NOVEL MEMBRANE ACTIVE PEPTIDES DERIVED FROM MARINE ORGANISMS AND THEIR MECHANISMS OF ACTION

ANJALI PRIYA

KUSUMA SCHOOL OF BIOLOGICAL SCIENCES

INDIAN INSTITUTE OF TECHNOLOGY DELHI

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by

ANJALI PRIYA

Kusuma School of Biological Sciences

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CERTIFICATE

This is to certify that the thesis titled "**Novel membrane active peptides derived from marine organisms and their mechanisms of action**", being submitted by **Ms. Anjali Priya** to the **Kusuma School of Biological Sciences, Indian Institute of Technology Delhi**, for the award of the degree of "**Doctor of Philosophy**" is a record of the bonafide research carried out by her, which has been prepared under my supervision and guidance in conformity with the rules and regulations of the 'Indian Institute of Technology Delhi'. The research report and the results described in it have not been submitted in part or full to any other University for the award of any other degree or diploma.

Dr. Archana Chugh
Professor
Kusuma School of Biological Sciences
Indian Institute of Technology Delhi
New Delhi 110016, India

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"Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less."

-Marie Curie

Joining a PhD program at IIT Delhi was the start of my journey towards expanding my understanding and doing so required a lot of courage and conviction. There are many individuals who helped me in this amazing journey because of whom this process became pleasant as well as exciting.

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ABSTRACT

In the current era of multi-drug resistance, the conventional antibiotics are proving inefficient in the treatment of serious life-threatening infections along with their debilitating side-effects and development of resistance. To tackle this major challenge, alternative therapeutic compounds are required and membrane active peptides (MAPs) are one such group of therapeutics that have gained attention due to their ability to interact with various biological membranes in both disruptive and non-disruptive manner. Concomitantly, the inability of pathogens to mutate their membrane components as frequently in response to MAPs, keeps the spread of antimicrobial resistance (AMR) under check. Two major classes of MAPs, namely cell penetrating peptides (CPPs) and antimicrobial peptides (AMPs) have been extensively studied and employed in therapeutics as drug delivery vectors and antimicrobial agents respectively. MAPs from marine organisms were chosen for this study because of their uniqueness and biochemical diversity that can be utilized for development of better peptide therapeutics with multi-functional properties.

The present thesis addresses the antimicrobial, anti-biofilm, cell penetrating and other functions of marine and toxin derived CPPs and AMPs along with elucidation of their mechanisms of action. It is hypothesized that the peptides used in this study will prove to be effective and multi-functional in their bioactivity with distinct mechanisms of action that can be utilized for the development of better peptide therapeutics.

The research questions targeted in the first objective of the study were: How the marine derived peptide Tachyplesin and snake-toxin derived peptide CyLoP-1 will fare as an anti-mycobacterial peptide? What will be the role of cysteine and arginine residues in imparting antimicrobial and cell penetrating activity to these peptides? What will be their mechanisms to inhibit or kill mycobacterium cells? Will they be able to enter macrophage cells infected with mycobacterium and kill the intracellular pathogen without disturbing the host cell? Will these peptides would also be able to inhibit or eradicate mycobacterium biofilms? To answer these questions, antimicrobial and cell culture based studies and assays were performed. For determination of anti-mycobacterial and anti-biofilm activity of peptides, minimum inhibitory

concentration (MIC), minimum bactericidal concentration (MBC), time-kill kinetics, co-culture and crystal violet assays were employed. Further, the mechanistic insights were gained by utilizing various microscopy and membrane-based assays such as transmission electron microscopy (TEM), membrane integrity and membrane depolarization assays. Intracellular reactive oxygen species (ROS) production was determined using fluorescent dye. The major outcomes of this objective were potent anti-mycobacterial and anti-biofilm activity of both the peptides with the ability to kill intracellular mycobacteria as well. Cysteine and arginine residues proved to be crucial for cell penetrating and anti-mycobacterial activity of both the peptides. Tachyplesin mainly followed membranolytic pathway whereas, CyLoP-1 employed intracellular ROS production as the mechanism to kill mycobacterium cells.

