ACIBADEM MEHMET ALI AYDINLAR UNIVERSITY
INSTITUTE OF HEALTH SCIENCES

ROLE OF INNOVATIVE PEPTIDE ANTIBIOTICS WITH KNOWN ANTIMICROBIAL ACTIVITY AND TOXICITY IN IMMUNE RESPONSE

SİNAN CEBECİ

M.Sc. THESIS

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DECLARATION

I declare that this thesis work is my own work, I had no unethical behavior at any stages from the planning to the writing of the thesis, I obtained all the information in this thesis in accordance with academic and ethicel rules, I cited all the information and comments that were not obtained with this thesis work, and I provided resources in the list of references. I also declare that there was no violation of any patents and copyrights during the study and writing of this thesis.

26/01/2024

Sinan Cebeci

PREFACE AND ACKNOWLEDGEMENT

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LIST OF ABBREVIATIONS AND SYMBOLS

ACN	Acetonitrile
AMP	Antimicrobial peptide
APC	Antigen presenting cell
APD	Antimicrobial peptide databese
APS	Ammonium Persulfate
BCA	Bicinchoninic acid assay
BSA	bovine serum albumin
C	Carbon
CAP18	18-kDa cationic antimicrobial protein
CPS	Capsular polysaccharides
DCM	Disupropylcarbodiamide
DMF	Dimethylformamide
DMSO	Dimethyl Sulfoxide
DNA	deoxyribonucleic acid
DC	Dendritic Cells
E. coli	*Eschericia Coli*
ELISA	enzyme-linked immunoassay
FBS	Fetal Bovine Serum
g	gravitational force
GMP	Good Manufacturing Process
HBD	Human Beta Defensins
HCI	Hydrogen Chloride
HNP	Human Neutrophil Peptides
HPLC	High Performance Liquid Chromatograhpy
HRP	horse radish peroxidase
i.e	That is
IFN-γ	Interferon-gamma
IgG	immunoglobulin G
IL-6	Interleukin 6
kDA	Kilo Dalton

LL-37	Human Cathelicidin
LPS	Lipopolysaccharide
MIC	Minimum Inhıbıtory Concentration
mRNA	messenger ribonucleic acid
MSSA	*Methicillin Sensitive Stahphylococus Aureus*
MRSA	*Methicillin Ressistant Stahphylococus Aureus*
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
P. Aeruginosa	*Pseudomonas aeruginosa*
NK	Natural Killer
NMR	Nuclear Magnetic Resonance
PBS	phosphate-buffered saline
Pen/Strep	Penicillin Streptomicin
pH	Power of Hydrogen
PVF	Polyvinyl fluoride
RPMI	Roswell Park Memorial Institute Medium
S.Aureus	*Stahphylococus Aureus*
SDS	Sodium Dodecyl Sulfate
SDS PAGE	SDS polyacrylamide gel
TBS	tris-buffered saline
TBST	Tris-buffered saline with 0.1% Tween 20
TFA	Trifluoraacetic acid
TNF-α	Tumor necrosis factor-alpha
V	Volt
2D	2 dimensional
3D	3 dimensional

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ÖZET

Antimikrobiyal Aktivite ve Toksisitesi Bilinen Yenilikçi Peptit Antibiyotiklerin İmmün Yanıttaki Rolü

Mikroorganizmaların, özellikle bakterilerin antimikrobiyal ajanlara karşı geliştirdiği direnç mekanizmaları son yıllarda hastalıkların tedavisinde önemli bir engel teşkil etmektedir. Hali hazırda enfeksiyonların tedavisinde kullanılan antibiyotikler ne yazık ki yetersiz kalmakta ve bakteri kaynaklı hastalıkların tedavisi güngeçtikçe zorlaşmaktadır. Bakterilerin antibiyotiklere karşı geliştirdiği direnç mekanizmaları ve kaçış sistemlerinin anlaşılması bu süreçte önem kazanmıştır. Bu kapsamda birçok alternatif ilaç çalışmaları ve antibakteriyel özellik gösteren yeni moleküller araştırılmaya başlanmıştır. Bu tez çalışması kapsamında, yeni nesil antimikrobiyal peptid molekülleri sentezlenerek, antibiyotik direnci geliştirmiş bakteriler üzerindekini etkileri incelenmiştir. Sentezlenen antimikrobiyal peptidlerin mikrobiyolojik etkileri araştırıldıktan sonra, hücre kültürü çalışamaları ile immün sistem üzerindeki etkileri araştırılmıştır. Bu kapsamda, farklı konsantrasyondaki antimikrobiyal peptidlerin, fare makrofaj hücreleri kullanarak, immün sistemin önemli yapıları olan ınterlökin-6 (IL-6), tumör nekroz faktör-α (TNF-α) ve Interferon-γ (IFN-γ) sekresyonunu nasıl etkilediği araştırılmıştır. Sonuçlar değerlendirildiği vakit çeşitli mikroorganizmalara karşı D ve L formlarını bir arada içeren NET1 ve NET3 peptidleri daha düşük konsantrasyonda inhibisyon etkisi göstermiştir. Buna karşılık hücre hattı üzerindeki sitotoksisite etkisi bakımından NET1 ve NET2 peptidleri düşük sitotoksisite göstermiştir. Her iki peptid grubunda da LPS ile uyarılmamış ortamda IL-6 ekspresyonu azalırken peptidlerin anti-inflamatuar yanıtı oluşturacağı gözlemlenmiştir. Ek olarak, her iki peptid grubunda da IFN-γ ve TNF-α yanıtı artmıştır. Bu durum, peptidlerin hem anti-inflamasyonda hem de pro-inflamasyon görev alabileceğini göstermiştir.

Anahtar Sözcükler: Antimikrobiyal peptidler, Peptid sentezi, Antibiyotik direnci, İmmün yanıt, İmmün sistem

ABSTRACT

Role of Innovative Peptide Antibiotics with Known Antimicrobial Activity and Toxicity in Immune Response

Resistance mechanisms developed by microorganisms, especially bacteria, against antimicrobial agents have become a significant obstacle in the treatment of diseases in recent years. Antibiotics currently used in the treatment of infections are unfortunately insufficient and the treatment of bacterial diseases is becoming more difficult day by day. Understanding the resistance mechanisms and escape systems developed by bacteria against antibiotics has become important in this process. In this context, many alternative drug studies and new molecules with antibacterial properties have begun to be investigated. Within the scope of this thesis study, new generation antimicrobial peptide molecules were synthesized and their effects on bacteria that have developed antibiotic resistance were examined. After investigating the microbiological effects of the synthesized antimicrobial peptides, their effects on the immune system were investigated through cell culture studies. In this context, it was investigated how different concentrations of antimicrobial peptides affected the secretion of Interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and Interferon-γ (IFN-γ), which are important structures of the immune system, using mouse macrophage cells. . When the results were evaluated, NET1 and NET3 peptides containing D and L forms together showed inhibition effects at lower concentrations against various microorganisms. On the other hand, NET1 and NET2 peptides showed low cytotoxicity in terms of their cytotoxicity effect on the cell line. In both peptide groups, it was observed that the peptides would create an anti-inflammatory response, while IL-6 expression decreased in the environment that was not stimulated with LPS. In addition, the response to IFN-γ and TNF-α was increased in both peptide groups. This showed that peptides can function in both anti-inflammation and pro-inflammation.

Keywords: Antimicrobial peptides, Peptide synthesis, Antibiotic resistance, Immune response, Immune system

1 INTRODUCTION AND AIM

In recent years, the increase in irregular use of antibiotics around the world has become unstoppable. The use of antibiotics has become commonplace and is seen as a routine practice. As a result of the increased use of antibiotics, resistance mechanisms for microorganisms have developed. Microorganisms that have developed resistance mechanisms have increased their ability to survive by disrupting the action pathways of antibiotics. Accordingly, antibiotics used in the treatment of many diseases are insufficient, and alternative treatments are sought. Many scientists have begun to search for innovative treatment areas and new molecules. There are many microorganisms in our environment that we are exposed to, and many of them are pathogenic. These pathogenic organisms are extremely dangerous to human health and have developed many resistance mechanisms against antibiotics. Antimicrobial peptides, a highly potent and naturally occurring structure, emerge as a defense against all these detrimental consequences.

Antimicrobial peptides (AMPs) are naturally occurring structures that are produced and synthesized by many living things, including humans, and that act against microbial sources. These structures play a crucial role in the innate immune system, contributing to non-adaptive defense mechanisms against various unsanitary microorganisms. One of the most common and important features of AMPs is their amphiphilic character. Due to these properties, they can directly affect the cell membranes of pathogenic microorganisms. Electrostatic interaction occurs between the positively charged groups of AMPs and the negatively charged groups of the bacterial cell membrane. As a result of this interaction, the hydrophilic groups in the AMP structure are directed towards the pathogenic microorganism. In addition, another advantage of AMPs is that they show broad-spectrum antimicrobial activity. Thus, they appear as innovative therapeutic molecules with high effectiveness for many microorganisms.

Examples of AMPs most found in nature are cathelicidin and defensins. These peptides are produced by mammals. Cathelicidins are positively charged peptides. The mechanism of action of cathelicidins starts directly on the cell membrane of the microorganism. They interact electrostatically with the cell membrane and directly kill microorganisms by creating pores in the cell membrane. LL-37 antimicrobial peptide, a cathelicidin derivative, is produced in humans. This peptide, which has an alphahelix structure, penetrates the cell membrane and directly kills the microorganism by disrupting the cell membrane.

In previous studies by Nihan Ünübol and her colleagues, LL-37-like peptides with mimicked amino acid sequences were synthesized. With this study, the effect of these peptides will be improved; they will be re-synthesized and used in a series of studies. The main goal of the thesis is to examine the properties of the immune system structures IL-6, TNF-α, and IFN-γ in the presence of these peptides. For this purpose, a series of experiments will be performed with the mouse macrophage cell line, and the results will be reported.

2 BACKGROUND

2.1 Antibiotics

Antibiotics are molecules found naturally or synthetically that are used against various diseases. Antibiotics that are effective against bacterial diseases are not effective against fungal infections or viral diseases. They inhibit bacterial pathogens with different mechanisms of action. Antibiotics show selective toxicity to bacteria, showing effects with minimal or no harm to host cells. Antibiotics can kill bacterial cells directly or prevent their growth. They work by selectively blocking certain important processes in microbial cells. Microorganisms are capable of producing antibiotics. The action of an antibiotic against microorganisms is selective in nature. Therefore, each antibiotic is characterized by a specific antimicrobial spectrum. There are great differences in the chemical and physical properties of antibiotics and their effects on living things. Consequently, several types of antibiotics are used to treat diverse microbial illnesses in living organisms and have the potential to be used as chemotherapeutic agents. (1,2).

In 1909, Alfred Bertheim, Paul Ehrich, and Sachachiro Hata discovered the compound called "Salvarsan". This compound was the first drug used against the syphilis infection. In the 1920s, studies on azo dyes examined the effects of these compounds on bacteria. These studies led to the emergence of a new compound referred to as "prontosil". These antimicrobial structures showed their effect against many gram-positive bacteria (1). In 1932, a group of antibiotics called sulfonamides, derived from Prontosil, was introduced to the market (1).

In 1928, penicillin, perhaps the most important antibiotic, was discovered. Alexander Fleming observed that a Petri dish contaminated with mold had antibacterial properties and thus discovered penicillin. Penicillins show an antibacterial effect by inhibiting the synthesis mechanism of the cell wall of bacteria. Penicillin antibiotics are classified according to the chemical structures found in their side chains (1,3,4).

In 1939, "thyrotricin" was isolated from soil bacteria by René Dubos. This antibiotic is the first to be studied on mice and has been shown to have strong antimicrobial properties (1). Selman Waksman used the term "antibiotic" in 1941 (1). In 1943, an antibiotic called streptomycin was isolated by Selman Schatz and Albert Schatz. This antibiotic is effective against tuberculosis and belongs to the aminoglycoside class (1). X-rays were used in 1945 to study the penicillin's three-dimensional structure (1). In 1948, the first tetracycline, "chlortetracycline", was discovered by Benjamin Duggar by analyzing global soil samples (1). Erythromycin, the first macrolide compound used by people allergic to penicillin, was discovered by Aguilar in 1949 (1).

Vancomycin, the first glycopeptide, was isolated from soil in 1952 (1). Penicillin was chemically produced by J.C. Sheeman in 1957. Numerous novel antibiotic-effective structures, like "ampicillin" and "carbenicillin," have emerged because of this research (1). Kanamycin, which is used to kill bacteria resistant to penicillin and streptomycin, was isolated by a group of scientists in Japan in 1957 (1).

2.1.1 The importance of antibiotics

Antibiotics were initially used to treat illnesses about 2000 years ago in ancient cultures. Moldy bread and soils that seemed to have therapeutic properties were used, although the practices were not conscious at the time. Today, millions of lives are saved with the correct use of antibiotics. The most important thing to consider when using antibiotics is whether the antibiotic used affects the disease. Antibiotics are only effective against bacterial infections. Unnecessary use of antibiotics and more than the specified dose cause more harm than good (8,9).

2.1.1.1 The relationship between *escherichia coli* and antibiotics

Escherichia coli (E. coli) is a gram-negative bacillus that commonly lives and survives in human and animal intestines, water, soil resources, and plants. There are hundreds of *E. coli* strains identified. While many of them are in the natural flora of humans, some of them cause many serious diseases. *E. coli* is among the bacteria that cause urinary tract infections, circulatory system disorders, pneumonia, nosocomial infections, and diarrhea and is pathogenic for humans. It is the most common cause of food and waterborne diarrhea in developing countries. It causes a high mortality rate in children aged 5 and under (10,11,12).

As a result of the unconscious use of antibiotics, these bacteria gain resistance and become insensitive to antibiotics. The resistance and susceptibility of *E. coli* to antibiotics vary between geographies. Nutritional habits, environmental factors, lifestyle, and genetic diversity change the effects of these bacteria on humans. The choice of antibiotics in the treatment of *E. coli* is extremely important.

Treatment depends on age and the strain of the bacteria. Examples of the most used antibiotics include fluoroquinolones, azithromycin, rifaximin, and common beta-lactam antibiotics (10,13).

2.1.1.2 The relationship between *staphylococcus aureus* and antibiotics

Staphylococcus aureus (S. aureus) is an important pathogen that causes hospital and community-acquired infections and causes numerous devastating disorders. It is seen as the primary source of hospital infections. It is the main cause of complications that develop due to blood circulation disorders, lower respiratory tract diseases, bone and joint infections, endocarditis, and damage to the skin and soft tissues. In addition, it can persist on the surfaces of many devices and catheters used in hospitals and penetrate directly into the patient. *S. aureus* infections have high morbidity and mortality (14-16).

Methicillin-resistant Staphylococcus Aureus (MRSA) or *Methicillin-sensitive Staphylococcus Aureus (MSSA)* is the primary cause of *S. aureus* infections. In 1959, methicillin began to be used against *S. aureus*. However, within 2 years, S. aureus began to develop resistance to methicillin. Resistance was acquired not only to methicillin but also to penicillin and cephalosporin. Treatment options include vancomycin. It can be given alone or in combination with different antibiotics. (17,18).

2.1.2 Antibiotics and their mechanism of action

Antibiotics are classified according to their various properties. This classification is generally classified according to parameters such as the mechanism of action of antibiotics against pathogens, the origins of antibiotics, and the structural features of antibiotics (1,5).

