DETERMINING THE ANTIMICROBIAL AND HEMOLYTIC ACTIVITY OF AUREIN-1.2 DERIVATIVES

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Abstract

Antibiotic-resistant bacteria are becominging increasingly common, posing a dire threat to global health, complicating the treatment of common infections and the performance of routine medical procedures. As a result, it is more important than ever to invest in research aimed at discovering novel, effective antimicrobial agents. One promising class of agents is antimicrobial peptides (AMPs), which are naturally occuring or synthetic peptides that exhibit potent antimicrobial bioactivity through a wide array of mechanisms. While these antimicrobial characteristics make AMPs a promising clinical antibiotic, unfortunately, AMPs often display significant cytotoxicity and hemolytic activity which limits their medical applications.

Aurein-1.2 is a natural occuring AMP synthesized by *Litoria raniformis* (the Southern Bell Frog), which has demonstrated strong antimicrobial and hemolytic activity (Ramezanzadeh 2020). In this study four derivatives of Aurein-1.2 (T1, T2, T3, and T4) were synthesized by substituting two lysine residues with arginine and two isoleucine residues with tryptophan at varying positions. To analyze the antimicrobial activity of the derivatives, a Minimum Inhibitory Concentration (MIC) assay was performed using the peptides at varying concentrations against *Escherichia coli* and multidrug resistant *Escherichia coli*, *Salmonella typhimurium*, *Candida albicans*, *Staphylococcus aureus*, and methicillin-resistant *Staphylococcus aureus* (MRSA). The results showed that T1 and T2 exhibit >90% cell death at 50 μg/ml , while T3 displayed similar activity at the same concentrations against all bacteria except for *Escherichia coli,* which required 100 μg/ml . T4 required 100 μg/ml for for >90% cell death for all tested organisms.

Hemolytic activity against mammalian erythrocytes was also analyzed in this study using hemolytic assays with whole horse blood. Cytoxicity was observed at all tested concentrations in all derivaties except for T1 and T3 at 10 μg mL^{-1} .

Introduction

For most of human history, mankind lacked the tools and innovations to combat bacterial infections, until the revolutionary discovery of the first antibiotic, penicillin. This marked a new era where bacterial infections could be effectively treated, saving countless lives. However, unbeknownst to scientists at the time, bacteria would evolve and develop mechanisms to resist these antibiotics.

Bacteria, like any organism, evolve from heritable mutations that provide advantages in survival and procreation. When the first antibiotics were administered, individual bacteria with antimicrobial-resistant traits survived the initial treatment and passed on their genes to their progeny, leading to the emergence of antibiotic-resistant strains. Ironically, antibiotic-resistant bacteria emerged from the very antibiotics designed to eradicate them. This created a cycle of antibiotic discovery and success followed by diminished efficacy and an increasing abundance of dangerious antibiotic resistant bacteria (Altarac 2021).

As penicillin and other novel antibiotics became less effective, scientists scoured the environment in search of new drugs, keeping us ahead of bacterial evolution for the next 60 years (Altarac 2021). However, the discovery of new naturally occuring antibiotics became an increasingly rare occurrence. This decline resulted in significant research investments being pulled during the 1990s and 2000s (Altarac 2021). Without a steady stream of new antibiotics, antimicrobial-resistant bacteria are causing a more deaths year after year. In 2019, the Center for Disease Control and Prevention (CDC) stated that antibiotic-resistant bacteria were responsible for “killing at least 1.27 million people worldwide and [were] associated with nearly 5 million deaths” (CDC 2019). This is because the common bacteria causing diseases like pneumonia,

tuberculosis, gonorrhoea, and salmonellosis (WHO 2018) are becoming resistant to even our most potent antibiotics, leading to devastating fatalities.

As bacteria continue to evolve, they will only become more dangerous, leading to even greater casualties. It is imperative that the scientific community accelerates work to discover, isolate, and begin production of new antibiotics.

Antimicrobial Peptides

AMPs are naturally occuring peptides that function in the innate immune system (Ganz 2003). These molecules are well conserved across several eukaryotic systems and serve as an efficient defense mechansims against a wide array of pathogens. The advantages of using AMPs are that they require less energy and time to produce than antibodies and can reach their targets more quickly than immunoglobins (Zhang 2021). In fact, some organisms do not possess an adaptive immune system and heavily rely on the productions of these molecules for survival.

