and very few crocodilian CAMPs have been discovered. Two groups have found CAMPs by fractionating the whole blood and white blood cell extracts of the Siamese crocodile, while another group found a CAMP by looking through ESTs in the NCBI database.

These approaches are technically difficult because the lack of available complete genomes, particularly for crocodilians, and more importantly, the lack of conservation at a nucleotide and amino acid level. However, we were able to find two CAMPs by searching ESTs in the NCBI database In the case of the Siamese crocodile hepcidin, the EST was deposited as a probable hepcidin by a group exploring genetic conservation in the animal kingdom. Because of the conserved pattern of Cys residues, hepcidins are an easier class of CAMP to identify. Cathelicidins are a more diverse class of CAMP, and access to encoding sequences from the propeptide are required to identify cathelicidins from ESTs. To find AM-CATH28, we identified a possible cathelin from *A. sinensis* while using the BLAST tool to look for sequences homologous to the cathelicidin gene from *O. hannah*, OH-CATH. From this, we used the BLAST tool to find an American alligator EST in the NCBI database that is homologous to that Chinese alligator

nucleotide sequence. These types of discoveries will become less difficult as genomes from more crocodilians are released, but currently limited genomic information is available.

The other two CAMPs was discovered using a bioprospecting process, in which negatively baited microparticles were used to harvest small cationic peptides, and after elution were subject to ETD mass spectrometry and subsequent *de novo* sequencing. Our initial research discussed a number of cationic peptides discovered using this process, some of which had strong antimicrobial activity. Two of these peptides were Apo5 and Apo6. This process is ideal for discovery in cases where the entire genome of a species is not known or the species is too small to gather large amounts of biological sample from without sacrificing the animal.

The Siamese crocodile hepcidin, AM-CATH, and the Apo derivatives seem to serve very different biological purposes and have very different characteristics. The Siamese crocodile hepcidin has 8 cysteines in a complex bonding pattern with a beta-sheet secondary structure. Siamese crocodile hepcidin is primarily a host defense peptide, though it does have some weak antimicrobial activity. In fish and mammals, hepcidins regulate the amount of iron in the blood. If PAMPs signal an immune response in the body, hepcidin production is upregulated and the concentration of extracellular iron is lowered, effectively "starving" the pathogen of this necessary metal (Ganz, 2002).

AM-CATH28 is a peptide with mixed structure: the N-terminal end is likely α-helical and the C-terminus is probably unstructured after a Pro hinge. AM-CATH28 has very strong antimicrobial activity, even in caMHB and NB. While some other

cathelicidins have shown strong activity in broth, such as NA-CATH and SMAP-29 from sheep (Blower, Barksdale, & van Hoek, 2015; Dean et al., 2011a, 2011b; M. Juba et al., 2013; M. L. Juba et al., 2015; Skerlavaj et al., 1999), LL-37 does not (Dean et al., 2011a, 2011b). LL-37, the human cathelicidin, is known to have a variety of immunomodulatory effects, such as increasing granulocyte migration. No research has been published about the role of cathelicidins in crocodilians or any possible immunomodulatory effect. Since cathelicidins are a conserved class of CAMPs, it is possible that AM-CATH has a similar role in host defense and controlling the immune system that LL-37 does. However, it has also been theorized that cathelicidins with stronger antimicrobial effect, such as AM-CATH, are less likely to aggregate and affect the immune system (Morgera et al., 2009).

Apo5 and Apo6 are nested peptides cleaved from an apolipoprotein. These peptides are highly α-helical, much like the parent protein. Though Apo5 and Apo6 are not active in broth, they are strongly active in phosphate buffer against a variety of bacteria, including MDR strains such as MDR *A. baumannii* and MRSA. It has been known that whole apolipoproteins have antimicrobial activity, as do synthetic derivations of the whole protein. Fragmentation of apolipoproteins in response to immune stimulation has not been discussed in literature, though this does happen in the case of other AMPs, such as LL-37 (Yamasaki et al., 2006). Apolipoproteins make up a large portion of circulating blood proteins, and it is possible that this fragmentation and antimicrobial activity is an important part of the innate immune system of crocodilians.

Many crocodilian species have been or are currently threatened with extinction due to overhunting, pollution, and habitat loss. These species play important roles in their

habitats, and many of them also have great value to our economy due to demand for their meat and skin. Learning more about their immune systems will allow for stronger conservation efforts and hopefully recovered populations in the most threatened species. In addition, because crocodilians are thought to be resistant to many bacterial infections, which could be partially due to CAMPs, they are an excellent source for the discovery of novel CAMPs. CAMPs from these species are shown in this work to have strong activity against MDR human pathogens, which are of great concern to the medical community. These CAMPs, in particular AM-CATH28, show great potential for a clinical product to use against these dangerous resistant pathogens.

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BIOGRAPHY

Stephanie Barksdale graduated from Potomac Falls High School in 2002. She received an A.A. in Liberal Arts from Northern Virginia Community College in 2009, and a B.S. in Biology from George Mason University in 2012. She has worked as a microbiology technician at George Mason University for three years while pursuing her M.S. in Biology.