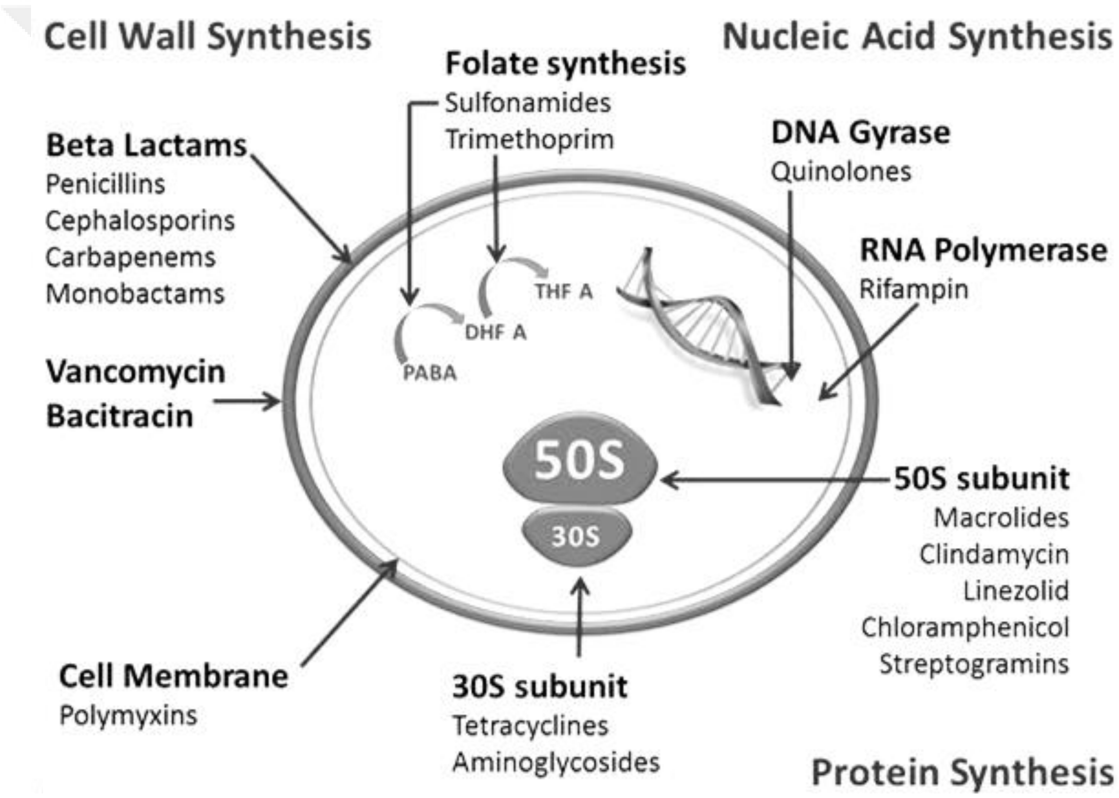


Figure 1. Antibiotic’s action mechanisms (7)

2.1.2.1 Inactivation of nucleic acid synthesis

Antibiotics called Rifampin and Quinolones can be given as examples of this group. These antibiotics show their effects by disrupting DNA and RNA synthesis.

Quinolones inhibit nucleic acid synthesis by targeting topoisomerase IV and topoisomerase II. Rifamycins stop RNA synthesis by inhibiting RNA polymerase. (5).

2.1.2.2 Interference with cell wall synthesis

The outer layer of bacterial cells is made up of a peptidoglycan layer made of sugar polymers. The peptidoglycan layer undergoes cross-linking of glycan strands by the action of trans glycosidases. The peptide chains in their structures extend from the sugar structures in the polymers and form cross-links from one peptide to another. The D-alanyl D-alanine portion of the peptide chain is cross-linked with glycine residues in the presence of penicillin-binding proteins. As a result of this cross-linking, the cell wall becomes stronger.

Antibiotics from the β-lactam family, called penicillin and cephalosporins, show their effects by inhibiting the synthesis of the peptidoglycan layer of bacteria. Glycopeptides directly target the bacterial cell wall by binding to the D-alanyl-D-alanine regions of the peptidoglycan chain and thus cross-linking steps. Examples of antibiotics that inhibit cell wall synthesis: Penicillin, Cephalosporins, Vancomycin, Beta-lactamase Inhibitors and Carbapenems can be given as examples. (6,7).

2.1.2.3 Inhibition of bacterial protein synthesis

Another important mechanism of action of antibiotics against bacteria is the inhibition of the protein synthesis pathway. Antibiotics in this group show their effects by targeting bacterial ribosomes. While the ribosomes in mammary cells are 80S units, the ribosomes in bacteria are 70S units. Antimicrobial agents show their effects by targeting the 30S and 50S subunits. Thanks to this mechanism of action, they only act on bacteria without damaging breast cells.

Aminoglycosides and tetracyclines are examples of antibiotics that affect the 30S subgroup. Macrolides, chloramphenicol, and lacosamide can be given as examples of antibiotics targeting the 50S subgroup (1,5,7).

2.1.3 Antimicrobial resistance

Bacteria have developed many mechanisms to escape any environmental factors in order to ensure the sustainability of their life cycle and to maintain their safety. One of these mechanisms is their resistance to antimicrobial agents. In nature, bacteria coexist with many other organisms that live. This situation sometimes becomes dangerous for bacteria.

Bacteria share the same domains as antimicrobial-producing organisms. They have acquired many resistance mechanisms in the evolutionary process to protect themselves from this negative factor. Evolutionarily, bacteria use two basic strategies against antibiotics. These are the horizontal gene transfers required for the coding and recognition of foreign DNA to determine mutations and resistance to the mechanisms of action it encounters (19).

2.1.3.1 Mutational resistance in bacteria

In mutational resistance, a subset derived from a bacterial population sensitive to antibiotics develops mutations in their genes that affect the drug's activity against the drug. As a result of this situation, bacteria manage to survive even in the presence of the antimicrobial molecule. When a new antibiotic-resistant mutant becomes dominant in the environment, the antibiotic-sensitive population disappears, and antibiotic-resistant bacteria become dominant. This mutation process is a difficult one in bacteria and is protected in the presence of antibiotics. As a result of this mutation, affinity for antibiotics decreases, drug uptake into the cell decreases, antibiotics are avoided by changing intracellular flow mechanisms, and bacterial metabolic pathways are changed. Resistance acquired because of mutation is very diverse and has many different mechanisms (19,20).

2.1.3.2 Horizontal gene transfer

Another mechanism of resistance is horizontal gene transfer. In this case, obtaining foreign DNA material leads to the acquisition of antibiotic resistance. The biggest factor in the occurrence of horizontal gene transfer is the antimicrobial resources found in nature. Because the source of many clinically applied antibiotics is soil and soil derived. This environmentally derived resistance is effective against many antibiotics.

Gathering genetic material from environmental sources by bacterial bacteria depends on three main strategies. These are transformation, transduction, and conjugation. Transformation is the most primitive and simple form of horizontal gene transfer. The emergence of resistance in the hospital environment is achieved by conjugation, a gene transfer method that requires cell-to-cell contact.

Plasmids, integrins, and transposons are important structures that play a role in the spread and development of antimicrobial resistance. These structures play a role in the development of mechanisms that allow new genes to be added to bacterial chromosomes (19,20).

2.1.3.3 Biochemical pathway of antibiotic resistance

The resistance mechanism that occurs in bacteria is generally achieved through more than one biochemical pathway. For instance, there are three distinct metabolic mechanisms that can lead to fluoroquinolone resistance. Furthermore, not all bacteria have the same antibiotic resistance mechanism. For example, gram-negative bacteria produce β-lactamases against β-lactam antibiotics, while gram-positive bacteria provide resistance thanks to modifications in the target region of penicillin-binding proteins (19).

The detailed classification of resistance mechanisms that develop against antibiotics is divided into four subheadings according to the biochemical pathway.

These are modifications of the antimicrobial molecule, preventing antibiotics from reaching the target site, changing target sites, and finally resistance resulting from the adaptive processes of the cell (19).

For both gram-positive and gram-negative bacteria, the development of enzymes that alter the chemical structure of the antibiotic molecule is an example of acquired antibiotic resistance. An example of this is β-lactamases. These enzymes destroy the amide bond of the β-lactam ring, rendering the antimicrobial molecule ineffective. To date, the existence of approximately 300 β-lactamases has been recognized (5, 19, 21). β-lactamases can be examined in four classes;

- Class A β-lactamases, also known as clavulanic acid-sensitive penicillinase,
- Class B β-lactamases, known as metallo-β-lactamases.
- Cephalosporinases are known as Class C β-lactamases. This group is produced by all gram-negative bacteria except *Salmonella* and *Klebsiella*.
- Class D β-lactamases: this group has oxacillin hydrolyzing enzymes. They are commonly found in *Enterobacteriaceae* and *P. aeruginosa* (5).

Another resistance mechanism is reducing drug uptake due to decreased outer membrane permeability. Drug molecules enter the cell through channels called porins. The decrease in porin channels due to developing resistance will prevent the drug from entering the cell (5,19). In another resistance mechanism, protection is provided by intervening in the target areas of the antibiotic. In this case, escape from the antibiotic is achieved by providing modifications that will reduce the affinity of the target region to the antimicrobial molecule (19).

2.1.4 Approaches against antibiotic resistance

Antimicrobial molecules have had a very important place in clinical medicine since the mid-20th century. It has saved millions of people from bacterial infections that threaten human life. However, with the development of antibiotic resistance in pathogenic bacteria that has emerged in recent years, the effectiveness of these antimicrobials has decreased. Vancomycin-resistant *Enterococcus*, *MRSA*, multidrug-resistant *Pseudomonas aeruginosa*, resistant *E. coli*, *Klebsiella pneumonia*, and broad-spectrum β-lactamase-producing pathogens pose a serious threat to public health around the world. The development of new antibiotic molecules is extremely important for these threats. Effective methods and approaches are needed to solve the problem of antibiotic resistance (22-25).

Among these approaches, emphasis is placed on adjuvants and innovative methods that provide hybrid therapy. The use of adjuvants in addition to antibiotics is crucial because of their distinct mechanisms of action.

Innovative solutions include not only chemical molecules but are also extremely important in applications such as bioinformatics to fully understand the formation of resistance in bacteria. In addition, preventing irregular use of antibiotics and creating social awareness are among the target strategies (28).

Other important building blocks are cytokines, which are the systems of innate immunity; cytokine-mediated interleukins; and, of course, antimicrobial peptides. These antimicrobial peptides structures, which are unique parts of the natural immune system, are extremely important in the development of new treatments against bacterial resistance (28, 29).

Probiotics, targeted drug delivery systems, and nanomedicine are also among the target strategies (26, 27,29)

2.2 Antimicrobial Peptides (AMP)

Pathogenic organisms' tolerance to current medicines has made the search for novel antimicrobial compounds crucial. There are peptides with antimicrobial activity that are a unique part of the natural immune system, produced by almost all living things in nature, from plants to bacteria to humans to insects. These structures, called antimicrobial peptides (AMP), are extremely important structures to prevent the proliferation of microorganisms in their own environment or to inhibit their harmful activities.

Numerous antimicrobial peptides exhibit broad-spectrum activity against bacteria, viruses, fungi, yeasts, parasites, and even cancer cells in addition to being efficacious against both gram-positive and gram-negative bacteria. Although AMPs are structurally simple, they are quite complex molecules in terms of their mechanism of action. (30-32).

Most antimicrobial peptides have structures with a net cationic charge. Cationic peptides generally have a length of 12–50 amino acids and are examined in four groups depending on their structure. These are: cationic peptides containing elongated peptide chains; cationic peptide structures containing β-sheet structures; circularly structured peptides formed with a single disulfide bond; and finally, peptide structures that can form amphipathic alpha-helical structures in the appropriate environment (33). These AMPs generally tend to be found in living things in areas that are most likely to come into contact with pathogen sources from the environment. Therefore, they are found in many structures and organs, including epithelial surfaces, skin, eyes, ears, nose, lungs, and intestinal flora (33).

There are several explanations put out for the structural variety observed in antimicrobial peptides. These include: (I) The fact that peptides' antibacterial spectrum does not encompass every pathogen that the species to which they belong may encounter; (II) the potential for peptides with various structural combinations to work in concert to either prevent or eliminate harmful germs; (III) Non-antimicrobial activities differ amongst peptides; (IV) The various metazoan tissues typically express a subset of the whole deposits of a particular species. This could be because different tissues have different needs for the non-antimicrobial properties of peptides, or it could be because the tissue is more sensitive to pathogen invasion (33).

Antimicrobial peptides' effects and the characteristics of the defense system can be influenced by structural diversity.

Longer precursor peptide sequences that are translated into proteins by genome-encoded areas give rise to AMP in all organisms studied thus far. The active peptide structure is formed by proteolytic cleavage of these precursor sequences from the N-terminal region. In the majority of biological structures, the precursor peptide has a signal sequence before the N-terminal region.

The signal sequence regulates both cell membrane targeting and posttranslational modifications that result in the production of the antimicrobial peptide. Mammals have homologous gene families located in certain chromosomal locations that express antimicrobial peptides. The universality of AMPs is evidenced by the fact that they have been identified in a wide range of organisms and have been preserved to this day (34).

A total of 3569 antimicrobial peptides have been isolated or discovered in six different life forms, according to the Antimicrobial Peptide Database (APD). 371 of these are found in plants, 2600 in mammals, 380 in bacteria, 5 in archaea, 8 in protists, and 25 in fungi (34, 35).

Not all parts of living things contain the same number of antimicrobial peptides. They are present in specific areas in high quantities. For instance, certain amphibians have specific structures in their epithelial layers that are used for AMP production and release. The immune system cells that reside in those glands release AMP, particularly in situations like skin injuries (36).

2.2.1 Mechanism of action of antimicrobial peptides

Most antimicrobial peptides have cationic properties. Their amphipathic α-helix properties allow them to modify the double fat layer in the cell membrane. In addition to operating via this route, AMPs' β-layer peptide architectures provide them strong antibacterial capabilities. Two common physicochemical features of AMPs are a cationic charge and a hydrophobic moiety. The negatively charged microbial membranes' selectivity is enhanced by this characteristic. In addition, it facilitates interactions with fatty acyl chains in the membrane structure (37).

AMPs basically show their antimicrobial effects by affecting the structural integrity of the bacterial cytoplasmic membrane at many points. It is characterized by antimicrobial mechanisms that target cellular functions such as inhibition of DNA and protein synthesis, alteration of protein folding, enzymatic activity, and cell wall synthesis (38).

A wide range of subgroups exist within the main categories of membrane-active AMPs and their mechanisms of action. These are: (I) barrel-stave model; (II) toroidal pore model; (III) carpet-like or detergent-like model; (IV) membrane thinning interaction; and (V) aggregate interaction.

The mechanism of action of each of these models is different. For example, in the Barrel-Stave model, AMP structures are positioned perpendicular to the membrane. In the carpet-like model, small sections of the membrane are coated with AMP molecules with the hydrophobic sides inward. In the toroidal-pore model, it is like the Barrel-stave model. However, differently, AMP molecules are always positioned to interact with the phospholipid head groups of the membrane (39).

The effect mechanisms of AMps against inflammation are generally twofold. These are anti-inflammatory effect and pro-inflammatory effect. The first response of the immune system against pathogens is acute inflammation and is an extremely important phenomenon against diseases. In this case, many immune system elements migrate to the inflammation area and constitute the first step of treatment. Examples of these include endothelial cells, cytokines, macrophages and neutrophils. It is involved in many signaling and effect pathways in this cell migration. For example, structures such as TLRs, MAPK and Nuclear factor kappa β are extremely important. These pathways and the immune system cells they mediate play a critical role in the course of inflammation. If pro-inflammatory structures and cytokines continue to migrate to the area of inflammation even after treatment, chronic inflammation is replaced by acute inflammation. Acute inflammation causes many diseases in the body. Crohn's disease, MS, psoriasis and rheumatic arthritis can be given as examples of autoimmune diseases due to chronic inflammation. In these diseases, various AMPs are upregulated or downregulated and take part in the course of the disease. For example, human α-defensins 5 is upregulated against Crohn's disease, while human LL-37 and human β-defensins 2 are upregulated against psoriasis. In addition to diseases, many synthetic or natural AMPs play an inhibitory role by showing anti-inflammatory effects against immune system regulators such as IL-12, IL-6, IL-1β, IL-8, NO, TNF-α, IL-15.

2.2.2 Antimicrobial peptide in humans

Alexander Fleming identified human lysozyme in saliva in 1922, and it was subsequently recognized as the first antimicrobial peptide and introduced to the scientific community (40). Human AMPs range in length from 10 to 149 amino acids. Peptides have net charges that range from -3 to +20. As we previously discussed, AMPs are essential components of the immune system and are present in a wide variety of vertebrate and invertebrate species. Immune system components recognize infectious organisms and eliminate them from human bodies.

AMPs boost the immune system and aid in the neutralization of pathogenic sources by directly interacting with the immune system's mechanism of action. AMPs support the defense system in two different ways. They both directly affect the pathogen source and increase the efficacy of the existing immune system by managing the immune system. We can classify the antibacterial peptides found in humans as cathelicidins, defensins, dermcidines, hepcidins, and histatins (34).

Individuals have diverse areas of expertise inside these structures. In general, AMPs are higher in locations where direct contact with pathogens occurs. For example, they can be discovered in the respiratory system, mouth, eyes, ear, skin, and intestines. The skin of newborn babies is more likely to contain the antimicrobial peptide cathelicidin LL-37, but the skin of elderly people is more likely to contain the antimicrobial peptide beta-defensin 2. Human oral cavity AMPs include defensins and cathelicidins. Human-produced AMPs have a variety of functions in addition to their antimicrobial properties, including immune system stimulation and control, apoptosis induction, and wound healing (41-43).

2.2.2.1 Human cathelicidin

Cathelicidin AMPs were isolated in 1989 (44). Cathelicidins are a family of proteins that contain a C-terminal cationic antimicrobial domain that becomes active upon release from the cathelin moiety of the N-terminal end of the holoprotein. These peptides are structures rich in proline and arginine and have various antimicrobial sequences (34, 45).

Human chalecidin is only characterized for the LL-37 form. Proteolysis is used to produce LL-37 from the human CAP18 protein's C-terminus, or hCAP18. The LL-37 peptide was synthesized in the following locations: it is expressed in skin and gastrointestinal tract epithelial cells, testis, as well as in leukocytes including T and B cells, NK cells, neutrophils, and monocytes. (46-48).