AMPs exist in a wide variety of sizes, structures, and functions and are organized into several subgroups based on characteristics such as amino acid sequence, net charge, secondary and tertiary structure, and source (Zhang 2021). Most AMPs contain the basic amino acids lysine and ariginie and lack acidic residues aspartic or glutamic acids, contrinbuting to a net charge of between +2 and +11. This cationic nature enables AMPs to interact with the anionic phospholipid head groups in the cell membrane of invading pathogens and disable the cell. It is worth noting however, that a positive charge greater than +7 does not necessarly increase activity further due to the formation of stronger electrostatic interactions between the peptide and the membrane which prevents penetration deeper into the hydrophobic interior of lipid membrane (Pasupuleti et al. 2012). Most AMPs exhibit approximately 50% hydrophobicity within their

amino acid sequence, which is critical for facillitating AMP penetration and disruption of the pathogen's membrane (Pasupuleti et al. 2012).

AMPs are produced and isolated from regions of the body that are most suseptible to infection, including mucus, blood, urine, phagocytic immune cells, epithelial cells in the skin, as well as the respiratory, digestive, and genitourinary tracts. They are also found in the heart and skeletal muscle (Zhang 2021). Additionally, AMPs are also present in other eukaryotes such as plants and invertebrates.

Since the discovery of the first AMP, nisin, studies investigating the antibiotic potential of these molecules has increased rapidly. Numerous novel AMPs have been discovered and tested for their potential clinical applications. With the development of advanced databases and computational simulations of peptide activity, scientists have also been able to design synthetic peptides or natural peptide deriviatives to either reduce human toxicity or enhance a peptide's antimicrobial bioactivity.

Aurein-1.2

Aurein 1.2 is a naturally occurng AMP secreted from the granular dorsal glands of the Green and Golden Bell frog, *Litoria aurea,* and the Southern Bell frog, *Litoria raniformis.* This peptide consists of only 13 amino acid residues, making it the smallast amphibian-derived peptide to exhibit antibiotic activity (Rozek 2022). Given the bioactivity of aurein 1.2, its derivatives could represent very powerful clinical AMPs in the future.

Table 1. Sequence and Properties of Aurein 1.2

Sequence	**GLFDIIKKIAESF**
Structure	Helix
Residue number	13
Monelcular weight	1479.76 Da
Net Charge	+1
Hydrophobic residue Percent	53%
Boman Index	0.12

Properties and data was obtained from the APD3 antimicrobial peptide database, Roztek 2000, and Madanchi 2022.

Structure and mechanism of Action

Aurein-1.2 adopts an amphipathic alpha helical structure with well defined basic, hydrophobic, and hydrophillic regions (Rozek 2022). This structure facillitates the peptide's attachment to the negatively charged lipid head groups in the phospholipid bilayer of bacterial membranes.

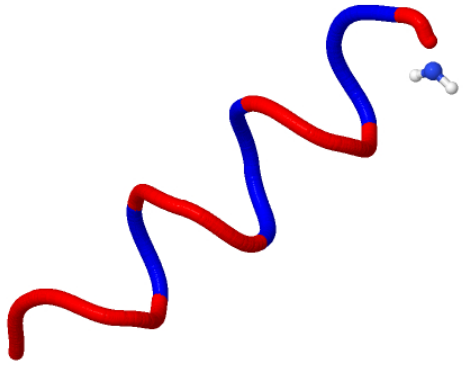

Figure 1. The Alpha Helical structure of Aurein 1.2 with red indicating hydrophillic regions and blue indicating hydrophobic regions. The structure was obtained from the RCSB Protein Data Bank.

