Faculty of Science

DETERMINING ANTIMICROBIAL SUSCEPTIBILITY AND HEMOLYTIC ACTIVITY OF INDOLICIDIN DERIVATIVES

2024 | ALIVIA LYN MONET MERCER-BRUNELLE

B.Sc. HONOURS THESIS – CHEMICAL BIOLOGY

THOMPSON RIVERS UNIVERSITY

DETERMINING ANTIMICROBIAL SUSCEPTIBILITY AND HEMOLYTIC ACTIVITY OF INDOLICIDIN DERIVATIVES

by

ALIVIA LYN MONET MERCER-BRUNELLE

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF

BACHELOR OF SCIENCE (HONS.)
in the
DEPARTMENTS OF BIOLOGICAL
AND PHYSICAL SCIENCES
(Chemical Biology)

This thesis has been accepted as conforming to the required standards by:
Heidi Huttunen-Hennelly (Ph.D.), Thesis Supervisor, Dept. Physical Sciences
Naowarat (Ann) Cheeptham (Ph.D.), Secondary Supervisor, Dept. Biological Sciences
Mark Rakobowchuk (Ph.D.), External Examiner, Dept. Biological Sciences

Dated this 24th day of April, 2024, in Kamloops, British Columbia, Canada

ABSTRACT

Antibiotic-resistant microorganisms have posed significant challenges to the development of novel antimicrobial agents. Antibiotic-resistant bacteria are increasingly being detected in humans and animals treated with antibiotics, making commercially available antibiotics less effective than they once were. Microbial compounds from various species are being studied extensively for potentially better and more effective antibacterial properties that may be used as an alternative. Antimicrobial peptides (AMPs) appear to be potential antibacterial medication candidates; however, the development of AMPs is impeded by their cytotoxicity. In the current study, indolicidin, a known antimicrobial originally isolated from bovine neutrophils, was modified by changing its tryptophan content. We predicted that replacing tryptophan with a less hydrophobic amino acid, alanine, would minimize hemolytic activity and maximize bioactivity. To address this question, we analyzed four indolicidin derivatives in which two of its tryptophan residues were replaced with alanine at different positions. We assessed their antibacterial and antifungal activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Candida albicans*, *Staphylococcus aureus*, and Methicillin-Resistant *Staphylococcus aureus* (MRSA). Antimicrobial testing identified two active derivatives with minimum inhibitory concentrations in the 250-500 $\mu g\ mL^{-1}$ range against all microbes, except *Pseudomonas aeruginosa*. Notably, the substitution of tryptophan residues at position 4 significantly affected antimicrobial activity. All derivatives exhibited a substantial decrease or complete removal of hemolytic activity at concentrations above their minimum inhibitory concentration (MIC). Additionally, derivatives containing the Proline-Tryptophan-Tryptophan-Proline (PWWP) domain showed DNA-binding capability, while those lacking this domain did not. These findings highlight the potential of modified indolicidin

derivatives as effective and less cytotoxic antimicrobial agents, offering a potential solution for combating antibiotic resistance.

Thesis Supervisor: Associate Professor Heidi Huttunen-Hennelly

ACKNOWLEDGEMENTS

First off, I would like to express my deepest appreciation to my thesis supervisor, Dr. Heidi Huttunen-Hennelly, for her unequivocal support throughout my research journey. Her expertise, teaching philosophy, and support will be something I remember as I continue my journey as a student outside of Thompson Rivers University. I would also like to thank Dr. Naowarat Cheeptham for access to her laboratory and its resources and for her knowledge and feedback. I am grateful to Dr. Mark Rakobowchuk for helping me with my research, being a source of knowledge with my project and for taking the time to read and provide feedback on my thesis. I appreciate all the help from Dr. Donald Nelson for my DNA binding assay. My sincere appreciation goes to the TRU UREAP for funding my research journey. Kathy Baethke has been instrumental in teaching me about all the equipment and has helped me gain confidence in the lab. Lastly, I want to recognize Ethan Furlong as my partner for this project. His support has been appreciated in overcoming challenges, celebrating successes, and advancing our research as a team.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Abbreviation	Meaning
AMPs	Antimicrobial peptides
MIC	Minimum inhibitory concentration
MHC	Minimum hemolytic concentration
TBE	Tris/Borate/EDTA
RBCs	Red blood cells
TA	Teichoic acid
EMSA	Electrophoretic mobility shift assay
PWWP	Proline-Tryptophan-Tryptophan-Proline
MSW	Mutant selection window
HDGF	Hepatoma-derived growth factors
LPS	Lipopolysaccharides
PBS	Phosphate buffer saline
Trp	Tryptophan
Ala	Alanine
Pro	Proline
MH	Mueller-Hinton
MRSA	Methicillin-resistant *Staphylococcus aureus*
mL	Millilitre
µL	Microlitre
CaM	Calmodulin
ASTM	American Society for Testing and Materials
HI	Hemolytic Index

1 INTRODUCTION

The discovery of penicillin started the current era of antibiotics (Lee, 2015). Since then, antibiotics have revolutionized contemporary medicine and saved millions of lives. With the introduction of antibiotic therapy, infectious bacteria have long been kept in check. In the conventional medical industry, antibiotics have been utilized for illness treatment and infection prevention in recent decades (Wang et al. 2019). However, the absence of early detection methods to identify the specific microorganisms causing infections and their susceptibility to antibiotics led to the excessive and often unnecessary use of broad-spectrum antibiotics (Frieri et al. 2017). Antibiotic-resistant bacteria are increasingly being identified in humans and animals treated with antibiotics, and resistance to commonly used medicines has become one of the world's most important public health issues (Wang et al. 2019). The World Health Organization estimates that over 95% of *Staphylococcus aureus* strains worldwide are resistant to penicillin (Briethaupt, 1999). Due to growing resistance and the rise of multi-drug resistant bacteria, the development of new antimicrobial treatments is critical to preserving reduced morbidity and mortality rates associated with harmful bacterial infections (Frieri et al. 2017). Fortunately, antimicrobial peptides (AMPs) offer a solution.

1.1 Antimicrobial Peptides

AMPs are small peptides that represent an important component of the innate immune system, serving as the first-line of defense against invading pathogens for many organisms (Pasupuleti et al. 2011) Found in all species such as bacteria, fungi, animals and plants, insects, amphibians, and all other mammalian species, these small peptides are between 12–50 amino acids long and 2–9 kDa in size (Zhang, 2022). They effectively kill a wide range of microorganisms

such as viruses, fungi, and both Gram-negative and Gram-positive bacteria (Swithenbank and Morgan 2017; Cunha et al. 2017).

Animals express AMPs in a wide variety of cells, including epithelial cells of the stomach, skin, and respiratory tracts, mast cells, monocytes, and neutrophils (Swithenbank and Morgan 2017). These peptides play a critical role in defending against pathogens at epithelial surfaces and in the immune system's cellular response to infection (Ganz 2003). Animal tissues exposed to microbes or cell types involved in host defense have the highest concentrations of AMPs. Epithelial surfaces secrete AMPs from both barrier epithelia and glandular structures. In invertebrates, AMPs are found in the fluid portion of blood (hemolymph) as well as in the granules of phagocytic cells (hemocytes), which contain various microbicidal substances and digestive enzymes (Ganz 2003). During phagocytosis, granules fuse to phagocytic vacuoles containing ingested microbes, exposing them to very high concentrations of microbicidal and digestive substances. Other granules are secreted into the extracellular fluid, where their contents kill microbes or inhibit their growth. Both types of granules contain abundant AMPs (Ganz 2003). AMPs are effective against multiple pathogenic bacteria, targeting both plasma membranes and intracellular components and demonstrate high effectiveness against drug-resistant strains (Zhang. 2021). As a result, AMPs represent a novel alternative to conventional antibiotics.

1.2 Pharmacodynamics of AMPs

Antibiotic resistance is common and occurs quickly. According to Yu et al. (2018), resistant strains often appear two years after a new antibiotic is introduced in the clinical setting. One of the alleged advantages of AMPs is that microbial resistance would occur much more slowly than against antibiotics (Yu et al. 2018). This reduced susceptibility of microbial resistance can be attributed to the rapid bactericidal action and broad-spectrum effects on microbial membranes and

intracellular processes, without targeting specific molecules or pathways (Zhang, 2021). Additionally, microorganisms treated with AMPs are less susceptible to evolution compared to those treated with antibiotics, largely due to the steepness of the pharmacodynamic curves (Figure 1). The concept of pharmacodynamics gives information about how drug dosage relates to bacterial death rates (El Shazely et al. 2020). Pharmacodynamic curves, particularly time-kill curves, play a crucial role in understanding the efficacy of antimicrobial agents against bacterial populations (El Shazely et al. 2020).

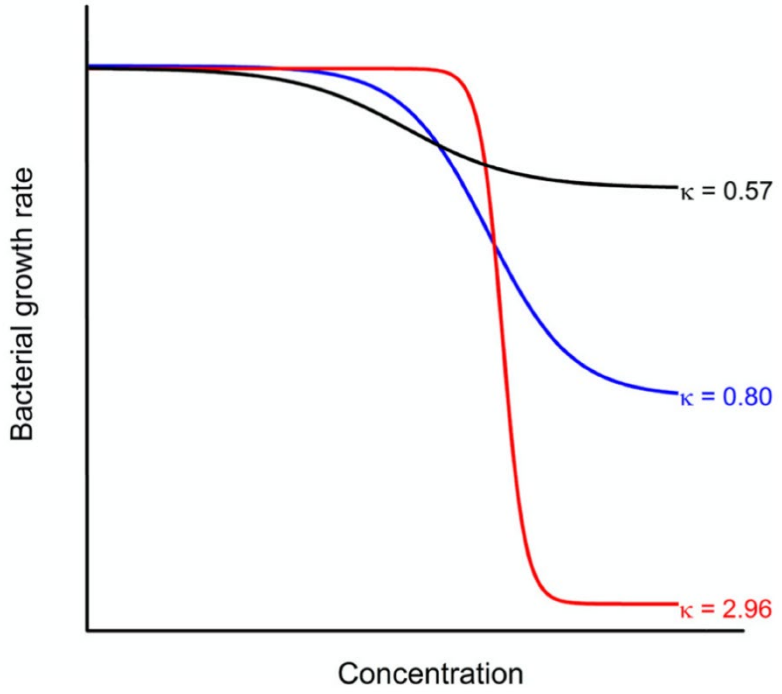

Figure 1. Pharmacodynamic curves of AMPs. The curves illustrate the effects (reflected as net bacterial growth rate) of increasing the concentrations of AMP(s). κ predicts the shape and slope of the pharmacodynamic curve; the higher the κ value, the steeper the pharmacodynamic curve (Yu et al. 2016).

