ABSTRACT

MURJI, AMYN ALY. Transcriptional Response of Zebrafish NK-lysin-like Genes to Infection. (Under the direction of Jeffrey Yoder).

Antibiotics have been used to treat infected patients over the last seventy years. While these drugs have mitigated mortality rates, misuse or prolonged use has led to an uprising of pathogens that have adapted to these drugs, making them increasingly more challenging to treat. For example, panaceas like penicillin were observed to be largely ineffective to strains of *Streptococcus pneumoniae* as early as the 1990s (Song et al., 1999). The emergence of antibiotic-resistant bacteria has created a demand for novel therapeutics, and with this demand comes renewed interest in antimicrobial peptides (AMPs), which are an integral part of the innate immune system and have been found in organisms from bacteria and plants to animals. Their range of killing activity includes not only bacteria, but also viruses, parasites, fungi and tumor cells.

NK-lysin is a membrane-disrupting AMP that was identified two decades ago in the pig intestine (Andersson et al., 1995; Ruysschaert et al., 1998). The pig NK-lysin is expressed in NK cells and cytotoxic T cells and upon immune activation, transcript levels of this gene were observed to increase dramatically (Andersson et al., 1995). In 2004, Yoder reported the first NK-lysin-like cDNA in zebrafish which, nearly a decade later, was characterized along with three other NK-lysin peptides (Pereiro et al., 2015; Yoder, 2004).

While Pereiro et al. characterized the transcriptional response of zebrafish NK-lysin genes to infection by spring viraemia of carp virus, I examined the transcriptional response of zebrafish NK-lysin genes to bacterial infection with the fish pathogen, *Streptococcus iniae*. Transcript levels of NK-lysin genes varied from tissue to tissue and across time points, suggesting their responses reflect a degree of specificity.
Transcriptional Response of Zebrafish NK-lysin-like Genes to Infection.

by
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A thesis submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the degree of Master of Science in Physiology

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DEDICATION

To my mother and father without whom I would not have the privilege of a first-rate education.
BIOGRAPHY

Amyn Aly Murji was born and raised in Nairobi, Kenya with his sister, Shaila, and their parents, Aly and Shelina Murji. His parents’ search for better education has always ruled foremost in their lives, and as a result the Murjis eventually found themselves in Charlotte, North Carolina. After graduating from high school in 2009, Amyn continued his education at the University of North Carolina at Chapel Hill, where he majored in biology with a double minor in chemistry and Islamic studies. While at UNC Chapel Hill, he received the unique opportunity to work for a small pharmaceutical company, Vascular Pharmaceuticals, Inc., where he cultivated his passion for research and drug development under Dr. Laura Maile. Her tutelage opened his mind to a career in industry and he went on to pursue a Master’s degree at North Carolina State University under the direction of Dr. Jeffrey Yoder. Though Amyn is still fiercely loyal to the Tar Heels and enjoys watching their college basketball games, he has come to love the outdoor activities NC State has to offer such as kayaking on Lake Johnson. After completing his Master’s degree Amyn intends on continuing his education further by pursuing a PhD at Vanderbilt University.
ACKNOWLEDGMENTS

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My interest in research began at Vascular Pharmaceuticals, Inc. under Dr. Laura Maile. Her tutelage and guidance inspired me to receive my Master’s Degree and, soon, my PhD. Thank you so much for opening my mind to this beautiful and exciting field. Finally, I want to thank my friends and family for constantly supporting me and nurturing me into the man I am today. Specifically, I want to thank my mother and father who sacrificed so much so that I could receive this education. I also want to thank my sister for all her unconditional love and support. There is still a lot I can learn from her and I hope I make her proud.
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CHAPTER 1: Literature Review

The human body’s defense against microbial agents can be broken into two large categories – innate immunity, which is a broadly specific or general, immediate response, and adaptive immunity, in which the body’s response is continually being modified to efficiently combat particular targets. The cellular mediators of innate immunity – often called the first line of defense – include macrophages, neutrophils and NK cells, each equipped with unique mechanisms to kill target cells. Once activated, NK cells can interact with cytotoxic T cells in particular to release antimicrobial peptides that actively work against bacteria, viruses, parasites, fungi and even tumor cells.

ANTIMICROBIAL PEPTIDES

By culturing nasal secretions from a sick patient on an agar plate, Alexander Fleming showed that lysozyme presence in the mucus inhibits bacterial growth (Fleming, 1922). Though it has since then been discovered that the structure and enzymatic mechanism of action of lysozyme place it in a different category than antimicrobial peptides, Fleming’s discovery marks what is arguably the first discovery of a peptide with antimicrobial properties. Shortly thereafter, in 1929, Fleming set aside plates cultured with staphylococcus variants and discovered that over time, these colonies were undergoing cell lysis. He subsequently discovered that Penicillium – which had contaminated the plate – has bacteriolytic properties (Fleming, 1929). These findings pioneered the “Golden Age of Antibiotics.”
A decade later, true antimicrobial peptides were discovered in prokaryotes when R.J. Dubos isolated Gramicidins from soil-dwelling bacteria (Dubos, 1939a; Phoenix et al., 2013). It was shown that these peptides attack Gram-positive microorganisms, but have no activity against Gram-negative bacteria; furthermore, when these peptides are applied in vivo in murine models, treatment with the peptides protects against infection with virulent pneumococci (Dubos, 1939a, 1939b). In 1980, Hultmark et al. purified three peptides – P7, P9 A and P9 B – from immunized insect pupae. Of these, P7 closely resembles lysozyme whereas the other two were thought to represent a novel class of bacteriolytic proteins; all three possess killing and lytic activity against *Escherichia coli*. It is important to highlight that all three peptides do not kill bacteria universally and each has greater success with particular pathogens (Hultmark et al., 1980). Further study of the two P9 peptides showed that they share strong homology, indicating probable gene duplication. In addition, they both have a basic N-terminal region and hydrophobic central region, which may be integral in understanding their bactericidal mechanisms (Steiner et al., 1981). These peptides were the first to be categorized under the new class of antimicrobial peptides named cecropins, which contain α-helices and have the ability to adopt amphipathic structures to carry out their bactericidal activity (Fu et al., 2004; Steiner et al., 1981). Finally, what was considered the first animal antimicrobial peptide was isolated in 1962 when Kiss and Michl isolated Bombinin from the frog species *Bombina variegata* (Kiss and Michl, 1962; Phoenix et al., 2013).
Amphipathic, α-helical structures are one of a few different peptide structural motifs that dictate the function of antimicrobial peptides. These kinds of peptides were first isolated in the insect but have also been found in mammals and amphibians, suggesting their presence throughout the animal kingdom. These peptides are generally characterized by highly amphipathic helices with hydrophobic and positively-charged residues on the surface, thus allowing them to bind to negatively-charged peptidoglycan and liposaccharide structures. Additionally, these peptides – including cecropins and magainins – are synthesized as pre-pro-peptides and then activated through proteolysis.