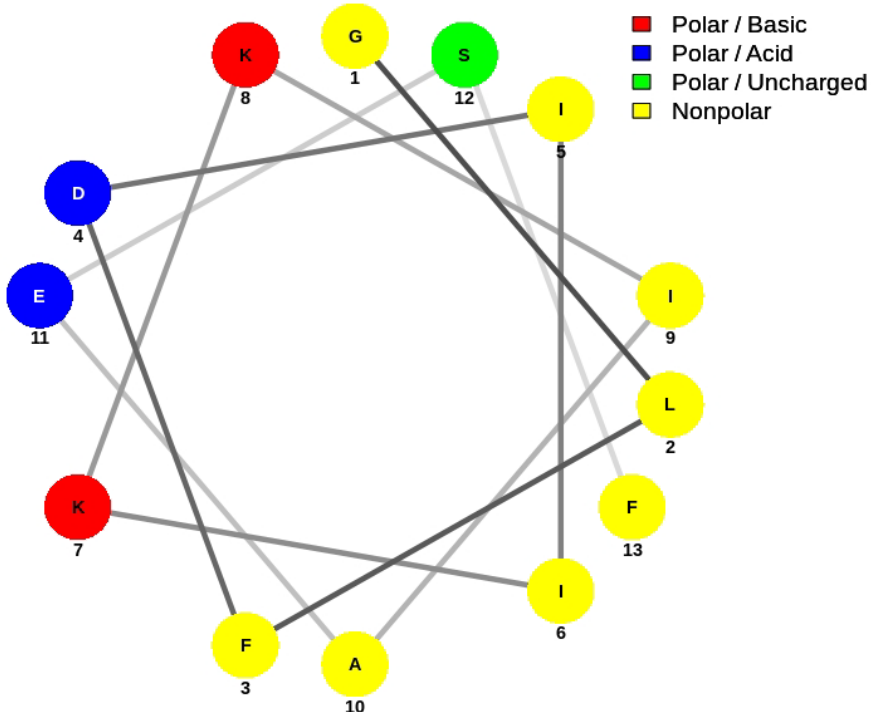

Figure 2. Edmunson Projection of Aurein 1.2 . The firgure was produced using NetWheels: Peptide Helical Wheel and Net Projections Maker.

Research has shown that Aurein 1.2 contains crucial residues required for its bioactivity. Through extensive antimicrobial assays in which alanine residues sequencially replaced key amino acids, it has been determined that phenylalanine 13, C-terminus amidation (Boland and Separovic 2006), and lysine 7 and 8 (Rozek 2022) are essential for the peptide's antimicrobial activity.

Aurein 1.2 can function through two potential mechanisms: the barrel±stave mechanism and the carpet mechanism.

The Barrel±stave mechanism

In this mechanism, the peptides assemble on the surface of the membrane forming bundles of amphipathic aplha-helices. The hydrophobic surfaces of the helices interact with the membrane's lipid core while the hydrophilic regions reside on the cytosolic and extracellular sides of the mebrane (Shai 1999). This mechanism produces aqueous pores in the membrane that destabilize the cell leading to its death.

The Carpet Mechanism

The carpet mechanism involves binding of the peptide to phospolipid head groups which align themselves on the surface of the membrane, ensuring hydrophillic regions orient toward head groups or extracellular water. As the peptiode rotates to move the hydrophobic regions to the hydrophobic interior of the membrane the membrane is disrupted, resulting in the breakdown of the mebrane and cell death (Shai 1999).

