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THE EFFECTS OF MODIFYING HYDROPHOBIC TRYPTOPHAN RESIDUES IN NOVEL INDOLICIDIN DERIVATIVES WITH PHARMACEUTICAL POTENTIAL

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THE EFFECTS OF MODIFYING HYDROPHOBIC TRYPTOPHAN RESIDUES IN NOVEL INDOLICIDIN DERIVATIVES WITH PHARMACEUTICAL POTENTIAL

by

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ABSTRACT

The creation of new antimicrobial agents is a significant area of research needed to be examined due to the era of antimicrobial resistance. Various microbial products are being studied to combat this issue. One approach to tackle this problem is the use of antimicrobial peptides (AMPs) which are the main focus of this study. However, using AMPs as antimicrobial medication is hindered due to their potential toxic and hemolytic activity. This study will analyze the AMP indolicidin which is known to have exceptional antimicrobial activity. Although a wide variety of microbes are susceptible to indolicidin, it would make a poor pharmaceutical due to its high hemolytic activity. To counter this, different derivatives of indolicidin will be examined that have tryptophan amino acid residues replaced with alanine residues in an attempt to reduce hemolytic properties, while also maintaining bioactivity. In this study, the indolicidin derivatives Δ4,8, Δ4,9, Δ6,11, and Δ8,11 underwent antibiotic sensitivity testing to inspect their potential therapeutic use. The cytotoxicity properties of the peptides was also analyzed via a hemolytic and apoptotic/necrosis assay.

Thesis Supervisor: Associate Professor Heidi Huttunen-Hennelly

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INTRODUCTION

Antibiotic Resistance

Antibiotics are based on natural products and used to fight microbial infections. They are mainly derived from natural secretions of soil-living bacteria and fungi. Microorganisms in their native environment have to compete with each other by generating antimicrobial compounds and need resistance strategies for survival. These antimicrobial agents are great candidates for antibiotics, however the organism creating these molecules are usually resistant to these agents themselves (Durand et al., 2019). For 70 years such antibiotics have been used for a wide selection of medical applications and have been revolutionary, saving millions of lives (Uddin et al., 2021). Antibiotics are unique products as they act selectively on microbes while leaving human cells unharmed, thus being of great medical potential (Skld, 2001). Medical disciplines including oncology and organ transplantation surgeries would be useless without the use of antimicrobial agents (Skld, 2001).

Although antibiotics have been medically revolutionary, over the years the microbial resistance crisis has been building due to the accumulated misuse of these medications. The misuse of these agents has allowed horizontal gene transfer amongst microbes, giving rise to microbial evolution. Such evolution has led to a plethora of antibiotic resistant pathogens (Jones et al., 2010). This has caused a major increase in multidrug resistant (MDR) strains of bacteria which are the most troublesome bacteria to deal with in healthcare systems. Additionally, there is also a lack of antifungal agents, creating a high demand for such products (Váradi et al., 2022). Some studies predict that in coming years millions of people will be killed from drug resistant microbes (Durand et al., 2019). This problem has stumped healthcare systems and researchers worldwide as new strategies are required to tackle bacterial and fungal infections (Jones et al., 2010; Uddin et al., 2021). To act against this issue, new sources of drugs that are easy to make, cheap and safe are of focus. Antimicrobial peptides (AMPs) match these criteria and are in need of further investigation (Podorieszach & Huttunen-Hennelly, 2010).

Antimicrobial Peptides

Animals, plants, and humans have a unique fast acting mechanism of protection against microbial infections. This process works separately from the adaptive immune system which requires time to produce cells capable of creating antibodies (Skld, 2001). This first line of defense is known as innate immunity, where AMPs can be released by tissues and cells to act on intruders (Skld, 2001). AMPs are host defense peptides that are existent in a wide variety of eukaryotic and prokaryotic organisms (Durand et al., 2019; Lei et al., 2010). These small chains of amino acids were first discovered in the 1980's and display antimicrobial activity against bacteria, fungi, and even viruses (Lei et al., 2010). There are four main classes of AMPs based on their secondary structure. They are generally displayed as an α-helix, β-sheet, non-structured with a lack of α-helix and β-sheet properties (extended), or as a combination of these three structures, as shown in Figure 1 (Biljana & Jenssen, 2015). Unlike antibiotics, AMPs have unique, fast acting mechanisms which make AMPs capable of killing various microbes, including drug-resistant ones (Lei et al., 2010). AMPs are usually cationic and amphiphilic and can be isolated from almost all living things (Durand et al., 2019; Lei et al., 2010). The cationic property allows AMPs to interact with negatively charged bacterial cell membranes to induce damage to the cell (Lei et al., 2010). AMPs have also been shown to interact with intercellular targets after penetrating into the cytoplasm (Durand et al., 2019). Thus, AMPs are a great contender to combat microbial resistance.

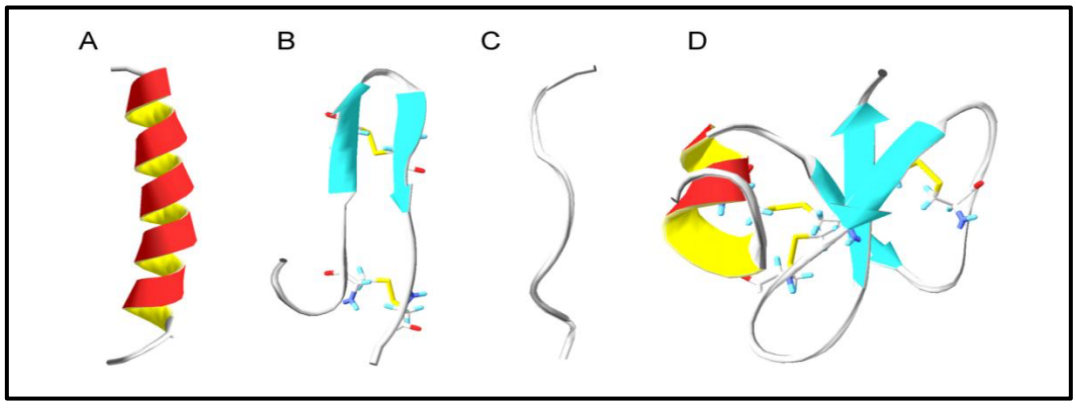

Figure 1. The generalized structures of AMPs (Biljana & Jenssen, 2015). α-helical peptide conformation (A); β-sheeted peptide (B); extended peptide (C); mixed structured peptide (D).

Indolicidin

An AMP of particular interest is indolicidin (ind) as it displays antimicrobial activity against a variety of microbes such as Gram-positive bacteria, Gram-negative bacteria, fungi, protozoa, and viruses (Jones et al., 2010). It also shows promising activity towards biofilms as it has been proven to both inhibit formation of biofilms and destruct already formed biofilms. This peptide belongs to the cathelicidin family that is found in a variety of mammalian species and was originally isolated from bovine neutrophils (Araujo et al., 2022). Ind has a special thirteen amino acid sequence (ILPWKWPWWPWRR-NH_2) which is 39% tryptophan (Figure 2). Peptides that are rich in tryptophan and arginine, and highly active despite their short length can be classified under the cathelicidin family (Ando et al., 2010). Structurally, based on circular dichroism and ^{1}H NMR spectroscopy, ind can be described as an unordered peptide with a lack of structure, as shown in Figure 1C (Jones et al., 2010; Podorieszach & Huttunen-Hennelly, 2010). Ind has an overall charge of 4+ coming from the positively charged arginine residues and the amidated C-terminus. Before amidation the peptide would have an overall 2+ charge. The C-terminus amidation replaces the negatively charged carboxylate that is present in the natural zwitterionic form. By adding an amide to the C-terminus it is not only removing a negative charge, but it also adds an additional positive charge. Thus, amidation is largely responsible for the 4+ charge of ind as net increases the cationic character by two positive charges. Amidation is significant for shorter peptides such as ind as it enhances electrostatic binding to cell membranes and protects the peptide from enzymatic degradation (Chaudhary et al., 2023). This bovine AMP contains multiple tryptophan residues which have a natural attraction for the cell membrane interface, a critical feature for antimicrobial activity (Ando et al., 2010). However, the five highly hydrophobic amino acids present in ind give it high hemolytic activity, limiting its usefulness as an antimicrobial agent (Jones et al., 2010; Podorieszach & Huttunen-Hennelly, 2010). Several studies have been performed to better understand the roles of the amino acid residues and how synthesizing analogous peptides via residue replacements/modifications can be used to improve the activity of ind (Ando et al., 2010; Podorieszach & Huttunen-Hennelly, 2010).

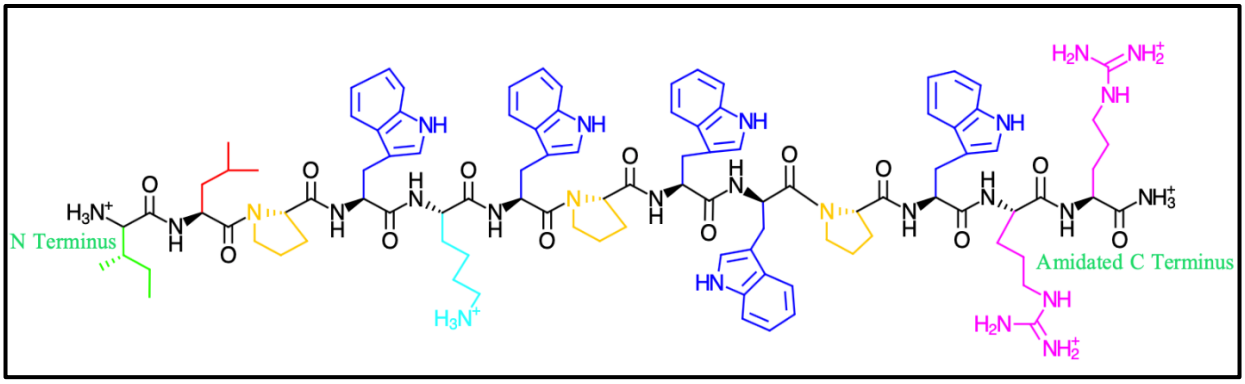

Figure 2. The amino acid sequence of indolicidin from N terminus (left) to the amidated C terminus (right).