Peptides can also be characterized by their cysteine density. Cysteine-rich peptides, first isolated from human neutrophil granules, bear resemblance to amphipathic, α-helical peptides in that they also are synthesized as pre-pro-peptides. The antimicrobial class of peptides known as defensins falls under this category, and is characterized by disulfide bridges that possess a particular fold. β-sheets are another common peptide structural motif. Often linked by disulfide bonds, β-sheets provide amphipathic character as well as structural stability to a peptide. Ultimately, the cationic character of antimicrobial peptides allows them to bind to pathogens and their amphipathic structure allows them to remain soluble in aqueous and lipid environments and thus insert themselves into a membrane (Hwang and Vogel, 1998). However, not all antimicrobial peptides fit neatly into these categories.

Human granulysin is a good example of an antimicrobial peptide that incorporates several of the aforementioned motifs. In 1987, a gene was discovered that was suggested to have a role in the growth and/or differentiation of T cells (Jongstra et al., 1987). It was later
discovered that gene 519, also known as NKG5, is expressed in natural killer cells and T cells in two forms, yielding a 15 kDa and a 9 kDa protein. The 519 protein displays lytic activity against tumor cells, Gram-positive and Gram-negative bacteria, parasites and fungi (Krensky, 2000; Peña et al., 1997). Because of its function and location in cytolytic granules, the peptide was named granulysin (Peña et al., 1997). Granulysin possesses conserved cysteine residues and amphipathic α-helices. It is a member of the saposin-like (SAPLIP) family despite having five cysteine residues, as opposed to the conserved six found in the family.

Natural killer-lysin (NK-lysin) is a peptide homologous to granulysin. Discovered in 1995, NK-lysin possesses structural motifs similar to granulysin and is believed to have antimicrobial properties (Andersson et al., 1995). This thesis details studies of NK-lysin homologues in zebrafish (Danio rerio) and findings are highlighted in Chapter 2.

**FULL-LENGTH NK-LYSINS**

**NK-lysin in Mammalian Species**

In 1995, a 78-residue, basic peptide with three intrachain disulfide bonds was isolated from the pig (Sus scrofa) small intestine and characterized (Andersson et al., 1995). A research group at the Karolinska Institutet identified antibacterial (E. coli) activity from protein fractions of thermostable intestinal polypeptides of the pig and resolved the antibacterial activity into two peptide components: NK-lysin and a smaller, less abundant version of NK-lysin lacking a glycine on the N-terminal end. (Andersson et al., 1995, 1996a).
It was observed that NK-lysin displays high antibacterial activity against both *E. coli* and *Bacillus megaterium*, and moderate activity against *Acinetobacter calcoaceticus* and *Streptococcus pyogenes*. In addition to its antibacterial activity, NK-lysin also displays antitumor activity against the YAC-1 mouse tumor cell line. Andersson et al. observed an increase in NK-lysin transcripts in an interleukin-2 (IL-2) activated population of T and NK cells. Additionally, a lymphocyte fraction isolated from porcine whole blood enriched in T and NK cells was incubated with or without IL-2, harvested after 6 days and subsequently tested for cytotoxic activity against YAC-1 cells. As expected, as the lymphocyte concentration increased, cytolytic activity increased until concentration was too high and cell lysis dropped off. Additionally, cells stimulated by IL-2 showed an increase in cell lysis activity when compared to unstimulated cells (Andersson et al., 1995).

Flow cytometric and immunohistochemical methods – both used to analyze the same lymphocyte fraction – showed that IL-2 incubation induced proliferation of T and NK cell populations that contained 90% CD2⁺, 6% CD4⁺, 75% CD8⁺ and 22% Nk1.1⁺ T and NK cells (Andersson et al., 1995). Immunostaining with anti-NK-lysin polyclonal and monoclonal antibodies directed against the CD2, CD4 and CD8 surface proteins demonstrate that NK-lysin is present in granules of CD2⁺, CD4⁺ and CD8⁺ cells. The presence of NK-lysin in these cells suggests that NK-lysin and other similar peptides are synthesized in cytotoxic T cells and likely in NK cells (Andersson et al., 1996a).

With regards to antibacterial activity, pig NK-lysin is active against Gram-positive and Gram-negative bacteria. As mentioned previously, NK-lysin displays high activity
against *E. coli* and *B. megaterium* and moderate activity against a porcine pathogenic strain of *E. coli*, *A. calcoaceticus* and *S. pyogenes*. NK-lysin is also effective against the fungal organism *Candida albicans*. Interestingly, NK-lysin displays no activity against *Salmonella*, a pathogen that is typically susceptible to NK cell activity. However, it also does not display activity against sheep red blood cells. Purified NK-lysin efficiently lyses YAC-1 cells under multiple experimental conditions resembling physiological conditions (Andersson et al., 1995).