The pharmacodynamic properties of both susceptible and resistant bacterial strains can be used as indicators for predicting the emergence of resistance (Yu et al., 2018). These predictions are based on a principle known as the 'mutant selection window' (MSW). The width of the MSW

is determined in part by the steepness of the pharmacodynamic curve. The concentration range within which AMPs exhibit no killing to maximal killing is much narrower compared to that of antibiotics, resulting in a much steeper curve (see red curve in Figure 1) (Yu et al., 2018). The maximum killing rate of AMPs is much higher than that of antibiotics, as reflected in quicker killing time (Yu et al. 2018). Consequently, AMPs have a narrower MSW compared to antibiotics, making the occurrence of resistance toward AMPs less likely (El Shazely et al., 2020).

1.3 Structures and Characteristics of AMPs

Typically, AMPs are composed of cationic and hydrophobic amino acids, giving them both positively charged and amphiphilic characteristics (Lei et al., 2019). These peptides contain hydrophobic and hydrophilic regions, contributing to their amphiphilic nature. Cationic AMPs are often short polypeptides, typically less than 100 amino acid residues, and are rich in positively charged amino acids such as lysine and arginine, which favours the interaction between these peptides and microbial cytoplasmic membranes (Lei et al., 2019; Cunha et al. 2019). In eukaryotes, AMPs are encoded by specific genes, which are expressed at basal levels and rapidly transcribed after induction by contact or exposure to invading pathogens (Cunha et al. 2019).

AMP molecules exhibit α-helical and β-sheet structures characterized by both hydrophobic and hydrophilic regions, which contribute to their amphiphilicity (Figure 2) (Lei et al., 2019). This enables them to interact effectively with microbial cell membranes. Such interactions play a crucial role in the mechanism by which AMPs exert their antimicrobial activity. Positively charged AMPs engage with negatively charged cell membranes through electrostatic interactions, leading to membrane adsorption and conformational changes (Lei et al., 2019). The peptides anchor onto membrane surfaces with their hydrophobic sides embedded within the lipid core of the bilayer (Lei

et al., 2019). Notably, these peptides have a high concentration of basic amino acids at their N-terminal ends, contributing to their strong alkalinity, while their C-terminal ends are amidated. The antimicrobial activity of specific peptides correlates with the number of cationic charges they possess, whereas their hydrophobicity aligns with their hemolytic activity (Lei et al., 2019).

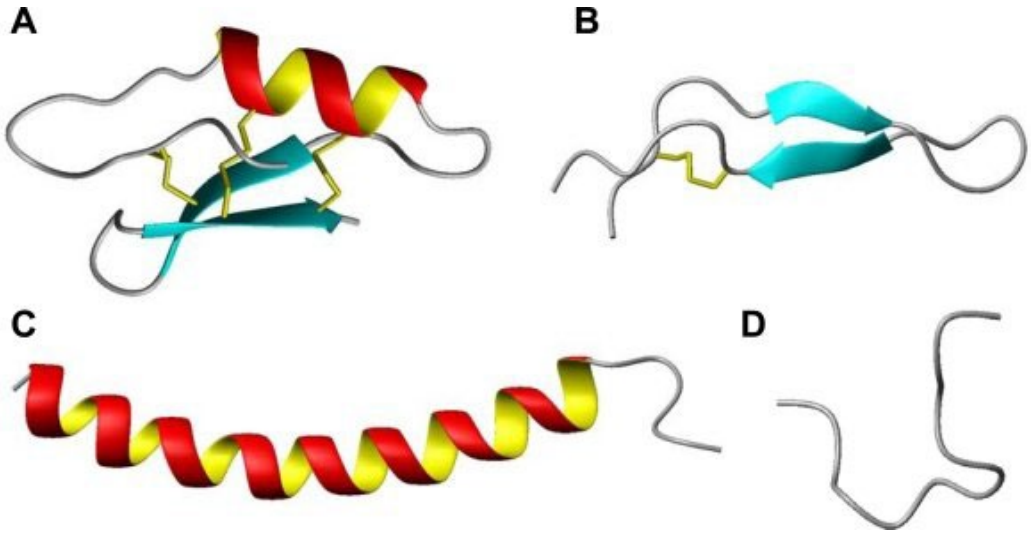

Figure 2. Structural classes of antimicrobial peptides; (A) Mixed structured peptide; (B) β-sheeted peptide; (C) α-helical peptide; (D) Extended peptide (Jenssen 2009).

1.4 Pathogen Specificity

1.4.1 Mechanism of Action of AMPs

AMPs have diverse mechanisms of action, primarily focusing on disrupting bacterial membranes or intracellular targets (Cuhan et al. 2019). The interaction between cationic AMPs and anionic bacterial membranes is the initial step, driven by electrostatic forces. Gram-positive and Gram-negative bacteria respond differently due to variations in cell wall composition (Cuhan et al. 2019). Several models have been proposed to explain the membrane targeting mechanism including the Toroidal pore, Carpet, Aggregate, and Barrel-Stave model, as shown in Figure 3

(Cuhan et al. 2019). Another mechanism of action involves non-membrane targeting. In this case, AMPs enter the cell and interact with its interior components, for example, disrupting DNA, RNA, and protein synthesis, affecting protein folding, enzyme function, and cell wall formation, ultimately resulting in cell death (Mazurkiewicz-Pisarek et al. 2023).

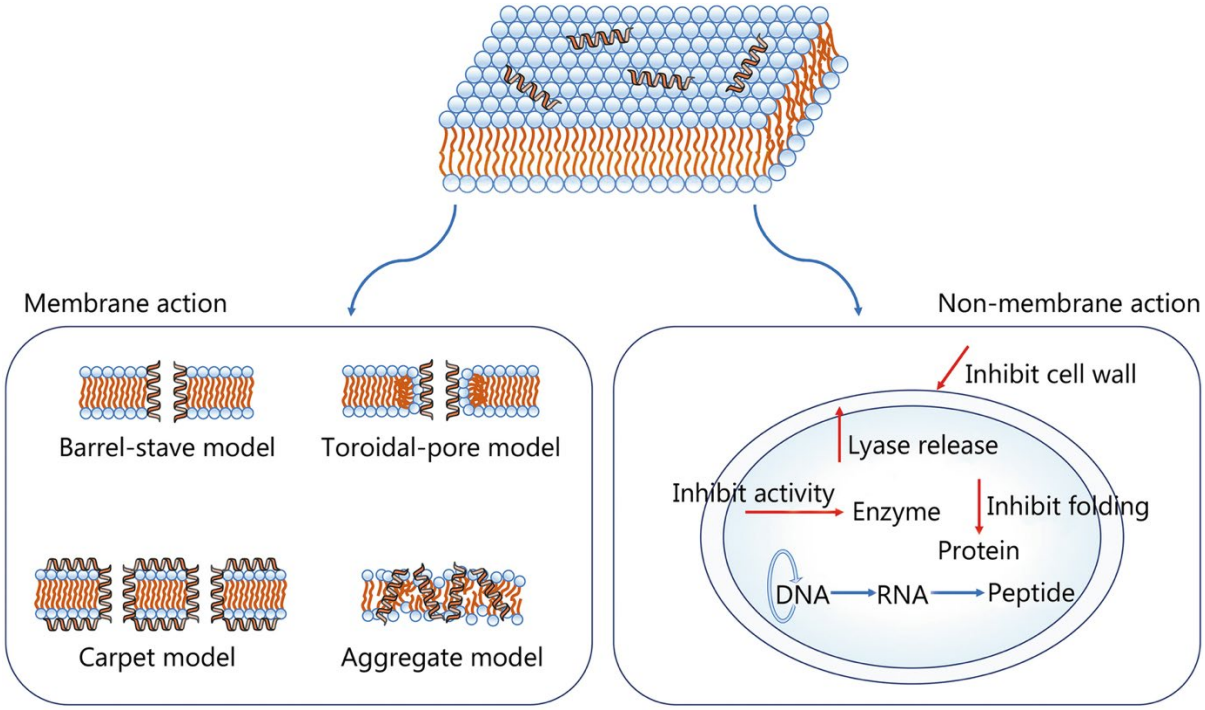

Figure 3. Models of antibacterial mechanisms of AMPs (Zhang et al. 2021).

1.4.2 Gram-positive/Gram-negative Bacteria and Fungi

AMPs are essential components of the innate immune system, playing crucial roles in defending against bacterial infections. Bacteria are categorized as either Gram-positive or Gram-negative, each characterized by distinct differences in their cell envelopes (Li et al. 2017). While the inner or cytoplasmic membranes of both bacterial groups exhibit similarities, their outer cell envelopes differ significantly. In Gram-positive bacteria, AMPs encounter a layer of cross-linked peptidoglycan. Anionic glycopolymers called teichoic acids (TAs) are covalently bound to the

peptidoglycan and surround the cytoplasmic membrane (Swoboda et al. 2009). This matrix provides a robust barrier that maintains cellular rigidity, yet nano-sized pores within the peptidoglycan layers allow AMP diffusion (Li et al. 2017).

Gram-negative bacteria, on the other hand, have a thinner and less cross-linked peptidoglycan layer, with an outer membrane beyond this layer (Figure 4) (Li et al. 2017). The inner leaflet of this membrane comprises predominantly phosphate lipids, while the outer leaflet is primarily composed of lipopolysaccharides (LPS). LPS molecules contain negatively charged phosphate groups that form salt bridges with divalent cations, creating an electrostatic network. This charged region serves as a significant barrier to most hydrophobic antibiotics, resulting in low permeability (Mazurkiewicz-Pisarek et al. 2023). Consequently, AMPs have different mechanisms for penetrating Gram-positive and Gram-negative bacteria due to their distinct cell envelope structures. In Gram-positive bacteria, AMPs first cross the peptidoglycan matrix before acting on the cytoplasmic membrane. Conversely, targeting Gram-negative bacteria involves disrupting both the outer and cytoplasmic membranes (Mazurkiewicz-Pisarek et al. 2023). Failure to permeabilize or penetrate the outer membrane results in the loss of antimicrobial activity (Li et al. 2017).

Moreover, AMPs are generally "electrostatically specific" for prokaryotic cells as the negatively charged membrane components (LPSs and TAs) are absent in mammalian cells (King et al. 2014). This electrostatic interaction between the positively charged AMPs and the negatively charged microbial membranes, facilitated by the absence of similar components in mammalian cells, enhances the selectivity of AMPs for targeting bacterial cells while minimizing cytotoxic effects on host cells.