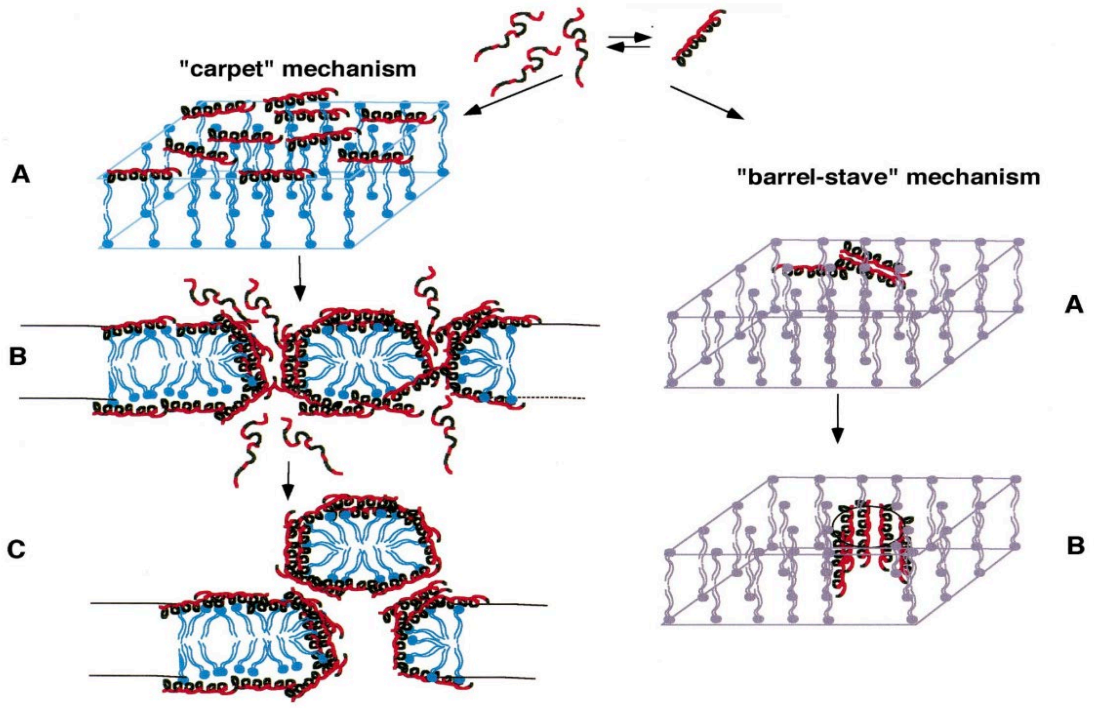

Figure 3. Models of the carpet mechanism and the barrel-stave mechanism (Shai 1999).

Materials and Methods

AMP Design

The design process began by first identifying an Aurein-1.2 derivative capable of maintaining the parent peptide's antimicrobial properties while reducing its hemolytic activity. This brought us to the work of Maryam Ramezanzadeh et al at Damghan University in Iran. Their studies tested the antimicrobial properties of several Aurein derivatives, one of which substituted the aspartic acid (Asp) and glutamic acid (Glu) residues for lysine residues (Lys). These alterations maintained the peptides hydrophobicity but increased the total net charge from +1 to +5. Results of the study found that derivative exhibited a significant decrease in hemolytic activity and an increase in antimicrobial efficacy (Ramezanzadeh 2021). The study concluded that replacing the Asp and Glu residues with cationic residues increased the peptide's

antimicrobial activity. This formed the basis for the design of peptide T1, in which we substituted Asp4 and Glu11 with another cationic residue, arginine (Arg).

Arginine was chosen because it appears in a very high frequency in many natural AMPs. As a cationic amino acid, the positive charge of the arginine residue facilitates interactions between the AMP and the target membrane. Furthermore, Arginine also able to strengthen the peptide-membrane interactions by forming hydrogen bonds between the peptide and bacterial membrane components such as lipopolysaccharides in gram-negative bacteria and teichoic acid in gram positive bacteria (Chan 2006). Other basic residues like lysine cannot form these hydrogen bonds, giving arginine a greater effect in increasing antimicrobial activity.

Peptides T2, T3, and T4 have two tryptophan residues (Trp) substituted at varying positions: Isoleucine 5, 6, and 9. Tryptophan residues are also commonly found in naturally occurring AMPs along with arginine. Trp serves multiple functions in peptides. The first being the addition of hydrophobic bulk to the peptide via the large uncharged side chain. This can act as an anchor for the peptide in the hydrophobic core of a membrane. Trp residues also possess an extensive π-electron system in the aromatic indole sidechain. This enables them to form a unique quadruple dipole moment extending from each side of the ring which will interact with the peptide's arginine residues forming cation- π interactions (Chan 2006). These interactions help shield the arginine groups from the hydrophobic environment, facilitating the peptides entr into the hydrophobic membrane. In peptides T2, T3, and T4 the Trp residues were substituted along with arginine to try to identify the optimal sequence locations of interacting residues that would maximise the antimicrobial efficacy while minimizing the hemolytic activity.

The Database of Antimicrobial Activity and Structure of Peptides (DBAASP) was used to predict the antimicrobial and hemolytic activity of the four derivatives along with the

Antimicrobial Peptide Database (APD3) which provided the characteristics of the peptide including total net charge, hydrophobicity, bowman index, and GRAVY values. These databases guided the peptide design, helping to achieve ideal characteristics of the derivatives that align with established knowledge of effective antimicrobial peptides.