NK-lysin’s efficacy against Gram-negative bacteria likely relies on its ability to bind lipid A, a conserved glycolipid region of lipopolysaccharides (LPS) that comprises the outer membranes of Gram-negative bacteria. LPS from *E. coli* binds NK-lysin in a dose-dependent manner on microtiter plates. Binding decreases if LPS is pre-incubated with (i.e. protected by) polymyxin B – an antibiotic to which it has high affinity – or if NK-lysin is pre-incubated with LPS or lipid A prior to being added to LPS on the microtiter plate. In addition, incubation of NK-lysin with LPS inhibits NK-lysin-mediated lysis of EL4 lymphoma cells in a dose-dependent manner. Similarly, incubation of NK-lysin with LPS inhibits LPS binding to granulocytes. The physical interaction between LPS and NK-lysin inhibits LPS-induced sepsis *in vivo* (Andersson et al., 1999).

Speculation about the structure of NK-lysin includes predicted amphipathic α-helices and β-sheets, supported by circular dichroism analyses (Hong et al., 2008). NMR spectroscopy (see Figure 1) verified that NK-lysin contains five amphipathic α-helices folded into a globular domain in an aqueous solution, with a hydrophobic core and hydrophilic
surface (Ruysschaert et al., 1998). More important to its function are the three intrachain disulfide bonds without which NK-lysin would be largely inactivated (Andersson et al., 1996b). Its membrane-binding activity, perhaps working in conjunction with perforin, is also key to its function as an effector molecule of the innate immune system. In an attempt to analyze its pore-forming capacity, NK-lysin was added to large unilamellar vesicles containing the dye calcein. As calcein release was observed, it was concluded that NK-lysin indeed disrupts the lipid bilayer. The electric current through a planar lipid bilayer was measured to verify that the observed behavior was general pore formation and not channel-forming activity. Integral to this finding is that Trp$^{58}$, the only tryptophan residue in NK-lysin, is generally externally exposed and has a fluorescence emission spectrum of 350 nm at the most. That value drops to 325 nm once incubated with asolectin large unilamellar vesicles (LUV), suggesting that Trp$^{58}$ enters a more hydrophobic environment (Ruysschaert et al., 1998).

The globular structure of NK-lysin contains arginine and lysine – both of which have positively-charged side chains – at the equator of the protein. Amino acids with negatively-charged side chains such as asparagine and glutamate reside in the upper half of the molecule, or the half ultimately exposed to the extracellular environment, and hydrophobic amino acids reside in the lower half of the molecule. The proposed mechanism of NK-lysin activity begins with the insertion of the hydrophobic bottom half into the lipid bilayer with the equatorial ring of arginine and lysine interacting with the lipid head groups and the negatively-charged top half protruding outside the cell. Later, the peptide may presumably
unfold and immerse itself deeper in the lipid bilayer. The folding of the peptide has been dubbed the “saposin fold,” and can be described as a long, centrally located helix surrounded by helices 2 and 3 opposing each other and helices 4 and 5 opposing each other (Liepinsh et al., 1997).

The mechanisms utilized by NK and cytotoxic T cells destroy target cells generally fall in two categories – apoptosis activation or perforin-mediated cell lysis. However, NK-lysin also has the ability to lyse NK-sensitive tumor cell lines by itself. Coupled with data which suggests its bacteria killing properties, NK-lysin may interact uniquely as part of the cytolytic mechanism. Its uniqueness is further substantiated by data displaying no NK-lysin lytic activity against *Salmonella*, which is unexpected because NK cells have robust activity against this pathogen. It is once again important to note that NK-lysin does not lyse sheep red blood cells, demonstrating its potential as a therapeutic *in vivo* (Andersson et al., 1995).

The relevance of NK-lysin to human biology lies in its similarity to granulysin (Andersson et al., 1996a). In fact, granulysin shares 35% identity and 66% similarity with NK-lysin (Peña et al., 1997). Furthermore, NK-lysin falls within a family of sequence-related proteins including saposins, surfactant-associated protein B, domains of human acid sphingomyelinase and acyloxyacylhydrolase, pore-forming amoeba proteins and aspartic proteinases in plants. It has therefore been included in the SAPLIP family (Wang et al., 2006). Its saposin fold is a common feature of all the peptides in the saposin family and in fact, NK-lysin was the first in the family to be characterized (Liepinsh et al., 1997).
Figure 1: NMR spectroscopy revealed the following structure for porcine NK-lysin revealing the saposin fold (Liepinsh et al., 1997).
Many researchers sought to identify and characterize homologs in other organisms as soon as the characterization and analysis of the porcine NK-lysin’s cytolytic properties were published. In 2004, the bovine (*Bos taurus*) homologue of granulysin and NK-lysin named Bo-lysin was discovered, which shares 74.6-74.8% nucleotide similarity with the porcine NK-lysin. Bo-lysin was detected in CD3+ , CD4+ , CD8+ and WC1+ γδ T cells but not in CD21+ B cells or CD14+ monocytes stimulated with lipopolysaccharide (Endsley et al., 2004).