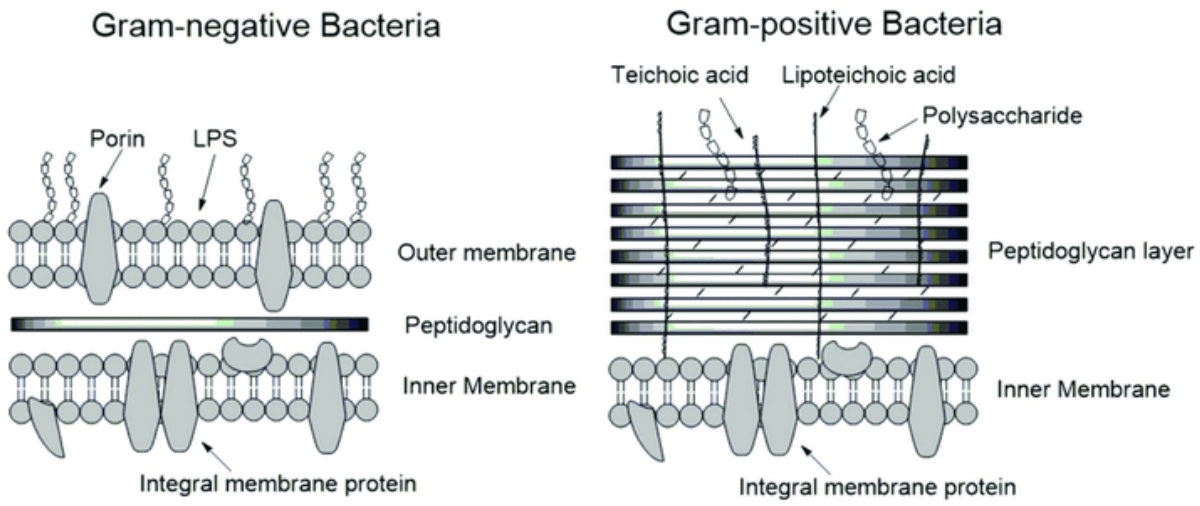

Figure 4. Schematic membrane structures of Gram-positive and Gram-negative bacteria. Gram-positive bacteria have a thick layer of peptidoglycan surrounding the cytoplasmic membrane, while Gram-negative bacteria have a thinner layer of peptidoglycan and an additional outer membrane (Li et al. 2017).

While peptidoglycan forms the cell wall in bacteria, fungal cell walls have more complex structures primarily composed of β-1,3-glucan, β-1,6-glucan, and chitin (Figure 5) (Lozančić et al., 2021). Mannoproteins and fibrous β-1,3-glucan are the predominant components of the walls (Lipke and Ovalle, 1998), with branched β-1,6-glucan linking other wall components. Chitin, although a minor component, contributes to the insolubility of the fibers. The β-1,3-glucan–chitin complex constitutes the main component of the inner wall, while β-1,6-glucan connects the inner and outer wall components. Mannoproteins, heavily O and N glycosylated, are located on the outer surface of the cell wall, closely spaced to restrict solute permeability (Lipke and Ovalle, 1998). Recent studies found that AMPs can bind to chitin and induce cell wall fragility (Peng et al., 2022). Moreover, certain AMPs can penetrate the fungal cells and interact with nucleic acids and fungal mitochondria, resulting in cell death.

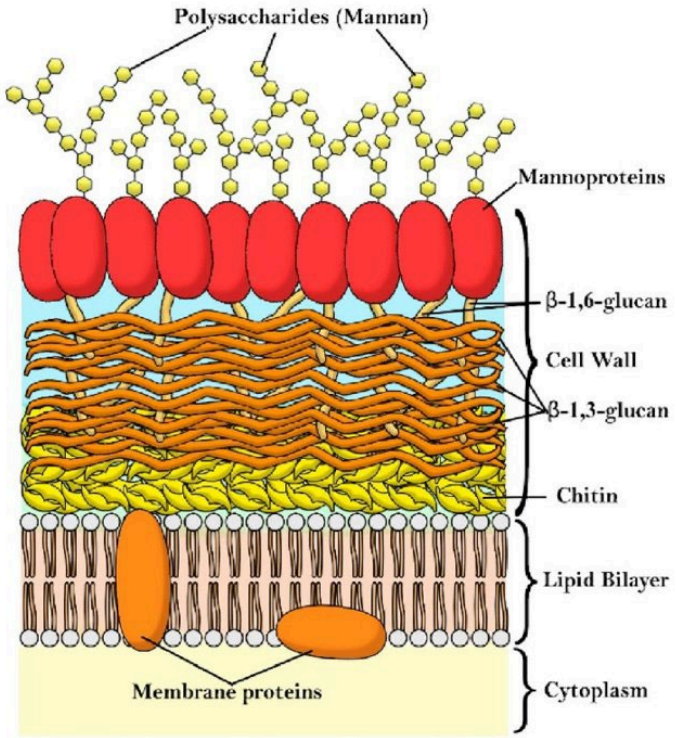

Figure 5. Fungal cell wall of *Candida albicans* (Aroso et al. 2021).

1.5 Indolicidin

Indolicidin, a natural cationic peptide found within the cathelicidin family, is found in cytoplasmic granules of bovine neutrophils (Friedrich et al., 2001). With a composition of 13 amino acid residues, it has a unique amino acid sequence (ILPWKWPWWPWRR-NH_2), containing 39% tryptophan (Trp) and 23% proline (Pro), with an amidated carboxyl terminus (Friedrich et al., 2001). Approximately half of its amino acid residues are hydrophobic, including the five Trp residues (Araujo et al., 2022). Remarkably, it has the highest Trp content among all known AMPs (Falla et al., 1996). Additionally, its broad-spectrum antimicrobial activity against various gram-positive and gram-negative bacteria and fungi has drawn significant interest for further research (Friedrich et al., 2001). The sequence and key physicochemical characteristics of indolicidin are detailed in Table 1.

Table 1. Sequence and physicochemical properties of indolicidin.

Sequence	**ILPWKWPWWPWRR-NH_2**
Structure	Multiple spatial conformation
Residue number	13
Molecular weight	1906.3 Da
Net charge*	+4
Isoelectric point	14
Solubility	1 mg/mL (water)

*Net charge at pH 7: Lys (K), Arg (R), and C-terminal amidation (NH_2) were assigned with +1 charge (Araujo et al. 2022).

1.5.1 Trp and Pro

Trp and Pro are significant in indolicidin's sequence as they are both important in the assembly and structure of membrane proteins (Ladokhin et al. 1997). Trp is one of the most hydrophobic among the 20 canonical amino acids, as assessed by the Wimley-White scale for peptides (Wimley and White 1996). This hydrophobicity impacts indolicidin's interactions with the hydrophobic interior of phospholipid membranes (Mishra et al. 2018). Additionally, Trp has a strong tendency to embed itself within membranes and localize near the interface between the membrane and water phases, showing a preference for the lipid-carbonyl region (Mishra et al. 2018). Because of its aromatic indole side chain, Trp readily forms hydrogen bonds with components of the lipid bilayer and the NH group on the indole ring. The unique structure of the indole side chain in Trp leads to a specific orientation preference when it resides at the membrane interface (Mishra et al. 2018). The bulky indole side chain, resembling a large paddle and approximately one-third the thickness of a phospholipid monolayer, disrupts the cohesive hydrophobic interactions among the lipid acyl chains when it penetrates deep into the hydrocarbon

core of the bilayer (Mishra et al. 2018). The presence of Trp in indolicidin residues facilitates its insertion and partitioning with biological membranes, contributing to its hemolytic activity (Subbalakshmi et al., 1996).

Moreover, Pro residues tend to introduce bends or kinks in peptides, which can interfere with the formation of secondary structures (Figure 6) (Podorieszach and Huttunen-Hennelly, 2010). Consequently, despite having five Trp residues that contribute to its high hydrophobicity, indolicidin's hydrophilic regions (with a net charge of +4) are not easily segregated into distinct amphipathic regions. As a result, indolicidin tends to adopt a disordered conformation or contains β-turn structures when free or bound to bilayers (Podorieszach and Huttunen-Hennelly, 2010).

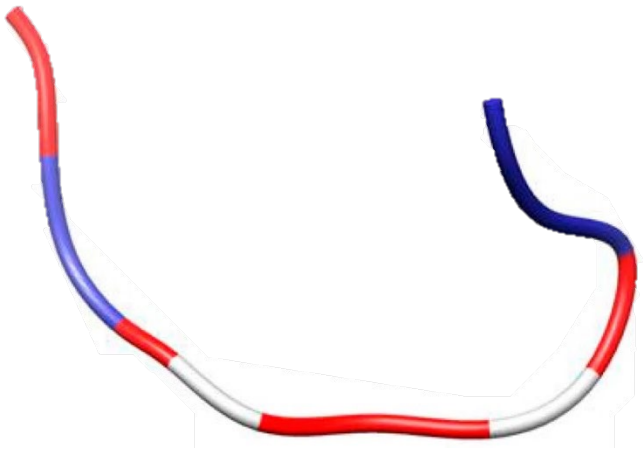

Figure 6. Theoretical shape of indolicidin with red indicating hydrophobic regions and blue indicating positively charge residues; the molecule is oriented from N-terminus (left) to amidated C-terminus (right) (Kumar et al. 2018).

1.5.2 Structure and Mechanism of Action

The charge distribution of indolicidin reveals a hydrophobic core flanked by positively charged areas positioned near both ends of the peptide, with three out of four charges oriented in the same direction (Rozek et al., 2000). This molecular arrangement creates a wedge shape,

facilitated by four of the five Trp indole rings being closely packed against the peptide backbone (Figure 7). Such a shape is well-suited for insertion between lipid molecules within a bilayer (Rozek et al., 2000).

There are two possible membrane orientations that are proposed: (i) insertion into one leaflet of the bilayer, resembling a "boat" structure with the positively and negatively charged regions directed toward one side of the membrane, and (ii) a transmembrane orientation where the two pairs of charges project onto opposite sides of the membrane (Figure 7b). Indolicidin predominantly binds at the membrane interface, as demonstrated by Rozek et al. (2000), suggesting penetration into one leaflet of the bilayer. This preference is likely due to the strong affinity of the Trp indole rings for this arrangement.

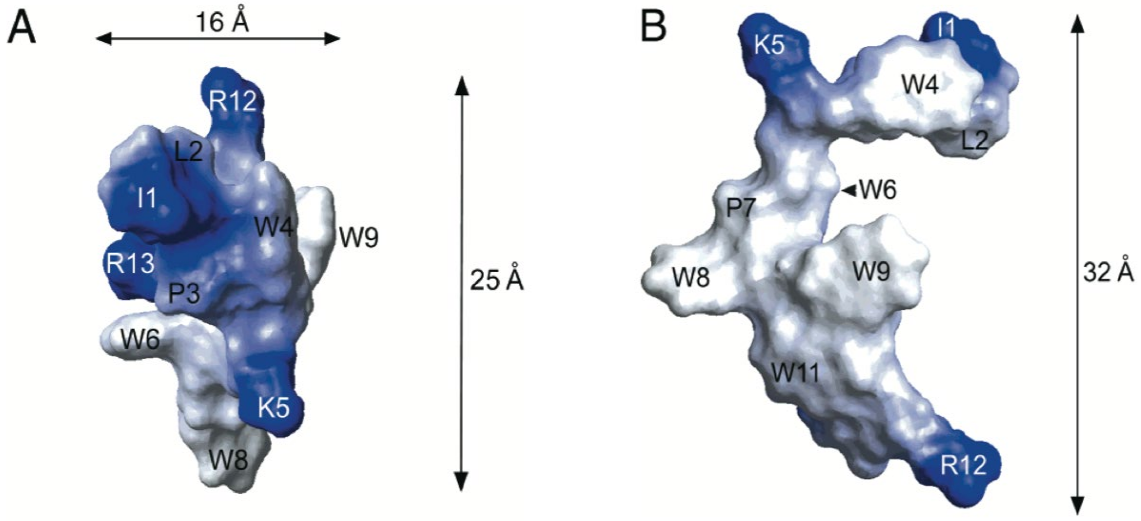

Figure 7. Charge distribution on the surface of indolicidin. Positive and neutral potentials are coloured blue and white, respectively. (A) End view looking down from the N-terminus (B) Side view with the N-terminus pointed to the top (Rozek et al. 2000).