Table 1. Sequence of Aurein-1.2 and the four tested derivatives

Amino Acid Sequence	**Abbreviations**
GLFDIIKKIAESF-NH_2	Aurein-1.2
GLFRIIKKIARSF-NH_2	T1
GLFRWIKKWARSF-NH_2	T2
GLFRIWKKWARSF-NH_2	T3
GLFRWWKKIARSF-NH_2	T4

Note: Single letter amino acid abbreviations include: Glycine (G), Luecine (L), Phenylalanine (F), Aspartic Acid (D), Isoluecine (I), Lysine (K), Alanine (A), Glutamic Acid (E), Serine (S), Arginine (R), and Tryptophan (W). Green arginine residues indicate the where argine has replaced aspartic acid and red tryptophan residues indicate where isoleucine has been replaced.

AMP stock preparation

29 mg of each peptide with a purity exceeding 95% was ordered through GenicBio. AMP concentrations were prepared using a NanoDrop One Spectrophotometer. Initially, 1 ml of deionized water (18 mΩ) was added to a 15 ml centrifuge tube and combined with the desired amount of powdered peptide. The mixture was then vortexed to homogenize the solution. To achieve the desired concentration, 2 µL of the solution was pipetted onto the pedestal of the

Nanodrop One Spectrophotometer and analysed using the Protein A205 Scopes Method, which measures the absorbance of 205 nm due to the presence of peptide bonds.

Minimum Inhibitory Concentration Assay

The minimum inhibitory concentrations (MICs) of the four peptide derivatives were measured against five microorganisms including: gram-negative bacteria (*Salmonella typhimurium, Escherichia coli,* multidrug resistant *Escherichia coli*), gram-positive bacteria (*Staphylococcus* aureus and Methicillin-Resistant *Staphylococcus aureus),* and yeast (*Candida albicans).* Test organisms were inoculated in Mueller Hinton (MH) broth inside 1.5 ml microcentrifuge tubes at 37°C for 16-18 hours at 200 RPM using an orbital shaker .

After the allotted time, the turbidity of the inoculated broth was measured using a spectrophotometer set at 621 nm wavelength over a 1-cm light path. Excess MH broth was used to dilute the solution to achieve an absorbance of 0.08-0.1 equivalent to the McFarland 0.5 standard (~1 x 10^8 CFU mL^{-1}). After recording the desired absorbances the solutions were diluted 100-fold to 1 x 10^6 CFU mL by combining 33 µL of the organism in a new microcentrifuge tube containing 967 µL of MH broth.

20 µL of the diluted solution was pipetted into a 96 well plate and combined with the desired peptide concentration to achieve a final volume of 200 µL. Peptide treatments from T1-T4 were tested at concentrations of 500 ppm, 250 ppm, 100 ppm, 50 ppm, and 10 ppm. 200 µL of MH broth was used as a blank, while a mixture of organisms with MH broth and no peptide served as a negative control, and 250 ppm of indole was used as a positive control. Given the 54% hydrophobicity of the peptides, there was a slight increase in solution turbidity at higher concentrations, so additional wells were prepared with MH broth and peptides but no organisms.

The plate was incubated overnight at 37°C using a orbital shaker at 160 rpm for 16 hours. The optical density of the wells was recorded using a using a Thermofisher Multiskan Ascent instrument at 621 nm. Measurements were taken in triplicate and were used to calculate the mean percent cell death. Minimum inhibitory concentration is considered ≥ 90% killing of the initial inoculum. The equation used to determine the percent cell death is displayed in Equation 1.

Equation 1. Percent death equation

$$Percent\ Cell\ Death = 1 - \frac{(Sample - Peptide\ Blank)}{(Negative\ Control - Peptide\ Blank)} \times 100$$

Hemolytic Assay

Whole horse blood samples were obtained from the TRU Veterinary Clinic from three different horses. 1 ml of the blood was centrifuged at 3000 RPM for 10 minutes at 4°C. The supernatant was discarded, and the pellet was combined with 10 ml 1 X phosphate-buffered saline (PBS). After being resuspended, the PBS-blood solution was centrifuged again under the same conditions. Once again, the Supernatant was removed, and the process was repeated twice more.