Modification of Indolicidin

In aims to reduce the hydrophobicity and maintain or improve the antimicrobial activity of ind, modification of the peptide is of interest. Previous studies have also followed this idea of modification by changing ind amino acid residues. According to research the removal of arginine can greatly reduce the antibacterial activity of ind, proving that the cationic property is important for activity (Ando et al., 2010). However, if the positive charge is increased in ind analogues using arginine or lysine, there will be more activity against both Gram-negative and Gram-positive bacteria and yeast, while decreasing toxicity (Falla & Hancock, 1997). According to Ando et al. (2010), tryptophan or large ring aromatic structures are also crucial to an AMPs activity. They found that specific tryptophan residues in ind were linked to hemolysis and antimicrobial activity. Tryptophan at positions 9 and 11 were linked to hemolysis, and positions 4 and 11 are crucial for antimicrobial activity as displayed in Figure 3 (Ando et al., 2010). In another study it has been found that reducing ind's hydrophobic tryptophan residues and replacing them with the amino acid alanine has led to lower hemolytic activity and an increase in antimicrobial activity (Podorieszach & Huttunen-Hennelly, 2010). However, this is only true if no more or no less than 2 tryptophan's are replaced starting from the C-terminus, so that one WPW motif can be retained. If more than 2 tryptophan's are removed starting from the C-terminus, then ind will begin to lose antimicrobial activity, as beyond this threshold there is a complete loss of both WPW motifs. On the other hand, replacing less than 2 tryptophan's with alanine beginning at the C-terminus kept the hemolytic activity. Thus, the replacement of 2 tryptophan's had the most significant results as there was the

least hemolytic activity and the most antimicrobial activity making it an ideal AMP (Jones et al., 2010; Podorieszach & Huttunen-Hennelly, 2010). Overall, it is important to investigate derivatives of ind to potentially discover AMPs that can be used as new antimicrobial agents, eliminating drug-resistant microbes.

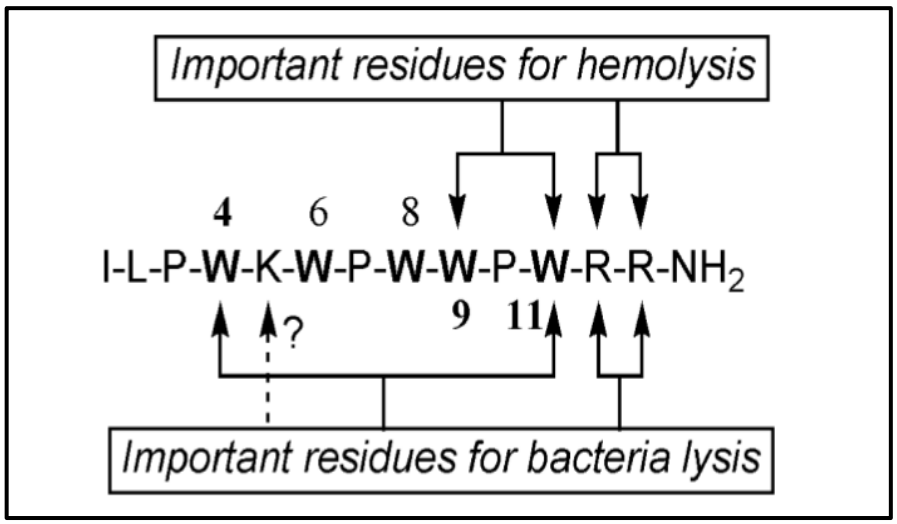

Figure 3. Ind's important amino acid residues linked to hemolysis and bacterial destruction (Ando et al., 2010).

Pathogen Specificity

AMPs, including ind, are naturally specific to pathogens such as Gram-positive bacteria, Gram-negative bacteria, fungi, and viruses that they are disruptive towards. All AMPs are membrane interactive, yet some mechanisms seem to involve the attack on intracellular targets (Dennison et al., 2005). Due to ind's cationic properties it is able to target the anionic lipopolysaccharides (LPS) in Gram-negative bacteria. This displaces cationic molecules such as Ca^{2+} and Mg^{2+} ions that stabilize the membrane and leads to membrane expansion. As for Gram-positive bacteria, the negative charge on teichoic acids, teichuronic acids, and carboxylic acid groups on the peptidoglycan layer attract cationic AMPs. These charged groups allow ind passage through the peptidoglycan layer and give access to the anionic phospholipids on the cytoplasmic membrane (Dennison et al., 2005). As for viruses, AMPs such as ind have a broad-spectrum of

activity against enveloped viruses as they are able to target negatively charged membrane glycoproteins. Although there is little information on the subject, AMPs can also be specific to fungi which is believed to be due to interactions with the anionic mannans on the cell wall of fungal cells (Zhang et al., 2021). Even though AMPs are acting on cells *in vivo*, host cells tend to not be at risk. Human and other mammalian eukaryotic cells may be safe from host invasion due to cholesterol content in the cell membranes (Lai & Gallo, 2009). Since cholesterol decreases membrane permeability, and condenses the lipid bilayer, this could make it more difficult for AMPs to penetrate the membrane. Another property of eukaryotic cells is that phospholipids are distributed asymmetrically. The outer leaflet consists of neutral phospholipids, whereas the inner leaflet contains most of the negatively charged phospholipids. This characteristic gives the AMPs little affinity to bind to the host mammalian cells (Lai & Gallo, 2009). On the contrary, AMPs still display hemolytic activity and may likely disrupt the osmotic balance, leading to red blood cell rupture (Rokitskaya et al., 2011).

Potential Mode of Action

Elucidating the potential mode of actions of AMPs is important as it allows one to understand the microscopic processes that lead to microbial death. This can be significant for developing peptide analogs and to further understand them (Araujo et al., 2022). Ind is an amphiphilic molecule. It contains a core nonpolar sequence (WPWWPW) which is flanked by cationic amino acids. This core section of ind is believed to be essential for partitioning into pathogen's membranes while the cationic residues electrostatically interact with the lipid headgroup region of the microbial cell membrane (Dennison et al., 2005).

One potential mechanism of ind is described as transient membrane perturbation (Figure 4a). Once ind disturbs the membrane, it is believed that ind can act intracellularly. According to Hsu et al. (2005), at a concentration higher than the minimum inhibitory concentration (MIC), ind can perturb the microbial cell membrane, leading to cell death by lysis. However, at concentrations at or smaller than the MIC ind is believed to kill bacteria through other methods such as inhibiting DNA synthesis. Ind is capable of changing conformations, supported by NMR structural data,

which is believed to aid ind's ability to partition through the lipid bilayer and bind to DNA or proteins involved in synthesizing DNA (Hsu et al., 2005). Thus, in theory, when there is a large amount of ind molecules present, it will be more probable for multiple ind molecules to disrupt the integrity of the cell membrane, causing the cell to lyse, which does not always lead to cell death. However, as this concentration decreases, the less likely there will be enough ind to cause cell lysis, meaning that ind has to rely on intracellular mechanisms. This likely explains why Hsu et al. (2005) found that lower concentrations were the most successful for killing pathogens as ind had to rely on inhibiting DNA synthesis or by binding to proteins and disrupting their function.

Related to the transient membrane perturbation, there is also a mode of action called the Barrel stave mechanism which creates pores in a microbial membrane (Figure 5). Where instead of relying on conformational changes to pass through the lipid bilayer, ind forms a pore allowing other ind molecules to pass through the cell membrane or by causing the membrane to lyse. In this model an AMP binds to the lipid headgroup of a pathogen's cell membrane. Multiple peptides will aggregate in the same area because of hydrophobic interactions. Then, the hydrophobic faces of these peptides can align with their hydrophilic domains penetrating through the membrane forming a pore (Dennison et al., 2005). This mechanism is likely more probable at higher concentrations of peptide as this would increase the chance of peptide aggregation in one area. Based on research by Falla et al. (1996), regardless of the fact that ind is a small molecule and lacks a defined structure, it is still able to form such pores as it can be in an extended conformation allowing it to span the lipid bilayer.

Another one of ind's potential mode of actions is believed to be from its ability to bind to LPS (Figure 4b). The LPS is an endotoxin and is part of the outer membrane of Gram-negative bacteria. LPS activates an inflammatory response in the body by causing cytokines and nitric oxide to be secreted by inflammatory cells. By binding to LPS ind can neutralize this effect and inhibit septic shock of the host (Nan et al., 2009). Furthermore, this property is linked to ind's ability to halt the production and adhesion of biofilms. Similar to LPS, lipoteichoic acid in Gram-positive bacteria seems to play a similar role and is another target of ind (Araujo et al., 2022).

According to Rokitskaya et al. (2011), ind can permeabilize membranes through organic anionic transport (Figure 4c). Ind can form complexes with anions and transport across membranes. This mechanism is thought to be linked to the hemolysis of erythrocytes as ind may disturb anionic metabolites and the surrounding environment (Rokitskaya et al., 2011).