Shortly thereafter, equine (*Equus caballus*) NK-lysin was identified and cloned (Davis et al., 2005). The equine homologue was found in CD4+ and CD8+ T cells and that gene expression for the equine NK-lysin was inducible following mononuclear cell stimulation with Concanavalin A. The sequence, which was compared to the bovine and porcine sequences, verified that the six cysteine residues are conserved between pig, cow and horse NK-lysins but that the first cysteine residue is absent in human granulysin. In fact, the equine homologue bears 67% identity with the porcine homologue and 53% identity with the bovine homologue. One feature that sets the equine NK-lysin apart from those discovered previously is its relatively positive charge. The equine NK-lysin has nearly double the number of lysine residues as porcine and bovine NK-lysin, and it seems as though these residues are crucial in disrupting a pathogen’s membrane (Davis et al., 2005; Wang et al., 2000).
NK-lys in Avian Species

NK-lys was identified in bird species, although with slightly different characteristics than mammalian NK-lysins. Ho et al. isolated a cDNA prepared from chicken (Gallus gallus) intestinal intraepithelial lymphocytes that has a deduced amino acid sequence that is less than 20% identical to mammalian NK-lysins. Using RT-PCR, it was determined that chicken NK-lys transcript levels are high in intestinal intraepithelial cells, moderate in splenic and peripheral blood lymphocytes and minimal in thymic and bursa lymphocytes after oral infection with either of three strains of coccidiosis-causing Eimeria parasites – Eimeria tenella, Eimeria acervulina and Eimeria maxima. It was further reported that NK-lys in the intestine is highest in the jejunum and lowest in the caeca. Different species of Eimeria acted upon different areas of the intestine. The kinetics of NK-lys expression for all three infections were shown to be biphasic, following the production patterns of parasite oocysts, leading to the suggestion that an increase in NK-lys transcript levels occurs when a pathogen reaches maximum infectivity. It is noteworthy that a full-length recombinant chicken NK-lys protein was expressed by and purified from mammalian cell culture (Ho et al., 2006).

In determining the cytotoxic activity of chicken NK-lys on bacteria and parasites, it was seen via an inhibition-zone assay that neither purified recombinant NK-lys nor unpurified recombinant NK-lys present in cell culture media are effective against Gram-positive or Gram-negative bacteria, but are effective against parasite strains E. acervulina and E. maxima (Hong et al., 2008). In contrast, Lee et al. discovered that a synthetically
derived peptide “corresponding to the mature intact chicken NK-lysin” possesses antibacterial properties (Lee et al., 2014). While Hong et al. utilized a simple inhibition-zone assay, Lee et al. exposed plated bacteria to serial dilutions of their peptides and then, using fluorescence microscopy, determined if the bacteria survived (Hong et al., 2008; Lee et al., 2014). In characterizing the cytolytic activity of chicken NK-lysin on an avian tumor cell line, cell culture medium containing recombinant NK-lysin was reported to inhibit uptake of $[^3$H] thymidine by LSCC-RP9 chicken lymphoid tumor cells, which marks the cytotoxic effect of NK-lysin on tumor cell viability (Ho et al., 2006). Chicken NK-lysin also has varied expression based on infection with viral pathogens. While the gene is upregulated upon infection with Marek’s Disease Virus, infectious bursal disease was observed to down-regulate NK-lysin transcription from 30- to 45-fold (Rauf et al., 2011; Sarson et al., 2008).

Though NK-lysin has not been explored much further in avian species, CjNKL was identified in the Japanese quail (Coturnix japonica), which has 81% identity with the chicken NK-lysin (Ishige et al., 2014).

**NK-lysin in Fish Species**

The search for NK-lysin homologues extended into fish species in 2004, when it was reported a NK-lysin-like cDNA from zebrafish had been sequenced (Yoder, 2004). However, the strong sequence identity observed between mammalian homologues is not conserved in fish. In 2005, a NK-lysin gene in channel catfish (Ictalurus punctatus) was characterized; less than 25% identity was observed between catfish and any other mammalian species, yet
the catfish protein possesses the six conserved cysteine residues that are characteristic of the SAPLIP family, barring granulysin (Wang et al., 2006). The NK-lysin gene structures were mapped for the first time in catfish, showing five exons and four introns. Channel catfish were challenged via immersion exposure to the Gram-negative fish pathogen *Edwardsiella ictaluri* and organs were harvested thereafter. NK-lysin gene RNA levels were initially reduced at four hours after challenge but increased three days after challenge. Over a seven-day period after the challenge, transcript levels continued to increase. The extent to which NK-lysin transcripts increased varied from tissue to tissue. NK-lysin was detected at high levels in gill, head kidney, intestine, spleen and trunk kidney tissues; at low levels in the skin and liver; and not at all in muscle tissue. High expression of NK-lysin in the gill was speculated to result from the particular mode of infection used – immersion into water containing the pathogen (Wang et al., 2006). While other organisms were observed to have only one NK-lysin gene, it was later discovered that channel catfish possess three (Pridgeon et al., 2012).

In 2006, NK-lysin was discovered and characterized in Japanese flounder (*Paralichthys olivaceus*). Similar to channel catfish, it holds little identity with the porcine, bovine, and equine homologs but displays 46% identity with zebrafish NK-lysin-like protein, containing six cysteine residues and consisting of five exons and four introns. While Andersson et al. induced expression of porcine NK-lysin with IL-2, Hirono et al. sought to induce flounder NK-lysin expression using lipopolysaccharide (LPS); however, expression
did not change with LPS induction. *In situ* hybridization showed that flounder NK-lysin is expressed in the same cells that express the CD8 gene (Hirono et al., 2007).

CsNKL1 is an NK-lysin discovered from the half-smooth tongue sole (*Cynoglossus semilaevis*). Tongue sole NK-lysin RNA expression varies in tissues in response to viral and bacterial infection. For example, CsNKL1 expression levels are low in the intestine but high in the spleen and head kidney, with maximum levels at 4 and 24 hours after infection with bacterial fish pathogen *Vibrio anguillarum*. To investigate the activity of CsNKL1, the peptide was overexpressed by intramuscularly injecting a plasmid expressing the peptide in adult tongue sole. The presence of the plasmid and expression of CsNKL1 both revealed that the plasmid is transported from the site of injection to other tissues, where the CsNKL1 gene is then transcribed. Overexpression of CsNKL1 led to the upregulation of IL-1 and IL-8 as well as other chemokines, indicating the induction of a pro-inflammatory response. When exposed to a pathogen, tongue sole that expressed recombinant CsNKL1 had significantly lower bacterial and viral loads in their tissues compared to control fish, suggesting that CsNKL1 enhances the immune response and/or is directly involved in killing the pathogen (Zhang et al., 2013).