The Blood samples were then washed 3 times using PBS centrifuging again at 1000 RPM for 10 minutes. At this point the washed cell pellet contained only isolated red blood cells (RBCs). The samples were resuspended in 1 X PBS to achieve a cell concentration of 5 x 10^8 cells/mL, confirmed by a hemocytometer. Peptide treatments were prepared by dissolving the peptides in 1 X PBS at the same concentrations as the MIC assay (500 ppm, 250 ppm, 100 ppm, 50 ppm, 10 ppm). 200 µL of the blood PBS solution was combined with 800 µL in 1.5 ml microcentrifuge tubes. Positive controls consisted of 800 µL of Triton X-100 (at a final concentration of 1 %),

while 800 µL of 1 X PBS was used as a negative control. Samples were inverted several times and incubated for one hour at 37 °C and 50 RPM using an orbital shaker.

Following incubation, the tubes were centrifuged at 14,000 rpm for 5 minutes and 200 µL of the supernatant was pipetted into a 96 well plate. The presence of free hemoglobin in the supernatant, indicative of hemolysis, was measured by recording the absorbance at 539 nm using a Thermofisher Multiskan Ascent instrument. The percent hemolysis was calculated using Equation 2, where a hemolytic percent exceeding 5% was deemed as cytotoxic.

Equation 2. Equation used to determine percent hemolysis

$$Percent\ Hemolysis = \frac{Sample\ absorbance - PBS\ absorbance}{Triton\ X}\ x\ 100$$

Results

MIC assay

All of the peptides tested exhibited antimicrobial activity against the various organisms tested. Antimicrobial activity was defined as at least a 90% reduction in optical density compared to the control according to equation 1. T1 and T2 displayed antimicrobial activity against yeast and all the tested gram-positive and negative bacteria at 50 µg /mL. T3 exhibited similar activity, however the peptide was active against non-drug resistant *Escherichia coli* at 100 µg /mL. T4 was active at 100 µg /mL against all tested microorganisms.

The MIC assay was performed in triplicate and were recorded as mean values and exhibited a standard deviation of less than 10 percent.

Table 2. Minimum inhibitory concentrations of peptide derivatives against various test organisms. Active concentrations are classified by reducing optical density by ≥ 90% compared to the control culture based on equation 1.

Active Concentration µg /mL						
Peptide	*Salmonella tryphimurium*	*Staphylococcus aureus*	*MRSA*	*Escherichia coli*	*MDR Escherichia coli*	*Candida albicans*
	Gram-negative	Gram-positive	Gram-positive	Gram-negative	Gram-negative	Yeast
T1	50	50	50	50	50	50
T2	50	50	50	50	50	50
T3	50	50	50	100	50	50
T4	100	100	100	100	100	100

Hemolysis Assay

Hemolytic activity was assessed by measuring the amount of free hemoglobin in peptide and blood test supernatants. Free hemoglobin was measured using a spectrophotometer at 539 nm wavelength.

Hemolytic activity was tested at the same concentrations as the MIC assay (10 ppm, 50 ppm, 100 ppm, 250 ppm, and 500 ppm) and the results are summarized in figure 1. The American Society for Testing and Materials (ASTM) states that hemolysis of less than 5% is considered non-cytotoxic (Luna-Vázquez-Gómez et al. 2021). T1 and T3 were non-hemolytic at 10 ppm exhibiting 3.82 ± 0.535 and 2.00 ± 0.569 percent hemolysis respectively. T1 and T3 at any higher concentrations were above the cytotoxic threshold and peptides T2 and T4 were cytotoxic at all tested concentrations.

The hemolysis assay was performed in triplicate and the results were reported using the mean values.