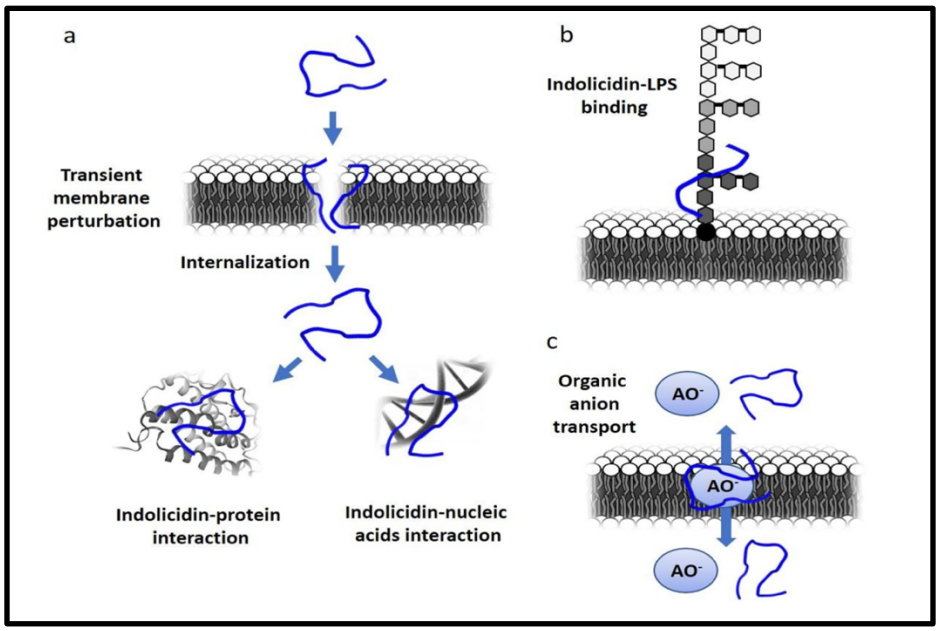

Figure 4. The three main proposals for ind's mode of action (Araujo et al., 2022). Transient membrane perturbation followed by interaction with either proteins or DNA to inhibit DNA synthesis (a); Ind's mechanism involving anti-sepsis, and prevention of biofilm production/adhesion (b); Ind's ability to bind to organic anions across cell membranes (c).

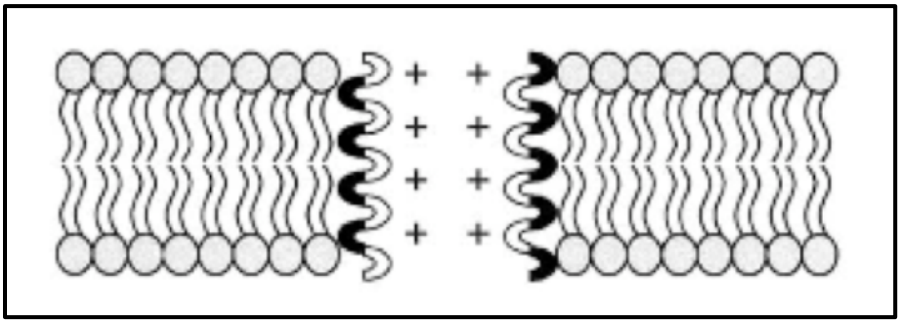

Figure 5. Schematic representation of pore formation via the Barrel stave mechanism (Dennison et al., 2005).

Project Objective

This project is meant to address the pharmaceutical potential of four ind derivatives (Figure 6). Analogs with two alanines replaced, Δ4,8, Δ4,9, Δ6,11, and Δ8,11 (Δ denoting the position of the tryptophan that is replaced by alanine), alongside ind will be analyzed (Table 1). Each of these

derivatives are identical physiochemically and only differ by the order of their amino acids. To understand the antibiotic potential of these AMPs they will each be tested against a series of microorganisms including Gram-positive bacteria, Gram-negative bacteria, and yeast (Table 2). Bioactivity against these microbes will be determined via the Kirby-Bauer assay as well as the MIC assay. The hemolytic activity of the derivatives will also be measured and compared to ind to see if this cytotoxic trait will be reduced. Lastly, since AMP mechanisms are not well understood, experiments will be performed to aid in understanding a potential mode of action. A necrosis/apoptosis assay will be performed in aims of figuring out a mechanism as well as to determine if nucleated mammalian cells are at risk.

Table 1. Ind and derivative sequences with their abbreviations.

Peptide Sequence	Abbreviation
ILPWKWPWWPWRR-NH_2	Ind
ILPAKWPAWPWRR-NH_2	Δ4,8
ILPAKWPWAPWRR-NH_2	Δ4,9
ILPWKAPWWPARR-NH_2	Δ6,11
ILPWKWPAWPARR-NH_2	Δ8,11

Note: Highlighted W's are the tryptophan's of interest. Highlighted A's are the alanine's in replace of tryptophan. Other amino acids include isoleucine (I), leucine (L), proline (P), and arginine (R), lysine (K).

Table 2. Microorganism used for bioactivity assays, the type of cell, and antibiotics that can be used as controls to kill them.

Microbe	**Microbe Type**	**Antibiotic Control**
Pseudomonas aeruginosa	Gram-negative	Tetracycline
Salmonella typhimurium	Gram-negative	Tetracycline
Escherichia coli	Gram-negative	Ampicillin
Candida albicans	Yeast	Nystatin
Methicillin-resistant *Staphylococcus aureus*	Gram-positive	Ampicillin
Staphylococcus *aureus*	Gram-positive	Ampicillin

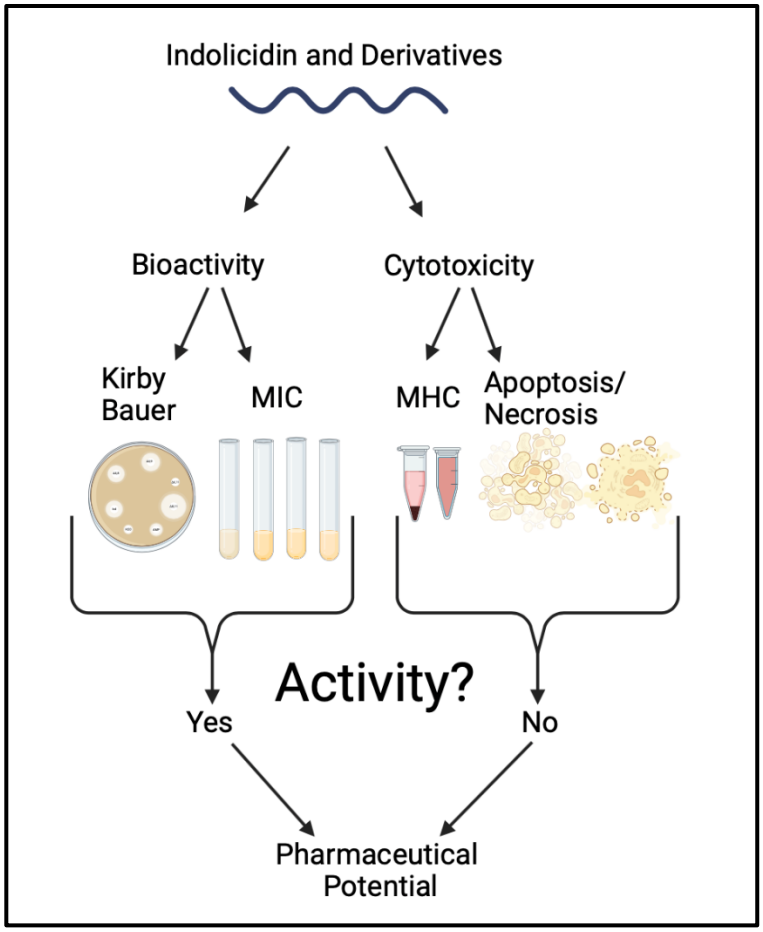

Figure 6. The general overview of the project and the assays that will be used to determine the pharmaceutical potential of the peptide derivatives.

MATERIALS AND METHODS

Peptide Synthesis

Each peptide was ordered in 20 mg vials and synthesized by Biomatik Corporation. All five of the peptides came with quality control as they are analyzed on MS and HPLC before shipment. All peptides were guaranteed approximately ≥ 95% purity and can be stored for long periods at -20°C. Solid phase synthesis is the method used by Biomatik to generate such peptides.

Bioactivity

To confirm bioactivity of each peptide, the Kirby-Bauer assay was performed (Figure 7). At various concentrations, 25 µL of each peptide was soaked onto 6 mm sterilized paper disks three times with an hour of drying time in between intervals. Additionally, all six microorganisms mentioned in Table 2 were grown in 1 mL Eppendorf tubes of Mueller Hinton broth and incubated for approximately 16 hours until a turbidity of 0.600 – 1.000 was obtained. This was determined by a spectrophotometer set to a wavelength at 600 nm. Six Erlenmeyer flasks containing 20 mL of Mueller Hinton agar were placed in the water bath at 55°C to keep from solidifying. 0.2 mL of the microbes from the Eppendorf tubes were pipetted into one of the Erlenmeyer flasks which were set aside. Immediately after, these bacteria containing flasks were poured into Petri dishes. Once the nutrient agar settled in the Petri dishes, all the Kirby-Bauer discs containing each peptide were placed evenly around the plate (Figure 7; Figure 11) alongside water (negative control) and an antimicrobial disc (positive control) containing the bacteria's respective antibiotic (Table 2). The plates were then incubated for 24 hours at 37°C and if any zones of inhibition appeared around the paper discs, they would be measured by a ruler and recorded.