It has been demonstrated that indolicidin functions as an antimicrobial agent via a different mechanism than the well-researched lytic peptides (Subbalakshmi and Sitaram 1998). Although its main mode of action is by interaction with bacterial membranes, specifically the cytoplasmic membrane, recent studies indicate other aspects of its mode of action (Figure 8). Indolicidin permeabilizes both the outer and cytoplasmic membranes of bacteria by displacing divalent cations from their binding sites on surface LPS (Falla et al. 1996). To achieve this, it uses a self-promoted uptake pathway, in which indolicidin penetrates bacterial membranes directly, facilitating its internalization into the cells (Falla et al. 1996). Inner membrane permeabilization by indolicidin occurs rapidly following outer membrane permeabilization and is characterized by the formation of discrete channels with relatively stable conductance (Falla et al. 1996).

Indolicidin disrupts fungal membranes similarly to bacterial membranes. Initially, it binds to the membrane via electrostatic interactions, which are influenced by salt concentrations and stabilized by Na^+ and Mg^{2+} ions (Lee et al. 2003). This binding is followed by the formation of ion channels in the membrane, leading to membrane destabilization and increased permeability. Overall, indolicidin's fungicidal effect is driven by its ability to disrupt fungal membranes and disrupt their integrity (Lee et al. 2003).

Indolicidin’s mode of action extends beyond membrane disruption. The mechanism of action of indolicidin has been determined to primarily function through intracellular targets. The most noteworthy action of indolicidin may be its capacity to inhibit uridine and thymidine from being incorporated into RNA and DNA, respectively, hence preventing the synthesis of RNA and DNA (Subbalakshmi and Sitaram 1998). Furthermore, this inhibition of DNA synthesis leads to filamentation of the treated cells, suggesting that indolicidin's mechanism of action is aimed at inhibiting cell replication (Hsu et al. 2005). Researchers also discovered that indolicidin binds to

a calcium-binding protein called Calmodulin (CaM), which is widely distributed in all eukaryotic cells and plays a crucial role in Ca^{2+}-mediated cellular processes like cell division, cycle, cytoskeletal organization, and ion channel regulation (Sitaram et al. 2003). Bacteria have also been found to express a CaM-like mutant protein called CalM, which is the protein product of the CaM-like gene (Michiels et al. 2002). This protein shares similar properties to CaM and binds Ca^{2+}. Although the exact mechanism by which indolicidin works remains unclear, it is reasonable to assume that the peptide enters the cytoplasm to exert its action with intracellular targets. Its multifaceted mechanisms underscore its potential as an effective antimicrobial agent with broad applications in combating microbial infections.

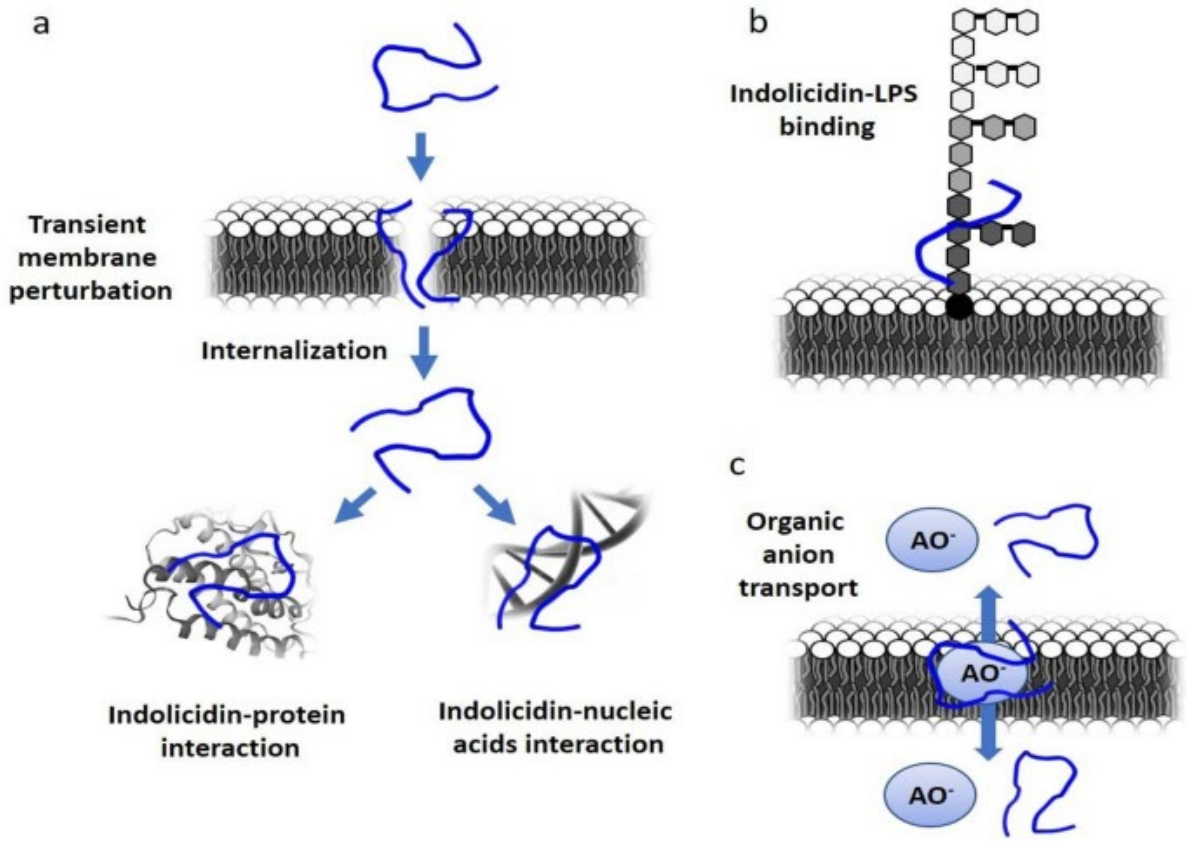

Figure 8. Illustration of indolicidin's mode of action following brief membrane disruption: (a) proteins and nucleic acids are intracellular potential targets; (b) the indolicidin–LPS interaction; (c) indolicidin anion carrier mechanism (Araujo et al. 2022).

1.5.3 Hemolytic Activity

The hemolytic activity of indolicidin poses a challenge to its potential use as a pharmaceutical. Rokitskaya et al. (2011) found that indolicidin induces erythrocyte lysis by disrupting the metabolic regulation of osmotic balance, potentially leading to increased erythrocyte permeability. This disruption could occur through the induction of either anionic metabolite efflux or the influx of the peptide itself into the cytoplasm, ultimately resulting in hemolysis (Rokitskaya et al., 2011). Previous research demonstrated that indolicidin's hemolytic activity decreases when the number of Trp residues is reduced, attributed to the peptide's reduced hydrophobicity and overall membrane-interface propensity (Podorieszach and Huttunen-Hennelly, 2010; Subbalakshmi et al., 2000). This reduction in hydrophobicity may result in a decreased affinity for lipid membranes and lower the tendency to disrupt them, leading to reduced hemolytic effects (Subbalakshmi et al., 2000).

Numerous studies have explored the role of specific residues in increasing the activity of indolicidin through the synthesis of analogous peptides. For instance, Ando et al. (2010) demonstrated that analogs with a single Trp at positions 4, 8, or 11, and others replaced by leucine, effectively reduced hemolytic activity while maintaining antibacterial efficacy (Figure 9). Additionally, Podorieszach and Huttunen-Hennelly (2010) found that replacing more than two Trp residues, starting from the C-terminus in indolicidin sequences, eliminated the peptide's antimicrobial activity. These studies indicate that (i) Trp residues or large aromatic moieties play important but complex roles in acting as AMPs, and (ii) some specific Trp analogs can exhibit lower hemolytic activities than indolicidin. Despite these findings, no comprehensive investigation has been conducted to develop indolicidin derivatives with high antimicrobial activity against both Gram-positive and Gram-negative bacteria and fungi, while minimizing hemolytic effects.

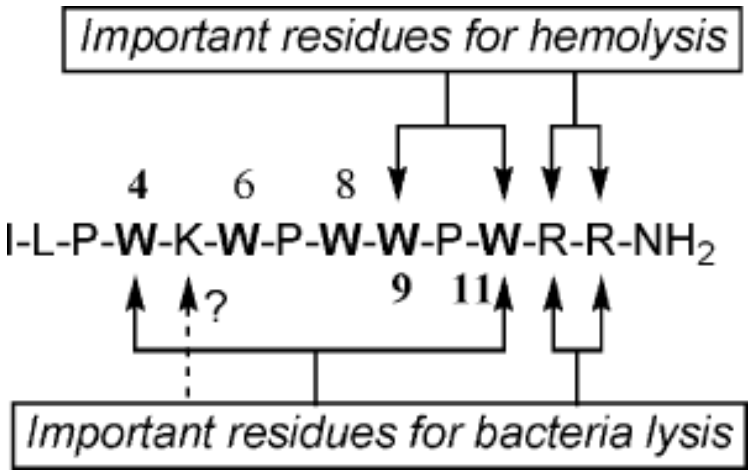

Figure 9. Predicted Key Tryptophan Residues for Bacterial Lysis and Hemolysis (Ando et al., 2010)

1.6 Objective

The significance of Trp and hydrophobicity must be considered when designing novel AMPs. Hydrophobicity plays a critical role in the peptide's membrane transience and hemolytic activity. Thus, in the present study, the impact of hydrophobicity will be evaluated by testing indolicidin derivatives. These derivatives substitute two Trp residues with the less hydrophobic, non-polar amino acid alanine (Ala) at different positions, thereby reducing the peptide's hydrophobicity. This approach will enable investigation of variations in the hydrophobic residue position within the peptide with respect to antimicrobial activity and hemolysis. Consequently, indolicidin derivatives listed in Table 2 were designed and studied to hopefully optimize bioactivity and reduce hemolysis.

Table 2. Sequence of indolicidin and other derivative peptides with abbreviations.

Sequence of Designed Peptides	Abbreviations
ILPWKWPWWPWRR-NH_2	Indolicidin
ILP**A**K**A**PWWPWRR-NH_2	Δ4,6
ILP**A**KWPWWP**A**RR-NH_2	Δ4,11
ILPWK**A**P**A**WPWRR-NH_2	Δ6,8
ILPWKWPW**A**P**A**RR-NH_2	Δ9,11

Note: Single-letter amino acid abbreviations include: Isoleucine (I), leucine (L) proline (P), tryptophan (W), lysine (K), arginine (R), and alanine (A). Bolded red alanine residues are the position at which Trp is being replaced with Ala.