The research questions to be addressed in the second objective were: How to design a novel CPP from marine antimicrobial peptide and improve its functionality? What will be the overall bioactivity and toxicity profile of the novel peptides and will they be suitable candidates for further drug development pipeline? These research questions were answered using various *in silico* and *in vitro* techniques. In this direction, a novel peptide from marine AMP clavanin was designed *in silico* and an immunomodulatory sequence was added to its C-terminal. The peptides were named clavanin derived peptides (CDPs) with 3 variants - CDP-1 (with immunomodulatory sequence), CDP-2 (without immunomodulatory sequence) and CDP-3 (mutated version of CDP-2). The most significant results were excellent cell penetrating, antimicrobial and anti-biofilm activity without considerable cytotoxicity demonstrated by CDP-1 as compared to the other two variants. However, cargo delivery abilities and cytotoxicity profile of all the three peptides were similar.

In conclusion, novel AMPs and CPPs from marine sources displayed multi-functionality that can be translated into viable therapeutics. This study highlights the importance of understanding structure-activity relationship and mechanistic pathways of peptides against a host of cells and microorganisms so as to develop antimicrobial peptide therapeutics for better management of diseases.

सार

बहु-दवा प्रतिरोध के वर्तमान युग में, पारंपरिक एंटीबायोटिक्स अपने दुर्बल करने वाले दुष्प्रभावों और प्रतिरोध के विकास के साथ-साथ गंभीर जीवन-धमकाने वाले संक्रमणों के उपचार में अक्षम साबित हो रहे हैं। इस बड़ी चुनौती से निपटने के लिए, वैकल्पिक चिकित्सीय यौगिकों की आवश्यकता होती है और मेम्ब्रेन एक्टिव पेप्टाइड्स (एमएपी) चिकित्सीय का एक ऐसा समूह है, जिसने विघटनकारी और गैर-विघटनकारी तरीके से विभिन्न जैविक झिल्लियों के साथ बातचीत करने की अपनी क्षमता के कारण ध्यान आकर्षित किया है। सहवर्ती रूप से, एमएपी के जवाब में अक्सर अपने झिल्ली घटकों को बदलने के लिए रोगजनकों की अक्षमता, रोगाणुरोधी प्रतिरोध (एएमआर) के प्रसार को जांच के दायरे में रखती है। एमएपी के दो प्रमुख वर्ग, अर्थात् सेल पेनेट्रेटिंग पेप्टाइड्स (सीपीपी) और एंटीमाइक्रोबियल पेप्टाइड्स (एएमपी) का बड़े पैमाने पर अध्ययन किया गया है और क्रमशः दवा वितरण वैक्टर और रोगाणुरोधी एजेंटों के रूप में चिकित्सीय में नियोजित किया गया है। इस अध्ययन के लिए समुद्री जीवों के एमएपी को उनकी विशिष्टता और जैव रासायनिक विविधता के कारण चुना गया था जिसका उपयोग बहु-कार्यात्मक गुणों के साथ बेहतर पेप्टाइड चिकित्सीय के विकास के लिए किया जा सकता है।

वर्तमान थीसिस एंटीमाइक्रोबियल, एंटी-बायोफिल्म, सेल पेनेट्रेटिंग और समुद्री और टॉक्सिन व्युत्पन्न सीपीपी और एएमपी के अन्य कार्यों के साथ-साथ उनकी क्रिया के तंत्र की व्याख्या के साथ संबोधित करती है। यह परिकल्पना की गई है कि इस अध्ययन में उपयोग किए गए पेप्टाइड्स अपनी जैव-सक्रियता में प्रभावी और बहु-कार्यात्मक साबित होंगे, जिसमें क्रिया के विशिष्ट तंत्र होंगे जिनका उपयोग बेहतर पेप्टाइड चिकित्सीय के विकास के लिए किया जा सकता है।

अध्ययन के पहले उद्देश्य में लक्षित शोध प्रश्न थे: समुद्री व्युत्पन्न पेप्टाइड टैचीप्लेसिन और साँप-विष व्युत्पन्न पेप्टाइड CyLoP-1 एक एंटी-माइकोबैक्टीरियल पेप्टाइड के रूप में कैसे काम करेगा? इन पेप्टाइड्स को रोगाणुरोधी और सेल पेनीट्रेटिंग गतिविधि प्रदान करने में सिस्टीन और आर्जिनिन अवशेषों की क्या भूमिका होगी? माइकोबैक्टीरियम कोशिकाओं को बाधित करने या मारने के लिए उनका तंत्र क्या होगा? क्या वे माइकोबैक्टीरियम से संक्रमित मैक्रोफेज कोशिकाओं में प्रवेश करने और मेजबान सेल को परेशान किए बिना इंट्रासेल्युलर रोगज़नक़ को मारने में सक्षम होंगे? क्या ये पेप्टाइड माइकोबैक्टीरियम बायोफिल्म्स को बाधित या मिटाने में भी सक्षम होंगे? इन सवालों का जवाब देने के लिए, रोगाणुरोधी और सेल कल्चर आधारित अध्ययन और परीक्षण किए गए।