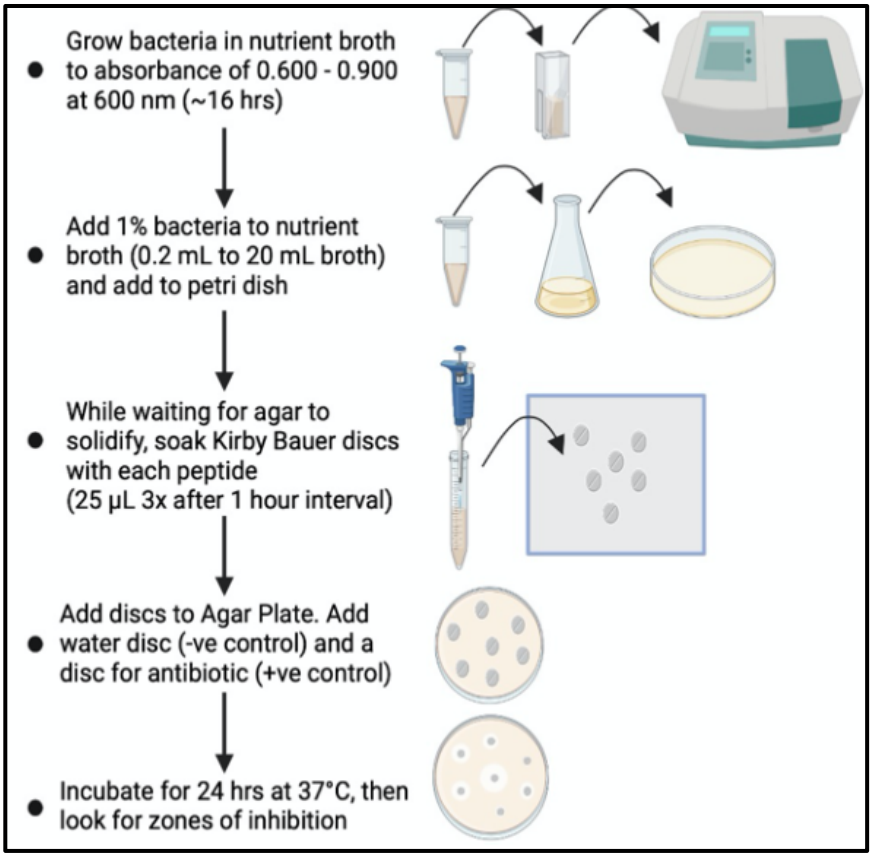

Figure 7. A visual overview of the Kirby Bauer assay.

The next bioactivity assay performed was the MIC assay (Figure 8). Peptides were made up to known concentrations in water and diluted to the desired concentrations using Mueller Hinton broth. The various bacterial and yeast species mentioned in Table 2 were inoculated into Mueller Hinton broth using multiple isolated culture colonies. These samples were placed on a shaker incubator for 30 minutes at 37°C and microbes were grown to mid-logarithmic phase. A 0.5 MacFarland standard was used to analyze the cultures and if they were too cloudy, they would need to be diluted with Mueller Hinton broth. From here, the microbes were transferred to the broth containing known amounts of peptides via a sterile loop. Cultures were then incubated at 37°C for 18-24 hours and optical density was determined using a spectrophotometer at 600 nm. Mueller Hinton broth was used as a blank. Broth containing 0 µg/mL of peptide was used as a negative control. The MIC was defined as $\geq$85% of the initial inoculum resulting in death.

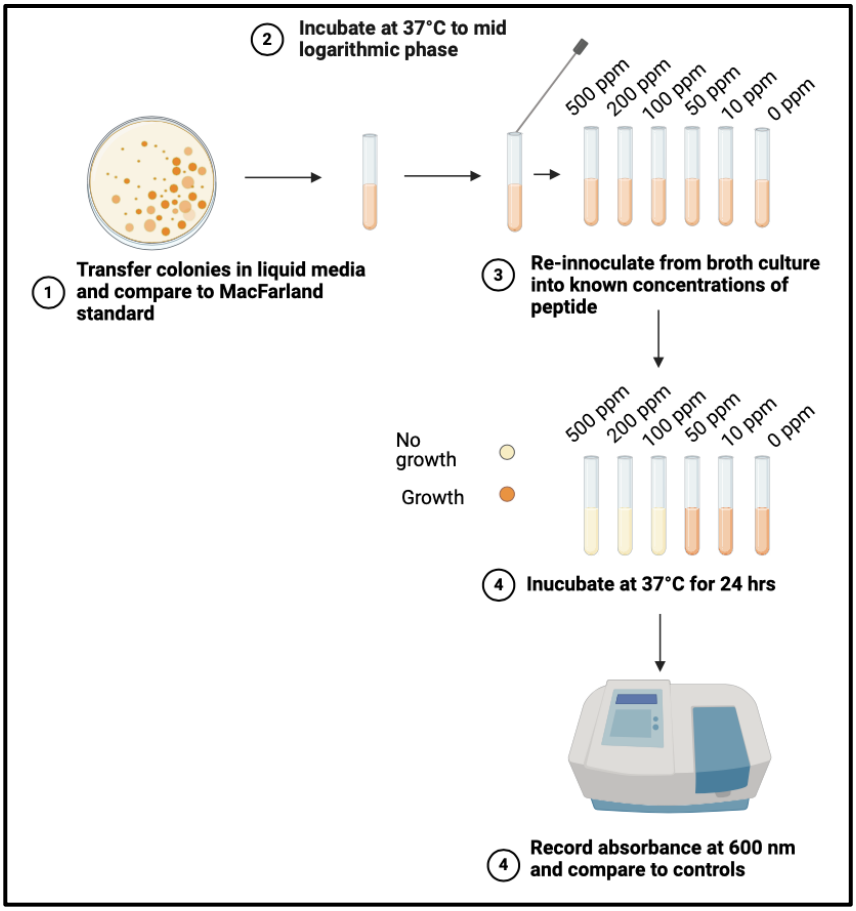

Figure 8. A visual map of the MIC assay performed on each peptide.

Hemolysis Assay

For the hemolysis assay (Figure 9), samples of horse whole blood were donated for this research by the TRU veterinary technician program. All peptides were prepared at various concentrations in 1 x PBS (phosphate buffer saline) using the nanodrop to confirm concentration values. To isolate the red blood cells for the hemolysis assay, whole blood was centrifuged at 3000 rpm for 10 minutes at 4°C, causing the supernatant to be aspirated. The supernatant was discarded using a pipette as it is easy to disrupt the erythrocyte pellet. The pellet was then resuspended in 1 x PBS (pH 7.4) at ten times the volume of the pellet and centrifuged again under the same conditions. This washing process was performed three times, before bringing the blood back to whole blood volume. 1 x PBS was then added at a volume 10 times the whole blood volume. This process will give approximately $5x10^8$ cells/mL and can be confirmed by using a hemocytometer

to count a portion of the cells. Of this sample, 200 µL of the blood sample was added to 800 µL of 1 x PBS at known peptide concentrations. 800 µL of 1% Triton X-100 was used as a positive control, whereas 800 µL of 1 x PBS was used as the negative control. Each sample vial was inverted several times to ensure homogeneity. Next, each sample was placed on a shaker incubator at 37°C on a slow rocking speed of 50 rpm. The samples were incubated for a total of 1 hour and were inverted multiple times after the first 30 minutes of incubation. From here, each vial was placed in centrifuge and was centrifuged for 5 mins at 4°C and 14,000 rpm to release hemoglobin from dead erythrocytes into solution. The hemoglobin solutions were detected on the VWR® M4, UV/Visible Spectrophotometer at 541 nm, using 1 x PBS as the blank. Assuming Triton X-100 causes 100% red blood cell lysis and 1 x PBS causes 0% lysis, the following equation can be derived to determine the percent hemolysis:

$$\frac{(Sample\ Absorbance) - (Negative\ Control\ Absorbance)}{(Positive\ Control\ Absorbance)}\ x\ 100 = \ Percent\ Hemolysis$$

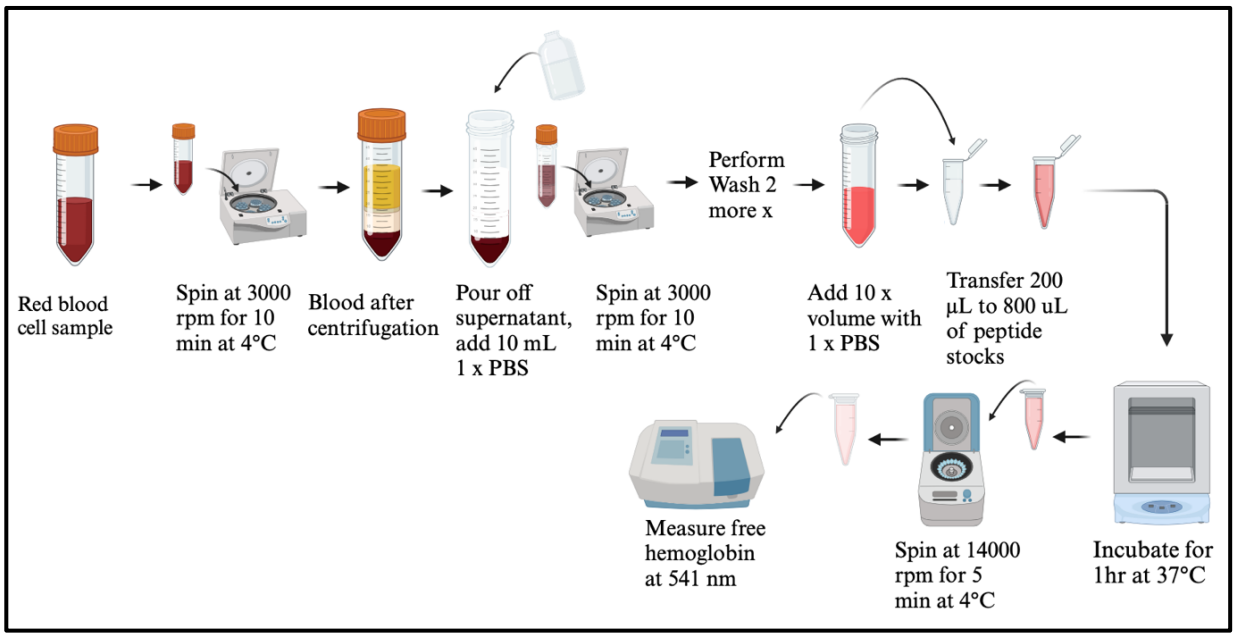

Figure 9. The general procedure of the hemolysis assay.