Recently, an unprecedented set of four NK-lysins were described in zebrafish and their transcriptional response to viral infection was characterized (Pereiro et al., 2015). Further information regarding this finding is presented below.
PHARMACEUTICAL IMPLICATIONS OF NK-LYSINS

Full length (~78mer) NK-lysin peptides have been challenging to chemically synthesize, and efforts to pinpoint the peptide domains responsible for cytolytic activity have led to the syntheses and screening of shortened NK-lysin peptide fragments, each with varying degrees of cytotoxic activity. The smallest length necessary for activity has been explored, paving the way to a better understanding of how NK-lysin works and, more importantly, how these synthetic, shortened peptides could be utilized as therapeutics in the future.

To investigate the properties of the membrane-docking portion of the NK-lysin peptide, or more specifically, the predicted helix-loop-helix region defined by helices 2 and 3, 29- and 22-residue peptides with an internal disulfide bond were synthesized based on the porcine NK-lysin. The peptides were named NKLF1 and NKLF2, respectively, and are just as effective as NK-lysin against *B. megaterium* and *E. coli*. NKLF1 even has greater activity against *Psuedomonas aeruginosa* and *Staphylococcus aureus*. It was surmised that they both have similar mechanisms of killing because similar dose-dependent activity was observed between the synthetic and actual peptide. Furthermore, these peptide fragments are cytolytic against K562 tumor cells and inhibit the growth of *Mycobacterium tuberculosis*, thus verifying the importance of the loop region for anti-mycobacterial activity. (Andreu et al., 1999). Further emphasizing the importance of this loop region, shortened porcine and bovine NK-lysin along with shortened human granulysin were used to demonstrate that helices 2 and 3 display the most antibacterial activity, killing *E. coli*, *S. aureus*, *Salmonella enteritidis*, and
*Myobacterium bovis* BCG. Other protein helices, namely 4 and 5, do not display antimicrobial activity against the aforementioned pathogens (Endsley et al., 2004). The shortened, synthetic porcine NK-lysin known as NK-2 (~27mer) displays activity against *Trypanosoma cruzi*, a human parasite. NK-2, which represents helices 3 and 4 of pig NK-lysin, is not only active against extracellular *T. cruzi*, but it also prevents the replication of the parasite intracellularly without destroying the host cells (Brandenburg et al., 2010; Jacobs et al., 2003). NK-2 is a linear synthetic peptide bearing resemblance to cecropins and magainins (Jacobs et al., 2003). Yet another anti-tumor porcine peptide, NK-18, was synthesized. NK-18 (~18mer), also from the cationic core region of NK-lysin, was shown to decrease the viability of human bladder cancer cell lines (Yan et al., 2012).

Synthetic peptides were created from chicken NK-lysin (cNK-1, cNK-2, cNK-3 and cNK-4) to determine the mechanism of anti-parasitic function and whether it overlaps or corresponds with the helix-loop-helix region of helices 2 and 3. These peptides were tested for cytotoxic activity against *E. acervulina*; it was found that cNK-2, which corresponds most closely with NK-2 of the pig NK-lysin, holds the greatest activity (Lee et al., 2013). The cNK-2 peptide was tested for cytotoxic activity against *E. tenella* and it was concluded that cNK-2 decreases parasite viability in a dose-dependent manner. A single nucleotide polymorphism (SNP) from adenine to guanine, which alters the asparagine in position 29 to aspartate, was isolated in the coding sequence between breeds of chicken. The peptides resulting from the two alleles were given the designations N29N and N29D, referring to the amino acids that were altered. Interestingly, N29D was discovered to have a reduced helical
profile. To explore these variations, shortened peptides comprised of helices 2 and 3 were synthesized from each full-length variant; while both are cytotoxic to tumor cells and bacteria, N29N has greater antibacterial and antitumor activity than N29D (Lee et al., 2012).

In the Japanese flounder, a 27 amino acid peptide (JF-NK-2) homogenous to the pig NK-2 (P-NK-2) was synthesized. JF-NK-2 displays biological activity against *E. coli, Klebsiella pneumoniae, P. aeruginosa and Photobacterium damselae*. Interestingly, flounder NK-lysin does not seem to display antimicrobial activity against Gram-positive bacteria. As mentioned previously, the charge of the peptide is posited to be integral to its biological activity. Both JF-NK-2 and P-NK-2 have positive net charges of +4 and +10, respectively. Despite its positive charge, JF-NK-2 has little or no activity against Gram-positive cells as well as some Gram-negative cells, including *E. tarda* and *Serratia marcescens*. In comparison, P-NK-2 displays activity against all of these, suggesting that the Japanese flounder peptide may possess different cytotoxic mechanisms than P-NK-2. Finally, these short peptides do not lyse Japanese flounder erythrocytes, which parallels the lack of cytolytic activity of porcine NK-lysin on sheep red blood cells (Andersson et al., 1995; Hirono et al., 2007).

In essence, the difference of one amino acid has been shown to have varied effects on cytotoxicity and could therefore be exploited to optimize and/or fine tune future therapeutics. Furthermore, it was found that shortened peptides have, at times, more robust activity than their full-length counterparts and that the loop region containing helices 2 and 3 and regions including helices 3 and 4 are important for any antimicrobial activity. These peptides could
be synthesized and manipulated more efficiently and could someday prove useful in the future for combatting the rise of drug-resistant bacteria or cancer.