2 MATERIALS AND METHODS

2.1 AMP Stock Solution Preparation

Each peptide was synthesized and analyzed using MS and HPLC by Biomatik Corporation. All peptides were guaranteed to have a purity of approximately >95% and were synthesized using solid-phase synthesis. The AMP stock solutions were prepared by dissolving lyophilized AMPs into sterile deionized water (18 mΩ) and their concentrations were determined by the NanoDrop One Spectrophotometer. To create a 1000 μg mL^{-1} AMP stock, a 15 mL centrifuge tube was used, into which 1 mL of sterilized deionized water was added. A small amount of AMP powder was added to the deionized water, followed by vortexing. The NanoDrop One Protein A205 Scopes method was selected. After zeroing the NanoDrop with deionized water, 2 μL of the AMP solution was transferred onto the pedestal and the concentration was read using the A205 Scopes method. This method monitors the absorbance of the peptide bond at 205 nm. The scopes method provides

a more accurate ε_{205} value, as it considers the substantial absorbance at 205 nm attributed by the aromatic side chains of Trp.

2.2 Antimicrobial Susceptibility

2.2.1 Kirby-Bauer Disc Diffusion Assay

Each peptide's bioactivity was qualitatively confirmed using the Kirby-Bauer assay. At various concentrations, 25 µL of each peptide was pipetted and absorbed into 6 mm sterilized paper discs three times, totaling 75 µL, with an hour of drying time between each interval. Additionally, bacterial and fungal species (Table 3) were incubated for approximately 16 hours to grow to a turbidity of 0.600–1.000 in Muller Hinton (MH) broth. The turbidity was determined using a spectrophotometer set to a wavelength at 600 nm. From the MH broth containing the microbes, 0.2 mL of the broth was pipetted into 20 mL of MH agar, to get a bacterial/fungal concentration of 1%. Immediately after, the bacteria/fungi containing agar was poured into Petri dishes. Once the agar solidified, the peptide-concentrated paper discs were evenly placed on the agar plates, alongside the negative control (water) and a positive control (antimicrobial disc). The plates were then incubated at 37°C for 24 hours and then were examined for the presence of clear zones, indicating inhibition of bacterial growth around the antibiotic discs. Antimicrobial activity was evaluated by measuring the diameter of inhibition zone against each bacterial/fungal strain.

Table 3. Bacteria and fungi species used for antimicrobial susceptibility assays, cell type, and antibiotic used for positive control.

Microbe	Microbe type	Antibiotic Control
Pseudomonas aeruginosa	Gram-negative	Tetracycline
Salmonella typhimurium	Gram-negative	Tetracycline
Escherichia coli	Gram-negative	Ampicillin
MRSA	Gram-positive	Ampicillin
Staphylococcus aureus	Gram-positive	Ampicillin
Candida albicans	Yeast	Nystatin

2.2.2 Minimum Inhibitory Concentration Assay

The minimum inhibitory concentrations (MICs) for the five AMPs were measured against various bacterial and fungal species (Table 3) using the broth microdilution method in triplicate. The test organisms were inoculated into a 2-mL microcentrifuge tube containing 1.5 mL of MH nutrient broth by touching three to four different isolated cultured colonies with a flame sterilized loop. The organisms were then grown to mid-logarithmic phase by incubating at 37°C with constant mixing at 200 rpm for 16–18 hours on an orbital shaker. The bacterial and fungal cultures were measured using the spectrophotometer set at 621 nm wavelength and a 1-cm light path. The cultures were adjusted by diluting with MH broth to get an absorbance reading within the range of 0.08 to 0.10, indicative of the McFarland 0.5 standard (~1 x 10^8 CFU mL^{-1}). The bacterial and fungal cultures were diluted 100-fold to get an approximate cell density of ~1 x 10^6 CFU mL^{-1}. In a standard tray containing 96-wells, 20 µL of the 1 x 10^6 CFU mL^{-1} culture was transferred to each well and the appropriate AMP concentration of 10–500 µg mL^{-1} was added and diluted to a final volume of 200 µL. The final inoculum size was ~5 x 10^5 CFU mL^{-1}. The 96-well plates were

incubated overnight for 16–18 hours at 37°C on an orbital shaker, providing sufficient time for bacterial growth and observation of the antimicrobial effect of the peptides. The optical density was measured using the ThermoFisher Multiskan Ascent plate reader at 621 nm. The instrument was calibrated using MH broth as a blank. Bacterial/fungal cultures incubated without peptide were used as negative controls, whereas antimicrobial activity exhibited by unaltered indolicidin was taken as a positive control to demonstrate pathogen death with a known AMP. All samples were prepared in triplicate. The lowest concentration of the derivatized AMP that prevented growth represented the MIC. The MIC was defined as ≥85% killing of the initial inoculum.

2.3 Microbial Viability Assay

To elucidate the antimicrobial properties (bactericidal or bacteriostatic) of indolicidin and its derivatives, 10 µL aliquots from the MIC wells were transferred into a 96-well plate and incubated overnight for observable growth. The optical density was measured again using the ThermoFisher Multiskan Ascent plate reader at 621 nm. If bacteria failed to resume growth in MH broth after overnight incubation, the AMPs were bactericidal in the mechanism of cell death, otherwise, it was considered bacteriostatic.

2.4 Hemolytic Activity

All horse whole blood samples were donated from the TRU Veterinary Technician program. All peptides were prepared at in 1X phosphate-buffer saline (PBS). Whole blood samples were centrifuged at 3000 RCF for 10 minutes at 4°C and the supernatant was poured off to isolate the pellet containing the red blood cells (RBCs). The pellet was resuspended in 1X PBS at ten times the volume of the pellet and centrifuged again, with the same conditions. The blood samples were washed three times. Samples were then brought back up to whole blood volume and diluted ten-fold to a final cell concentration of ~5 x 10^8 cells/mL, which was confirmed with a

hemocytometer. Of this, 200 µL was added to 800 µL of 1X PBS (pH 7.4) containing known peptide concentrations in 10-fold serial dilutions. Triton X-100 (1% final concentration) was added as a positive control, whereas 1X PBS was used as a negative control. Samples were then inverted several times to ensure homogeneity and incubated for one hour at 37°C on the orbital shaker at 50 RPM, with further inversions after 30 minutes. After incubation, samples were centrifuged at 14000 RPM for five minutes at 4°C and the absorbance of the supernatant was measured on the VWR® M4, UV/Visible Spectrophotometer at 541 nm to detect free hemoglobin in solution. The instrument was blanked against 1X PBS, and the amount of hemolysis exhibited by 1% Triton X-100 was taken to be 100% hemolysis (positive control). Thus, the percent hemolysis was taken to be:

$$\frac{(Sample\ absorbance) - (phosphate\ buffer\ absorbance)}{(Triton - X\ absorbance)}\ x\ 100\%$$

The minimum hemolytic concentration was taken to be the concentration of peptide that caused 100% hemolysis (relative to Triton X-100).

2.5 Electrophoretic Mobility Shift Assay

To evaluate the DNA binding capabilities of indolicidin and its derivatives, an electrophoretic mobility shift assay (EMSA) was performed. The plasmid pDN5, derived from pRc/CMV, was purified using a QIAfilter plasmid midi kit. The plasmid concentration was determined by UV spectrophotometry on the Nanodrop at 260 nm and quantitative analysis on an agarose gel. The plasmid DNA pDN5 (200 ng) was mixed with increasing amounts of peptides (50-1000 ng) with 2 µL of 10X digestion buffer and sterilized deionized water such that the final volume of the reaction was 20 µL. For the negative control, 200 ng of pDN5 was used. The

mixtures were incubated at room temperature for 30 minutes. The peptide:DNA mass ratios were 0:1, 0.25:1, 0.5:1, 2.5:1, 5:1, respectively. After adding 5 µL of loading buffer (5X loading dye), complexes of DNA and peptides were resolved by electrophoresis on a 1% agarose gel in 1X Tris/Borate/EDTA (TBE) buffer (40 mM tris borate and 1 mM EDTA, pH 8.0) containing ethidium bromide for visualization of the DNA bands. The gel was run for 60 minutes at 80 V and the migrated DNA was visualized under UV light.

3 RESULTS

3.1 Bioactivity Characterization

3.1.2 Kirby-Bauer Disc Diffusion Assay

Kirby-Bauer disc diffusion assays were performed on nutrient agar with all target organisms listed (Figure 10). Zones of inhibition occurred one day post-inoculation and ranged from 5.5 mm to 8 mm in diameter (Table 4). All derivatives demonstrated activity against *Escherichia coli*, except for indolicidin and Δ4,11. Δ4,11 demonstrated activity against *Salmonella typhimurium*. Indolicidin was not active in the Kirby-Bauer assay against any microbes tested.

Figure 10. Kirby-Bauer assay of the different indolicidin derivatives against *Escherichia coli.*

Table 4. Zone of inhibition diameter measurements of the indolicidin derivatives tested on various bacteria and fungi.

	Escherichia coli	*Pseudomonas aeruginosa*	*Salmonella typhimurium*	MRSA	*Staphylococcus aureus*	*Candida albicans*
Δ0	–	–	–	–	–	–
Δ4,6	++	–	–	–	+	–
Δ4,11	–	–	++	–	+	–
Δ6,8	++	–	–	–	++	–
Δ9,11	++	–	–	–	++	–

Note: – indicated no zones of inhibition, + indicates inhibition zones < 7 mm, ++ indicates zones ≥ 7 mm.

3.1.3 MIC Assay

The MIC values at a 90% reduction in optical density for indolicidin and the analogs are shown in Table 5. All the peptides listed in Table 4, except for Δ4,6, exhibited antibacterial activities against Gram-positive bacteria. Interestingly, compared to indolicidin, all peptide derivatives demonstrated a >10-fold decrease in antimicrobial activity against both Gram-positive and Gram-negative bacteria and fungi. Substitution of Trp residues at 4,11 resulted in the loss of activity against Gram-positive bacteria. Substitution of the Trp residues at 4,6 resulted in a loss of activity against Gram-positive and Gram-negative bacteria, only being active against *Candida albicans*. The derivative Δ4,11 was only active against the Gram-negative bacteria *Salmonella typhimurium.* The assay was conducted in triplicate, resulting in consistent findings with variations of less than 5% between replicates.

Table 5. MICs of peptide derivatives against a variety of bacterial and fungal pathogens, where active concentrations are determined by exhibiting at least 90% reduced optical density compared to each control culture incubated without peptide; a dash (—) indicates that the peptide was not active at the concentrations tested.