Apoptosis/Necrosis Assay

To elucidate ind and ind derivatives potential mechanisms and cytotoxicity an apoptosis/necrosis assay was performed (Figure 10). First, peptide stocks were made at concentrations near the MIC values in FACS (Fluorescent Activated Cell Sorting) buffer (1% BSA, 1 x PBS, 5 mM EDTA). This was done by adding peptides into 1 x PBS until reaching double the desired concentration at half the volume, based on nanodrop readings. Then the stocks were diluted with FACS buffer with 2% BSA to double the volume and reach the desired BSA and peptide concentration. Whole blood samples were ethically obtained by human participants. 1 mL of the whole blood was washed with 10 mL of 1 x lysis buffer in a centrifuge tube. The blood sample was then incubated at room temperature for 10 minutes with rocking at 50 rpm. The cells were centrifuged at 300 RCF for 8 minutes at 4°C and the supernatant was removed. Next, the pellet was resuspended in 10 mL of 1 x FACS buffer by gently pipetting the solution. Washing was done at 300 RCF and 4°C for 8 minutes and was repeated 2 more times. After the last wash, the pellet was resuspended in 5 mL of FACS buffer. This gave approximately $1x10^6$ cells/mL which can be confirmed by a hemocytometer or by the flow cytometer. The 5 mL of the white blood cell sample was divided into 5 new tubes at 1 mL each to give $1x10^5$ cells/mL. The 1 mL tubes were centrifuged at 300 RCF and 4°C for 8 minutes, the supernatant was discarded, and 1 mL of the peptide stock was added. Samples were incubated for 1 hour with gentle inversions throughout this time. After incubation, the samples were centrifuged, and the peptide stock was removed. From here, the cells were resuspended in 500 µL of annexin-binding buffer and transferred to flow cytometer tube. 5 µL of annexin V-FITC was added, followed by 1 µL of SYTOX green dye. This was performed in the dark and the tube was wrapped in tinfoil and incubated at room temp for 5-10 minutes. Samples were then ran through the flow cytometer using the FITC laser to measure the fluorescence emission at 530 nm. Cell populations were separated into three groups: live cells with low green fluorescence, apoptotic cells with moderate green fluorescence, and necrotic cells with high-intensity green fluorescence. 2 mM hydrogen peroxide was used as a positive control and FACS buffer was used as a negative control.

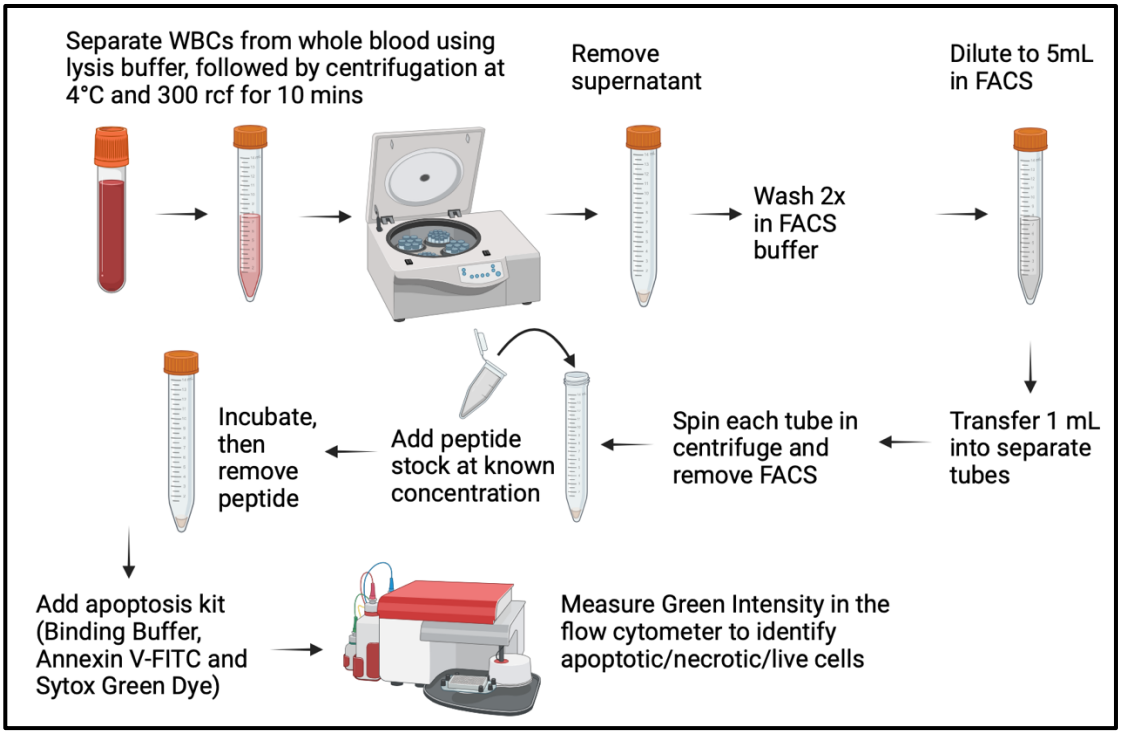

Figure 10. Isolation of white blood cells and apoptosis/necrosis assay.

RESULTS AND DISCUSSION

Antimicrobial Activity

In this study, multiple microbes, including bacteria and fungi (Table 2) were tested against ind and derivatives to determine antimicrobial potential. This assay was performed with the objective of only confirming antimicrobial activity and is why multiple concentrations were not used. Concentrations of 500 µg/mL were applied to the Kirby-Bauer discs three times at 25 µL. This meant that there was approximately 500 ng of peptide per aliquot and thus 1500 ng total in the small 6mm paper discs. It was confirmed through this assay that the derivatives Δ4,8, Δ4,9, Δ6,11, Δ8,11 were all active against microbes, and no inhibition zones were obtained for ind (Table 3). This assay confirmed that derivatives Δ4,8, Δ4,9 were both moderately active against *Escherichia coli* and *Staphylococcus aureus.* Δ6,11, Δ8,11, were also active against *Escherichia coli* and *Staphylococcus aureus*, except the zones of inhibition were much larger for these derivatives. Δ6,11 was also the only derivative that showed inhibition for *Salmonella typhimurium.* These results were surprising because data suggests that at least one WPW motif is to remain when

substituting tryptophan with alanine or else the effectiveness of bacteria lysis will be reduced (Jones et al., 2010; Podorieszach & Huttunen-Hennelly, 2010). This data also contradicts Ando et al. (2010) in that the 4th and 11th tryptophan residue is the most significant for killing microbes. In this assay ind ended up having no zones of inhibition, despite being known to have activity against each of the microorganisms it was tested against. Even though results were obtained they must not be taken too literally because this method is purely qualitative, especially when the zone of inhibition sizes are not compared to a standard curve (Patel et al., 2021). Additionally, by looking at inhibition zones one cannot distinguish whether the effects of microbial inhibition are bactericidal or bacteriostatic. Another consideration is that the inhibition zones can be affected by the solubility, diffusion range, and evaporation. Molecules that are less polar tend to diffuse more slowly in aqueous agar (Bubonja-Šonje et al., 2020). This may explain why the most hydrophobic peptide ind did not result in any inhibition zones. Even though no zones were found necessarily against each microbe, this does not deny their chances of being active against them as the peptides are non-polar and are of high molecular weight. Thus, it is important to perform the MIC assay to determine the true activity of these peptides and what concentrations are ideal for antimicrobial action.

Table 3. Inhibitory zones obtained from Kirby-Bauer assay using peptides at 500 µg/mL.

Peptide	*Escherichia coli*	*Staphylococcus aureus*	*Salmonella typhimurium*
Ind	-	-	-
Δ4,8	+	+	-
Δ4,9	+	+	-
Δ6,11	++	++	+
Δ8,11	++	++	-

Note: - indicates no zones of inhibition, + indicates inhibition zones < 9 mm, ++ indicates zones ≥ 9 mm.