2.2.2.2 Human defensins

A family of mature α-defensins was identified from human neutrophils by Lehrer and colleagues in 1985 (34). Defense peptides in host cells generally appear as precursor proteins and are released into the environment in mature form by protease treatment. These isolated peptides were named HNP-1, HNP-2, and HNP-3 according to their properties. These structures are approximately 29–30 amino acids long and have the same sequences except for the amino end. These peptides, which lack carbohydrate and sulfhydryl groups, are rich in cysteine, arginine, and aromatic structures (49).

HNP-4, the fourth human neutrophil defensin with 33 amino acids, was reported because of research in 1989 (50). Similarly, the fifth and sixth human α-defensin genes were cloned (51, 52). In addition to α-defensins, hBD-1, the first β-defensin with a 36 amino acid length and a basic structure, was obtained and reported from human plasma by Bensch and colleagues in 1995 (53).

2.2.2.3 Human dermcidines

Defensins and cathelicidins are generated by inflammation or injury, but dermcidines are naturally expressed in human sweat. Dermcidins are peptides that display an anionic defense system by being released by the sweat glands of the skin surface to demonstrate their antimicrobial activity. (54).

2.2.2.4 Human hepcidins

Hepcidins are antimicrobial peptides that are expressed in the human liver and are 25 amino acids long and high in cysteine. It was found in 2000 using ultrafiltrate of human blood. Additionally, it has been discovered that this AMP is crucial for the use of iron (55,56).

2.2.2.5 Human histatins

Histatins, which are rich in histidine, were obtained from human saliva in 1988. It was isolated using size-exclusion chromatography and then HPLC method. They are extremely important structures in neutralizing Candida Albicans, a type of yeast (57).

2.2.3 Resistance mechanism of bacteria to antimicrobial peptides

One of the most important reasons that makes it difficult to treat pathogenic microorganisms encountered by the body is the resistance mechanisms shown by these harmful microorganisms. These resistance mechanisms against AMPs are extremely dangerous. It is vital to resolve these resistance mechanisms in terms of managing the treatment process for the disease. Among the resistance mechanisms of pathogen sources, proteolytic degradation, biofilm layer formation, cell surface modifications, and the removal of AMPs from the cell membrane because of modifications of channels and vesicles on the cell membrane can be given as examples.

The resistance that AMPs encounter during their first interaction with pathogen sources is protease. Proteases cause the enzymatic degradation of AMP (58). Proteases, which are structural elements of the host cell, can directly affect macroglobulin and AMPs. or they secrete dermatan sulfate, a structure that neutralizes AMPs. Aspartate protease, which is a highly effective protease found in the outer membrane of gram-negative bacteria, is involved in the breakdown of many AMPs. In addition, another structure involved in the protection of gram bacteria against AMPs is metalloprotease (59).

Another defense mechanism that bacteria use against AMPs is biofilm. The biofilm structure of bacteria is a structure consisting of extracellular proteins, extracellular DNA, and exopolysaccharides (EPS). This structure is a layer of surface-attached bacterial cells embedded in the matrix. Bacteria with a biofilm layer show higher resistance to AMPs and antibiotics (60, 61). The reason for this strong resistance faced by AMPs is seen as the difficulty experienced in penetrating the biofilm layer in bacteria (61-62).

EPS and capsular polysaccharides (CPS) show activity by removing AMPs from the region. This mechanism of action is based on removing AMP from the region by trapping it or pushing the AMP molecule (63). These capsular structures possessed by some bacteria have an important role in the sequestration of AMPs. Hyaluronic acid capsules and M protein, which are important building blocks of these structures, eliminate the antibacterial activities of AMPs. Despite all the negative effects of this biofilm layer of bacteria, AMPs are much more advantageous than classical antibiotics in the treatment of infection. One of the reasons for this is that the mechanism of action of AMPs is bactericidal. However, many traditional antibiotics generally show bacteriostatic properties. (64,65).

2.2.4 Mechanism of action of human antimicrobial peptides

AMPs, which are generally cationic in nature, act by targeting the anionic membranes of bacteria.

2.2.4.1 Targeting bacterial internal membranes

Human cathelicidins work by focusing on the inner membranes of bacteria. The LL-37 protein disrupts the bacterial cell membrane in at least three ways. First, this cationic peptide recognizes and surrounds the anionic surface structure of bacteria. The peptide, which has a classical amphipathic helical structure, exerts its effect by directly targeting anionic bacterial membranes.

In another case, the LL-37 protein binds to and crosses the outer membrane of the membrane. In the last case, the peptide molecule reaches the inner membrane directly and exerts its effect. Peptides present in high concentrations can disrupt membranes through micellization. Alternatively, peptides can also take a vertical position to form a pore on the membrane surface (72, 73).

Another AMP other than LL-37 is the hepcidin peptide. Hepcidin 25, which acts depending on pH, and hepcidin 20, an isoform of which is dependent on the ability to disrupt bacterial membranes These structures containing histidine are rich in cysteine. The effects of peptides with β-sheet structures in an acidic environment are more effective than in a neutral environment (74).

2.2.4.2 Targeting the bacterial cell wall

The bacterial membrane is not the only target for AMP molecules. The following are a few instances of how AMPs work against the bacterial wall. The human α-Defensin 6 structure attaches to the surface proteins of bacteria and acts on them via producing fibrils and nanonet structures through the process of self-assembly, rather than directly killing them (66).

HNP-1, on the other hand, targets lipid II to inhibit the biosynthesis of the bacterial cell wall. Similarly, HBD-3 also shows its effect by targeting lipid II (67, 68). RegIII is a lectin family protein that impacts the bacterial cell wall by selectively recognizing the portion of the bacterium that contains carbohydrates. Of these, RegIIIα kills gram-positive bacteria by targeting the peptidoglycan layer. Furthermore, the gram-negative bacteria's lipid A layer interacts with the mouse RegIIIβ protein (69, 70).

Human lysozyme peptide also works by inhibiting the bacterial cell wall from growing. It attaches itself to the bacterial peptidoglycan chains in the process (71).

2.2.4.3 Targeting inside the cell

We have stated that the human cathelicidin LL-37 peptide acts on the bacterial membrane. The effect of this peptide is not only this, but it also shows its effect by interacting with bacterial DNA. Buforin can be given as another example of a peptide that causes DNA damage (75).

AMPs not only directly kill bacterial infections but also work together with immune system cells to exert their effects against pathogenic microorganisms. This effect is generally due to an increase in the activation of immune system cells. For example, peptide molecules derived from chromogranin A can act on neutrophils. These peptides can bind to cytoplasmic calmodulin and increase neutrophil activation by inducing Ca2+ influx.

An increase in immune system cells occurs due to increased neutrophil activation. Granulysin, an effector molecule found in the cytotoxic granules of cytotoxic T lymphocytes and natural killer (NK) cells, can kill intracellular pathogens in infected cells in the presence of perforin and create a cytotoxic effect against tumor cells (76, 77).

2.2.5 Protection of human cells from AMP action

The cell membranes of eukaryotic cells that synthesize AMP have a different lipid composition than the cell membranes of bacterial cells. Due to this difference, they are protected from AMPs that act by targeting the cell membrane. While there are anionic groups on the outer part of bacterial cell membranes, these structures are located in the cytoplasm of the eukaryotic cell membrane.

Another reason why peptides cannot target the eukaryotic cell membrane is that the crude precursor form of the peptide remains bound to the cationic groups of the peptide until it is proteolytically removed. Storage of peptides in inactive precursor forms in neutrophil granules is thought to be an additional mechanism for protection (33).

AMP-generating microorganisms Unlike eukaryotes, AMP-producing bacteria do not require a defensive mechanism since their cytoplasmic membranes are resistant to peptides (78). This bacterial process is composed of one or two components, each of which has a distinct purpose. The first structure entails an immune protein that binds to the cell membrane through a fat modification, obstructing the peptide's ability to disrupt normal membrane structure and function.

The mechanism underlying this binding is unclear, though it is thought to work in this way. In the second scenario, the immune system consists of an extroverted ABC transporter system, which, upon binding to the environment, quickly expels the peptide from the cytoplasmic membrane, thereby preventing the peptide from building up within the membrane (31).

2.2.6 Structural visualization and classification of AMPs

The structure of AMPs is ascertained via nuclear magnetic resonance (NMR) spectroscopy and X-ray crystallography. 2D solution NMR spectroscopy has been used to determine many of the 3D structures of AMPs. For peptides with short amino acid lengths, this approach works well. However, this approach is regrettably insufficient in more intricate constructions like LL-37 (79).

AMPs' three-dimensional structure is analyzed in four groups. Peptides are categorized into four groups based on their secondary structures: α, β, αβ, and non-αβ. Magainin and LL-37 are members of the α family, which has a helical structure. There are β strands in the β family, which includes human α-defensins. The three-dimensional structures of the αβ family comprise β-strands and α-helices β-defensins can be given as an example of AMPs in this group. In the non-αβ AMP group, there are no α-helix and β-strands (79).

2.3 Immune System

The immune system can be defined as the set of reactions of the body against diseases and infections. All the cells, tissues, and molecules that provide defense against infections are called immune system cells. The regular and simultaneous reactions of these cells, tissues, organs, and molecules in our body against microorganisms that cause diseases are called immune responses. The most important task of the immune system is to prevent infections from entering the body and, if they have, to eliminate these infections without harming the body. Infections that enter the body inactivate the immune system and cause many diseases. Our body's immune system consists of three basic defense layers.

The first of these layers are mechanical, chemical, and biological barriers that protect the body. The skin, respiratory tract, and mucosal layer are examples of the components of this line of defense. If microorganisms overcome these barriers, which are the primary line of defense, they encounter the second line of defense, which is the natural immune system. The innate immune system cells possess genetic programming and encoding that expedites their response to internal infections. Natural immune system components play a crucial role in the body's defense because of their inflammatory qualities, ability to quickly identify and eliminate pathogenic organisms, and stimulation of phagocytic elements (80).

The adaptive immune system is the last line of protection. Through their complementary roles and simultaneous involvement, the innate and adaptive immune systems contribute to protection in an efficient manner. The adaptive immune system also uses natural immune system cells to carry out its functions. When adaptive immunity encounters more than one infectious organism, it differs from the innate immune system by responding in different ways, including receptor-specific discrimination ability, specificity, and rapid response times. By creating memory against infections, it has encountered before, it offers a more advanced and stronger example of defense against an infection that occurs at another time in another cell and tissue (80).

Our body's defensive mechanisms function in harmony to perfectly protect us from the millions of infectious organisms that we come into contact with during the day. Removing a line of defense from this interaction affects and disrupts the entire defense system. No two defensive elements can be considered completely separate from each other, and their effects cannot be ignored (80).

2.3.1 Innate immunity

The first line of protection that multicellular organisms have against hazardous sources is innate immunity. Compared to the adaptive immune response, the innate immune response is far more evolved. It is responsible for defending the host organism against many invasive species. Its most important task is to protect the organism. It is to protect against bacteria, fungi, infectious agents such as viruses and parasites, and toxic structures from the surrounding environment (81).

The immune system can be examined as two complex structures that complement each other. Natural barriers and fluids from the body come first. Targeting a particular bacterium or antigen that has been previously encountered and is now identified triggers adaptive immunity, which is the other type. The innate immune system of the host cell uses this simultaneously operating mechanism to identify a particular pathogen source, which subsequently triggers the adaptive immune response. The initial line of protection for the host is innate immunity. Prioritizing infection control over pathogen neutralization is the key objective. This non-specific immune response happens very fast.

Acquired immunity lasts much longer. Among the innate immune structures are physical barriers such as mucus and epithelial layers, anatomical barriers, epithelial and phagocytic cell enzymes, phagocytes such as neutrophil monocytes and macrophages, complement structures, surface and phagocyte granule antimicrobial peptides such as C-reactive proteins, mannose-binding lectins, defensins and cathelicidins, cytokines, mast There are many structures, such as cells and NK cells. The innate immune response begins gradually when interacting with pathogenic structures. This reactive response is basically established to prevent infection from entering the body, to eliminate pathogens if they enter the body, and finally to activate the acquired immune response. IL-6, one of the cytokines, is a pro-inflammatory cytokine and has broad biological effects that are closely related to tumor growth. Dysregulation of this cytokine is associated with chronic inflammation and multifactorial autoimmune diseases. Additionally, IFN-gamma is the most important macrophage-stimulating cytokine group. Its pro and anti-inflammatory effects are extremely important for the course of diseases. It plays an important role in the innate and acquired immune system. Another important immune system element is TNF-alpha. The relationship with autoimmune diseases plays an important role. It plays an effective role in the initiation of the immune system when induced by LPS. (82-84).

Innate immune system cells consist of many elements. They are neutrophils that are collected and characterized in the inflammation area after chemotactic stimuli and are involved in the processes of inflammation and phagocytosis (84).

Eosinophils are found in the respiratory, gastrointestinal, and urinary tracts, and their effector functions are mediated by the degranulation and release of chemotactic factors such as histamine, cationic proteins, and major basic proteins. Eosinophils' fundamental duty is to eliminate infections caused by microorganisms and parasites.

Moreover, they also function effectively in allergic reactions together with mast cells (85).

AMPs are peptides secreted by innate cells and epithelial cells that play a role in host defense. They have a broad-spectrum effect against many pathogen sources. In addition to their direct effects, AMPs act as inducers of many cellular activities, such as cell migration, cell proliferation and differentiation, cytokine production, angiogenesis, and wound healing (84).

Basophils and mast cells are non-phagocytic granulocytes. They have a variety of receptors, including IgE receptors. Mast cells are found in tissues, especially the mucosa, and contain heparin, serotonin, and histamine. They can secrete various cytokines that increase the inflammatory process. These cells also play a role in allergic and viral processes (84).

Dendritic cells (DC) and monocytes/macrophages are engaged in both innate and adaptive immunity. Peripheral blood contains monocytes that are able to move to the site of inflammation. They change into macrophages and start acting upon entering the tissues. Macrophages possess a microbicidal function and are phagocytic. By exposing lymphocytes to antigens, they contribute to boosting their activity. Following activation, they participate in cytokine release and become more potent. They take part in regulating the immune response. They also act against tumor cells (84, 86).

DCs function as antigen-presenting cells (APCs). They are commonly observed on mucosal surfaces and skin. They are crucial in the T cell activation mechanism against microbial infections. They present antigens to other immune cells after acquiring them by processes including phagocytosis and endocytosis. They are involved in carrying antigens from the peripheral region to the primary lymphatic nodes, where antigen presentation occurs (84, 87).

Natural killer (NK) cells play a regulatory role in cell-mediated immune responses thanks to their cytotoxic activities (84).

2.3.2 Acquired (adaptive) immunity

The adaptive immune system is an immune system response that occurs between T and B lymphocytes and antigen-presenting cells, specific to the pathogen source. After this response, an immunological memory emerges, unlike the natural immune response. T and B lymphocytes are developed and activated by lymphoid organs. During this activation, gene regulation, specifically against antigens, also occurs. With this emerging rearrangement mechanism, a diverse receptor pool is produced that can recognize components of many pathogens. During the first encounter with a pathogen's antigen source, long-lived memory T and B cells are formed. On the next encounter with the same pathogen source, these memory cells are rapidly activated compared to the previous time to mount a faster and stronger protective response (88).

T cells develop in the thymus. The bone marrow is the source of B lymphocytes, which are important in the manufacture of antibodies. Lymphocytes possess a movable structure. Following their development in main lymphoid organs, they go to secondary lymphoid organs by blood and lymph, where they are able to collect circulating antigens (88).

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 RAW 264.7 macrophage cell line

The TIB-71 macrophage cell line, which belongs to the Mus musculus mouse, was used in this study, and this cell line was supplied by ATCC. The cell line that arrived frozen at -80 degrees was received. Passaging was performed in cell culture, and stock was created for the continuity of the cell culture. The content of the culture medium is given in Table 1. The ingredients in the solution required to freeze the cell line are given in Table 2.

Table 1. 100 ml culture medium component

Component	Amount
Roswell Park Memorial Institute 1640 (RPMI 1640)	89 ml
Heat Inactivated Fetal Bovine Serum (HI- FBS)	10 ml
Penicillin Streptomycin (10000U/ml) (Pen/Strep)	1 ml

Table 2. 10 ml freezing mix content

Component	Amount
RPMI 1640	8,5 ml
HI FBS	1 ml
Dimethyl Sulfoxide (DMSO)	0,5 ml

3.1.2 Commercial kits

Commercial kits and their supplier company were used in this study are shown in Table 3.