**ZEBRAFISH NK-LYSINS**

Zebras possess the largest number of NK-lysin genes of any organism characterized thus far. In 2015, four zebrafish NK-lysin genes were described on chromosome 17 (nkla, nklb, nklc, nkld). This includes a sequence (nkld) mentioned, but not described in detail, a decade earlier by Yoder (Pereiro et al., 2015; Yoder, 2004). The 2015 report details transcriptional response to viral infection, mRNA expression during ontogeny, NK-lysin presence in the kidney leukocyte population and mRNA expression in recombination activation gene 1 deficient (rag1^-/-) mutant zebrafish. In healthy zebrafish, nkla is found predominantly in the muscle, nklb in the intestine, nklc in the spleen and nkld “showed less tissue-specificity” but has higher prevalence in gill, kidney and spleen tissue. In the kidney leukocyte population, the highest levels of NK-lysins are found in leukocyte precursor cells. The next highest level of NK-lysin transcripts is found in lymphocytes and the lowest levels of NK-lysins are found in myeloid cells (Pereiro et al., 2015). The rag1^-/- mutant zebrafish lack functional immunoglobulin and T cell receptors and therefore do not have mature B and T lymphocytes. Consequently, the innate immunity – including NK-like cells and non-specific cytotoxic cells – would hypothetically be the main source of NK-lysin. Interestingly, transcript levels of all zebrafish NK-lysins, with the exception of nkla, are higher in rag1^-/- zebrafish. Overall, rag1^-/- mutants exhibit more than double the total NK-
lysin mRNA when compared to wild-type zebrafish, with nkla displaying significantly lower expression, suggesting that cytotoxic T lymphocytes may be responsible for nkla production and NK-like cells for the other three NK-lysins. During ontogeny, it was discovered that while nkla transcription levels increased during the duration from one day post-fertilization (dpf) through twenty-nine dpf, nkld levels decreased, with the highest transcript levels recorded one dpf. This discovery is noteworthy because it is well known that cell-mediated and humoral immunity in zebrafish is fully competent several weeks after hatching and nonfunctional during early larval stages. Both nklb and nklc display fluctuations in transcript levels (Pereiro et al., 2015).

Finally, zebrafish at different stages of life were challenged with spring viraemia of carp virus (SVCV). In larvae 3 dpf, mRNA levels of NK-lysin did not significantly change 24 hours post-challenge. Only nkla and nkld significantly increased based on kidney samples from adult zebrafish. Ultimately, the presence of more than one NK-lysin gene could indicate a level of specificity to a particular pathogen and/or preferential expression of these genes by particular cell types (Pereiro et al., 2015).
Figure 2: Comparison of Human Granulysin and NK-lysin Homologues across Species. Conserved residues are shaded in black. Residues that share similarity are shaded in light gray. Conserved cysteine groups are highlighted in red to denote that their conservation spans all species except human granulysin which lacks one cysteine. Hosa refers to Homo sapiens (human), Cyse to Cynoglossus semilaevis (tongue sole), Paol to Paralichthys olivaceus (Japanese flounder), Dare to Danio rerio (zebrafish), Icpu to Ictalurus punctatus (channel catfish), Gaga to Gallus (chicken), Coja to Coturnix japonica (Japanese quail), Bota to Bos taurus (cow), Susc to Sus scrofa (pig) and Eqca to Equus caballus (horse).
CHAPTER 2: Transcriptional Response of Zebrafish NK-lysin-like Genes to Infection.

ABSTRACT

Though it was shown that the transcript level of porcine NK-lysin increases in cytotoxic lymphocytes after immune activation, it was unknown whether transcripts of zebrafish NK-lysin genes similarly increase after bacterial infection. To test NK-lysin transcriptional response to a bacterial infection, zebrafish were infected with *Streptococcus iniae* and multiple organs were harvested 2, 4, 12, 24 and 36 hours post-infection. After RNA isolation and reverse transcription, qRT-PCR was performed to determine fold-change of transcripts. As a model bacterial pathogen, it was hypothesized that infection with *S. iniae* would increase transcript levels of zebrafish NK-lysin genes over the course of the infection. Transcript levels of the four published zebrafish NK-lysin genes – *nkla, nklb, nklc, and nkld* – as well as a fifth zebrafish NK-lysin sequence, *nklc-like (nklcl)*, which has been identified, but is currently unplaced in the reference genome, were examined. Transcript levels of each NK-lysin both increased and decreased in all tissue types over the course of infection, with the greatest number of increases occurring at 2 hours post-infection and the greatest number of decreases occurring at 12 hours post-infection.
METHODS

Animals

Zebrafish were purchased from EkkWill Wildlife Resources in Ruskin, Florida and maintained in a recirculating aquarium system at 28º C. All experiments involving live zebrafish were performed in accordance with relevant institutional and national guidelines and regulations, and were approved by the North Carolina State University Institutional Animal Care and Use Committee.

Streptococci Preparation

The *Streptococcus iniae* strain 9117 was generously provided by the laboratory of Dr. Melody Neely at Wayne State University School of Medicine. *S. iniae* was grown overnight in 10 mL of Todd-Hewitt medium supplemented with 2% peptone (TP) in a 15 mL conical tube without shaking for approximately 14 hours at 37º C. The culture was vortexed to resuspend the bacteria and diluted 1:100 into a new tube with 10 mL of fresh TP. The culture was grown to mid-log phase and 1 mL was removed, pelleted by centrifugation and resuspended in 1 mL fresh TP for infecting zebrafish.