Active Concentration/μg mL^{-1}						
Peptide	*Escherichia coli*	*Pseudomonas aeruginosa*	*Salmonella typhimurium*	*Candida albicans*	MRSA	*Staphylococcus aureus*
	Gram-negative	Gram-negative	Gram-negative	Yeast	Gram-positive	Gram-positive
Indolicidin	50	250	50	50	50	50
Δ 4,11	—	—	500	—	—	—
Δ 4,6	—	—	—	500	—	—
Δ 9,11	500	—	500	500	250	500
Δ 6,8	250	—	500	500	250	500

The MIC values at a 50% reduction in optical density for indolicidin and its analogs are presented in Table 6. Interestingly, when tested for a 50% reduction instead of 90% in optical density, Δ4,11 exhibited activity against *Candida albicans* at 500 μg mL^{-1}, showing a significant reduction in optical density. Additionally, Δ4,6 demonstrated activity at 50 μg mL^{-1} against *Candida albicans*. Similarly, Δ9,11 displayed activity at 50 μg mL^{-1}, reducing the optical density by 58%. Moreover, Δ6,8 was the only analog that showed activity against *Pseudomonas aeruginosa* at 500 μg mL^{-1}.

Table 6. MICs of peptide derivatives against a variety of bacterial and fungal pathogens, where active concentrations are determined by exhibiting at least 50% reduced optical density compared to each control culture incubated without peptide; a dash (—) indicates that the peptide was not active at the concentrations tested.

Active Concentration/μg mL^{-1}						
Peptide	*Escherichia coli*	*Pseudomonas aeruginosa*	*Salmonella typhimurium*	*Candida albicans*	MRSA	*Staphylococcus aureus*
	Gram-negative	Gram-negative	Gram-negative	Yeast	Gram-positive	Gram-positive
Indolicidin	50	250	50	50	50	10
Δ 4,11	—	—	500	500	—	—
Δ 4,6	—	—	—	50	—	—
Δ 9,11	250	—	500	500	250	50
Δ 6,8	250	500	250	250	250	250

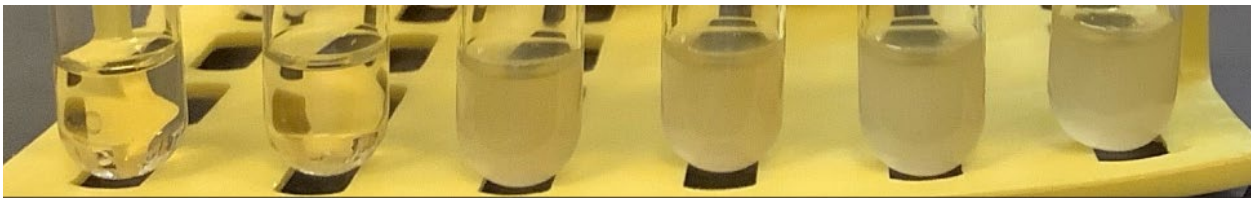

Figure 11. Serial dilution results with decreasing concentrations of Δ6,8 (from left to right: 500 μg/mL to 0 μg/mL) against *Candida albicans.*

3.1.2 Microbial Viability Assay

The bacteriostatic (BS) and bactericidal (BC) activities of the indolicidin derivatives were assessed by subculturing the broths used for MIC determination into fresh MH broth. Among the derivatives, Δ4,11 exhibited BS activity against *Salmonella typhimurium* at its MIC of 500 μg mL^{-1}, while Δ4,6 demonstrated BS activity against *Candida albicans* at its MIC of 500 μg mL^{-1} (Table 7). Additionally, both Δ6,8 and Δ9,11 displayed BS activity at their respective MICs for all tested microorganisms. However, Δ6,8 demonstrated BC activity above its MIC at 500 μg mL^{-1}

against *Escherichia coli* and MRSA. Similarly, Δ9,11 exhibited BC activity against MRSA above its MIC at 500 μg mL^{-1}.

Table 7. Bacteriostatic/Bactericidal (BS/BC) activity of indolicidin derivatives.

Peptide	Δ 4,11		Δ 4,6		Δ9,11		Δ 6,8	
	MIC (μg mL^{-1})	BS/BC action	MIC (μg mL^{-1})	BS/BC action	MIC (μg mL^{-1})	BS/BC action	MIC (μg mL^{-1})	BS/BC action
Escherichia coli	—	—	—	—	500	BS	250	BS
Pseudomonas aeruginosa	—	—	—	—	—	—	—	—
Salmonella typhimurium	500	BS	—	—	500	BS	500	BS
Candida albicans	—	—	500	BS	500	BS	500	BS
MRSA	—	—	—	—	250	BS	250	BS
Staphylococcus aureus	—	—	—	—	500	BS	500	BS

3.1.3 Hemolysis

The hemolytic activity of the five structurally related peptides was evaluated in erythrocytes from horses. The subsequent release of hemoglobin was used to assess hemolytic activity as function of peptide concentration by measuring to absorbance of the supernatant at 541 nm to detect free hemoglobin in solution. Comparing the analogues to indolicidin, the hemolysis test findings show more than 16-fold reduction in hemolysis, or decreased cytotoxicity (Table 8). However, since the hemolytic concentrations for these derivatives were beyond the measurable range, the hemolytic index (MHC/MIC) could not be calculated (NA). These results indicate significantly lower hemolytic activity when the two Trp's were substituted for Ala in all

derivatives compared to indolicidin. According to the database, DBAASP, it was predicted that all peptides, except for the peptide Δ9,11, would have substantial hemolytic activity. However, the data in table 8 indicates that this prediction is only valid for indolicidin.

Table 8. Minimum hemolytic concentrations (MHCs) of indolicidin and derivatives compared to the AMP hemolytic activity predictor. In cases of high MHCs, the highest concentration tested is given with its percent hemolysis at that concentration. Hemolytic Index (HI) is defined here as the MHC divided by the lowest MIC derived.

	Hemolytic concentration (μg mL^{-1})	**Hemolytic Index (MHC/MIC)**	**Database Hemolytic Concentration Prediction (μg mL^{-1})**
Indolicidin	30	39	<25
Δ4,6	700 (2.92%)	NA*	<25
Δ4,11	700 (0.84%)	NA*	<25
Δ9,11	700 (4.2%)	NA*	>100
Δ6,8	700 (2.2%)	NA*	<25

Note: *Not available; For hemolytic activity predictions, the DBAASP was used (https://dbaasp.org/tools?page=property-calculation).

3.2 DNA Binding Assay

To determine Trp's effect on indolicidin's mechanism, DNA binding was indirectly measured by monitoring the effect of increasing peptide amount on the migration of purified pDN5 plasmid DNA. Specifically, indolicidin and its derivatives were tested to see if they could interact with DNA molecules, which would lead to altered mobility in agarose gel electrophoresis. The plasmid migration of the derivatives is shown in Figure 12. The assay revealed distinct binding patterns among the tested peptides. Δ9,11 and Δ6,8 demonstrated the weakest ability to inhibit plasmid migration since strong DNA bands were still observed at peptide:plasmid weight ratios of

5:1. As shown in Figure 12, Δ4,11 and Δ4,6 appeared to inhibit plasmid migration at a peptide:plasmid ratio of 2.5:1 and 5:1, as the bands travelled less far compared to the control. Indolicidin had the highest DNA affinity, inhibiting plasmid migration at a weight ratio as low as 0.5:1. These findings suggest that certain structural modifications within indolicidin and its derivatives, particularly with Trp, influence their ability to interact with DNA.

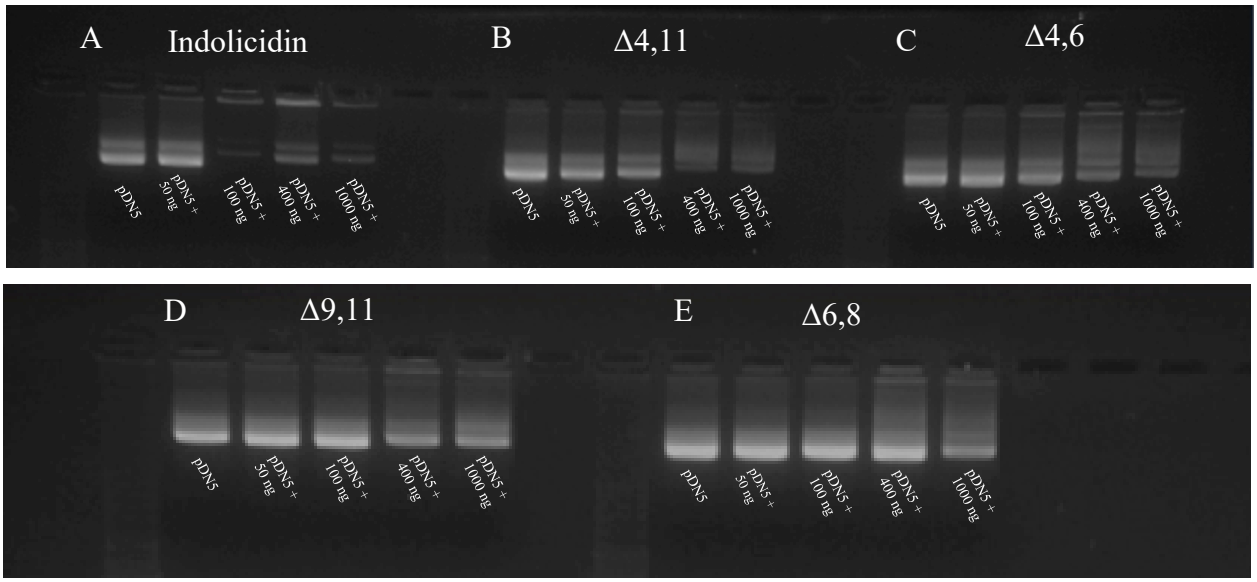

Figure 12. Electrophoretic mobility assay of DNA binding to peptides. Different amounts of indolicidin (A), Δ4,11 (B), Δ4,6 (C), Δ9,11 (D), and Δ6,8 (E) was incubated with 200 ng of pDN5 plasmid DNA. The binding affinity was assessed by the inhibition of the electrophoretic migration of the DNA by the peptides. The peptide:DNA mass ratios for the different lanes from left to right.

4 DISCUSSION

The relationships of the structural features of indolicidin and functional properties of Trp with cell lysis activities can be derived from the evaluations of a series of indolicidin derivatives: their antimicrobial activities, and DNA binding capacity. This study assessed both the changes in antimicrobial and hemolytic activity of indolicidin and its derivatives, when Trp was replaced with the less hydrophobic amino acid Ala, as well as the DNA binding capabilities of indolicidin and its derivatives. By replacing Trp in indolicidin's sequence, its antimicrobial activity decreased by >10-fold, however its hemolytic activity was almost completely removed, decreasing by >16-fold. Furthermore, indolicidin, Δ4,11, and Δ4,6 were found to bind DNA, while Δ9,11 and Δ6,8 did not.

4.1 Antimicrobial Activity

In the Kirby-Bauer assay, indolicidin was not active even though indolicidin has known activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Candida albicans*, *Staphylococcus aureus*, and MRSA. A possible explanation for why indolicidin may not have shown any zones of inhibition is that perhaps indolicidin was too hydrophobic to diffuse well from the disc into the aqueous agar. One known shortcoming of the Kirby-Bauer assay is that the antimicrobial agent on the disc needs to be able to diffuse from the disc into the agar plate. Due to the inconsistencies with the Kirby-Bauer assay and due to its qualitative nature, we moved on to the MIC assay.