Table 3.Commercial kits and their supplier company

Commercial Kit	Supplier Company
Cell Proliferation Kit I (MTT)	GeneMark
Pierce™ Quantitative Peptide Assays & Standards	Zymo Research
Mouse Tumor Necrosis Factor α ELISA Kit	Sigma
Mouse IFN-γ ELISA Kit	Elabscience
Clarity Western ECL Substrate	BIO-RAD
Pierce™ BCA Protein Assay Kit	Thermo Scientific
Protein Extraction Kit	Abcam

3.1.3 Antibodies

Antibodies, their supplier company, and dilution rates used in this thesis study are shown in Table 4.

Table 4. Antibodies, their target protein sizes with the supplier company and dilution rates

Antibody	Target Protein Size (kDA)	Supplier Company	Dilution Rates
IL-6	35 kDa	ABCAM	3 µg/ml

Table 4. Antibodies, their target protein sizes with the supplier company and dilution rates (continued)

Beta- actin	41 kDA	ABCAM	1:1000
HRP Anti-rabbit IgG antibody (secondary antibody)	-	ABCAM	1:20000

3.1.4 Antibiotics and antimicrobials

The antibiotics and supplier companies used in cell culture are given in Table 5.

Table 5. Antibiotics with the supplier company

Materials	Supplier Company
Pen/Strep	Gibco
Magainin II	MCE

3.1.5 General biological and chemical materials

General biological and chemical materials that were used in this study are given with the supplier company in Table 6.

Table 6. General biological and chemical materials and their supplier company

Materials	Supplier Company
F moc L-aminoacids	SIGMA-ALDRICH
F moc D-aminoacids	Novabiochem
Rink Amide Resin	SIGMA-ALDRICH

Table 6. General biological and chemical materials and their supplier company (continued)

Oxyma	SIGMA-ALDRICH
N,N’-Dimethylformamide (DMF)	Merck
N,N’-Disopropylcarbodiimide (DCM)	Merck
Trisopropylsilane (TIS)	Gibco
Piperidine	Merck
Diethyl Ether	Carlo Erba
Dicloromethane	Carlo Erba
Acentonitrile HPLC Grade	Merck
Trifluoroacetic acid (TFA)	SIGMA-ALDRICH
Tetramethylethylenediamine (TEMED)	BioFroxx
Ammonium Per Sulfate (APS)	BioFroxx
Bovine Serum Albumin (BSA)	SIGMA-ALDRICH
RPMI	SIGMA-ALDRICH
LPS	SIGMA-ALDRICH
Tween-20	SIGMA-ALDRICH
Glycine	BIO-RAD
Acetone ≥99%	Tekkim
Trizma® Base	SIGMA-ALDRICH

Table 6. General biological and chemical materials and their supplier company (continued)

Sodium Dodecycle Sulfate (SDS)	SIGMA-ALDRICH
FBS	Gibco
Acrylamide-bis-acrylamide 30% Solutions	SIGMA-ALDRICH
Acetic Acid ≥99%	SIGMA-ALDRICH
4X Laemmli Sample Buffer	BIO-RAD
Tripsin/Edta	SIGMA-ALDRICH
Dry Milk Powder	PINAR
Ethylenediaminetetra aceteic acid (EDTA)	SIGMA-ALDRICH
Ethanol 96%	ISOLAB
Ethanol 100%	Merck
Methanol 100%	Merck
2-Propanol	Merck

3.1.6 Equipments

Equipments and their supplier company were used in this study, are listed with their supplier company in Table 7.

Table 7. Equipment with the supplier company

Equipment	**Supplier company**
Biosafety Class II Cabinet	Thermo Scientific

Table 7. Equipment with the supplier company (continued)

Light Microscopy	Leica DM500
Autoclave	Nüve steamArt
Water Purification System	Merck Millipore, Milli-Q® Advantage A10
NanoDrop	Thermo Scientific One^{C}
Peptide Synthesizer	CEM Liberty Blue
Imaging System	BIO-RAD, ChemiDoc™ MP
Razor Peptide Cutting Device	CEM Liberty Blue-Razor
pH meter	Bench Meter Xs
Electrophoresis System	BIO-RAD, Mini Protean
Power Supply	BIO-RAD, Power™ Pac Basic
Mini-Puroteain® Glass Plates	BIO-RAD, 1mM spacers
Mini-Puroteain® Short Plates	BIO-RAD
TransBlot Turbo™ Transfer System	BIO-RAD
Microcentrifuges	Thermo Scientific, MICROCL 21R and Thermo Scientific, MICROCL 17
Centrifuges	Beckman Coulter™ Allegra 64R and Thermo Scientific, SL16R
Vortex	bıoSan Combi-Spin FVL-2400N
Incubator	Thermo Scientific, HERATHERM
Neubauer	SIGMA-ALDRICH
T75 Flask	Corning

Table 7. Equipment with the supplier company (continued)

Magnetic Shaker with Heat	Thermo Scientific, CIMAREC
Dry Heat Sterilizer	Nüve FN120
Petri Dishes	ISOLAB
Pipettes	Eppendorf ResearchPlus, Ergo One and Thermo Scientific
Pipette Tips	RatioLab
Serological Pipette Tips	Isolab
Pipet Controller	Thermo Scientific
Water Bath	EMCO, ESM-3710
Dry Block Heating Thermostat	BIOSAN, Bio TDB-100
Weighing Machine	SHIMADZU UW620H
Fridges	ARCTIKO and Kirsch
Homogenizer	BioSpec, Mini Bead Beater 16
Laboratory Hood	Wesemann, System Delta 30
Membrane	BioRad, Immun-Blot PVDF Membranes for Protein Blotting
Plate reader	BioTek, Power Wave xs2

3.2 Methods

3.2.1 Solid phase peptide synthesis and cleavage

The chemical synthesis of peptides intended to have antibiotic properties was done in solid phase. The "CEM Liberty Blue® peptide synthesizer" instrument was utilized within the parameters of the experiment to create peptides. Amino acids, resin, activator base (oxyma), activator (DIC), washing solution (DMF), and protection inhibitor (piperedine) were determined using the device's program and the peptide's sequence. The peptide's molecular mass was used to compute the ratios of amino acids and resins. After the synthesis was finished, the produced peptide coupled to the resin was separated from the resin using the Razor® cutting instrument. The cutting device was heated to 38 °C before the synthesized peptide was added.

The synthesized peptide was added to a cutting solution including 4.75 ml of trifluoroacetic acid (TFA), 125 µl of triosopropyl silane (TIS), and 125 µl of distilled water, which was then placed in the cutting apparatus and heated to 38 °C for 30 to 45 minutes. Following the cutting step, 15 ml of cold (-20 °C) diethyl ether was then added to the mixture, and separation was accomplished using centrifugation. The precipitate containing the peptide was gathered, and the supernatant was discarded.

Peptides NET1, NET2, NET3, and NET4 were previously synthesized and reproduced during peptide synthesis. The purpose of choosing these peptides was that their microbial activities were determined and re-evaluated in this study. As a result of this study, we continued with NET1 and NET2 of the peptides we had. Although the amino acid sequences of the peptides are the same, the amino acid forms used are different.

Table 8. Synthesized antimicrobial peptides

Antimicrobial Peptides	**Sequence**	**Leucine Isomer Types**	**Arginine Isomer Types**	**Molecular Weight (g/mol)**
NET1:	RLLLRLLRRLLRLLLR	D type	L type	2085,75
NET2:	RLLLRLLRRLLRLLLR	L type	L type	2085,75
NET3:	RLLLRLLRRLLRLLLR	L type	D type	2085,75
NET4:	RLLLRLLRRLLRLLLR	D type	D type	2085,75

3.2.2 Peptide purification

The synthesized peptide structures were examined and purified by HPLC. For this purpose, as the mobile phase, solutions A (0.05% TFA dissolved in distilled water) and B (0.25% TFA dissolved in acetonitrile) were used. Before loading the peptide structures into the system, the HPLC column was cleaned by passing 100% B solution through the system to remove contamination. The peptide was then loaded into the system, and a wide range of hydrophobicity was selected (solution B was adjusted to go from 5% to 80% for 30 min).

At the end of the process, the purity of the peptide was evaluated by examining the peaks belonging to the peptide. When peaks belonging to the peptide were seen, these parts coming out of the column were collected, and the peptide was obtained in pure form. Although the device used during peptide synthesis was suitable for the GMP environment and yielded 95% impurities, it was still purified by HPLC. The peptide collected in this way was subjected to evaporation to separate it from the organic solvent before the lyophilization process.

Table 9. HPLC Device Application Conditions

Time (min)	**A (%)**	**B (%)**	**Flow (mL/min)**
0.00	0.0	100.0	2.000
5.00	95.0	5.0	4.000
5.01	95.0	5.0	4.000
35.00	20.0	80.0	4.000
35.01	0.0	100.0	4.000
40.00	0.0	100.0	4.000
40.01	95.0	5.0	4.000

After all processes were completed, if the peptide in powder form was soluble, it was dissolved in deionized water; if it was not soluble in water, it was dissolved in acetonitrile, and its concentration was calculated. The concentrations of the peptides were determined with the "Pierce™ Quantitative Peptide Assays & Standards Catalog Number: 23290" kit and applied in accordance with the conditions specified in the method.

3.2.3 Lyophilization

The pure peptide solution, which was purified and collected by HPLC, was subjected to lyophilization to remove the organic solvents present in it. The lyophilization process is a process that allows the substance to be dried by freezing the product in solution and then removing the gas phase formed by sublimation. The peptide solution was kept at −80 °C and then placed in the device once the lyophilizer reached −80 °C. The vacuum of the system was turned on, and the peptides were kept for 2 days. When they were completely dried, the peptides were removed from the device. Peptides, now in powder form, were stored at +4°C or -20°C for use in experiments.

3.2.4 Determination of peptide concentration

After the lyophilization process, the stored peptides are processed to determine their concentration before being used in experiments. For this purpose, the peptides weighed on a precision balance are dissolved in 1 ml of distilled water. It is kept in the sonicator for 5–10 minutes to complete the dissolution process. Peptide concentrations were measured using "Pierce™ Quantitative Peptide Assays & Standards Catalog Number: 23290". Since the kit used was not suitable for water, the samples were diluted 1:1 with 10 µl 100% ACN. 10 µl of standard, blank, and peptides were placed in each well of a 96-well black plate. 70 µl Fluorometric Peptide Assay Buffer was added to each well and pipetted. 20 µl Fluorometric Peptide Assay Reagent was added and incubated for 5 minutes in a dark environment at room temperature.

After incubation, the samples were measured fluorometrically with the "Thermo Varioskan" device. Standards and graphics were created in line with the information on the kit. Peptide concentration was calculated by entering the results obtained on the created graph. Peptide concentrations were calculated with the formula below.

$$\text{Concentration}\ \left(\frac{\text{mg}}{\text{ml}}\right) = \frac{[\text{Peptide Molecular Weight (Da) xConcentration }(\mu\text{m})]}{1000}$$

3.2.5 Detection of antibacterial activity (minimum inhibitor concentration assay)

Minimum Inhibitory Concentration (MIC) is an assay that determines the lowest concentration of material or molecule at which the growth cycle of microorganisms is completely inhibited. In this experiment, the lowest concentration required for the targeted molecule to have an effect is determined. *Staphylococcus Aureus (S. aureus), Escherichia Coli (E. Coli), Candida Albicans* and *Methicillin-resistant Staphylococcus Aureus* (MRSA) were used throughout the experiment. Before the experiment, the necessary environmental conditions for the growth of microorganisms were created. While MRSA was provided as a patient isolate, other bacteria were provided as lyophilized strains.

Mueller Hinton Agar and Broth were used for this. The media weighed in appropriate amounts were autoclaved. When the agar to be poured into petri dishes cooled to 50–55 °C, it was poured into petri dishes. Petri dishes were stored at +4°C to be used when completely cooled. To check whether there was contamination during the pouring process, two petri dishes were left in the incubator at 37°C. When the other prepared liquid medium reached room temperature, it was stored at +4°C.

Before starting the MIC experiment, it is necessary to seed the petri dishes to revive the organisms to be used. For this purpose, all materials to be used were brought to room temperature. Microorganisms taken from the microorganism samples taken from the stock with the help of a loop were planted in petri dishes as line cultivation and incubated at 37°C. It was incubated for one night. The next day, single colony samples were taken from petri dishes and inoculated into liquid medium.

Samples taken into liquid medium were kept at 37°C for 1 day. After incubation, the microorganisms taken from the liquid samples were brought to a 0.5 McFarland value and planted in a 96-well plate. To perform the MIC test, peptides at 1024 mg/ml were seeded into each well by serial dilution and incubated at 37°C for 1 day. The results were evaluated after incubation.

Table 10. Mueller Hinton Agar Content

Components	**Volume (Final 1000 ml)**
Mueller Hinton Agar	38 g
Distilled Water	1000 ml

Table 11. Mueller Hinton Broth Content

Components	**Volume (Final 1000 ml)**
Mueller Hinton Broth	21 g
Distilled Water	1000 ml

3.2.6 Revitalization and passage of stock cells

Cell passaging is extremely important to ensure the continuity of the cell line. The first passage of the frozen cell line was carried out under appropriate environmental conditions. TIB-71 mouse macrophage cell line was used in cellular studies. The cell line was purchased as a secondary. The biggest reason for choosing this cell line is that it is used in many studies in the literature to represent the immune system. It is frequently preferred especially in inflammation studies. For this purpose, RPMI medium containing 10% FBS and 1% Pen/Strep was prepared. All chemical materials to be used were brought to room temperature. 5 ml of medium was taken into a falcon tube. The stock cell culture line was added as soon as it dissolved. The obtained suspension was centrifuged at 500 g for 5 minutes. After centrifugation, the supernatant was discarded.

The remaining cellular pellet was dissolved by pipetting approximately 2–3 ml of medium. 10 µl of the thawed cell line was taken and mixed with trypan blue at a ratio of 1:1. The mixture was placed on a Neubauer slide and viewed under a microscope, and counting was performed. By calculating the appropriate cell amount, cell seeding was carried out in a T25 flask. After the sowing process, it was incubated for 1 day in a 37°C incubator with 5% CO2. The next day, the flask was checked under a microscope. Since the cell line used maintains its viability by adhering to the surface, the appearance of cells adhering to the surface is expected. The medium in the flask was aspirated, and the same amount of medium was added and incubated again under the same conditions. When the cell confluency in the flask reached 80–85%, the passage process was appropriate. The continuity of the cell line was ensured by passaging, and the cells were stored as stock.

For the passage process, first the medium in the flask was withdrawn. Approximately 1 ml of 0.25% Trypsin-EDTA solution was added to the cells adhered to the surface and kept at 37°C with 5% CO2 for 5–10 minutes. After this process, the cells cut off their connection with the surface. Gather the entire solution by adding 2–3 ml of fresh medium to the flask. The resulting suspension was centrifuged at 500g for 5 minutes. After centrifugation, the supernatant was discarded, and the cellular pellet was dissolved in an appropriate amount of medium. After the procedure, viability was checked by counting cells again. Before transplanting into the flask again, the cells must be frozen to be stored in stock.

For this process, a freezing mix was prepared as previously described, mixed with the thawed cells, and placed in a screw-capped Eppendorf tube. Eppendropf tubes were frozen under appropriate conditions and stored for use in other experiments.

3.2.7 Performing cytotoxicity analysis

A cytotoxicity assay is a test used to evaluate the damage that the molecules to be examined will cause on eukaryotic cells. In this test, peptides synthesized using the MTT Cell Proliferation Kit, were evaluated. The implementation of the assay is a colorimetric test method based on the reduction of the yellow tetrazolium salt to purple formazan crystals by metabolically viable active cells. The TIB-71 RAW 264.7 macrophage cell line, a mouse macrophage line, was used in this study. The cell line was revived in a suitable culture medium and made ready for the experiment.

Cells were incubated in RPMI medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin in a 5% CO2 incubator at 37 °C. After being removed from the stock, cells were first cultivated in a T25 flask. When the cells had grown to 80% saturation, passage was done. The purpose of this process is to ensure the continuity of the cell line and to increase the number of cells by transferring them to the T75 flask. Since the cell line used proliferates by adhering to the surface, the cells must be removed from the flask environment. Respectively: The medium in the flask was aspirated. After this the flask (i.e. cells) was washed with phosphate buffer. 2 ml of 0.25% Trypsin-EDTA was added to the cells and kept at 37 °C in a 5% CO2 incubator for 5–10 minutes. After incubation medium containing FBS and RPMI was added to the flask, and cells were harvested. The collected cells were centrifuged at 500 g for 5 min. After centrifugation, the supernatant was discarded. The remaining cell pellet was dissolved with FBS and RPMI solutions.

Following these procedures, the thawed pellet was subjected to certain procedures for cell counting and viability determination. For cell counting, cells were stained with trypan blue at a ratio of 1:1 and counted under a microscope. Following this process, the cells were seeded in 96-well plates with 50,000 cells in 100 µl of medium in each well. It was incubated for 24 hours at 37 °C in an incubator with 5% CO_2. After 24 hours of incubation, the medium in the 96-well plate was aspirated. The prepared peptide solutions were diluted serially from 64 µg /ml to 0.125 µg/ml and added to the wells as 10 µl. The final volume was completed with RPMI containing FBS in 1 ml.