पेप्टाइड्स की एंटी-माइकोबैक्टीरियल और एंटी-बायोफिल्म गतिविधि के निर्धारण के लिए, न्यूनतम निरोधात्मक एकाग्रता (एमआईसी), न्यूनतम जीवाणुनाशक एकाग्रता (एमबीसी), टाइम-किल कैनेटीक्स, सह-संस्कृति और

क्रिस्टल वायलेट परख कार्यरत थे। इसके अलावा, विभिन्न माइक्रोस्कोपी और झिल्ली-आधारित जांच जैसे ट्रांसमिशन इलेक्ट्रॉन माइक्रोस्कोपी (टीईएम), झिल्ली अखंडता और झिल्ली विध्रुवण जांच का उपयोग करके यंत्रवत अंतर्दृष्टि प्राप्त की गई। फ्लोरोसेंट डाई का उपयोग करके इंट्रासेल्युलर प्रतिक्रियाशील ऑक्सीजन प्रजातियों (आरओएस) का उत्पादन निर्धारित किया गया था। इस उद्देश्य के प्रमुख परिणाम इंट्रासेल्युलर माइकोबैक्टीरिया को मारने की क्षमता के साथ दोनों पेप्टाइड्स की शक्तिशाली एंटी-माइकोबैक्टीरियल और एंटी-बायोफिल्म गतिविधि थे। दोनों पेप्टाइड्स की कोशिका भेदन और एंटी-माइकोबैक्टीरियल गतिविधि के लिए सिस्टीन और आर्जिनिन अवशेष महत्वपूर्ण साबित हुए। टैचीप्लेसिन ने मुख्य रूप से मेम्ब्रेनोलिटिक मार्ग का अनुसरण किया, जबकि CyLoP-1 ने माइकोबैक्टीरियम कोशिकाओं को मारने के तंत्र के रूप में इंट्रासेल्युलर आरओएस उत्पादन को नियोजित किया। दूसरे उद्देश्य में संबोधित किए जाने वाले शोध प्रश्न थे: समुद्री रोगाणुरोधी पेप्टाइड से एक उपन्यास सीपीपी कैसे डिजाइन करें और इसकी कार्यक्षमता में सुधार करें? उपन्यास पेप्टाइड्स की समग्र जैव-सक्रियता और विषाक्तता प्रोफ़ाइल क्या होगी और क्या वे आगे की दवा विकास पाइपलाइन के लिए उपयुक्त उम्मीदवार होंगे? सिलिको और इन विट्रो तकनीकों में विभिन्न का उपयोग करके इन शोध प्रश्नों का उत्तर दिया गया था। इस दिशा में, समुद्री एएमपी क्लैविनिन से एक उपन्यास पेप्टाइड को सिलिको में डिजाइन किया गया था और इसके सी-टर्मिनल में एक इम्यूनोमॉड्यूलेटरी सीक्वेंस जोड़ा गया था। पेप्टाइड्स को क्लैविनिन व्युत्पन्न पेप्टाइड्स (सीडीपी) नाम दिया गया था जिसमें 3 प्रकार थे - सीडीपी-1 (इम्यूनोमॉड्यूलेटरी अनुक्रम के साथ), सीडीपी-2 (इम्युनोमॉड्यूलेटरी अनुक्रम के बिना) और सीडीपी-3 (सीडीपी-2 का उत्परिवर्तित संस्करण)। अन्य दो रूपों की तुलना में सीडीपी-1 द्वारा प्रदर्शित काफी साइटोटोक्सिसिटी के बिना सबसे महत्वपूर्ण परिणाम उत्कृष्ट सेल मर्मज्ञ, रोगाणुरोधी और एंटी-बायोफिल्म गतिविधि थे। हालांकि, तीनों पेप्टाइड्स की कार्गो डिलीवरी क्षमता और साइटोटोक्सिसिटी प्रोफाइल समान थे।