According to the MIC results of indolicidin and the analogs, there was no antibacterial activity observed against Gram-negative and Gram-positive bacteria in the Trp analog substituted at positions 4,6. Δ4,6 was only microbially active against the eukaryotic fungi *Candida albicans.* Conversely, the Trp analog substituted at 4,11 was no longer active against *Candida albicans* and only against the Gram-negative bacteria *Salmonella typhimurium.* On the other hand, the Trp

analogs substituted at positions 6,8 and 9,11 exhibited the highest activity among the analogs. This demonstrates the important role of the Trp residue at position 4 for the antimicrobial activity of indolicidin, while indicating that position 6 is not crucial for activity.

The absence of antibacterial activity observed in the Trp analogs substituted at position 4 suggests the critical importance of the Trp residue at that specific position for effective antimicrobial action. This is consistent with previous findings by Ando et al. (2010), which also highlighted the significance of the Trp residue at position 4 in indolicidin's activity. However, they found the Trp residue at position 11 was important to the antimicrobial activity of indolicidin (Ando et al. 2010). Conversely, the derivative in which the Trp analog was substituted for Ala at positions 9,11 had strong antimicrobial activity against Gram-negative and Gram-positive bacteria, as well as fungi, consistent with the findings of Podorieszach and Huttunen-Hennelly (2010). The current results further highlight the significance of the Trp residue at position 4 for antimicrobial activity, as its replacement led to a significant decrease or complete loss of antimicrobial efficacy.

While Δ6,8 and Δ9,11 exhibited antimicrobial activity against most of the microbes tested, both had a reduction in activity by ~10-fold, compared to indolicidin. The decrease in antimicrobial activity observed in all derivatives compared to indolicidin can be attributed to the reduction in overall hydrophobicity by removing two of the Trp residues. Hydrophobicity plays a crucial role in the interaction of AMPs with bacterial membranes. When hydrophobicity is reduced, AMPs may have decreased affinity for the lipid bilayer, leading to less efficient membrane disruption and ultimately reduced antimicrobial activity. Additionally, Trp residues are known to contribute to the amphipathic nature of AMPs, allowing them to insert into the lipid bilayer and disrupt membrane integrity. The substitution of Trp residues in our derivatives may have disrupted this

amphipathic structure, impairing their ability to interact with and permeabilize bacterial membranes effectively.

A 90% reduction in optical density was stated to be the MIC. At concentrations of 500 μg mL^{-1}, above their MIC, for Δ6,8 and Δ9,11, cell membranes were completely solubilized, resulting in a 100% decrease in optical density. These Δ6,8 and Δ9,11 experimental cultures were re-inoculated, and no growth was observed as confirmed by no absorbance at 621 nm, supporting completed bacterial lysis and complete cell death. However, at lower concentrations near the MIC, growth was severely limited by over 90%, yet the initial inoculum often remained intact, yielding a slight absorbance. Additionally, the initial inoculum remained viable, as demonstrated by re-inoculation of the pathogens in peptide-free media. The re-inoculated solutions appeared clear, but still exhibited a slight absorbance, indicating their bacteriostatic and bacteriolytic properties. Growth inhibition that does not compromise membrane integrity would also suggest an intracellular mode of action, which may be related to the peptide derivatives' reduced hydrophobicity (Podorieszach and Huttunen-Hennelly 2010) and hence preference to be inside the aqueous cytosol. While studies have reported that the primary mechanism of action involves self-promoted uptake through electrostatic interactions with bacterial/fungal cell wall and LPS components (Falla et al. 1996), our study suggests that other factors, such as hydrophobicity and Trp substitution location, also play crucial roles.

4.2 Hemolytic Activity

In this study, we observed a reduction in hemolysis with each Trp substitution (Table 5). This reduction in hemolysis can be attributed to Trp's significant contribution to the hydrophobicity of the peptides and their positioning at the membrane interface, as well as its

involvement in protein/DNA binding. These results provide important insights into the hemolytic nature of cationic AMPs.

The reduction in hemolytic activity observed in our derivatives indicates that the mechanisms of membrane interaction are more complex than previously thought. Rather than solely relying on electrostatic associations, peptides with reduced Trp content may interact differently with cell membranes, leading to decreased cytotoxicity. This suggests that AMPs can potentially be designed or modified to minimize hemolytic effects while maintaining antimicrobial efficacy.

According to the American Society for Testing and Materials (ASTM), hemolysis of less than 5% is considered negligible (Luna-Vázquez-Gómez et al. 2021). All the peptide derivatives exhibited ≤5% hemolysis, indicating their minimal cytotoxicity. This, coupled with their antimicrobial activity, suggests that these peptides have significant potential as pharmaceutical agents, particularly Δ6,8 and Δ9,11. These two peptides demonstrated broad-spectrum activity against Gram-negative and Gram-positive bacteria, as well as eukaryotic yeast cells, all while having minimal hemolytic effects.

4.3 DNA Binding by Inhibition of Plasmid Migration

To evaluate the DNA binding abilities of indolicidin and its derivatives, plasmid migration was monitored in an agarose gel with increasing peptide concentrations. Indolicidin exhibited the strongest affinity for DNA binding, followed by Δ4,6 and Δ4,11, which also displayed significant DNA binding capabilities. Conversely, Δ9,11 and Δ6,8 showed no detectable DNA binding. This differential binding pattern suggests that specific structural features and ordering of the amino acids in a sequence influence the DNA binding activity and mechanisms of these peptides.

Several possible factors may impact the DNA binding activity of these peptides. This may be explained by the presence of the PWWP motif present in indolicidin's sequence. Indolicidin, Δ4,6, and Δ4,11, which include the PWWP domain in their sequence, bind DNA more strongly than Δ6,8 and Δ9,11, which lack the PWWP domain. PxxP motifs consist of a proline (P) followed by any two amino acids (x) and another proline (P) (Lee et al. 2023). Studies have shown that central PxxP sequences are crucial for bacterial selectivity and efficient interaction with negatively charged membranes (Lee et al. 2023). Specifically, PWWP motifs are known to recognize and bind DNA and form a helical turn structure (Ghosh et al. 2014). PWWP motifs are found in nuclear proteins such as hepatoma-derived growth factors (HDGF) and DNA methyltransferase proteins (Marchand et al. 2006). Another example of a PWWP domain-containing protein is the lens epithelium-derived growth factor (LEDGF/p75), which directly binds HIV-1 and facilitates its tethering to chromosome (Marchand et al. 2006). The results of this study demonstrate that the removal of this PWWP motif from the Δ6,8 and Δ9,11 derivatives, affected their capacity to bind to plasmid DNA. This suggests that the presence of the PWWP domain enhances the DNA binding affinity of indolicidin and its derivatives.

Although the derivatives Δ4,11 and Δ4,6 exhibited DNA binding capacities, this study found no correlation between the peptide's ability to bind DNA and its antimicrobial activity. Surprisingly, peptides Δ6,8 and Δ9,11, lacking the PWWP domain, exhibited better antimicrobial activity compared to Δ4,11 and Δ4,6 that retained the PWWP motif and bound DNA. This suggests that mechanisms other than DNA binding, such as membrane disruption or intracellular targeting, may play a significant role in determining the antimicrobial efficacy of these peptides. Once these analogs enter the cell, they may disrupt various intracellular lipid-bound organelles in eukaryotic cells, including the nucleus (exposing DNA to cytosolic DNases), the endoplasmic

reticulum/Golgi body (disrupting protein synthesis), mitochondria (impairing cellular respiration), or vesicles (releasing proteolytic enzymes or disrupting storage compartments). An apoptosis/necrosis assay can help distinguish between these possibilities in animal cells, where DNA fragmentation or mitochondrial disruption would indicate apoptosis, while cellular permeabilization would suggest necrosis. This assay would also assess the cytotoxicity against nucleated animal cells.

5 CONCLUSIONS AND FUTURE WORK

This study demonstrates the significant impact of Trp substitution on the antimicrobial and hemolytic activities of indolicidin and its derivatives. The findings reveal that while substitutions at specific Trp positions led to a loss or reduction in antimicrobial activity, they also resulted in a significant decrease in hemolytic activity, suggesting a more nuanced role for Trp in both functions. Furthermore, the substitution of Trp for Ala at position 4 resulted in a significant loss or complete removal of antimicrobial efficacy against Gram-positive and Gram-negative bacteria and fungi, which is consistent with previous studies. Furthermore, it was found that derivatives lacking the PWWP domain, known for DNA binding, exhibited better antimicrobial activity than those with strong DNA binding capacity. This suggests that mechanisms other than DNA binding may play a significant role in determining the antimicrobial efficacy of these peptides. Further studies are needed to elucidate the specific mechanisms involved in the antimicrobial action of these peptides. Additional future work should include cytotoxicity testing against nucleated animal cells to ensure safety and testing the effects of indolicidin derivatives on biofilm formation to provide insights into their potential as anti-biofilm agents.

LITERATURE CITED

Ando S, Mitsuyasu K, Soeda Y, Hidaka M, Ito Y, Matsubara K, Shindo M, Uchida Y, Aoyagi H. 2010. Structure-activity relationship of indolicidin, a Trp-rich antibacterial peptide. Journal of Peptide Science. 16(4):171–177. doi:https://doi.org/10.1002/psc.1217.

Araujo JB, Souza GS de, Lorenzón EN. 2022. Indolicidin revisited: biological activity, potential applications and perspectives of an antimicrobial peptide not yet fully explored. World Journal of Microbiology & Biotechnology. 38(3). doi:https://doi.org/10.1007/s11274-022-03227-2.

Aroso RT, Schaberle FA, ArnautLG, Pereira MM. 2021. Photodynamic disinfection and its role in controlling infectious diseases. Photochemical and Photobiological Sciences. 20(11):1497–1545. doi:https://doi.org/10.1007/s43630-021-00102-1.

Breithaupt H. 1999. The new antibiotics. Nature Biotechnology. 17(12):1165–1169. doi:https://doi.org/10.1038/70705.

da Cunha NB, Cobacho NB, Viana JFC, Lima LA, Sampaio KBO, Dohms SSM, Ferreira ACR, de la Fuente-Núñez C, Costa FF, Franco OL, et al. 2017. The next generation of antimicrobial peptides (AMPs) as molecular therapeutic tools for the treatment of diseases with social and economic impacts. Drug Discovery Today. 22(2):234–248. doi:https://doi.org/10.1016/j.drudis.2016.10.017.

El Shazely B, Yu G, Johnston PR, Rolff J. 2020. Resistance Evolution Against Antimicrobial Peptides in Staphylococcus aureus Alters Pharmacodynamics Beyond the MIC. Frontiers in Microbiology. 11. doi:https://doi.org/10.3389/fmicb.2020.00103.

Falla TJ, Karunaratne DN, Hancock REW. 1996. Mode of Action of the Antimicrobial Peptide Indolicidin. Journal of Biological Chemistry. 271(32):19298–19303. doi:https://doi.org/10.1074/jbc.271.32.19298.

