APPLICATION OF CELL PENETRATING PEPTIDES IN THE MANAGEMENT OF ANTERIOR SEGMENT DISEASES OF THE EYE

S SUJITHRA

Kusuma School of Biological Sciences

Indian Institute of Technology Delhi

October, 2022

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by

S SUJITHRA

Kusuma School of Biological Sciences

Submitted

in fulfilment of the requirements of the degree of

Doctor of Philosophy

to the

Indian Institute of Technology Delhi

October, 2022

This thesis is dedicated to my beloved parents.

For their endless love, support and encouragement.

CERTIFICATE

This is to certify that the thesis entitled **"Application of Cell Penetrating Peptides in the management of anterior segment diseases of the eye"** being submitted by **Ms. Sujithra Shankar** 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 the research work carried out by her, prepared under my supervision, in conformity with the rules and regulations of the 'Indian Institute of Technology Delhi'. The research report and the results present in the thesis have not been submitted to any other University or Institute for the award of any other degree or diploma.

Date:

Place:

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

ACKNOWLEDGEMENTS

Thirukkural # 102

காலத்தி னாற்செய்த நன்றி சிறிதெனினும்
ஞாலத்தின் மாணப் பெரிது.

"A favour conferred in the time of need, though it be small (in itself), is (in value) much larger than the world."

— Thiruvalluvar (200 BC)

The ideas behind this research have become a reality with the kind support and help of many individuals. I would like to extend my sincere gratitude to all of them.

Joined as a MS(R) student under my supervisor, I was the youngest in my lab and hence was called the baby of our lab. The warmth provided by my supervisor and with growing interest in the research carried out in my lab made me opt for a conversion from MS(R) to Ph.D. program. First and foremost, I would like to sincerely thank my Ph.D. ***supervisor Dr. Archana Chugh***, for guiding me throughout this wonderful journey and being there alongside me in both peaks and valleys of my Ph.D. and my life during these 6 years away from home. The valuable suggestions, insights and constant motivations have helped me to frame my research work and to achieve the objective of my Ph.D. in a well-rounded manner. Her doors are always open for discussions and brainstorming the issues faced in experiments. She has always given us an immense freedom to execute our thoughts to try out different experiments and has never let any fund crunch affect our work in any manner. Besides work, she has also been there in my difficult times during Ph.D., instilling hope (*always listen to your gut feeling)* and pushing me to achieve greater heights. I even got an opportunity to know and interact with the fun side of ma'am during our travel to Estonia for an international conference. I have admired her personality and have learnt a lot from her to become a better researcher indeed a better person. I really hold her in high esteem for wonderfully managing all the aspects of life.

I would like to extend my deepest gratitude to our collaborators, ***Dr Sushmita G Shah***, Ophthalmologist and ***Dr Shikha Yadav***, National Institute of Biologics, NOIDA. Sushmita ma'am helped us in providing clinical inputs to our study. Her assistance in animal studies and constant optimism has motivated me throughout our *in vivo* studies. The

clinical point of view for our research has helped me to refine the objective and experiments towards a much more translational work. Shikha ma'am being the head of the animal facility in NIB, NOIDA has really helped us to provide animals and successfully carry out our *in vivo* studies at NIB. I would like to thank her for training me to make meticulous animal study plans and executing them without any hiccups.

I would like to thank Dr Shilpi Minocha for her expert advice in the histological studies of animal tissues and for providing the microtome for sectioning. Also, I would like to thank her Ph.D. student, Priyanka Prakash Srivastava, for helping us in tissue sectioning and H&E staining.

I am really thankful to my SRC members, chairperson, ***Prof Bishwajit Kundu***, internal expert ***Prof. Vivekanandan Perumal*** and external expert ***Prof. Pramit Kumar Chowdhury*** for their valuable inputs and suggestions during the progress presentations.

I would like to extend my thanks to all the faculty members of KSBS for allowing me to use instruments or facilities whenever required for the study. Specially would like to thank ***Prof. Aditya Mittal*** for allowing me to use confocal microscope and ***Prof. Tapan K Choudhuri*** for the fluorescence spectrometer, CD spectrometer and GFC.