अंत में, समुद्री स्रोतों से उपन्यास एएमपी और सीपीपी ने बहु-कार्यक्षमता प्रदर्शित की जिसे व्यवहार्य चिकित्सीय में अनुवादित किया जा सकता है। यह अध्ययन कोशिकाओं और सूक्ष्मजीवों के एक मेजबान के खिलाफ पेप्टाइड्स के संरचना-गतिविधि संबंध और यंत्रवत मार्गों को समझने के महत्व पर प्रकाश डालता है ताकि रोगों के बेहतर प्रबंधन के लिए रोगाणुरोधी पेप्टाइड चिकित्सीय विकसित किया जा सके।

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ABBREVIATIONS AND SYMBOLS

α	Alpha
β	Beta
Da	Dalton
µ	Micro
µg	Microgram
µL	Microlitre
µM	Micromolar
%	Percent
°C	Degree Celcius
®	Registered
w/w	weight/weight
w/v	weight/volume
rpm	rotations per minute
ACE	Angiotensin Converting Enzyme
AMP	Antimicrobial Peptide
AMR	Antimicrobial Resistance
ANOVA	Analysis of Variance
APD	Antimicrobial Peptide Database
BEC	Biofilm Eradication Concentration
BHI	Brain Heart Infusion
BIC	Biofilm Inhibitory Concentration
CD	Circular Dichroism
CDP	Clavanin Derived Peptide
CFU	Colony Forming Unit
CLSI	Clinical and Laboratory Standards Institute
CLSM	Confocal Laser Scanning Microscope

CPP	Cell Penetrating Peptide
CyLoP-1	Cytoplasmic Localizing Peptide-1
DAPI	4', 6-diamidino-2-phenylindole
DCF	2',7'-dichlorofluorescein
$DiSC_3(5)$	3,3'-Dipropylthiadicarbocyanine Iodide
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
ECP	Eosinophilic Cationic Protein
ESKAPE	*Enterococcus faeceum, Staphylococcus aureus, Klebsiella pneumonia, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* species
EU	European Union
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FITC	Fluorescein Isothiocyanate
Fmoc	Fluorenylmethyloxycarbonyl
H_2DCFDA	2',7'-dichlorodihydrofluorescein diacetate
HDPs	Host Defence Peptides
HEK	Human Embryonic Kidney
HEPES	N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid
HR-TEM	High Resolution-Transmission Electron Microscope
IL-1β	Interleukin-1 β
INH	Isoniazid
LC	Lethal Concentration
LPS	Lipopolysaccharides
MAP	Model Amphipathic Peptide
MAPs	Membrane Active Peptides
MBC	Minimum Bactericidal Concentration

MBF	Moderate Biofilm Former
MDR	Multi-drug Resistance
MIC	Minimum Inhibitory Concentration
miRNA	micro Ribonucleic Acid
MNPs	Marine Natural Products
MOI	Multiplicity of Infection
MRSA	Methicillin-resistant *Staphylococcus aureus*
MTCC	Microbial Type Culture Collection and Gene Bank
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NAC	N-Acetyl Cysteine
NBF	Non-biofilm Former
NO	Nitric oxide
OADC	Oleic acid, Albumin, Dextrose, Catalase
PBS	Phosphate Buffered Saline
PI	Propidium Iodide
PrP	Prion Protein
PS	Phosphatidylserine
PTD	Protein Transduction Domain
PTMs	Post-translational Modifications
qPCR	Quantitative Polymerase Chain Reaction
RFU	Relative Fluorescence Unit
RIF	Rifampicin
ROS	Reactive Oxygen Species
SAP	Sweet Arrow Peptide
SBF	Strong Biofilm Former
SDS	Sodium Dodecyl Sulfate
SEM	Scanning Electron Microscope
siRNA	small-interfering Ribonucleic Acid

SVM	Support Vector Machine
SV40	Simian Virus 40
TAT	Trans Activator of Transcription
TB	Tuberculosis
TFE	Trifluoroethanol
Tpl	Tachyplesin
XDR	Extremely Drug Resistant
WBF	Weak Biofilm Former
WHO	World Health Organization