Friedrich CL, Rozek A, Patrzykat A, Hancock REW. 2001. Structure and Mechanism of Action of an Indolicidin Peptide Derivative with Improved Activity against Gram-positive Bacteria. Journal of Biological Chemistry. 276(26):24015–24022. doi:https://doi.org/10.1074/jbc.m009691200.

Frieri M, Kumar K, Boutin A. 2017. Antibiotic resistance. Journal of Infection and Public Health. 10(4):369–378. doi:https://doi.org/10.1016/j.jiph.2016.08.007. https://www.sciencedirect.com/science/article/pii/S1876034116301277.

Ganz T. 2003. The Role of Antimicrobial Peptides in Innate Immunity. Integrative and Comparative Biology. 43(2):300–304. doi:https://doi.org/10.1093/icb/43.2.300.

Ghosh A, Kar RK, Jana J, Saha A, Jana B, Krishnamoorthy J, Kumar D, Ghosh S, Chatterjee S, Bhunia A. 2014. Indolicidin Targets Duplex DNA: Structural and Mechanistic Insight through a Combination of Spectroscopy and Microscopy. ChemMedChem. 9(9):2052–2058. doi:https://doi.org/10.1002/cmdc.201402215.

Goyal T, Schmotzer CL. 2015. Validation of Hemolysis Index Thresholds Optimizes Detection of Clinically Significant Hemolysis. American Journal of Clinical Pathology. 143(4):579–583. doi:https://doi.org/10.1309/ajcpdude1hra0ymr.

Hsu C-H, Huang W-T, Chen C, Jou M-L, Wu S-H, Lee AY-L, Lin Y-C, Yu Y-P. 2005. Structural and DNA-binding studies on the bovine antimicrobial peptide, indolicidin: evidence for multiple conformations involved in binding to membranes and DNA. Nucleic Acids Research. 33(13):4053–4064. doi:https://doi.org/10.1093/nar/gki725.

Jenssen H. 2009. Therapeutic Approaches Using Host Defence Peptides to Tackle Herpes Virus Infections. Viruses. 1(3):939–964. doi:https://doi.org/10.3390/v1030939.

King AA, Chakrabarty S, Zhang W, Zeng X, Ohman DE, Wood L, Abraham S, Rao RD, Wynne KJ. 2014. High Antimicrobial Effectiveness with Low Hemolytic and Cytotoxic Activity for PEG/Quaternary Copolyoxetanes. Biomacromolecules. 15(2):456–467. doi:https://doi.org/10.1021/bm401794p.

Kumar P, Kizhakkedathu J, Straus S. 2018. Antimicrobial Peptides: Diversity, Mechanism of Action and Strategies to Improve the Activity and Biocompatibility In Vivo. Biomolecules. 8(1):4. doi:https://doi.org/10.3390/biom8010004.

Ladokhin AS, Selsted ME, White SH. 1997. Bilayer Interactions of Indolicidin, a Small Antimicrobial Peptide Rich in Tryptophan, Proline, and Basic Amino Acids. Biophysical Journal. 72(2):794–805. doi:https://doi.org/10.1016/s0006-3495(97)78713-7.

Lee DG, Kim HK, Kim SA, Park Y, Park S-C, Jang S-H, Hahm K. 2003. Fungicidal effect of indolicidin and its interaction with phospholipid membranes. Biochemical and Biophysical Research Communications. 305(2):305–310. doi:https://doi.org/10.1016/s0006-291x(03)00755-1

Lee H, Yang S, Shin S-H. 2023. Effect of central PxxP motif in amphipathic alpha-helical peptides on antimicrobial activity and mode of action. Journal of analytical science & technology. 14(1). doi:https://doi.org/10.1186/s40543-023-00398-x.

Lei J, Sun L, Huang S, Zhu C, Li P, He J, Mackey V, Coy DH, He Q. 2019. The antimicrobial peptides and their potential clinical applications. American Journal of Translational Research. 11(7):3919–3931. https://pubmed.ncbi.nlm.nih.gov/31396309/.

Li J, Koh J-J, Liu S, Lakshminarayanan R, Verma CS, Beuerman RW. 2017. Membrane Active Antimicrobial Peptides: Translating Mechanistic Insights to Design. Frontiers in Neuroscience. 11. doi:https://doi.org/10.3389/fnins.2017.00073.

Lipke PN, Ovalle R. 1998. Cell wall architecture in yeast: new structure and new challenges. Journal of bacteriology. 180(15):3735–40. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC107352/.

Lozančić M, Žunar B, Hrestak D, Lopandić K, Teparić R, Mrša V. 2021. Systematic Comparison of Cell Wall-Related Proteins of Different Yeasts. Journal of Fungi. 7(2):128. doi:https://doi.org/10.3390/jof7020128.

Luna-Vázquez-Gómez R, Arellano-García ME, García-Ramos JC, Radilla-Chávez P, Salas-Vargas DS, Casillas-Figueroa F, Ruiz-Ruiz B, Bogdanchikova N, Pestryakov A. 2021. Hemolysis of Human Erythrocytes by Argovit^{TM} AgNPs from Healthy and Diabetic Donors: An In Vitro Study. Materials. 14(11):2792. doi:https://doi.org/10.3390/ma14112792.

Marchand C, Krajewski K, Lee H-F, Antony S, Johnson AA, Amin R, Roller P, Kvaratskhelia M, Pommier Y. 2006. Covalent binding of the natural antimicrobial peptide indolicidin to DNA abasic sites. Nucleic Acids Research. 34(18):5157–5165. doi:https://doi.org/10.1093/nar/gkl667. [accessed 2021 Nov 2]. https://academic.oup.com/nar/article/34/18/5157/3112128?login=true.

Mazurkiewicz-Pisarek A, Baran J, Ciach T. 2023. Antimicrobial Peptides: Challenging Journey to the Pharmaceutical, Biomedical, and Cosmeceutical Use. International Journal of Molecular Sciences. 24(10):9031. doi:https://doi.org/10.3390/ijms24109031. [accessed 2023 Jun 9]. https://www.mdpi.com/1422-0067/24/10/9031.

Michiels J, Xi C, Verhaert J, Vanderleyden J. 2002. The functions of Ca2+ in bacteria: a role for EF-hand proteins? Trends in Microbiology. 10(2):87–93. doi:https://doi.org/10.1016/s0966-842x(01)02284-3.

Mishra A, Choi J, Moon E, Baek K-H. 2018. Tryptophan-Rich and Proline-Rich Antimicrobial Peptides. Molecules. 23(4):815. doi:https://doi.org/10.3390/molecules23040815.

Pasupuleti M, Schmidtchen A, Malmsten M. 2011. Antimicrobial peptides: key components of the innate immune system. Critical Reviews in Biotechnology. 32(2):143–171. doi:https://doi.org/10.3109/07388551.2011.594423.

Peng C, Liu Y, Shui L, Zhao Z, Mao X, Liu Z. 2022. Mechanisms of Action of the Antimicrobial Peptide Cecropin in the Killing of Candida albicans. Life. 12(10):1581. doi:https://doi.org/10.3390/life12101581.

Podorieszach AP, Huttunen-Hennelly HEK. 2010. The effects of tryptophan and hydrophobicity on the structure and bioactivity of novel indolicidin derivatives with promising pharmaceutical potential. Organic & Biomolecular Chemistry. 8(7):1679. doi:https://doi.org/10.1039/b921248e.

Qiu C, Sawada K, Zhang X, Cheng X. 2002 Feb 11. The PWWP domain of mammalian DNA methyltransferase Dnmt3b defines a new family of DNA-binding folds. Nature Structural Biology. doi:https://doi.org/10.1038/nsb759.

Rokitskaya TI, Kolodkin NI, Kotova EA, Antonenko YN. 2011. Indolicidin action on membrane permeability: Carrier mechanism versus pore formation. Biochimica et Biophysica Acta (BBA) - Biomembranes. 1808(1):91–97. doi:https://doi.org/10.1016/j.bbamem.2010.09.005.

Rozek A, Friedrich CL, Robert. 2000. Structure of the Bovine Antimicrobial Peptide Indolicidin Bound to Dodecylphosphocholine and Sodium Dodecyl Sulfate Micelles†. Biochemistry. 39(51):15765–15774. doi:https://doi.org/10.1021/bi000714m.

Rozek A, Powers J-PS, Friedrich CL, Hancock REW. 2003. Structure-Based Design of an Indolicidin Peptide Analogue with Increased Protease Stability. Biochemistry. 42(48):14130–14138. doi:https://doi.org/10.1021/bi035643g.

Sitaram N, Subbalakshmi C, Nagaraj R. 2003. Indolicidin, a 13-residue basic antimicrobial peptide rich in tryptophan and proline, interacts with Ca2+-calmodulin. Biochemical and biophysical research communications. 309(4):879–884. doi:https://doi.org/10.1016/j.bbrc.2003.08.095.

Subbalakshmi C, Bikshapathy E, Sitaram N, Nagaraj R. 2000. Antibacterial and Hemolytic Activities of Single Tryptophan Analogs of Indolicidin. Biochemical and Biophysical Research Communications. 274(3):714–716. doi:https://doi.org/10.1006/bbrc.2000.3214.

Subbalakshmi C, Krishnakumari V, Nagaraj R, Sitaram N. 1996. Requirements for antibacterial and hemolytic activities in the bovine neutrophil derived 13-residue peptide indolicidin. FEBS Letters. 395(1):48–52. doi:https://doi.org/10.1016/0014-5793(96)00996-9.

Subbalakshmi C, Sitaram N. 1998. Mechanism of antimicrobial action of indolicidin. FEMS Microbiology Letters. 160(1):91–96. doi:https://doi.org/10.1111/j.1574-6968.1998.tb12896.x. [accessed 2021 Nov 2]. https://academic.oup.com/femsle/article/160/1/91/515578.

Swoboda JG, Campbell J, Meredith TC, Walker S. 2009. Wall Teichoic Acid Function, Biosynthesis, and Inhibition. ChemBioChem. 11(1):35–45. doi:https://doi.org/10.1002/cbic.200900557.

Wimley WC, White SH. 1996. Experimentally determined hydrophobicity scale for proteins at membrane interfaces. Nature Structural & Molecular Biology. 3(10):842–848. doi:https://doi.org/10.1038/nsb1096-842.

Yu G, Baeder DY, Regoes RR, Rolff J. 2016. Combination Effects of Antimicrobial Peptides. Antimicrobial Agents and Chemotherapy. 60(3):1717–1724. doi:https://doi.org/10.1128/aac.02434-15.

Yu G, Baeder DY, Regoes RR, Rolff J. 2018. Predicting drug resistance evolution: insights from antimicrobial peptides and antibiotics. Proceedings of the Royal Society B: Biological Sciences. 285(1874):20172687. doi:https://doi.org/10.1098/rspb.2017.2687.

Zhang Q-Y, Yan Z-B, Meng Y-M, Hong X-Y, Shao G, Ma J-J, Cheng X-R, Liu J, Kang J, Fu C-Y. 2021. Antimicrobial peptides: mechanism of action, activity and clinical potential. Military Medical Research. 8(48):48. doi:https://doi.org/10.1186/s40779-021-00343-2. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8425997/.