I would like to thank KSBS staff members, ***Ms. Mini Sharma, Mr Praveen, Mr Pradeep, Mr Vijaypal***, ***Pushpalta Ma'am*** *and* ***Garg Sir*** for their assistance during TA duty in various administrative proceedings whenever required.

I would like to acknowledge, ***IIT Delhi*** for providing financial assistance (GATE fellowship) during my Ph.D. program and a safe campus environment for a peaceful stay. I am extremely thankful to ***IIT Delhi*** for providing me with the travel assistance to present my work at international conference in University of Tartu, Estonia in 2018.

This journey would not have been as fruitful as it has turned out without a friendly lab environment for which I am really lucky to be part of. To start with I would like to thank ***Dr. Anupama*** for helping me to effortlessly gel in with the lab and department. She is one of most giving and helping personalities I have ever met and have always made the lab environment filled with energy and liveliness with her charisma. I would like to extend my gratitude towards ***Dr Nisha*** and ***Dr Deepthi*** for guiding me in the initial phase of my Ph.D. with their valuable suggestions and tips. Stressful attempts of experiments were bearable because of my friendly lab mates, ***Dr. Vivek, Dr. Harsha, Dr. Pankhuri,*** my batchmate ***Anjali, Saurabh, Sai, Prasan, Aditi, Gagan, Malay and Mayank***. The delightful discussions about

hot topics and favorite series, tea breaks and lunch treats were a complete stressbuster during the work hours. I would like to give special thanks to two lifelong friendships that I have gained during my stay at IIT Delhi, ***Pankhuri*** *and* ***Harsha.*** These two people have always been by my side professionally as well as personally and I share a special bond with them. I thank Pankhuri for constantly inspiring me, for the shopping outings, chai times, for surprise gifts which really made my day and for all the memorable moments which brought smile even during the toughest times. I would like to specially thank Harsha for her constant guidance and help during my project and for the unforgettable moments of our first animal handling experience together at animal facility. Started off being partner in science working on same project, have ended up being friends for life. I would like to specially appreciate the help and assistance provided by ***Aditi and Prasan*** during the rabbit studies of my project. I would also like to thank ***Dr Anusha*** and ***Dr Nirupama*** for their expert suggestions for experiments and career advice.

I am extremely thankful to ***Devanshu Mehta*** for being there along by my side and helping me throughout this journey in both good and bad times. He has been there for me as a pillar of support and indeed will be a constant companion for lifetime. I would like to mention special thanks for his assistance in carrying out biophysical experiments including CD spectroscopy, fluorescence spectrophotometry and his help in learning SDS PAGE gels which really boosted up my confidence. In addition, I would like to thank my friends ***Ramesh***, ***Chandrashekar***, ***Akanksha Saini*** and ***Shubham*** who have aided me whenever in need even in short notice.

I am grateful to the ***Central research facility, IIT Delhi*** for Scanning electron microscopy with special thanks to ***Mr. Kuldeep*** and ***Mr. Dinesh*** for accommodating numerous animal tissue samples for SEM imaging.

I would like to extend my acknowledgements to ***Dr Vivek Singh*** from LV Prasad Eye Institute, Hyderabad for his timely help in providing the Human corneal epithelial cell line for this study.

I also would like to appreciate help by Sahil from CBME department, for his help in fluorimetry required for *ex vivo* and *in vivo* studies.

Also, I want to thank the members of IIT Delhi Tamil Mandram committee and their efforts to keep the Tamil culture and festivities alive in spite of staying at IIT Delhi, so far

away from home. I want to specially thank my Tamil friends Gautam, Kalidas, Venkat, Jyothi, Ashwin and Jayalakshmi for making me feel at home even miles away.

I would like to mention special gratitude towards my childhood best friends ***Dr Namita*** and ***Dr Kanchan*** for their constant emotional and moral support that has helped me to sail through all the tough times of my life.