After adding appropriate doses of peptide to the wells, the plate was incubated for 18–24 hours at 37 °C in a 5% CO2 incubator. The experimental set was set up as three replicates. Cells without peptide under the same conditions were used as controls. Magainin II, a natural antimicrobial peptide known to have no cytotoxic effect, was used as a positive control.

After incubation, the protocol of the MTT cytotoxicity kit was applied. 10 µl of MTT labeling reagent was added to each well. The plate was incubated at 37 °C in an incubator containing 5% CO2 for 4 hours. After 4 h, 100 µl of solubilization solution was added to each well, and the plate was allowed to stand overnight in the incubator at 37 °C with 5% CO2. After all these steps, it was analyzed at appropriate wavelengths (550 and 690 nm) to evaluate the result. Following the MTT results, experiments were continued with 2 peptides with appropriate cytotoxicity out of 4 different peptides we had.

3.2.8 Investigation of TNF-alpha expressions by elisa

Within the scope of the Elisa study, the effect of varying concentrations of the peptides we synthesized on the mouse macrophage cell line was examined. Within the scope of this study, the effects of different concentrations of peptides on the cell line induced by different concentrations of LPS were examined. As previously described, the cell line was dissolved from the stock, and the cells were seeded onto the flask. After incubation, cells were seeded into a 96-well plate to perform the experiment. It was incubated for 1 day at 37 °C in a 5% CO2 environment. After incubation, antimicrobial peptides of different concentrations were added to the cells and incubated under the same conditions for 2 hours. After 2 hours, 50 ng/ml LPS was added to the cells treated with antimicrobial peptides and incubated overnight.

In this study, a cell line that was not treated with antimicrobial peptides and LPS, a cell line treated only with antimicrobial peptides, a cell line treated only with LPS, and a cell line treated with both LPS and antimicrobial peptides were used. To find the changing TNF-α ratio after incubation, the kit named "Mouse Tumor Necrosis Factor α ELISA Kit-RAB0477" was applied according to the protocol.

3.2.9 Investigation of IFN-gamma expressions by elisa

Similarly, the Elisa study was applied to measure the change in IFN-γ. This test was applied to examine the effect of different concentrations of peptide on the cell line and to observe the results in the presence of LPS. Likewise, cells were taken from the stock and first grown under appropriate conditions, and then the cell seeding process was carried out on a 96-well plate to perform the Elisa test. It was incubated for 1 day at 37 °C with 5% CO2. After incubation, it was treated with peptides in different concentrations. After 2 hours of peptide treatment, 50ng/ml LPS was added and incubated again under the same conditions for 1 day. In order to see the change of IFN-γ after incubation, "Mouse IFN-γ ELISA Kit-E-EL-M0048" was applied according to the specified kit protocol, and the results were evaluated.

3.2.10 Protein extraction

Protein was extracted from the TIB-71 cells to be used for Western blot analysis. For this purpose, the supernatant of the cells grown in the appropriate environment was separated for Elisa, while protein was extracted from the cellular pellets. Proteins obtained according to the Protein Extraction Kit instructions were made ready for western blot analysis.

The protein samples' concentration was determined using the Pierce BCA protein assay kit. The absorbance readings were measured at 565 nm in a BioTek plate reader following the treatment of the standards and proteins extracted from patient tissue samples using kit reagents. The absorbance values of the standards were used to generate a standard curve, and the resulting equation allowed for the determination of the sample concentrations. The recovered proteins were kept at -20°C and their concentrations were expressed in µg/mL.

3.2.11 IL-6 expressions nalysis by Western Blot

IL-6 were conducted on 12% (w/v) SDS-polyacrylamide gel (SDS-PAGE) and transferred onto the PVDF membrane using the wet transfer technique to compare the expression levels of the markers. Tables 12 and 13 list the elements of each of the SDS-page gel's phases.

Protein samples were diluted 4:1 with an Laemmli buffer mixture containing 10% beta-mercaptoethanol, and then heated at 95˚C for five minutes to denature them. Samples were evenly distributed into each well in 12 µl. Using a pre-stained marker (5–180 kDA) the proteins were tracked as they moved over the gel. Following the removal of the protein samples from the stacking gel, 160 volts was applied to the voltage. The samples were previously run at 80 volts. The contents of the running buffer used while running are listed in Table 14.

Table 12. Separating gel content

Components	**Volume**
Distilled Water	3,2 ml
30% Acrylamide Bis-acrylamide	4 ml
TEMED	10 µl
10% SDS	100 µl
10% APS	100 µl
1.5 M Tris HCl pH 8.8	2,6 ml

Table 13. Stacking gel content for 5 ml

Components	**Volume**
Distilled Water	2,975 ml
TEMED	5 µl
10% SDS	50 µl
30% Acrylamide Bis-acrylamide	670 µl
10% APS	50 µl
0.5 M Tris HCl pH 6.8	1,25 ml

Table 14. 10X Running buffer content

Components	**Amount**
Tris Base	30 gr
Glycine	144 gr
SDS	10 gr
ddH_2O	up to 1L

To activate the PVDF membrane methyl alcohol, ddH_2O, and 1X transfer buffer were successively poured over it before to transfer. Table 14 lists the components of the transfer buffer. To activate the PVDF membrane methyl alcohol, ddH_2O, and 1X transfer buffer were successively poured over it before to transfer. Table 15 lists the components of the transfer buffer.

Table 15. 10X transfer buffer contents

Components	Amount
Tris Base	30 gr
Glycine	144 gr
ddH_2O	up to 1L

The transfer operation was done at 4°C and took 16 hours at 20 volts. Following the transfer, the membrane was blocked by soaking in TBS-T solution containing 5% (w/v) milk powder for an hour in an orbital shaker. Tables 16 and 17 show how to prepare 10X TBS buffer and 1X TBS-T. After that, the membrane was washed three times for five minutes using 1X TBS-T buffer. Parts of the membrane containing activated markers were sliced to express them, while beta-actin served as a positive control. The membrane was then rinsed three times for five minutes using 1X TBS-T buffer. After washing, the membrane was allowed to sit at room temperature for one hour. A secondary antibody, anti-rabbit IgG, was chosen and diluted to a 1: 1000 ratio using 1X TBS-T 5% milk solution. Membranes were cleaned as mentioned before. Illuminations made with a horseradish peroxidase (HRP)-based Clarity Western ECL substrate were read using ChemiDoc devices. The area of the bands obtained in the western blotting results was computed using Image Lab software.

Table 16. 10X TBS buffer contents

Components	Amount
Tris Base	28 gr
Sodyum Chloride (NACI)	88 gr
HCI	Up to the pH is equal to 8
ddH_2O	up to 1L

Table 17. 1X TBS-T buffer contents

Components	**Volume**
10X TBS buffer	100 ml
Tween 20	1 ml
ddH_2O	up to 1L

After the first blotting, antibodies on the membrane were removed by stripping and reblotted with beta-actin for normalization. The contents of the strip buffer used for the stripping process are given in Table 18.

Table 18. Strip buffer contents

Components	**Volume**
Glycine	15 gr
SDS	1 gr
Tween 20	10 ml
HCI	Up to the pH is equal to 2,2
ddH_2O	up to 1L

The membrane was washed three times with strip buffer for ten minutes after the first blotting. The membrane was subsequently immersed in the milk powder-TBS-T solution and the second blotting procedure began. The membrane had previously been cleaned twice with PBS and once with TBS-T buffer for ten minutes. The blotting procedure was followed as mentioned before.

Using Image Lab software, the volume occupied by the bands on the membrane was determined, and the band volumes IL-6 were normalized to beta-actin and examined to see how the protein expressions changed in a semi-quantitative manner.

Microsoft Excel was used for the semi-quantitative analysis. IL-6 secretion in mouse macrophages due to increased peptide concentration was examined by Graphpad Prism (9.0.0) was used for statistical analysis.

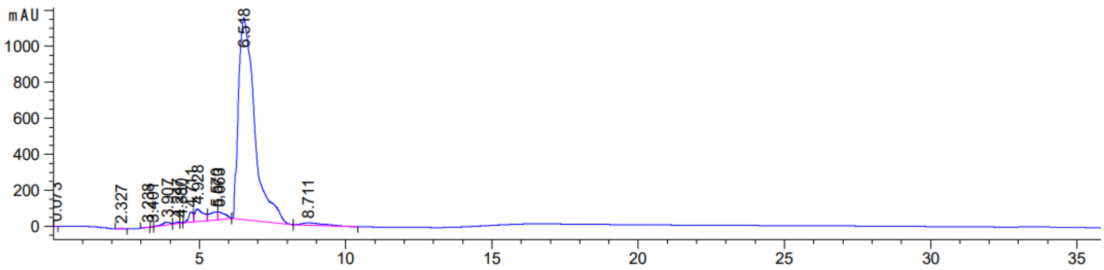
4 RESULTS

4.1 AMP Purification and Determination of Concentration

NET peptides were developed in previous studies by Nihan ÜNÜBOL and Tanıl KOCAGÖZ In addition, these peptides were used as the basis of this study. These peptides target the phospholipids in bacterial cell membranes by way of an alpha-helix shape made up of positively charged and hydrophobic amino acids. The peptides were designed using the human cathelicidin LL-37 C-terminal region. Long and massive positively charged side chains of arginine (Arg) and hydrophobic side chains of leucine (Leu) amino acids are present in the peptides in various configurations.

Peptides synthesized in a solid phase were purified before use in experiments. After the purification process was carried out by HPLC, peptide concentrations were measured.

A.



B.

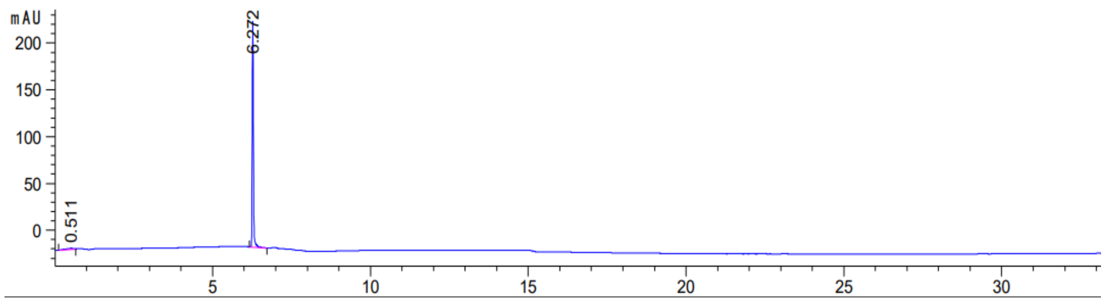
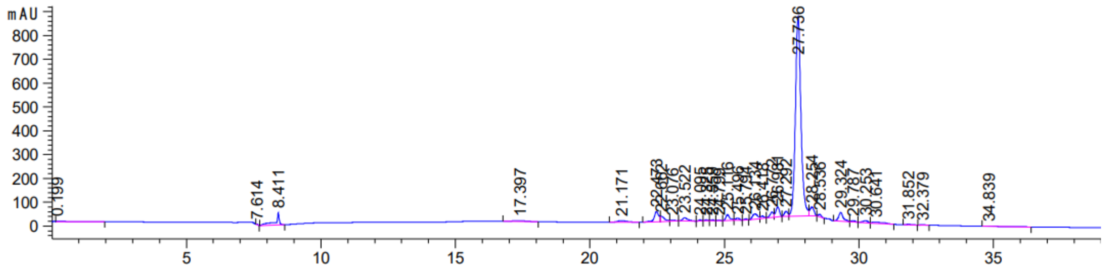


Figure 2. NET1 NET2 NET3 NET4 peptides HPLC results

C.



D.

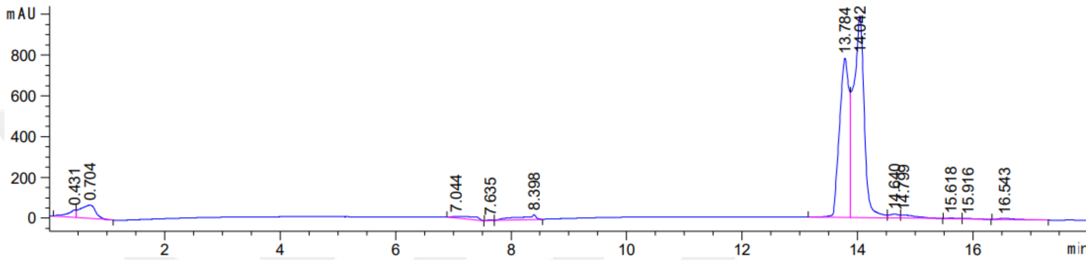


Figure 2. NET1 NET2 NET3 NET4 peptides HPLC results (continued)

The HPLC results in Figure 2 belong to A. NET1, B. NET2, C. NET3, D. NET4 peptides, respectively. The uptake time of NET1 peptide was 6 minutes 30 seconds after the HPLC process started. The uptake time of NET2 peptide was 6 minutes and 20 seconds. The retention time of NET3 and NET4 peptides is 27.7 minutes and 13.7-14 minutes, respectively. The presence of a single peak in the chromatography results indicates that the peptide we synthesized is pure and does not contain any other molecules.

Fluctuations seen as a result of chromatography indicate previous contamination (noise) in the device. As a result of the analysis, the peak areas were collected by HPLC and completely pure peptides were obtained. In this way, other pollution factors were eliminated.

While measuring peptide concentrations, the Pierce™ Quantitative Peptide Assays kit was used. After peptide concentrations were determined, it was stocked under appropriate conditions to be used in experiments.

Table 19. Peptide Concentrations

Peptide	Peptide Concentration (mg/ml)	Absorbance	Peptide Concentration (µm)
NET1	1,65	19,72	795,16
NET2	1,50	17,85	719,75
NET3	1,87	22,34	900,80
NET4	1,71	20,43	823,79
Mag2	2,14	21,56	869,35

4.2 MIC

MIC experiments were performed with different microorganisms to measure the antimicrobial effect. The microorganisms used in this study were selected from microorganism families that cause nosocomial infections and have become antibiotic resistant today. In this context, *Escherichia Coli ATCC 25922*, *Staphylococcus Aureus ATCC 29213*, *Candida Albicans ATCC 10231* and *MRSA* strains were used.

In this experiment using various organisms, it appears that the NET1 peptide acts at lower concentrations than other peptides. Similarly, NET2 peptide showed an inhibition effect at higher concentrations than other peptides. *S. Aureus MRSA* strain showed a more resistant effect against peptides than other microorganisms.

Table 20. Peptide concentrations showing minimum inhibition of bacterial strains included in the MIC study

	E. Coli 25922	***S. Aureus 29213***	***C. Albicans 10231***	***MRSA***
NET1	2 µg/ml	4 µg/ml	2 µg/ml	4 µg/ml

Table 20. Peptide concentrations showing minimum inhibition of bacterial strains included in the MIC study (continued)

NET2	**16 µg/ml**	**16 µg/ml**	**8 µg/ml**	**32 µg/ml**
NET3	2 µg/ml	2 µg/ml	4 µg/ml	4 µg/ml
NET4	8 µg/ml	8 µg/ml	8 µg/ml	16 µg/ml

4.3 Cytotoxicity Assay

In cytotoxicity studies, the interaction of the synthesized peptides with the mouse macrophage cell line TIB-71 was examined by the MTT method. The toxic effects of peptides at varying concentrations are extremely important for the vital activities of macrophages, which are members of the immune system.

Different peptides at different concentrations were used in the cytotoxicity study. MTT studies were conducted separately without and in the presence of LPS. In this study, only TIB-71 cells were used as negative control and TIB-71 cells containing magainin2 were used as positive controls. TIB-71 cell treated with different concentrations of different peptides and LPS-treated TIB-71 cell were used to examine in the experiment. Finally, the experimental line containing TIB-71 cell + Peptide molecule + LPS was established. MTT results without LPS are given in Table 21, 24 and Figure 3, 5. Table 22, 25-28 and Figure 4, 6-9 shows the MTT results in the presence of LPS.