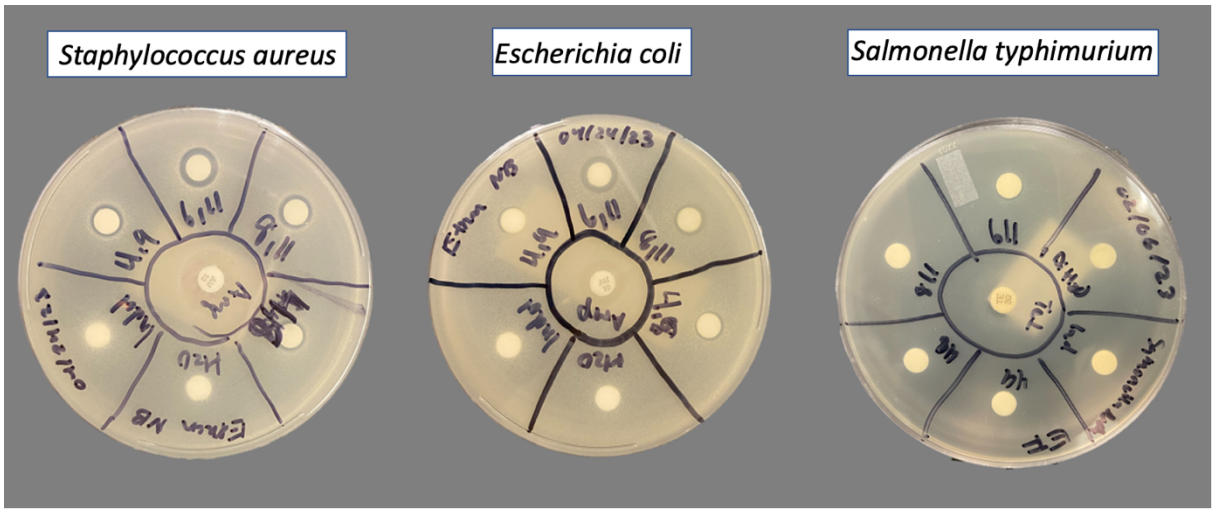

Figure 11. Inhibitory zones on cultured media from the Kirby-Bauer assay.

As for the MIC assay, it was found that ind was active against each microbe that it was tested against. Whereas, for the other four derivatives they were found to be active against mainly Candida *albicans* (Table 4). Derivative Δ6,11 had activity against *Staphylococcus aureus* at 500 µg/mL and derivative Δ4,8 likely does as well between 500-200 µg/mL. Besides ind, derivative Δ6,11 was the most active, there was high activity at 200 µg/mL and moderate activity at 100 µg/mL against *Candida albicans* meaning the MIC value against this organism is likely between 200 and 100 µg/mL. What was interesting about Δ6,11 is that at concentrations 10, 50, and 100 µg/mL it still partially inhibited *Candida albicans*. This may be explained by Hsu and colleagues' study (2005) on transient membrane perturbation. According to their data, ind at smaller concentrations is believed to inhibit DNA synthesis by ionically binding to DNA or protein. This may be the case for Δ6,11 as at the concentrations below 200 µg/mL it was seen to still inhibit the growth of *Candida albicans*. Therefore, at higher concentrations the main mechanism of cell death would be from cell permeabilization and cell lysis. This experiment should be repeated by reinoculating the successful MIC cultures into peptide free broth to see whether the initial inoculum remained viable. This would explain if the MIC concentrations were bacteriostatic/bacteriolytic against bacteria or fungicidal/fungistatic against *Candida albicans*. As mentioned by Váradi et al., there is also a lack of antifungal agents and a high demand for them (2022), meaning that these results against yeast were significant. Thus, it would be valuable to extend the study of these derivatives due to their antifungal properties.

By comparing the results to that of the machine learning database DBAASP (https://dbaasp.org/tools?page=property-calculation), it appears that there was quite a significant difference in the data (Table 5). This database works by screening through peer-reviewed articles containing potent information on AMPs. Selected articles are further screened for key terms, before being read manually by a human who then places the information found into the database. Data on topics such as terminal modifications, amino acids used, modifications to peptides, target organisms, source of peptide, as well as antimicrobial/cytotoxicity activity are all entered in the database. This means a large variety of information is used in order for the machine learning database to predict activity of AMPs and does not only rely on physiochemical properties (Pirtskhalava et al., 2021). The DBAASP database correctly predicted that the derivatives would be most active against *Candida albicans*. It also correctly indicated that derivatives Δ4,9 and Δ8,11 would be active against *Staphylococcus aureus* at concentrations higher than 100 µg/mL which was confirmed by the Kirby-Bauer assay. However, the DBAASP did not correctly predict the activity of the peptides towards other microorganisms. The chances that these AMPs are active against these microbes is still very possible. It may be that the MIC concentrations are higher than 500 µg/mL. As mentioned previously in the Kirby-Bauer assay all derivatives were capable of inhibiting the growth of *Escherichia coli* and *Staphylococcus aureus*. In addition, Δ6,11 was able to inhibit *Salmonella typhimurium.* What was captivating from this database was the suggestion that ind and the derivatives would be active against enveloped viruses such as HIV1 and Sars-Cov-2. Robinson et al., (1998) tested ind against HIV1 infected cells at various concentrations and found that it was able to inactivate the virus, supporting this antiviral prediction. It would be beneficial to test the ind and its derivatives against a plethora of lethal viruses. Unfortunately, this would not be possible at TRU as these viruses would be classed above the level 2 biohazardous class of the laboratory.

Table 4. The MIC for each peptide against bacterial and fungal microorganisms, where concentrations are considered active when having a reduced optical density at 85% when compared to the control culture.

Peptide	Microbes	500 µg/mL	200 µg/mL	100 µg/mL	50 µg/mL	10 µg/mL
Δ4,8	*Staph a*	X	X	X	X	X
	MRSA	X	X	X	X	X
	E. coli	X	X	X	X	X
	Salmonella	X	X	X	X	X
	Candida	✓	X	X	X	X
	Pseudo	X	X	X	X	X
Δ4,9	*Staph a*	X	X	X	X	X
	MRSA	X	X	X	X	X
	E. coli	X	X	X	X	X
	Salmonella	X	X	X	X	X
	Candida	✓	X	X	X	X
	Pseudo	X	X	X	X	X
Δ6,11	*Staph a*	✓	X	X	X	X
	MRSA	X	X	X	X	X
	E. coli	X	X	X	X	X
	Salmonella	X	X	X	X	X
	Candida	✓	✓	X	X	X
	Pseudo	X	X	X	X	X
Δ8,11	*Staph a*	X	X	X	X	X
	MRSA	X	X	X	X	X
	E. coli	X	X	X	X	X
	Salmonella	X	X	X	X	X
	Candida	✓	X	X	X	X
	Pseudo	X	X	X	X	X
Ind	*Staph a*	✓	✓	✓	✓	X
	MRSA	✓	✓	✓	✓	X
	E. coli	✓	X	X	X	X
	Salmonella	X	✓	X	X	X
	Candida	✓	✓	✓	X	X
	Pseudo	✓	✓	✓	X	X

Note: ✓ indicates that the peptide was active at that concentration; X indicates that the optical density was reduced to 0-35%; X indicates that the optical density was reduced between 35-85% when compared to the culture control.

Table 5. The DBAASP predicted MIC values for ind and derivatives against a variety of pathogenic microbes and viruses.

Peptide	Pathogen	MIC Prediction (μg/mL)	Predictive Index
Ind	*Escherichia coli*	<25	0.75
	Staphylococcus aureus	<25	0.65
	Pseudomonas aeruginosa	<25	0.87
	Candida albicans	<25	0.83
	HIV1	<25	0.60
	Sars-Cov2	<25	0.64
Δ4,8	*Escherichia coli*	<25	0.55
	Staphylococcus aureus	<25	0.62
	Pseudomonas aeruginosa	<25	0.72
	Candida albicans	<25	0.80
	HIV1	<25	0.58
	Sars-Cov2	<25	0.65
Δ4,9	*Escherichia coli*	<25	0.55
	Staphylococcus aureus	>100	0.67
	Pseudomonas aeruginosa	<25	0.78
	Candida albicans	<25	0.86
	HIV1	<25	0.58
	Sars-Cov2	<25	0.65
Δ6,11	*Escherichia coli*	<25	0.56
	Staphylococcus aureus	<25	0.71
	Pseudomonas aeruginosa	>100	0.65
	Candida albicans	<25	0.83
	HIV1	<25	0.58
	Sars-Cov2	<25	0.65
Δ8,11	*Escherichia coli*	>100	0.52
	Staphylococcus aureus	>100	0.67
	Pseudomonas aeruginosa	<25	0.52
	Candida albicans	<25	0.69
	HIV1	<25	0.62
	Sars-Cov2	<25	0.67

Note: For antimicrobial and antiviral activity predictions, the DBAASP was used (https://dbaasp.org/tools?page=property-calculation).

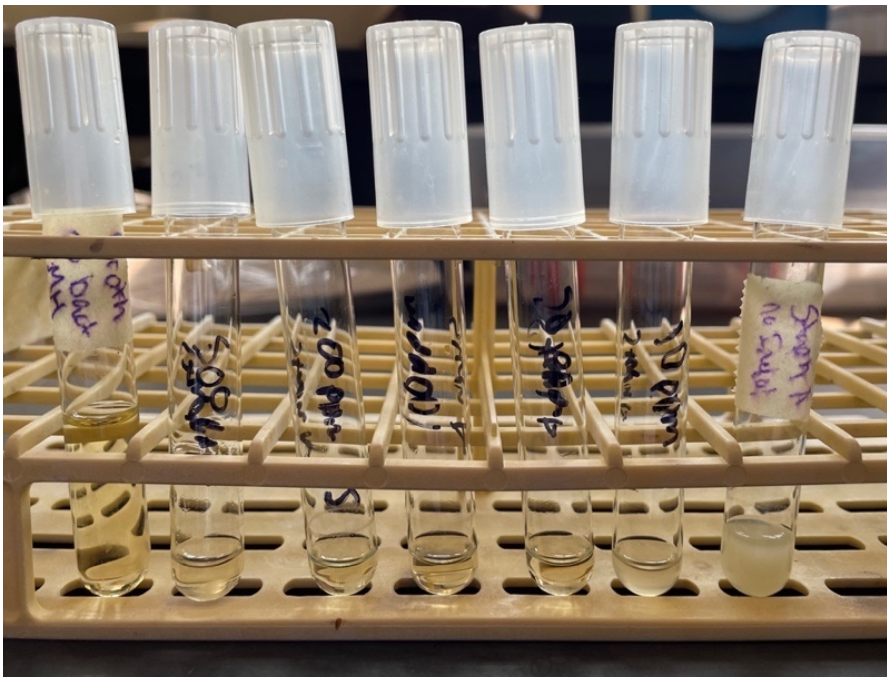

Figure 12. Example of an MIC for *Staphylococcus aureus*. On the very left is Mueller Hinton broth, followed by 500 µg/mL, 200 µg/mL, 100 µg/mL, 50 µg/mL, 10 µg/mL, 0 µg/mL solutions of ind.