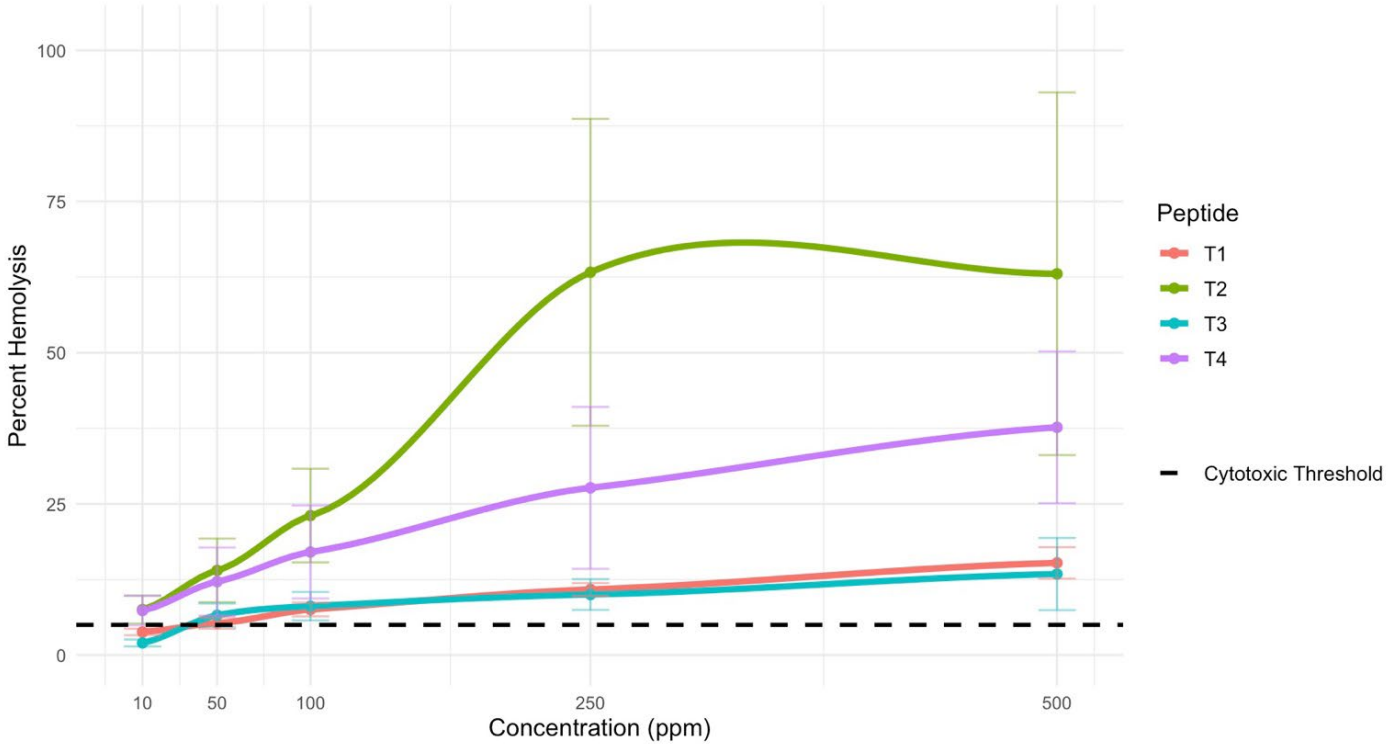

Figure 1. Percent hemolysis curves of the four tested peptides (n=3). The Dashed line represents the American Society for Testing and Materials (ASTM) standard for negligible hemolytic activity (≤ 5%). Any points above the line represent cytotoxic concentrations of the peptide.

Discussion

This study was designed to assess the antimicrobial and hemolytic activity of four Aurein-1.2 peptide derivatives. Arginine and tryptophan residues were strategically substituted into the peptide for aspartic acid, glutamic acid, and isoleucine residues to enhance antimicrobial efficacy while reducing hemolytic activity.

Antimicrobial Activity

The minimum inhibitory concentration (MIC) of Aurein-1.2 is reported as 25.00 μg/ml for gram positive bacteria Staphylococcus aureus and 200 μg/ml in gram negative bacteria Escherichia coli (Ramezanzadeh 2021).