Bacterial Infection

Sixty adult zebrafish were anesthetized with Tris-buffered tricaine methanosulfonate (168 µg/ml). After 2-3 minutes of exposure the fish were stationary and failed to maintain buoyancy. For each fish, approximately 10 µL of $3 \times 10^7$ CFU/mL of the bacterial
suspension was injected into the dorsal muscle at a 45° angle anterior and lateral to the dorsal fin. The sterile needle was held in the fish briefly to prevent the bacterial suspension from welling up from the injection site. Following injection, zebrafish were placed in a beaker with fresh water from the zebrafish holding facility. Sixty additional zebrafish were mock-injected with 10 μL of TP media at the same time, and following the same protocol. All fish were monitored until recovered from anesthetic. Fish were then maintained at 28º C and subsequently euthanized for dissection at 2, 4, 12, 24 and 36 hours post-infection. For RNA extraction, tissues (gill, intestine, kidney and spleen) were harvested from ten zebrafish per time point each from the infected and mock-infected fish. Despite including ten fish, only six fish survived in each of the bacteria-infected 24 and 36 hour time points and thus, four mock-injected fish from each time point were removed from the study in order to maintain the correct number of control individuals (Phelps and Neely, 2010).

Reverse Transcriptase – Quantitative Polymerase Chain Reaction (qRT-PCR)

Zebrafish tissues were homogenized using TRIzol® reagent (Life Technologies) and RNA was isolated and recovered in 10 μL water (RNeasy mini kit, Qiagen). A Nanodrop (ND-1000 Spectrophotometer) was used to determine RNA quantity and a Bioanalyzer (Agilent 2100 Bioanalyzer) determined quality (See Table 1). One μg RNA from each sample was reverse transcribed (SuperScript® III First-Strand System, Life Technologies) and diluted to a final volume of 100 μL. One μL cDNA was used with TaqMan® primer/probe sets (Life Technologies) designed to detect nklα, nklb, nklc, nklcl, nklδ and ef1α
in 20 μL quantitative PCR reactions (Bio-Rad MyiQ Thermal Cycler). Reactions were performed in triplicate. Reactions lacking cDNA were employed as negative controls. Thermal cycling parameters included: an incubation at 95°C for ten minutes followed by 50 cycles of 95°C for 15 seconds and 60°C annealing temperature for one minute. The infection-induced change in transcript levels of each NK-lysin was calculated with the $2^{-ΔΔC_t}$ method, normalizing to transcript levels of $ef1α$ (Livak and Schmittgen, 2001).

**RESULTS**

*S. iniae*-induced changes in transcript levels of *nkla*

After infection with *S. iniae*, transcript levels of *nkla* fluctuated in a similar pattern across all immune tissues sampled (gill, intestine, kidney and spleen). Increases in *nkla* transcript levels occurred only at 2, 4 and 24 hours post-infection (hpi). Transcript levels of *nkla* in spleen displayed the largest increases, with 16- and 98-fold increases at 2 and 24 hpi, respectively, while transcript levels in intestine displayed an overall smaller magnitude in fold change at all time points. Transcript levels of *nkla* in gill decreased the most at 12 hpi (22-fold decrease) and increased the most at 24 hpi (14-fold increase). The largest decrease in *nkla* transcript levels across all tissue types (67-fold decrease) was observed in kidney at 12 hpi (Figure 3).
**S. iniae-induced changes in transcript levels of nklb**

The pattern observed for changes in post-infection *nkla* transcript levels was not observed for *nklb*. Changes in *nklb* transcript levels in spleen and gill were the most similar to those observed for *nkla*, with a negative fold-change observed only at 12 hpi. Changes observed in kidney primarily reflected decreased *nklb* transcript levels, with only a minor increase (2-fold increase) or no change at all at 2 and 24 hpi, respectively (Figure 4). Positive fold-changes were observed for *nklb* in intestine only at 2, 4 and 12 hpi. The greatest decrease and increase in transcript levels of *nklb* paralleled those of *nkla*, with greatest magnitudes at 12 hpi in kidney (-11-fold) and 24 hpi in spleen (21-fold), respectively.

**S. iniae-induced changes in transcript levels of nklc**

Infection-induced changes in *nklc* transcript levels in gill were not dramatic, with changes ranging from -2-fold to 3-fold. In general, transcript levels of *nklc* in intestine decreased (-7-fold to -13-fold) with the exception of no change at 2 hpi. In kidney and spleen, *nklc* transcript levels were increased only at 2 and 4 hpi. The 12 hpi kidney displayed the largest negative change value (-20-fold) but the largest positive fold-change value (11-fold) was observed in the 2 hpi spleen (Figure 5).

**S. iniae-induced changes in transcript levels of nklcl**

Despite a similarity in sequence (not shown), the overall changes in transcript levels of *nklcl* after infection reflected those of *nklc* only in intestine. In gill, *nklcl* transcript levels increased
at 2 and 24 hpi with 8-fold and 18-fold changes, respectively. In kidney and spleen, the only decrease in *nklc1* was observed at 12 hpi. Gill, kidney and spleen all displayed consistent increases of *nklc1* at 2 and 24 hpi. Interestingly, the largest positive and negative fold-changes were observed in spleen at 2 and 12 hpi, respectively (Figure 6).

*S. iniae*-induced changes in transcript levels of *nkld*

The only observable increase in *nkld* transcripts in gill after infection was at 2 hpi (4-fold). Transcript levels in kidney and intestine followed a similar pattern, displaying increases in *nkld* transcript levels at 2 and 4 hpi and subsequent decreasing levels. Transcript levels of *nkld* in spleen increased at three time points – 2, 4 and 24 hpi. The greatest decrease in *nkld* transcript levels was observed in gill at 36 hpi (-128-fold), which was also the greatest decrease for all NK-lysins. The greatest increase of *nkld* levels (9-fold) was recorded from spleen at 24 hpi (Figure 7).
Table 1: NanoDrop values and RNA Integrity Number (RIN) values were obtained to verify RNA quality and quantity.