Last but definitely not the least ***MY FAMILY*** without whose love and support my existence would have been insignificant. No acknowledgements are equivalent to what they have provided for me. I thank my loving ***Daddy***, who has sacrificed a lot in life to give the best for me in every aspect and for continuously instilling hope and encouraging me to become a better person day by day. Daddy never ceases to give his extra efforts to provide me a comfortable life and always protecting me from any harm. ***Amma***, thank you for always showering immense love and care. I have never seen a stronger person than her. She is the reason behind for all my achievements in my academic career, her constant motivation and encouragement has always pushed me towards the excellence. Her every phone call conversation starts with *saaptiya (had food?)* which reminds me how much she cares for me and this thought helped me to get away with all the stress in the world. I bow upon for all the efforts and sacrifices made by my parents for which the person who I am today. I also would like to extend my thanks to ***Sakkaravarthi Chithappa, Prami Chithi, Selvi Periamma, Saranya akka*** and ***Indu akka*** for constantly encouraging me and providing me strength to plough through my Ph.D.

At last, I am immensely grateful to the Almighty for giving me this life, wonderful people in it and for giving me the strength to live and accomplish further.

Sujithra Shankar

ABSTRACT

Human eye is a highly complex organ that provides one of the crucial senses, the sense of sight, essential for interacting with the surrounding environment. It's complexity is justified by the accurate arrangement of tissues creating various anatomical and physiological barriers at multiple levels to restrict the entry of any exogenous substances thereby protecting the inner ocular tissues. In addition to the protective nature these barriers, they also limit entry of therapeutic molecules consequently leading to reduced bioavailability of the drugs at the site of action. The primary barrier encountered by any topically applied therapeutics is the cornea. As an avascular clear tissue, cornea allows the passage of light to retina in the eye. According to WHO, 2.2 billion people have visual impairment or blindness out of which 4.2 million account for corneal opacities. Multiple attempts have been made to provide effective and efficient treatment strategies for corneal diseases. One such approach is to enhance the penetration of the drugs to attain clinically relevant concentrations at the desired therapeutic site. A class of peptides known as cell penetrating peptides (CPPs) can traverse the plasma membrane carrying cargo molecules attached to it. Usually ranging from 5 to 40 amino acids in length, it has the potential to carry along different types of cargoes such as therapeutic agents, proteins, nucleic acids, plasmids and even nano particles such as liposomes.

This thesis presents novel strategies for therapeutic interventions for anterior segment disease management.

The first part introduces a novel CPP, designed to target the corneal tissue for drug delivery in corneal diseases. Corneal Targeting Sequence 1 (CorTS 1) has been developed by modifying a conserved leucine rich repeat (LRR) motif present in corneal proteins. The novel CorTS 1 peptide exhibits a promising cell penetrating activity with no notable cytotoxicity and an increased accumulation in corneal stroma than in aqueous humor *in vitro* and in *ex vivo* conditions, respectively. The peptide also delivers protein cargo (beta galactosidase) in its active biological form inside human corneal epithelial cell (HCE) line. Interestingly, antimicrobial activity has been also noted against MRSA and *Fusarium dimerum*. CorTS 1 also possesses anticollagenolytic activity thereby holding a promising potential in treatment of microbial keratitis and stromal melts.

The second part of the study demonstrates *in vivo* efficacy of a novel CPP drug conjugate based strategy for the treatment of Keratoconus (KC). KC is a common corneal disorder characterised by progressive thinning leading to cone shaped cornea with irregular astigmatism and impaired vision. Corneal collagen crosslinking (CXL) is the standard treatment employed to halt the disease progression. A concerning step of this procedure is epithelial debridement carried out to facilitate the entry of poorly permeable riboflavin (Rb). In this study the Rb is covalently conjugated with the dimer of a well known CPP (Tat dimer – Tat_2) resulting in Tat_2riboflavin or RiTe conjugate to enhance the penetration and thereby improve the efficiency of the CXL protocol. Approximately a 2 fold increase in tissue penetration was achieved in rabbit corneas upon conjugation with the CPP. The comparative analysis of RiTe conjugate mediated and standard CXL exhibited an equivalent extent in crosslinking in both types of CXLs as observed in enzymatic digestion of corneas. No endothelial damage and keratocyte loss in case of RiTe conjugate mediated CXL in contrast to standard CXL further establishes the safety of the proposed protocols along with the efficacy.