Table 21. MTT Cytotoxicity Results without LPS

% Cytotoxicity										
	64 µg/ml	**32 µg/ml**	**16 µg/ml**	**8 µg/ml**	**4 µg/ml**	**2 µg/ml**	**1 µg/ml**	**0,5 µg/ml**	**0.25 µg/ml**	**0,125 µg/ml**
NET1	96,45	96,38	88,76	32,51	7,88	11,74	13,61	6,91	1,98	0,36
NET2	62,82	60,82	46,40	25,40	5,40	7,88	4,71	2,22	1,16	2,22

Table 21. MTT Cytotoxicity Results without LPS (continued)

NET3	90,26	87,46	78,48	57,25	48,99	41,29	37,20	28,49	21,64	9,54
NET4	85,35	73,28	62,90	53,82	46,37	30,24	23,64	16,82	12,35	6,27
Mag2	29,51	25,16	21,57	18,61	4,47	4,40	4,12	1,71	2,33	0,81
Cell	0	0	0	0	0	0	0	0	0	0

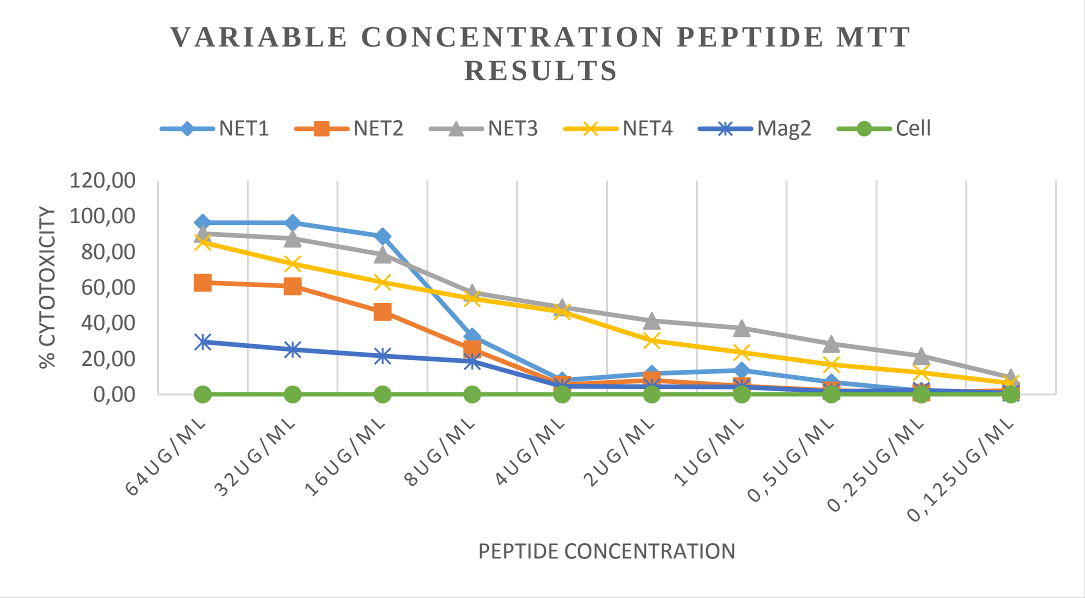


Figure 3. MTT Results of different peptides different concentration

Cytotoxicity values varying depending on decreasing peptide concentration are given in Table 21 and Figure 3. This experimental group was studied in an LPS-free environment.

Table 22. Selectivity Index for Antibacterial Activity and Cytotoxicity.

SI: TC50(µg/ml)/(MICµg/ml)				
	E. Coli 25922	***S. Aureus 29213***	***C. Albicans 10231***	***MRSA***

Table 22. Selectivity Index for Antibacterial Activity and Cytotoxicity (Continued)

NET1	5,24	2,62	5,24	2,62
NET2	1,25	1,25	2,5	0,625
NET3	2,24	2,24	1,12	1,12
NET4	0,78	0,78	0,78	0,39

When calculating the selectivity/safety index, the MIC concentration at which AMP affects the microorganism is related to the concentration of AMP at 50% toxicity on the cell. The important point to note here is that AMPs will show both low inhibition and low toxicity (89).

Table 23. Cytotoxicity of variable concentration LPS on cells

% Cytotoxicity			
	100ng/ml	**10ng/ml**	**1ng/ml**
Cell-LPS	61,46	59,47	57,94
Cell	0	0	0

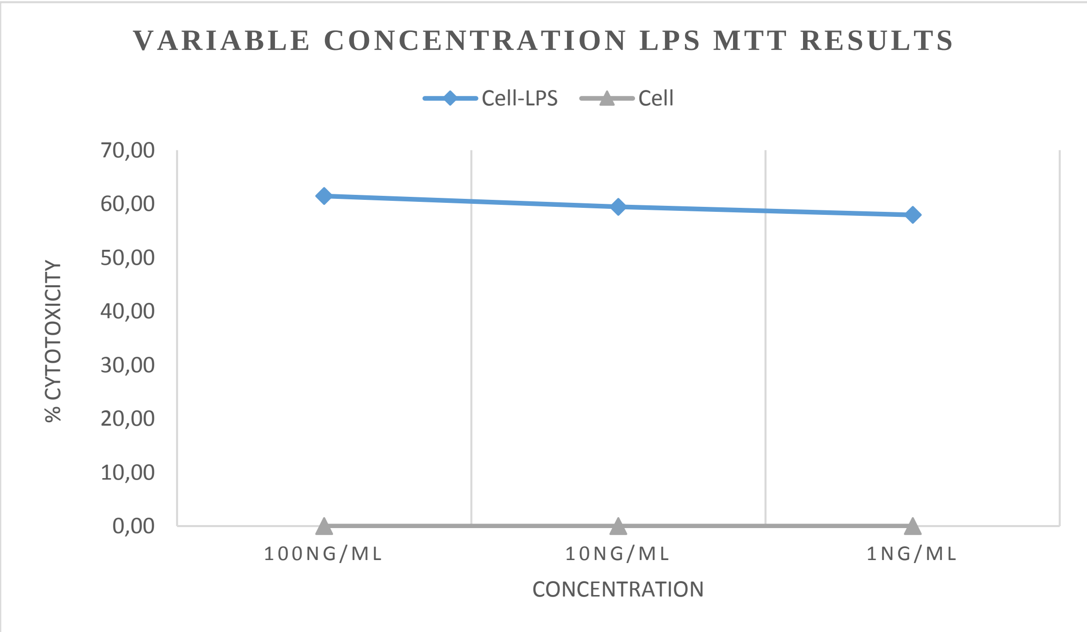


Figure 4. Cytotoxicity of variable concentration LPS on TIB-71 cell

Table 23 and figure 4 show the cytotoxicity values of the cell in the presence of LPS.

Table 24. Cytotoxicity of NET1, NET2 and Mag2 peptides at variable concentrations

% Cytotoxicity				
	4 µg/ml	**2 µg/ml**	**1 µg/ml**	**0,5 µg/ml**
Cell-NET1	14,64	12,99	8,97	6,41
Cell-NET2	6,12	5,82	3,75	1,01
Cell-Mag2	4,04	3,64	2,28	1,03

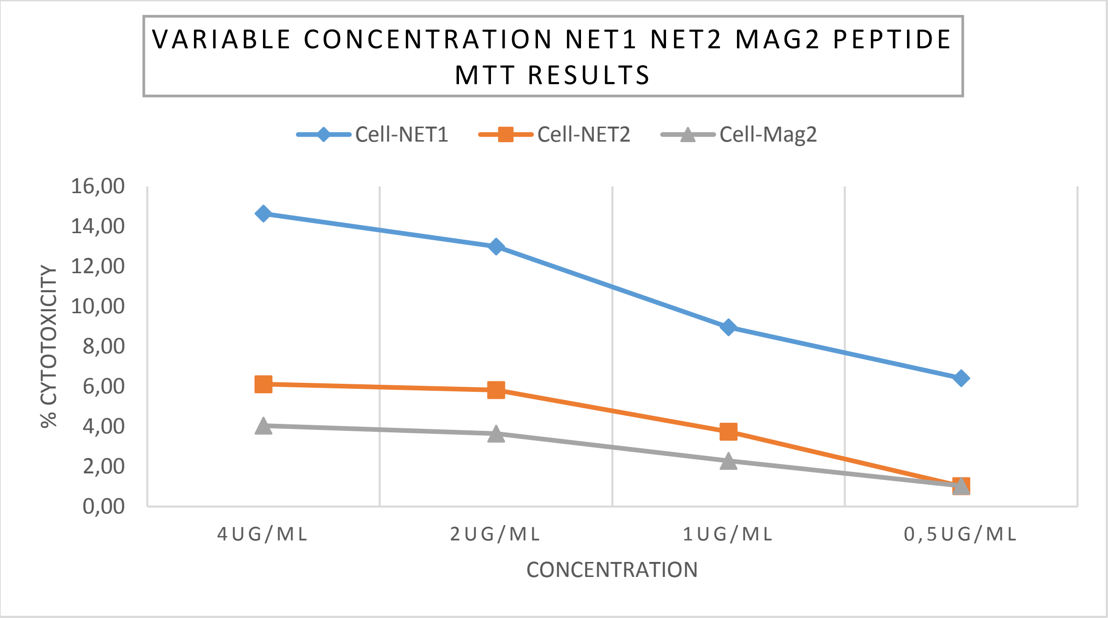


Figure 5. Cytotoxicity of NET1, NET2 and Mag2 peptides at varying concentrations

Table 24 and figure 5 show the cytotoxicity of NET1, NET2 and Mag2 peptides on the cell.

Table 25. Cytotoxicity results when the peptide concentration is 4 µg/ml and the LPS concentration is variable.

% Cytotoxicity			
	100ng/ml LPS	**10ng/ml LPS**	**1ng/ml LPS**
Cell-LPS-NET1	57,77	54,85	55,64
Cell-LPS-NET2	61,13	63,79	66,82
Cell-Mag2-LPS	63,47	65,78	66,98

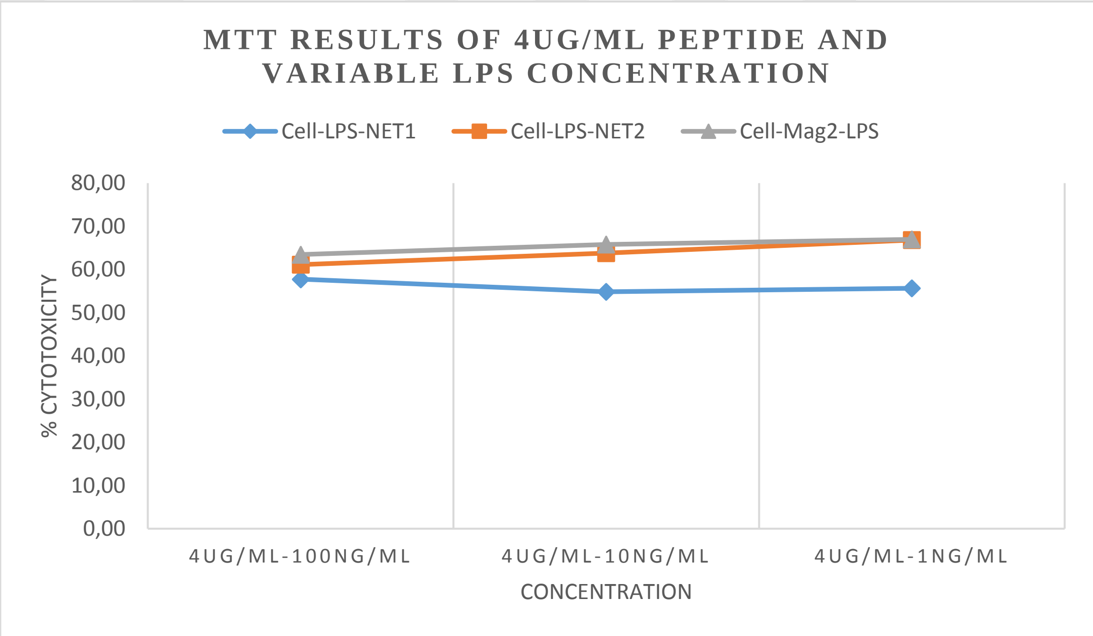


Figure 6. Cytotoxicity results when the peptide concentration is 4 µg/ml and the LPS concentration is variable.

Table 25 and Figure 6 show the cytotoxicity of NET1 and NET2 and Mag2 peptides at a concentration of 4µg/ml on cells treated with varying concentrations of LPS.

Table 26. Cytotoxicity results when the peptide concentration is 2 µg/ml and the LPS concentration is variable

% Cytotoxicity			
	100ng/ml LPS	**10ng/ml LPS**	**1ng/ml LPS**
Cell-LPS-NET1	57,40	54,15	59,64
Cell-LPS-NET2	53,89	63,61	70,09
Cell-Mag2-LPS	55,65	59,76	64,87

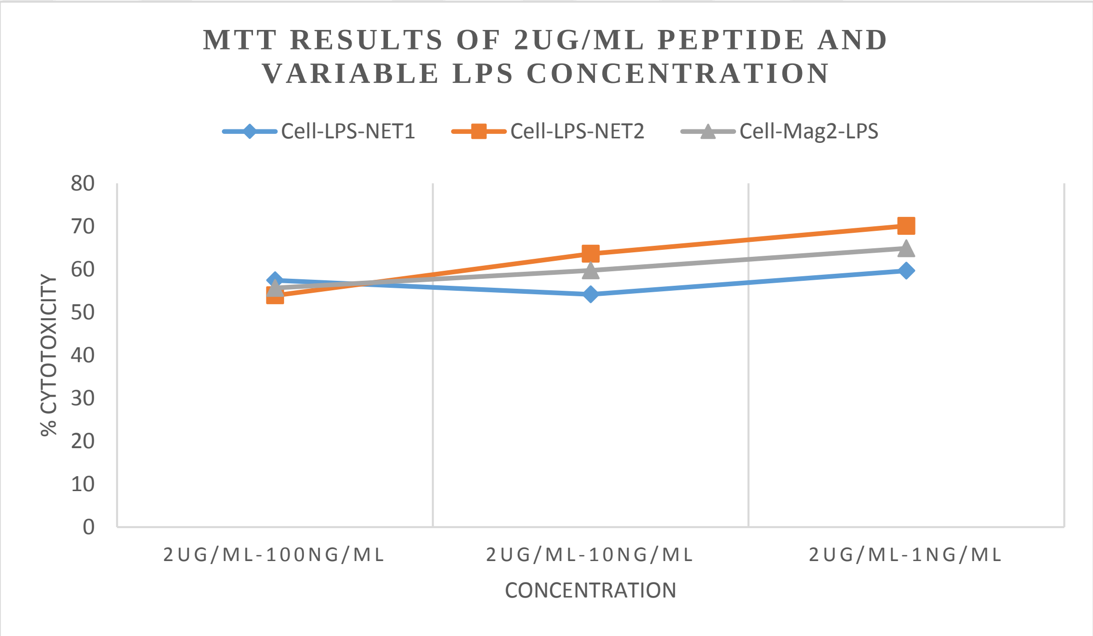


Figure 7. Cytotoxicity results when the peptide concentration is 2 µg/ml and the LPS concentration is variable.

Table 26 and Figure 7 show the cytotoxicity of NET1 and NET2 and Mag2 peptides at a concentration of 2µg/ml on cells treated with varying concentrations of LPS.

Table 27. Cytotoxicity results when the peptide concentration is 1 µg/ml and the LPS concentration is variable

% Cytotoxicity			
	100ng/ml LPS	**10ng/ml LPS**	**1ng/ml LPS**
Cell-LPS-NET1	53,51	57,78	67,61
Cell-LPS-NET2	64,33	64,06	70,68
Cell-Mag2-LPS	60,56	61,98	57,98

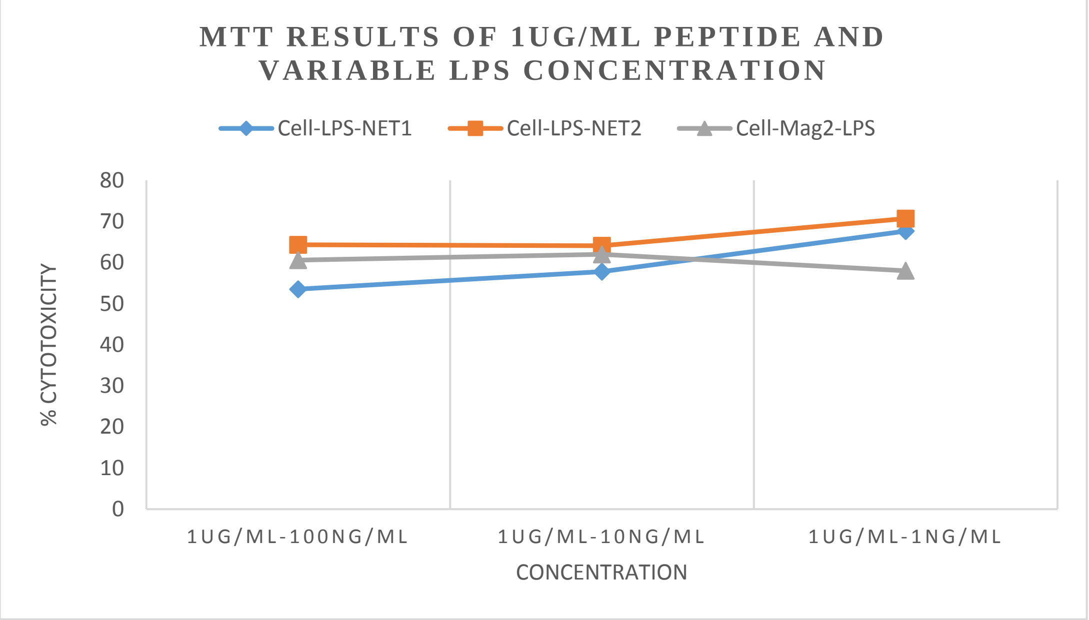


Figure 8.. Cytotoxicity results when the peptide concentration is 1µg/ml and the LPS concentration is variable.