Hemolytic Activity

Knowing that antimicrobial activity was determined for the peptides, it is significant to examine the cytotoxicity to investigate pharmaceutical potential and therefore perform the hemolytic assay. Results of this assay can be found in Table 6 which shows that there was negligible hemolytic activity in all peptide derivatives, whereas ind remained hemolytic. In a previous study, ind was found to have a minimum hemolytic concentration (MHC) at 30 µg/mL (Podorieszach & Huttunen-Hennelly, 2010). This data successfully lined up with that obtained in this lab. It was found that the MHC was somewhere greater than 10 µg/mL but equal or smaller than 50 µg/mL. They also found that the derivative Δ9,11 (9^{th} and 11^{th} tryptophan replaced with alanine), had more than a 100-fold decrease in hemolysis in comparison to ind (Podorieszach & Huttunen-Hennelly, 2010). Since the derivatives in this study also have 2 tryptophan's substituted for alanine, it can be predicted that the decrease in hemolytic activity of these peptides will be in a similar range. However, according to Ando et al. (2010), positions 9 and 11 in ind are the most important residues for hemolytic activity. Thus, the hemolytic concentration may vary amongst the peptides, and may be less than 100-fold since Δ9,11 lacks the two most hemolytic tryptophan

residues, whereas the derivatives in this study do not. Based on this information, it can be predicted that peptide Δ4,8 will likely be the most hemolytic out of the four derivatives studied.

According to the machine learning antimicrobial database, DBAASP, it was predicted that all peptides, except for the peptide Δ8,11 would have substantial hemolytic activity. The data in regard to Table 5 suggests that this is only true for ind. This peptide characteristic predictor has been found to be accurate, especially towards the bacteria listed in the database (Vishnepolsky et al., 2022). Nevertheless, it is possible that it is lacking critical information to determine correct hemolytic properties of AMPs. This may because there is a lack of information regarding the mechanism of hemolysis, especially for analogues with a successful decrease in hemolysis. Overall, the data concluded that each ind derivative was a worthy pharmaceutical prospect. Yet, further cytotoxicity testing is needed for nucleated eukaryotic cells especially considering *Candida albicans* is susceptive to the derivatives. To elucidate this, nucleated human cells will be tested against the peptides using an apoptosis/necrosis kit.

Due to a lack of published material on the mechanism behind the hemolytic characteristic of AMPs, it is challenging to give a definitive reason for the results obtained. Since hemolysis decreases at concentrations near the MIC of bacteria and fungi species, one can assume there is a special mechanism at play with red blood cells. Rokitskaya et al. (2011), explain that hemolysis is likely due to osmosis. They believe that ind disturbs the osmotic balance of the erythrocytes' environment. One explanation is that due to cationic properties, ind can interact with anionic metabolites causing the cell to efflux. The other could be influx of the peptide into the cell (Rokitskaya et al. 2011). Since ind is highly hydrophobic it is possible that it makes the cell membrane more permeable when it lyses the membrane, causing such an influx. Another thing to consider is that RBCs, unlike most eukaryotic cells, have net negative surface due to sialylated glycoproteins. These anionic proteins are designed to prevent RBCs from interacting with other cells and with each other (Fernandes et al., 2011). Being that there is a negative charge on the membrane, it is likely that ind is already attracted to the surface of the erythrocyte where it can perform its mode of action. In comparison to the transient membrane perturbation mechanism, there would be no lower concentration lethality, as RBCs do not contain DNA or a nucleus. Thus, if ind had an intracellular mechanism in erythrocytes, it is improbable that this would cause

hemolysis, since there is a lack of organelles. With this, it is most likely that hemolysis is due to permeability of the lipid bilayer.

But then why do the derivatives not cause the RBCs to lyse? The main difference between ind and the four derivatives is the decrease of hydrophobicity. In 2007 Chen and colleagues stated that peptides with higher hydrophobicity will penetrate deeper into the hydrophobic core of RBC membranes. This results in an increase in pores and channels created in the membrane, which would ultimately lead to lysis of the cell. Thus, the peptide derivatives are not hydrophobic enough to truly disturb the RBC cell membrane. The main focus of the peptide derivatives would likely be from binding to proteins or DNA.

Table 6. The hemolytic concentrations of ind and derivatives and the comparison to an AMP hemolytic activity predictor.

Peptide	Concentration of peptide tested (µg/mL)	Hemolytic Concentration (µg/mL)	Database Hemolytic Concentration Prediction (µg/mL)
Ind	10	≤50, >10	<25
	50		
	100		
	200		
Δ4,8	100	>700	<25
	300		
	500		
	700		
Δ4,9	100	>700	<25
	300		
	500		
	700		
Δ6,11	100	>700	<25
	300		
	500		
	700		
Δ8,11	100	>700	>100
	300		
	500		
	700		

Note: For hemolytic activity predictions, the DBAASP was used (https://dbaasp.org/tools?page=property-calculation).

Cytotoxicity to Nucleated Cells

In aims of further understanding the mechanisms of action, while also examining their potential cytotoxicity, the AMPS of interest underwent a necrosis/apoptosis assay (BioVision3

Annexin V-FITC Apoptosis Kit). This kit runs on the action of Annexin V-FITC and Sytox Green Dye. Annexin V is a phospholipid binding protein, whereas Sytox green is nucleic acid stain. Cell death by apoptosis results in a change in the plasma membrane structure. Phosphatidylserine is normally present on the inner leaflet of the lipid bilayer, but in apoptosis flips to the outer layer of the membrane. Annexin V has high affinity to phosphatidyl serine and can bind to it (Balaji et al., 2013). In this experiment Annexin V-FITC was used. FITC, meaning fluorescein isothiocyanate, is a fluorescent dye that can be coupled to annexin-V which can be detected by a flow cytometer. As for Sytox green stain, it has high affinity for nucleic acids. It can easily penetrate the plasma membrane of necrotic cells, bind to nucleic acids, giving an intense green stain (Lebaron, 1998). Overall, apoptotic cells fluoresce a moderate green, necrotic cells fluoresce green intensely and viable cells may have little or no green fluorescence which can be detected on a flow cytometer (Figure 14; Figure 15).

To the best of our knowledge these peptide derivatives are yet to be studied, let alone an apoptosis/necrosis assay for ind using white blood cells. Therefore, a time trial was performed to figure out what incubation time was needed. Ind was tested at concentrations near the MIC (200 µg/mL, 50 µg/mL, 10 µg/mL) to see whether it was cytotoxic. This was performed in triplicate at times 10 min, 30 min and 60 min as shown in Figure 13. All three time points ranged from about 17-21% viability, showing no gradual decrease in the amount of alive white blood cells. By comparing ind concentrations with the negative control (FACS Buffer) it seemed that as the ind concentration decreased there was a slight decrease of cells dead by necrosis paired with an increase in apoptotic cells (Table 7). Perhaps in this assay ind was relying more on intracellular mechanisms, as the concentration was low, rather than cell lysis that is more common at high AMP concentration, supporting Hsu et al.'s study (2005). However, it is hard to tell whether this data is significant as it was difficult to harvest a high percentage of viable white blood cells and there are other factors that may have led to cell death. In Podorieszach & Huttunen-Hennelly's research (2010), they found that ind caused a slight increase in the 400-500 µg/mL range. Thus, it would be worthwhile to test this experiment with more concentrations and perhaps with cells that are easier to remain viable. As for Δ6,11, Δ8,11, Δ4,9, and Δ4,8, these derivatives were tested at 600 µg/mL. It appears that there is not a significant difference between the cells viable and those dead

by apoptosis/necrosis (Table 7). However, more concentrations should be tested in multiplicity to have a more definitive conclusion.

Although it appears that the peptides do not have any significant cytotoxicity to nucleated mammalian cells, it has been proven that they can be destructive to yeast cells (*Candida albicans*). Literature suggests that AMPs are not selective to host eukaryotic cells due to cholesterol content in the lipid bilayer, as well as the lack of a negative net charge on the outer leaflet of the lipid bilayer (Lai & Gallo, 2009). Yeast do not carry cholesterol in their membrane but do however contain ergosterol. Ergosterol is very similar in structure and function and only differs by the extra double bond in the steroid ring, as well as an extra methyl group in the alkyl side chain (Hung et al., 2016). It seems that ergosterol is even bulkier than cholesterol which would make it just as hard, if not harder, for an AMP to permeabilize a yeast membrane. Like other eukaryotes, yeast also distributes phospholipids asymmetrically (Henderson & Block, 2016), making the membrane symmetry rationale difficult to support. Nevertheless, one difference between yeast and other eukaryotes is that they contain a cell wall. Yeast cell walls are usually 10-25% composed of fibrous β-1,3 glucan and mannoproteins. The phosphorylation of the mannosyl side chain on this protein give yeast an anionic surface charge (Kreigal et al., 2012). This may be why the AMPs were easily able to disturb Candida *albicans*. This negative charge could potentially draw the cationic peptides close to the cell where they can perform their mode of action. As one can see, the mechanism of AMPs on yeast is relatively unknown and will require further investigation.