The two above-mentioned interventions highlight the potential application of CPPs and CPP drug conjugates for treating anterior segment diseases or disorders of the eye.

सार

मानव की आंख एक अत्यधिक जटिल अंग है जो आसपास के वातावरण के साथ बातचीत करने के लिए आवश्यक महत्वपूर्ण इंद्रियों में से एक, दृष्टि की भावना प्रदान करता है। किसी भी बहिर्जात पदार्थों के प्रवेश को प्रतिबंधित करने के लिए कई स्तरों पर विभिन्न शारीरिक बाधाओं को बनाने वाले ऊतकों की सटीक व्यवस्था द्वारा इसकी जटिलता को उचित ठहराया जाता है जिससे आंतरिक ओकुलर ऊतकों की रक्षा होती है। सुरक्षात्मक प्रकृति के अलावा इन बाधाओं, वे चिकित्सीय अणुओं के प्रवेश को भी सीमित करते हैं जिसके परिणामस्वरूप कार्रवाई की साइट पर दवाओं की जैव उपलब्धता कम हो जाती है। किसी भी शीर्ष रूप से लागू चिकित्सा विज्ञान द्वारा सामना की जाने वाली प्राथमिक बाधा कॉर्निया है। एक संवहनी स्पष्ट ऊतक के रूप में, कॉर्निया आंख में प्रकाश को रेटिना तक जाने की अनुमति देता है। डब्ल्यूएचओ के अनुसार, 2.2 अरब लोगों को दृष्टि हानि या अंधापन है, जिनमें से 42 लाख कॉर्निया की अस्पष्टता के लिए जिम्मेदार हैं। कॉर्नियल रोगों के लिए प्रभावी और कुशल उपचार रणनीति प्रदान करने के लिए कई प्रयास किए गए हैं। ऐसा ही एक दृष्टिकोण वांछित चिकित्सीय स्थल पर चिकित्सकीय रूप से प्रासंगिक सांद्रता प्राप्त करने के लिए दवाओं के प्रवेश को बढ़ाना है। पेप्टाइड्स का एक वर्ग जिसे सेल पेनेट्रेटिंग पेप्टाइड्स (सीपीपी) के रूप में जाना जाता है, प्लाज्मा झिल्ली को पार कर सकता है जो इससे जुड़े कार्गो अणुओं को ले जाता है। आमतौर पर लंबाई में 5 से 40 अमीनो एसिड तक, इसमें विभिन्न प्रकार के कार्गो जैसे चिकित्सीय एजेंट, प्रोटीन, न्यूक्लिक एसिड, प्लास्मिड और यहां तक कि नैनो कण जैसे लिपोसोम ले जाने की क्षमता होती है।

यह थीसिस पूर्वकाल खंड रोग प्रबंधन के लिए चिकित्सीय हस्तक्षेप के लिए नई रणनीति प्रस्तुत करता है।

पहला भाग एक उपन्यास सीपीपी पेश करता है, जिसे कॉर्नियल रोगों में दवा वितरण के लिए कॉर्नियल ऊतक को लक्षित करने के लिए डिज़ाइन किया गया है। कॉर्नियल टारगेटिंग सीक्वेंस 1 (CorTS 1) को कॉर्नियल प्रोटीन में मौजूद एक संरक्षित ल्यूसीन रिच रिपीट (LRR) मोटिफ को संशोधित करके विकसित