All derivatives appeared to exhibit at least a two-fold decrease in antimicrobial activity against Staphylococcus aureus. T1, T2, and T3 displayed minimum inhibitory action at 50 μg/ml, while T4 showed this activity at 100 μg/ml. Specifically, T1 exhibited 98.47 ± 2.48% killing of *S. aureus* and 98.00 ± 3.31% of methicillin-resistant *S. aureus* (MRSA). T2 showed 95.74 ± 2.78% and 95.62 ± 3.49% killing of *S. aureus* and MRSA, respectively. T3 achieved 92.61 ± 2.37% for *S. aureus* and 90.04 ± 6.77% for MRSA, while T4 exhibited 91.68 ± 5.92% for *S. aureus* and 94.02 ± 2.81% for MRSA.. Although the results show that the antimicrobial activity decreased slightly in the four derivatives, it is possible that peptides were tested using a too wide range of concentrations. Future studies with smaller intervals between tested concentrations is recommended to determine the exact MIC of each peptide more precisely.

The T1 and T2 derivatives demonstrated a four-fold increase in antimicrobial activity against the tested gram positive bacteria, E.coli, MDR E.coli, and Salmonella *tryphimurium.* T3 displays similar increases in activity, except against E.coli where instead it shows a two-fold increase. T4 shows a two-fold increase in activity against all the tested gram-negative bacteria. Specifically, T1 killed 93.84 ± 3.78% of *E. coli*, 97.39 ± 4.44% of MDR *E. coli*, and 100.00 ± 0.44% of *S. typhimurium*. T2 killed 95.02 ± 3.77% of *E. coli*, 95.19 ± 3.86% of MDR *E. coli*, and 98.38 ± 2.93% of *S. typhimurium*. T3 demonstrated 93.98 ± 8.09% killing of *E. coli* at 100 μg/mL, 92.06 ± 7.05% of MDR *E. coli* at 50 μg/mL, and 90.98 ± 6.63% of *S. typhimurium* at 50 μg/mL. T4

achieved 91.49 ± 9.74% for *E. coli*, 92.98 ± 7.00% for MDR *E. coli*, and 99.01 ± 1.98% for *S. typhimurium*.

These changes in bioactivity against gram-positive and gram-negative bacteria may explain the mechanism of action of the peptide derivatives. Gram-negative bacteria have a much thinner layer of peptidoglycan than gram-positive bacteria and an outer membrane containing lipopolysaccharides. Arginine can form hydrogen bonds and electrostatic interactions with lipopolysaccharides which may explain the increase in activity. Added tryptophan groups can also assist in the anchoring of the peptide to the outer membrane which could also explain this increase.

Hemolytic Activity

Aurein-1.2 has been reported to exhibit hemolytic activity of less than 5% at a concentration of 12.5 μg/ml (Ramezanzadeh 2021). T2 and T4 failed to decrease the hemolytic activity even at lower concentrations of 10 μg/ml. T1 and T3 showed promise, however the tested concentrations do not reveal enough to come draw any definitive conclusions. At 50 μg/ml T1 exhibited a 5.27773691 ± 0.9378268 % hemolysis and T3 exhibited a 6.592486642 ± 1.94304433 % hemolysis. It is possible that the peptide concentration required to induce hemolysis has increased to a range between 12.5 μg/ml and 50 μg/ml.

Tryptophan and arginine substitutions should increase the selectivity of AMPs to bacterial membranes over erythrocyte membranes. Both residues are known to facilitate interactions between AMPs and negatively charged bacterial membranes, as opposed to less negatively charged cholesterol-rich mammalian red blood cells (Ruiz 2014). The arginine residues in T1 may reduce hemolysis through this mechanism and the added tryptophan residues in T3 may

have a similar effect. Furthermore, T3 tryptophan may also interact with the arginine residues to optimize antimicrobial activity and minimize hemolytic effects.

Conclusion and Future Work

This study investigated the antimicrobial and hemolytic activity of four Aurein-1.2 peptide derivatives containing various arginine and tryptophan substitutions. Antimicrobial activity was increased in all derivatives against gram negative bacteria and the results against gram positive bacteria remain inconclusive. Hemolytic activity was significantly increased in derivatives T2 and T4 but may have been reduced in T1 and T3. Future studies should analyze narrower peptide concentration intervals between 0 and 50 μg/ml to provide more precise data and allow for stronger conclusions regarding the effectiveness of the peptides.

Additionally, a cell viability assay and DNA binding assay will also provide us with greater insight into the mechanisms of action of the peptides and their potential viability as a pharmaceutical product.

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