Table 27 and figure 8 show the cytotoxicity of NET1 and NET2 and Mag2 peptides at a concentration of 1µg/ml on cells treated with varying concentrations of LPS.

Table 28. Cytotoxicity results when the peptide concentration is 0,5 µg/ml and the LPS concentration is variable.

% Cytotoxicity			
	100ng/ml LPS	**10ng/ml LPS**	**1ng/ml LPS**
Cell-LPS-NET1	66,36	60,37	58,82
Cell-LPS-NET2	55,54	60,82	65,27
Cell-Mag2-LPS	64,68	63,76	63,78

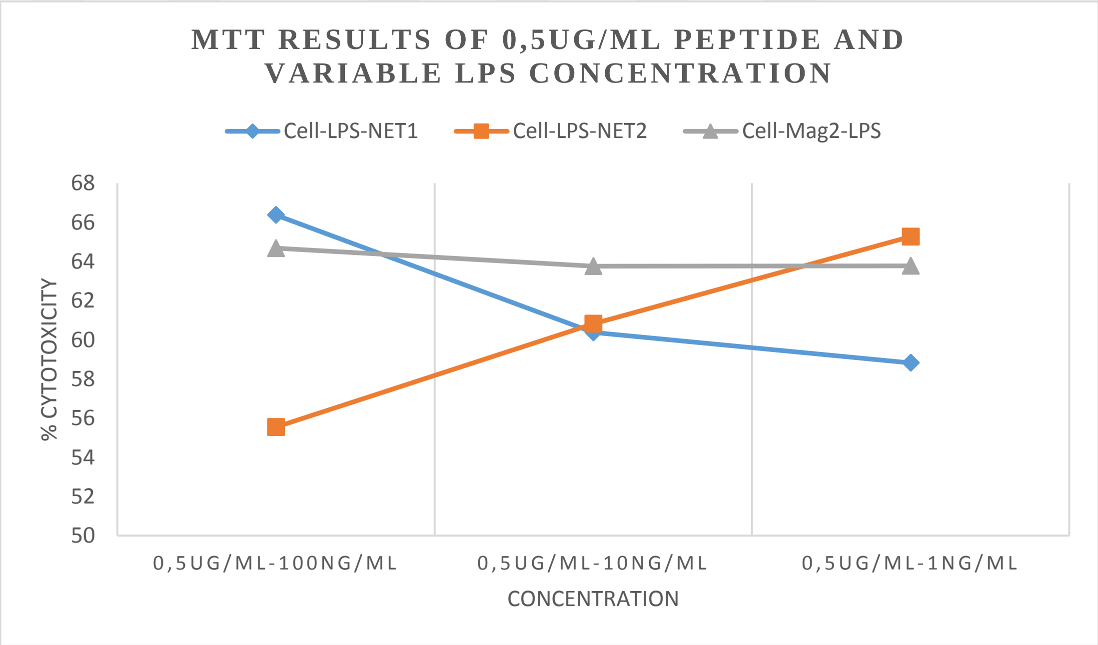


Figure 9. Cytotoxicity results when the peptide concentration is 0,5 µg/ml and the LPS concentration is variable.

Table 28 and figure 9 show the cytotoxicity of NET1 and NET2 and Mag2 peptides at a concentration of 0,5µg/ml on cells treated with varying concentrations of LPS.

4.4 Investigation of IFN-gamma Response

The ELISA method was used on cell supernatant to examine INF-gamma secretion in the presence of LPS (50 ng/ml) in TIB-71 macrophage cells treated with increasing concentrations of peptide. Whether the changing IFN-gamma response in these samples was statistically significant was examined with the one-way ANNOVA technique. GraphPad Prism (9.0.0) was used for statistical analysis. Samples with $p<0.05$ were found to be statistically significant. The graph showing the amount of IFN-gamma in the samples is shown in Figures 10 and 11. The symbol "*" means $p \leq 0.05$, the symbol "**" means $p \leq 0.01$, the symbol "***" means $p \leq 0.001$.

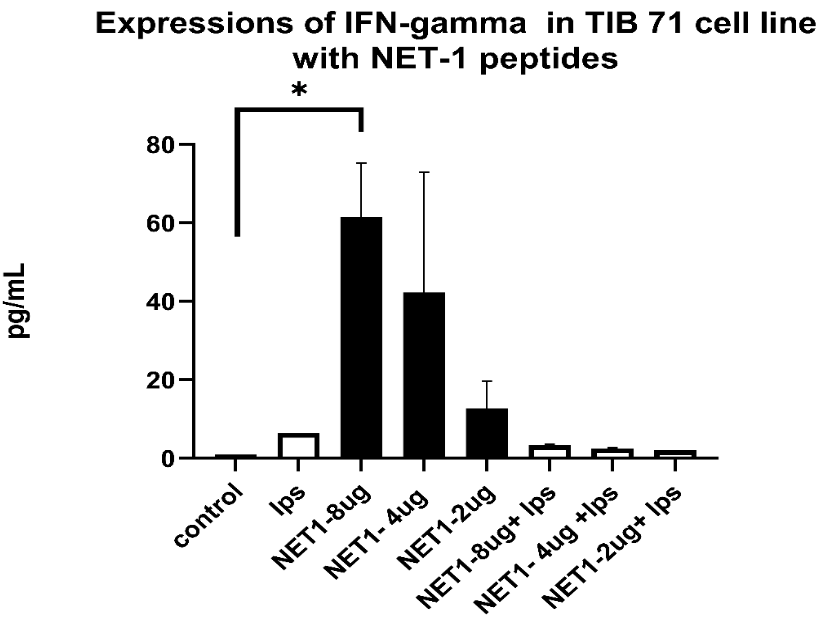


Figure 10. Increased concentrations of NET1 peptide and the amount of IFN-gamma expressed from TIB-71 macrophage cells treated with LPS.

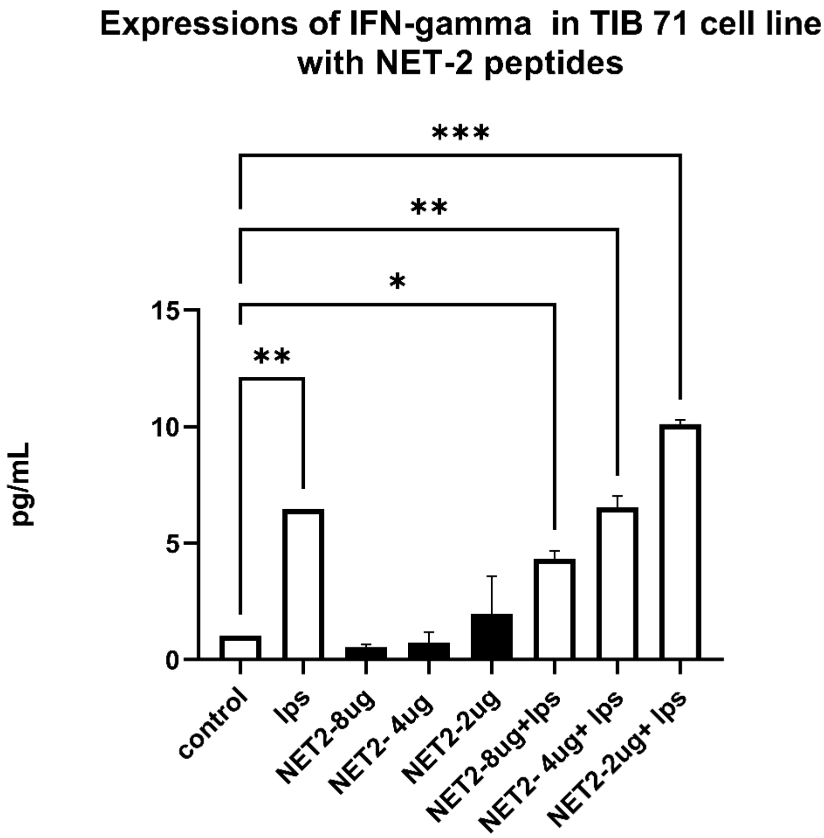


Figure 11. Increased concentrations of NET2 peptide and the amount of IFN-gamma expressed from TIB-71 macrophage cells treated with LPS.

4.5 Investigation of TNF-alpha Response

TNF-α secretion in the presence of LPS (50 ng/ml) in TIB-71 macrophage cells treated with escalating doses of peptide was examined using the ELISA method on cell supernatant. The one-way ANNOVA approach was used to determine whether the changing TNF-α response in these samples was statistically significant. Prism 9.0.0 from GraphPad was utilized for statistical analysis. The symbol "*" means $p \leq 0.05$, the symbol "**" means $p \leq 0.01$. Figures 12 and 13 display the curve that illustrates the concentration of TNF-α in the samples.

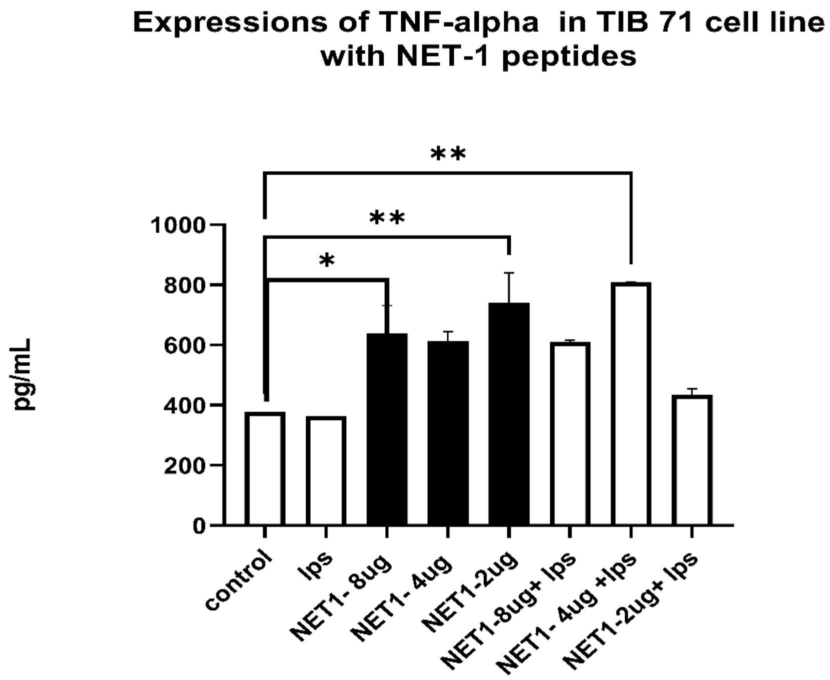


Figure 12. Changes in increasing amount of TNF-α and NET1 peptide in TIB-71 macrophage cells treated with LPS.

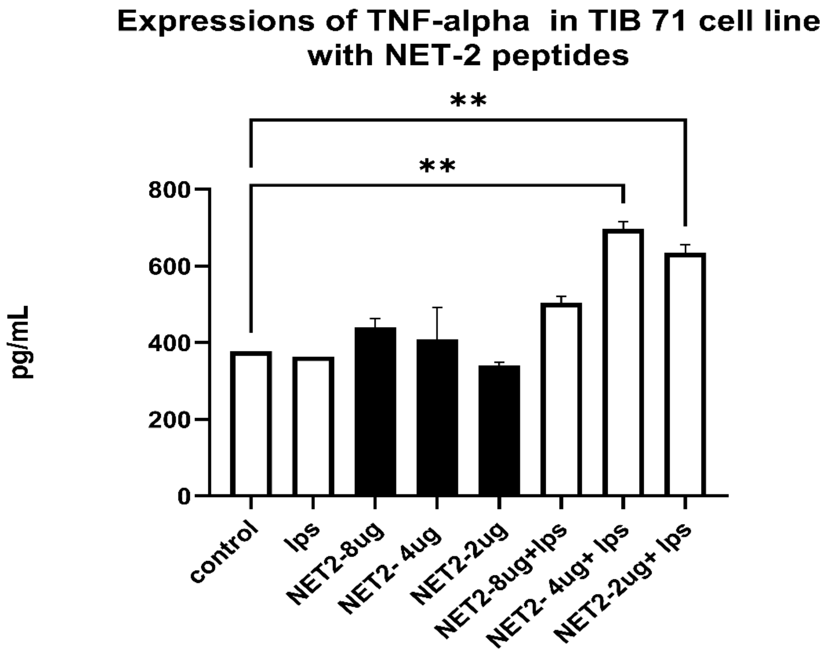


Figure 13. Changes in increasing amount of TNF-α and NET2 peptide in TIB-71 macrophage cells treated with LPS.

4.6 Investigation of IL-6 Response with Western Blot

Images of the IL-6 bands obtained as a result of the first blotting process and the band examples of the secondary blotting process performed after striping are given in Figure 14.

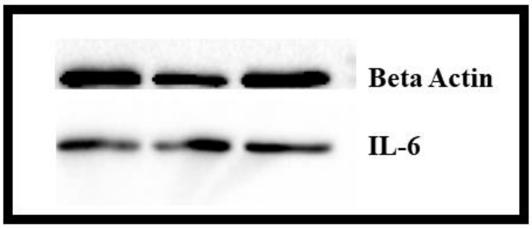


Figure 14. Beta-actin (41 kDA) and IL-6 (35kDA) bands

When Western blot results were examined, IL-6 expression was normalized to beta-actin expression and its expression level was compared to the control (unstimulated cell). The relationship between different experimental groups was examined statistically by applying the one-way ANNOVA test. In Figures 15 and 16, fold change values in cases containing different amounts of peptide and LPS are given. The symbol “*” means $p \leq 0.05$.

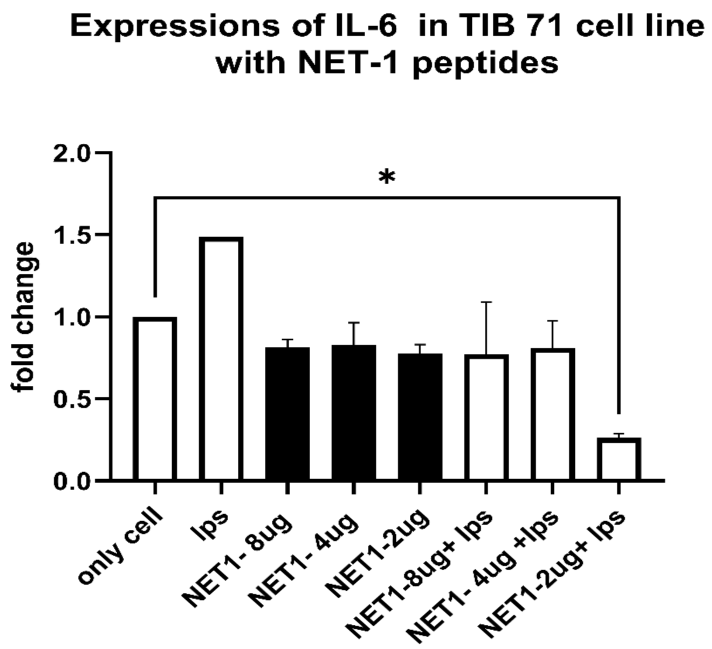


Figure 15. Change in increasing amount of IL-6 and NET1 peptide in TIB-71 macrophage cells treated with LPS

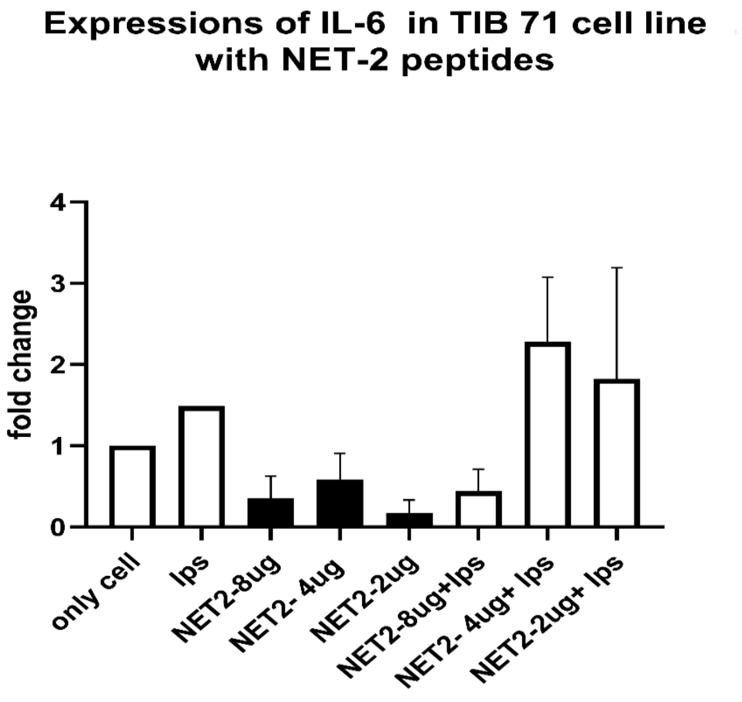


Figure 16. Change in increasing amount of IL-6 and NET2 peptide in TIB-71 macrophage cells treated with LPS

5 DISCUSSION

The basis of this research consists of from peptides with antimicrobial abilities, which are crucial components of the immune system and can be found in a wide variety of naturally occurring organisms. These peptides are one of the most important mechanisms of natural defense and work simultaneously with the secondary immune response. The emergence of AMPs and their rise to importance in recent years are also related to the resistance of microorganisms to antibiotics. This resistance mechanism acquired by microorganisms has increased the tendency for alternative drug or molecule candidates. The peptides used throughout this study include peptides previously developed and used by Nihan Ünübol and colleagues. The peptides used during the study were re-synthesized by the solid-state synthesis method and used during the experiments.