किया गया है। उपन्यास कॉर्ट्स 1 पेप्टाइड बिना किसी उल्लेखनीय साइटोटोक्सिसिटी के एक आशाजनक सेल मर्मज्ञ गतिविधि प्रदर्शित करता है और क्रमशः इन विट्रो और पूर्व विवो स्थितियों में जलीय हास्य की तुलना में कॉर्नियल स्ट्रोमा में वृद्धि हुई है। पेप्टाइड मानव कॉर्नियल एपिथेलियल सेल (एचसीई) लाइन के अंदर अपने सक्रिय जैविक रूप में प्रोटीन कार्गो (बीटा गैलेक्टोसिडेज़) भी वितरित करता है। दिलचस्प बात यह है कि एमआरएसए और फुसैरियम डिमेरम के खिलाफ रोगाणुरोधी गतिविधि भी नोट की गई है। CorTS 1 में एंटीकोलेजेनोलिटिक गतिविधि भी होती है जिससे माइक्रोबियल केराटाइटिस और स्ट्रोमल मेल्ट्स के उपचार में एक आशाजनक क्षमता होती है।

अध्ययन का दूसरा भाग केराटोकोनस (केसी) के उपचार के लिए एक उपन्यास सीपीपी दवा संयुग्म आधारित रणनीति की विवो प्रभावकारिता में प्रदर्शित करता है। केसी एक सामान्य कॉर्नियल विकार है जो प्रगतिशील पतलेपन की विशेषता है जो अनियमित दृष्टिवैषम्य और बिगड़ा हुआ दृष्टि के साथ शंकु के आकार का कॉर्निया की ओर जाता है। कॉर्नियल कोलेजन क्रॉसलिंकिंग (सीएक्सएल) रोग की प्रगति को रोकने के लिए नियोजित मानक उपचार है। इस प्रक्रिया का एक संबंधित कदम खराब पारगम्य राइबोफ्लेविन (आरबी) के प्रवेश की सुविधा के लिए किया गया उपकला मलत्याग है। इस अध्ययन में आरबी को एक प्रसिद्ध सीपीपी (टाट डिमर - टाट 2) के डिमर के साथ सहसंयोजी रूप से संयुग्मित किया गया है, जिसके परिणामस्वरूप पैठ को बढ़ाने के लिए टैट 2 राइबोफ्लेविन या रीट संयुग्मित होता है और इस तरह सीएक्सएल प्रोटोकॉल की दक्षता में सुधार होता है। सीपीपी के साथ संयुग्मन पर खरगोश के कॉर्निया में ऊतक प्रवेश में लगभग 2 गुना वृद्धि हासिल की गई थी। RiTe संयुग्म मध्यस्थता और मानक CXL के तुलनात्मक विश्लेषण ने दोनों प्रकार के CXL में क्रॉसलिंकिंग में एक समान सीमा का प्रदर्शन किया जैसा कि कॉर्निया के एंजाइमी पाचन में देखा गया है। मानक CXL के विपरीत RiTe संयुग्म मध्यस्थता CXL के मामले में कोई एंडोथेलियल क्षति और केराटोसाइट हानि नहीं, प्रभावकारिता के साथ-साथ प्रस्तावित प्रोटोकॉल की सुरक्षा को और स्थापित करता है।

उपर्युक्त दो हस्तक्षेप सीपीपी और सीपीपी दवा संयुग्मों के संभावित अनुप्रयोग को पूर्वकाल खंड रोगों या आंख के विकारों के इलाज के लिए उजागर करते हैं।

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ABBREVIATIONS AND SYMBOLS

α	Alpha
β	Beta
δ	Delta
µ	Micro
µg	Microgram
µL	Microlitre
mW/cm^2	Milli Watt per centimetre square
%	Percent
℃	Degree Celsius
®	Registered
a.a	amino acid
AMD	Age-related Macular Degeneration
AMPD	Antimicrobial Peptide Database
AMPs	Antimicrobial Peptides
ARVO	Association for Research in Vision and Ophthalmology
ATP	Adenosine triphosphate
BAB	Blood Aqueous Barriers
BCRPs	Breast Cancer Resistance proteins
BRB	Blood Retinal Barrier
CDE	Clathrin-Dependent endocytosis
CDI	Clathrin-Independent
CESC	Corneal Epithelial Stem Cells
CLSM	Confocal Laser Scanning Microscope
CorTS 1	Corneal Targeting Sequence 1
CPCSEA	Committee for the Purpose of Control and Supervision of Experiments on Animals guidelines
CPP	Cerebral Perfusion Pressure
CPPs	Cell Penetrating Peptides
CXL	Corneal Collagen Crosslinking