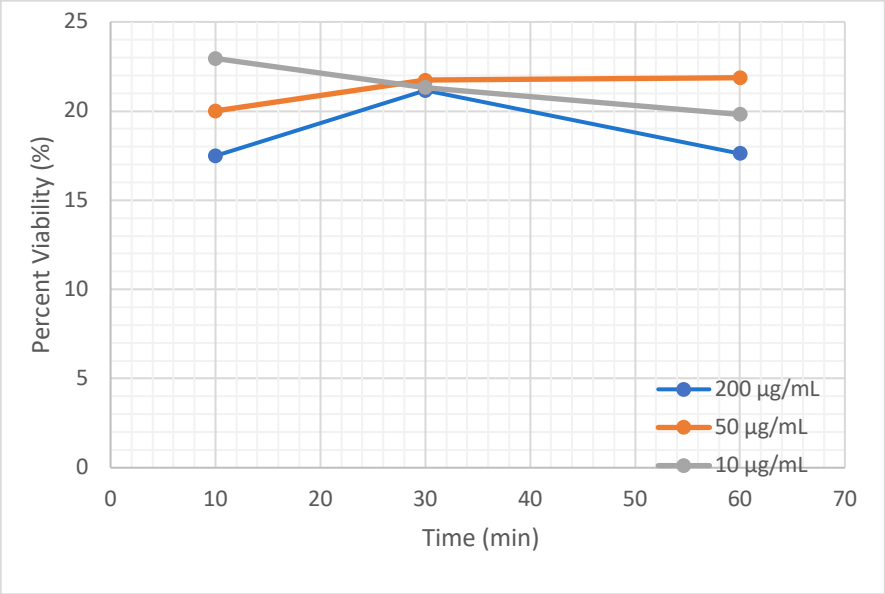

Figure 13. Time trial analysis of ind at 100, 50, and 10 µg/mL to see if time affected cell viability.

Table 7. Percent of live, apoptotic, and necrotic white blood cells at various ind concentrations (n=3).

Sample	Percent Viable (%)	Percent Apoptotic (%)	Percent Necrotic (%)
Ind 200	18.76 ± 2.08	41.13 ± 3.63	20.56 ± 3.60
Ind 50	21.20 ± 1.06	47.41 ± 1.05	16.94 ± 8.74
Ind 10	21.36 ± 1.57	49.95 ± 1.01	10.76 ± 1.69
FACS Buff (-ve control)	28.80	51.70	4.64
Hydrogen Peroxide (+ve control)	3.76	67.83	21.78

Table 8. Percent of live, apoptotic, and necrotic white blood cells for peptide derivatives at 600 µg/mL.

Sample	Percent Viable (%)	Percent Apoptotic (%)	Percent Necrotic (%)
Δ6,11	9.93	43.16	34.59
Δ8,11	7.95	51.03	30.42
Δ 4,9	7.00	52.37	29.61
Δ 4,8	7.50	45.34	32.56
FACS Buff (-ve control)	9.93	43.16	31.57
Hydrogen Peroxide (+ve control)	0.32	33.27	63.50

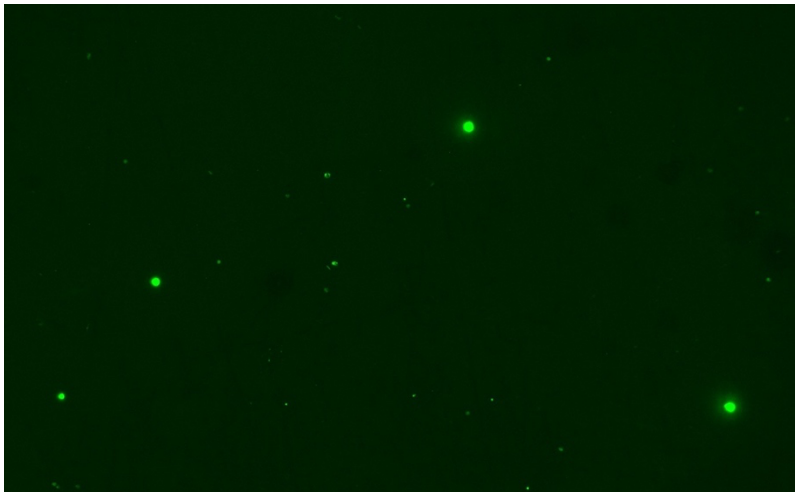

Figure 14. White blood cells under fluorescence microscope. Bright green represents necrosis; moderate green cells are apoptotic; cell with little to no green colour are viable.

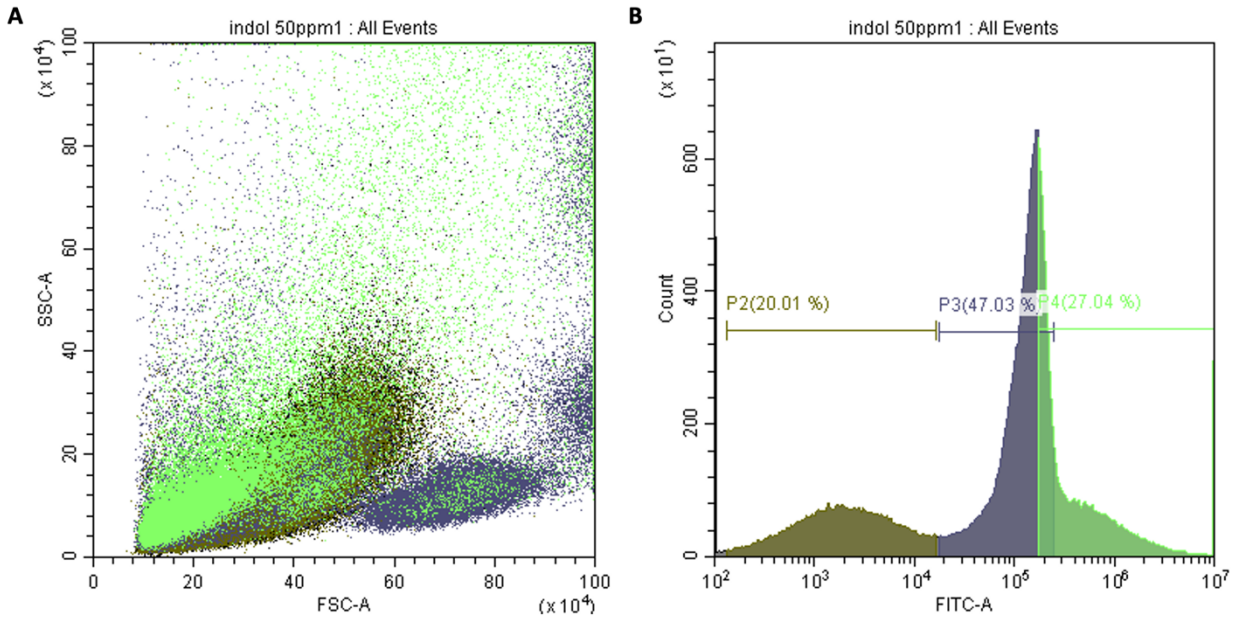

Figure 15. Example of the necrosis/apoptosis results obtained from a flow cytometer. The side scatter absorbance (SSC-A) versus the front scatter absorbance (FSC-A) showing size of white blood cells tested against ind at 50 µg/mL (A); The percentage of white blood cells that were viable (left – 20.01%), underwent apoptosis (middle – 47.03%), and experienced necrosis (right – 27.04%) (B).

CONCLUSION AND FUTURE WORK

To the best of our knowledge, this was the first time that these ind derivatives have been studied. Of the four derivatives examined, they all had bioactivity at higher concentrations in comparison to ind. While on the contrary, the hemolytic properties of these peptides were notably reduced. None of the peptide derivatives showed any sign of hemolysis near their MIC values. However, other possible ind derivatives with two alanine's replaced with tryptophan will have to be examined before determining if tryptophan location itself, or the overall hydrophobicity directly impacts ind's hemolytic and antimicrobial activity. What is notable about this research is that the peptides investigated have identical physiochemical properties, therefore hemolytic and bioactivity result from the subtle changes in the sequence order. While the derivatives were effective against *Candida albicans*, it seems based on the necrosis/apoptosis assay and literature data that they are unlikely cytotoxic to nucleated eukaryotic cells. Still, more research regarding the mode of action of the AMPs in this study will be required. Assays focused specifically on

elucidating the mode of action against yeast could be significant due to the demand of antifungal agents. The derivatives' mode of action is also vague against other microorganisms and requires further studying. One assay to aid in elucidating the mode of action would be a DNA gel shift assay. This would involve placing ind and derivatives at varying ratios with DNA in a gel electrophoresis set up and seeing if they have strong affinity for DNA. Furthermore, ^{1}H-NMR and circular dichroism spectroscopy are also needed to help determine the presence or absence of secondary structure to deduce molecular conformations and to further understand the derivatives' relatively unknown mechanism of action. Lastly, all microorganisms were tested in planktonic form, applying the peptides to biofilms would better represent human infections and thus the pharmaceutical potential of the derivatives.

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