The 16 amino acid-long peptides that serve as the study's backbone are structures that are composed of two distinct amino acid types, the D- and L-forms. We first tested the four different peptides we synthesized on various microorganisms. We evaluated bacteria that cause nosocomial infections as well as those resistant to antibiotics throughout the selection process. Once the minimal inhibitory concentrations on microorganisms were established, we used TIB-71 mouse macrophages in several tests to study the drug's effects on the immune system. We examined the interactions of the peptides we synthesized with IL-6, INF-gamma, and TNF-alpha, which are important structures of the immune system, through cytotoxicity, ELISA, and western blot studies.

First, we synthesized the peptides to be used throughout the study. We synthesized these peptides using the solid-state synthesis method. Although the four different peptides we synthesized contain the same amino acids, they have different D- and L-forms. After the synthesis process, we subjected the peptides to purification by HPLC to check their purity. HPLC results are shown in Figure 2. According to Figure 2A, the NET1 peptide peaked at 6 minutes, 54 seconds. The appearance of a single peak indicates that the peptide was synthesized purely and does not contain any external molecules. Fluctuations seen before the purification process indicate impurities. The reason for these contaminations can be attributed to the purification and analysis arm not being washed completely. In addition, the speed at which the peptide is introduced into the system during HPLC also causes contamination.

In order to eliminate these impurities, we collected the peptides given to the system after analyzing the impurity of the peptide. The liquid peptide solution we obtained was taken back from the system in completely pure form. Similarly, NET2 is shown in figure 2 B, NET3 is shown in Figure 2 C, and NET4 is shown in Figure 2 D. Each peptide gave a single peak in the system and was collected back from the system in pure form.

We load peptides into the system as liquids according to the working principle of the HPLC system. We subjected the peptides to the lyophilization process to remove the liquid from the peptides that we gave to the system as a solution and collected back from the system as a solution. After lyophilization, the liquid in the solution was removed, and solid peptide structures were obtained. The solidified peptides were stored at +4°C degrees to be used in experiments. To apply solid peptides at specific concentrations during the experiments, we measured their concentrations and raised them back to +4°C degrees. Peptides became soluble in pure water after HPLC and lyophilization.

MIC experiments constitute the first study with peptides. The purpose of this experiment is to show at what concentration the peptides are effective against microorganisms. *E. coli 25922*, *S. aureus 29213*, *C. albicans*, and *MRSA* were used as microorganisms during the experiment. The most important factor in choosing these is that they are the most important microorganisms that cause nosocomial infections and have antibiotic resistance. When we evaluated the results, the inhibition of NET1 and NET3 peptides on *E. coli* showed a lower dose effect than NET2 and NET4 peptides. Both peptides caused inhibition at concentrations 8 times lower than NET2 and 4 times lower than NET4. Similar results emerged in *S. aureus*. NET3 provided the lowest concentration of inhibition, followed by NET1. For *C. albicans*, NET1 provided the lowest concentration effect at 2 µg/mL. While NET3 had an effect with a two-fold higher concentration, NET2 and NET4 had the highest concentration values with a four-fold higher concentration. Generally speaking, the most resistant microorganism is *MRSA*. The required minimum inhibition value is higher compared to the other 3 microorganisms. However, NET1 and NET3 peptides provided the lowest effects. Promising results have been observed when compared to the effects of antimicrobial peptides and existing antibiotics, which are included in the literature and have been the subject of some studies, on various microorganisms. They gave similar results to antibiotics such as ampicillin and gentamicin, which are used especially against many infectious agents (90).

For cytotoxicity analysis, studies were conducted with the macrophage cell line, which is an immune system cell and plays an effector role in many infectious diseases. In this context, the mouse macrophage TIB-71 cell line was chosen. First, all peptides were applied at a certain concentration to see their effects on the cells. In Table 21 and Figure 3, mag2 peptide, known to be a natural antimicrobial peptide, was also chosen as a reference. In order to conduct a broad screening, peptides were started at a concentration of 64 µg/ml and reduced to 0.125 µg/ml. In this context, the Mag2 peptide we used as a reference showed low toxicity at almost all concentrations. NET1 and NET2 peptides showed their effects in the safe area, starting at a concentration. of 4 µg/mL.In particular, the NET2 peptide showed lower toxicity than other peptides, even at high concentrations. Due to the low concentration of NET1 and NET2 peptides compared to the others, subsequent studies continued with these two peptides, and the concentration range was applied between 4 µg/ml and 0.5 µg/ml.

To measure the cytotoxicity of LPS, both alone and its interaction with cells were observed. As seen in Table 22 and Figure 4, LPS, which had a highly toxic effect alone, as expected, showed lower toxicity when applied with cells.

Since we would continue with the NET1 and NET2 peptides, experiments were repeated with these peptides. The data obtained as a result of cell treatment with different concentrations of peptides and different concentrations of LPS are given in tables 23-27.

When the peptide concentration was 4 µg/ml, the groups containing NET1 showed the least toxicity. However, toxicity is still significantly higher. The toxicity of NET1 increased threefold compared to the medium without LPS. The amount of toxicity seen in NET2 increased 10 times compared to the environment without LPS. There were no significant changes in the cytotoxicity examined depending on the change in LPS concentration. The cytotoxicity of NET2 and Mag2 peptides increased due to decreasing LPS concentrations.

The results are similar when the peptide concentration is 2 µg/ml. The cytotoxicity values of NET2 and Mag2 peptides increased with decreasing LPS concentrations. On average, the NET1 peptide showed the lowest toxicity at all concentrations of LPS.

NET1 again showed the lowest toxicity in samples where the peptide concentration was 1 µg/ml. Similarly, in this analysis, there is increasing cytotoxicity due to a decreasing LPS concentration.

Finally, in the analysis results where the peptide concentration was 0.5 µg/ml, decreased NET1 cytotoxicity was observed in response to decreasing LPS concentrations, while increased NET2 cytotoxicity was observed. Mag2 showed a constant effect at all concentrations.

After cytotoxicity studies, the selectivity index among the peptides was created. In this context, toxicity values at that concentration are proportioned based on MIC values. These values are shown in table 22. The point to consider here is to evaluate the peptides on their own. When each peptide group is evaluated within itself, the higher this ratio is, the more effective it is. For example, for the NET2 peptide, it showed inhibition against C. albicans at lower concentrations and its toxicity on the macrophage cell line was lower than other concentrations. When we made a ratio for the SI value, it was higher against other microorganisms. The point to be considered here is that the safety/selectivity index of a molecule is both low concentration for the MIC value and low cytotoxicity. When the table is evaluated, NET1 peptide showed more effective results than other peptides, with both low toxicity and low concentration inhibition effect.

After cytotoxicity studies, ELISA tests were performed for NET1 and NET2 peptides and IFN-gamma secretion. In this context, the effects of peptides both with and without LPS on IFN-gamma secretion were observed. In experiments, it was observed that the presence of LPS caused a higher amount of IFN-gamma secretion than in unstimulated cells. When cells were treated with NET1 peptide in an LPS-free medium at three different concentrations, IFN-gamma secretion increased significantly. Depending on the decreasing peptide concentration, IFN-gamma secretion also decreased. When the same peptide was treated with cells in the presence of LPS, a decrease in the expression of IFN-gamma occurred. IFN-gamma expression was lower than secretion in the presence of LPS alone. Due to the decrease in peptide concentration, there was also a decrease in secreted IFN-gamma. It was observed that the amount of IFN-gamma in cells stimulated with 8 μg NET1 peptide increased significantly compared to the control.

In the NET2 peptide, the expression of IFN-gamma is lower than in NET1. Moreover, with increasing concentrations of NET1 peptide, IFN-gamma secretion increased, while the opposite was observed with NET2. Furthermore, in contrast to NET1, the IFN-gamma response was higher when NET2 and LPS were used as co-stimulators. In effector cells stimulated with NET2 and LPS, IFN-gamma secretion was significantly increased.

Normally, the major sources of IFN-gamma expression are T lymphocytes and NK cells. However, some studies showed that this situation may change and that IFN-gamma secretion may occur from macrophages or different structures (91). In this study, it was thought that NET1 and NET2 peptides significantly increased the IFN-gamma secretion, and especially the NET2 peptide showed high stimulatory properties in the presence of LPS. This situation, which changes in the presence and absence of

LPS, stands out in terms of its pro-inflammatory effect. Cytokine secretion, which comes to the fore during acute inflammation, is effective against many infections. Studies should also be conducted with different cytokines to observe the immune effect of these peptides against diseases.

TNF-α normally has a bidirectional mechanism of action. While it plays a role in inflammation against microorganism sources, in the case of chronic inflammation, it can continue this process and cause irreversible tissue damage. In this study, we examined how NET1 and NET2 peptides affect TNF-α expression at the cellular level in the presence and absence of LPS. There is no change in the cell line exposed only to LPS compared to the control. Different concentrations of NET1 peptide in non-LPS medium increased (statistically signicant) TNF-α released from the cellular environment by 50%. However, there is no significant change according to peptide concentration. There is no significant change between the TNF-alpha response in cells stimulated simultaneously with peptide and LPS and the response in cells stimulated only with peptide.

Similar results emerged in studies conducted with the NET2 peptide. TNF-α secretion decreased due to a decreasing peptide concentration in LPS-free medium. In the presence of LPS, the TNF-α response is higher than in the NET1 group. TNF-alpha secretion increased significantly, especially from the groups containing 4 μg and 2 μg NET2 and LPS. Studies to be conducted at lower peptide concentrations in an LPS-free environment have the potential to show anti-inflammatory effects (92-95).

Cytokines and their mediators are produced by macrophages during the inflammatory process. IL-6 has a wide-ranging effect on immune system cells and has the potential to trigger an acute inflammation-related response. IL-6 is involved in hematopoiesis, regulation of the immune response and inflammation. It has been reported that there is an increase in IL-6 levels in patients with rheumatoid arthritis, psoriasis and encephalomyelitis, therefore inhibition of IL-6 synthesis may be beneficial in the treatment of autoimmune diseases and inflammation. The findings we obtained as a result of the experiment are that the NET1 and NET2 peptides we used may have an anti-inflammatory effect. The results were evaluated based on the IL-6 value released from the cell into the medium alone. In this context, the amount of IL-6 released from LPS-treated cells into the environment increased by 50% and is consistent with the data in the literature (96-97). Long-term presence of IL-6 can cause chronic inflammation. As a result of treatment of cells with NET1 peptide, there was a decrease in the amount of IL-6 released into the medium. It is predicted that peptides may have anti-inflammatory effects. However, no concentration-dependent change was observed. The result is the same when the cells are treated with LPS and not treated with NET1. Again, IL-6 expression decreased. An important point here is that at the lowest concentration of 2 μg/ml, IL-6 expression is 3 times lower than others.

Similar results were seen in the study with the NET2 peptide. When NET2 peptide is used alone in cell therapy, IL-6 expression is 50% lower than NET1 peptide. However, there was no significant change as the peptide concentration decreased. When cells treated with LPS were treated with NET2 peptide, it showed an anti-inflammatory effect at a peptide concentration of 8μg/ml. However, as the concentration decreased, this effect disappeared and IL-6 expression increased significantly. Both peptides caused the highest IL-6 expression in the presence and absence of LPS in the presence of 4 μg of peptide. There is a statistically non-significant increase in cells treated with 4 and 2 μg/ml NET2 and stimulated with LPS compared to the others. This may be caused by aggregation of peptides at low concentrations or disruption of peptide structures upon stimulation with LPS. Detailed studies should be carried out by testing a wide range of concentrations.

I said that antimicrobial peptides have effects against many diseases. In addition, various peptides play a role against autoimmune diseases. For example, antimicrobial peptides such as human catelicidin LL-37 against psoriasis, defensins against multiple cyclorosis, human alpha defensins against Crohn's disease, and human neutrophil peptides against type 1 diabetes are used. In the face of diseases, these peptides are upregulated and play a role in treatment by affecting different immune system structures. (98) In addition to these peptides, the magainini II peptide produced by the African clawed frog named Xenopus laevis is also used in many studies and its potential effects are being investigated. This peptide directly disrupts membrane permeability and neutralizes the source of the pathogen. They show a broad spectrum of activity against gram-negative and gram-positive bacteria, fungi and protozoa (99). Another similar example can be given for Pseudomonas aeruginosa. P. aeruginosa, which causes acute or systemic infections, is a common pathogen in hospital-acquired pneumonia. Although various antibiotics are used in its treatment, these drugs are no longer sufficient due to developing antibiotic resistance. Therefore, various antimicrobial peptides are used both alone and together with antibiotics in the course of this disease. For example; AMPs such as Lynronne, acidocin, actifensin, defensin-d2, LL-37, BMAP-27 and dermaseptin are important structures used against this infection (100).

The aim of the thesis is to compare the effects of the peptide structures we synthesized on the immune system elements with peptide molecules of different origins in the literature and to evaluate their effectiveness. Many antimicrobial peptides are promising structures in the treatment of various diseases. It has two-way effects on the immune system. They show both pro-inflammatory and anti-inflammatory effects, especially in inflammation. In this context, the NET1 and NET2 peptides we synthesized cause anti-inflammation at certain concentrations against IL-6 and TNF-alpha, while IFN-gamma also causes pro-inflammation. This situation is compatible with the literature, and by increasing the diversity of experiments and improving the peptide structures we have obtained, it can lead to new generation therapeutic structures.

6 CONCLUSION

The present thesis is an initial investigation into the impact of peptides possessing antibacterial characteristics on the immune system. For this purpose, we completed the process, which started with the synthesis of peptides consisting of different amino acids and their forms, with in vitro experimental studies on mouse macrophage cells. Antimicrobial peptides are effective mechanisms of natural immunity and are peptide structures that work in harmony with the adaptive immune system. They defend against microorganism sources by taking an active role against many infections.

The synthesized NET1 and NET2 peptides are 3D-modeled peptides with D and L forms and cationic properties. These structures, which are 16 amino acids in total, consist of a sequence containing arginine and leucine. Peptides were synthesized by the solid-state synthesis method, which provides high impurities. Despite the high impurity of the device, extra purification by HPLC was still applied. While the peptides were low in water solubility after the first synthesis (50% water and 50% ACN), they became soluble in water after the lyophilization process. This creates an important point in terms of application and the purity of the peptide.

This will pave the way for us to obtain innovative antibiotics by testing them in different in vitro studies and in vivo. Consistent with the literature, antimicrobial peptides have generally come to the fore with both their inflammation-increasing effects and their anti-inflammatory properties in chronic inflammation situations. Since AMPs act against many pathogen sources, we performed experiments to observe their effects on important structures of the immune system.

The peptides we synthesized had a low concentration inhibitory effect against microorganisms that cause nosocomial infections and also showed a low toxicity effect on the mouse macrophage cell line. Our experimental results revealed that peptides increase inflammation. However, it is also among the results that it may have anti-inflammatory properties at decreasing peptide concentrations. In light of all this, innovative peptide structures with a broad mechanism of action and low toxicity emerge as a solution to increasing antibiotic resistance and as an important step in the discovery of promising drugs against many diseases.

When the experimental results are evaluated, we think it is important to apply these peptides we use in a wider concentration range and see how they affect different immune system elements. Seeing how it affects interleukin members working simultaneously with each other or evaluating its effects on different cytokine groups will offer wider application areas.

Some of the follow-up studies to be carried out as a result of this study include the application of peptide structures with antimicrobial effects to various structures used in hospital infections, pressure sores and tissue wounds. There are many materials used as wound dressings. Related to these, we think that the antimicrobial peptides we synthesized can be used in the treatment of these diseases and infections. In addition, microbial cellulosic structures come to the fore due to their origin from natural sources and their non-toxic effects. We think that the combined application of synthesized peptides and these cellulosic structures will have an effect on various wounds. We predict that product groups with high commercialization potential will emerge as a result of studies to be carried out under both in-vitro and in-vivo environmental conditions.

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