DALK	Deep Anterior Lamellar Keratoplasty
DAPI	4',6-diamidino-2-phenylindole
DED	Dry Eye Disease
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
EAU	Experimental automimmune uveitis
ECM	Extracellular matrix
EDTA	Ethylenediamine Tetraacetic Acid
EGFR	Epithelial Growth Factor Receptor
EIU	Endotoxin Induced Uveitis
EMA	European Medical Agency
FACIT	Fibril Associated Collagens with Interrupted Triple helices
FACS	Fluorescence-Activated Cell Sorting
FDA	Food and Drug Association
FGF	Fibroblast Growth Factor
FITC	Fluorescein Isothiocyanate
GAGs	Glycosaminoglycans
h	Hour/hours
H&E	Hematoxylin and Eosin
HB-EGF	Heparin-Binding-Epidermal Growth Factor
HCE	Human Corneal Epithelial
HDP	Host Defensin Peptides
HGF	Hepatocyte Growth Factor
HIV	Human immunodeficiency virus
HMDS	Hexamethyldisilazane
IAEC	Institutional Animal Ethics Committee
IGFR	Insulin-Like Growth Factor Receptor
IL-1	Interleukin - 1
KC	Keratoconus
kDa	Kilo Dalton
KGF	Keratinocyte Growth Factor

LASIK	Laser-Assisted in Situ Keratomileusis
LDH	Lactate Dehydrogenase
LESC	Limbal Epithelial Stem Cells
LRR	Leucine Rich Repeat
MAPs	Membrane Active Peptides
MDR	Multi Drug Resistance
MIC	Minimum inhibitory concentration
min	Minutes
MMP	Matrix metalloprotease
MRP	Multidrug Resistance Protein
MRSA	Methicillin-Resistant Staphylococcus Aureus
MTT	MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)
NBD	Nucleus Binding Domain
NIB	National Institute of Biologicals
NV	Neovascularisation
P-gp	P-glycoprotein
PAGE	polyacrylamide gel electrophoresis
PBS	Phosphate Buffered Saline
PDA	Potato Dextrose Agar
PDGF	Platelet Derived Growth Factor
PGE	Prostaglandin E
PGI	Prostaglandin
PIGF	Placental growth factor
PK	Penetrating Keratoplasty
POD	Peptide for Ocular Delivery
pVEC	peptide vascular endothelial-cadherin
QD	Quantum Dots
Rb	Riboflavin
RIPA buffer	RadioImmunoPrecipitation Assay buffer
RiTe conjugate	Riboflavin TransEpithelial conjugate

RNA	Ribonucleic acid
RPE	Retinal Pigment Epithelium
SDS	Sodium Dodecyl-Sulfate
SEM	Scanning Electron microscopy
SLC	Stabilized Liquid Creatine
SLC	Solute carrier
SLRPs	Small Leucine Rich Proteoglycan
SP	Substance P
SPF	Specific Pathogen Free
SVM	Support Vector Machine
TAT	Trans-Activator of Transcription
TBST	Tris Buffer Saline Tween 20
TCA	Trichloroacetic Acid
Tg	Transgenic
TGF	Transforming Growth Factor
TNF-alpha	Tumour Necrosis Factor alpha
TXA	Thromboxane
UV A	Ultraviolet A
VEGF	Vascular Endothelial Growth Factor
VRE	Vancomycin-Resistant Enterococcus
WHO	World Health Organization
ZO-1	Zonula occludens-1