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<th>Sample Name</th>
<th>ng/ul</th>
<th>RIN</th>
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<td>2 Intestines-Media-2hrs</td>
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Figure 1: The transcriptional response of nkla in zebrafish to bacterial infection. Values were obtained via qRT-PCR following infection with S. iniae. Mock-infected fish were used to determine fold-change. The housekeeping gene ef1α was used to normalize values.
Figure 2: The transcriptional response of *nklb* in zebrafish to bacterial infection. Values were obtained via qRT-PCR following infection with *S. iniae*. Mock-infected fish were used to determine fold-change. The housekeeping gene *ef1α* was used to normalize values.
Figure 3: The transcriptional response of *nklc* in zebrafish to bacterial infection. Values were obtained via qRT-PCR following infection with *S. iniae*. Mock-infected fish were used to determine fold-change. The housekeeping gene *ef1α* was used to normalize values.
Figure 4: The transcriptional response of nklcl in zebrafish to bacterial infection. Values were obtained via qRT-PCR following infection with S. iniae. Mock-infected fish were used to determine fold-change. The housekeeping gene efla was used to normalize values.
**Figure 5:** The transcriptional response of *nkld* in zebrafish to bacterial infection. Values were obtained via qRT-PCR following infection with *S. iniae*. Mock-infected fish were used to determine fold-change. The housekeeping gene *ef1α* was used to normalize values.
**DISCUSSION**

The work performed for this thesis builds upon a report by Pereiro et al. which characterized four NK-lysins in the zebrafish: *nkla, nklb, nklc* and *nkld* and includes a fifth *nklc-like* (*nklcl*) sequence. In an experiment exploring the transcriptional response of these genes to bacterial infection with *S. iniae*, it was found that responses varied at each time point and in each tissue. This parallels the findings of Pereiro et al., who observed that the transcript levels of zebrafish NK-lysins sometimes decreased after viral infection. Whereas *nkla* and *nkld* in kidney displayed the greatest increase after infection with spring viraemia of carp virus (Pereiro et al., 2015), these NK-lysins displayed differing responses to *S. iniae* infection. This may reflect NK-lysin specialization for different pathogens. The observed variations in post-infection transcript levels may also be explained by the amount of bacteria injected into the zebrafish. A published dose, $1 \times 10^7$ CFU/mL *S. iniae*, is typically lethal to zebrafish after three days; however, three times that dose was used to infect the zebrafish in this study, which may have had consequences, including transcriptional changes reflective of a more severe infection. It is important to highlight, however, that the observation of both increased and decreased levels of NK-lysin transcripts has been reported previously: While Bo-lysin transcript levels increased in peripheral blood mononuclear cells after activation with phorbol myristate acetate (PMA) and calcium ionophore, Bo-lysin transcript levels decreased in CD3+ lymphocytes after activation (Endsley et al., 2004). In addition, biphasic trends were observed in the transcriptional response of chicken NK-lysin after infection with parasite pathogens. According to Hong et al., the kinetics leading to increases and decreases
of chicken NK-lysin transcript levels coincide with parasite oocyst production, thus suggesting that transcription levels increase when more pathogens are present (Ho et al., 2006). Once *S. iniae* was injected intramuscularly in zebrafish, the pathogen may have travelled to surrounding tissues and induced transcription of the NK-lysin genes. This could have led to variations in NK-lysin response, as some tissues may have been exposed to the pathogen at different times. Additionally, inflammation of the muscle tissue at the injection site as well as antigen presentation could signal the recruitment of lymphocytes and transcription of NK-lysin genes from distal tissues as part of the adaptive immune response.
Chapter 3: Future Directions

Data detailing the transcriptional responses of zebrafish NK-lysin to a viral and a bacterial infection are now available; therefore, it will be important to conduct further studies testing the natural functions of NK-lysin in immune response and their potential therapeutic value. In order to provide a more comprehensive survey of which pathogens lead to changes in transcript levels of zebrafish NK-lysin, similar studies could evaluate the transcriptional responses of NK-lysin to additional bacteria, viruses, parasites and fungi.

Recombinant NK-lysin have recently been synthesized from chicken and pig resulting in structural and functional data (Ho et al., 2006; Jacobs et al., 2003). The expression of full-length, recombinant zebrafish NK-lysin would provide a means for evaluating zebrafish NK-lysin structures and mechanisms of action. If full-length NK-lysin peptides are produced and purified, biological activity assays could be performed to determine their effectivity on different pathogens, particularly drug resistant pathogens. Shortened peptide fragments derived from each zebrafish NK-lysin could also be synthesized to identify and isolate functional domains of NK-lysin. Shorter peptides can be chemically synthesized and are potentially more viable as future therapeutics. The engineering of peptide sequences could then be applied to optimize biological activity of the full-length and/or shortened peptides.

*In vivo* studies could also provide information regarding the role of NK-lysin genes during an infection. With the advent of CRISPR/Cas9 technology for zebrafish genome engineering, disrupting NK-lysin genes would result in NK-lysin knockout zebrafish (Hwang
et al., 2013). Breeding and subsequently infecting NK-lysin knockout zebrafish would provide a mechanism for defining their normal biological roles in immune response. By infecting knockout zebrafish with non-lethal doses of a variety of pathogens, percent mortality assays could be conducted to determine if absence of a specific NK-lysin leads to immunodeficiency. Furthermore, tissue samples from knockout zebrafish could be examined histopathologically to evaluate bacterial load and inflammation after infection. Infected tissues could also be homogenized and then plated to quantify bacterial concentration and viability. Finally, knockout zebrafish could be exposed to carcinogenic chemicals and monitored to quantify tumor incidence in order to determine if NK-lysins play a role in targeting tumor cells.
REFERENCES


