CPP MEDIATED DRUG DELIVERY FOR OCULAR DISEASE MANAGEMENT

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INDIAN INSTITUTE OF TECHNOLOGY DELHI

OCTOBER 2021

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by

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Submitted

in fulfilment of the requirements of the degree of Doctor of Philosophy

to the

Indian Institute of Technology Delhi

New Delhi-110016, India

OCTOBER 2021

CERTIFICATE

This is to certify that the thesis entitled **"CPP Mediated Drug Delivery for Ocular Disease Management"** being submitted by **Ms. Harsha Rohira** 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 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.

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ACKNOWLEDGEMENTS

"Believe in yourself and all that you are. Know that there is something inside you that is greater than any obstacle." – Christian D. Larson

Arduous journey of a PhD student starts as soon as one gets admission into this prestigious program that demands not only mental exhaustion but physical enervation too. Right from beginning, one person who also scratches his/her head along with you is your supervisor and I am very grateful to have one such supervisor who not only supported me professionally but personally also. Many people have helped me in one way or another throughout my journey, including my parents who cannot be thanked enough for their contribution.

First and foremost, I would like to express my deepest gratitude to my PhD guide, ***Dr. Archana Chugh****, whose constant support made me sail through this grueling journey of 7 years. Her ceaseless motivation and care gave me the spirit of confidence. In times of mental distress, when one misses their home, her words of encouragement proved to be a boon for me. There was a time when I was low on confidence to complete one of my work objectives, but her faith in me gave me a boost because of which I was able to handle immense pressure of work. She gave me freedom to work in whatever way I like and supported me whenever I got stuck in between. Words are not enough to pen down a feeling of thankfulness that I feel for her.*

I am also thankful to ***Dr. Sushmita G. Shah*** *and* ***Dr. Shikha Yadav*** *who helped me a lot during my animal studies. Without their support, I cannot imagine working with animals for my in vivo work. Being an Ophthalmologist, inputs from* ***Sushmita mam*** *were very valuable to polish my work. An important part of my work was arranging an animal house which was made possible with the efforts of* ***Shikha mam*** *who, at the last moment, arranged animal facility at her institution* ***(National Institute of Biologicals, NOIDA, India).*** *Their contributions in my work are as significant as carrying out experiments for successful results.*

I am also grateful to my ***SRC*** *members,* ***Prof. C. S. Dey, Prof. Vivekanandan Perumal and Prof. Ritu Kulshreshtha (Department of Biochemical Engineering and Biotechnology, IIT Delhi),*** *for their valuable inputs time to time that helped me carrying out my research in a right direction and refine my work. I would like to convey my special thanks to* ***Prof. Dey*** *whose*

inspirational talks made me remain motivated all throughout my research journey. I would also like to thank faculty members of Kusuma School of Biological Sciences for their constant guidance and allowing me to use their equipment or research facility whenever I needed.

My sincere thanks to non-academic staff also for giving me a smooth experience related to any kind of administrative work. I specially acknowledge the help provided by ***Ms Pushpalata*** *whenever I needed UG Lab facilities even when she was very busy.*

Working in a research lab is not possible without a friendly environment and I am very blessed to have such wonderful lab mates, ***Vivek, Pankhuri, Sujithra, Anjali, Saurabh, Prasan, Aditi and Gagan,*** *who helped me a lot at every step of my research, sometimes even by going out of their way. I cannot forget our chai/coffee breaks where our research discussions smoothly merged with humor, chit chats and plenty of jokes that were definitely needed to refresh ourselves. One of the admiring features of my lab is that every occasion, whether a small one or a big one, is celebrated with equal enthusiasm and I wish I get such environment in my future academic endeavors. Not only my current lab mates, but also my seniors* ***Dr. Aastha, Dr. Deepthi, Dr. Nisha and Dr. Anupama*** *made this place a perfect research environment. I would also like to thank* ***Dr. Anusha, Dr. Chayanika*** *and* ***Dr. Nirupama*** *for their kind help in my work, be it discussions or experiments. Their stay in our lab was short but very sweet and delightful. It is very rightly said that you need someone to share your success and failures in order to keep moving forward and I am lucky that I found "that someone" in two of my lab mates,* ***Pankhuri and Sujithra*** *with whom I could share my personal experiences also apart from my research work. They supported me, guided me and cared for me in my ups and downs. I cannot imagine my stay to be this happy and cheerful in this lab if they were not present. I cannot thank* ***Sujithra*** *enough for her contributions in my work. Whether it was staying at NIB for animal studies or going to Hyderabad for cell culture, I enjoyed every single bit of a time spent with her.*

"A friend in need is a friend indeed" aptly fits on one of my close friends ***Dr. Bhaskar.*** *I am very grateful that we have shared so many memories in last couple of years. He motivated me at every step and encouraged me to move forward crossing any hurdle I faced during my research. He used to patiently listen my tantrums, my boring talks and never complained. We celebrated every small and big achievement together. Listening to his songs in happy or sad*

moments was a gift for me that I can never forget. Our parties were sometimes incomplete without ***Devanshu*** *and* ***Sujithra.*** *Watching movies and dining out with these three crazy people were the most favorite activities of my PhD tenure. I will cherish those moments forever.*

Living in a hostel away from home is never an easy task but my roommate, my batchmate, my birthdate sharer and my very close friend, ***Kirtika****, made it possible for me to feel it like home. I cannot forget how she cared for me whenever I was sick or I was upset. She surely took care of me like an elder sister or a mother. She not only helped me in my hostel room, but also in lab. We shared a very healthy and cheerful professional and personal bond that I would like to cherish forever. Without her I don't feel I could have lived in that hostel. I cannot forget those night mess times, 'night wali' Maggie and our pillow fights. Our chai breaks were incomplete without hanging out with* ***Prashant****. Whenever I was unsatisfied with my experimental results and I needed a company, I counted on them. They were the best batchmates I have got till now who never declined any chai/coffee breaks.*

Talking about my batchmates, I am really very thankful to ***Anshu, Sabeeha, Akhilesh, Ashutosh,*** *and* ***Budhaditya*** *for their enlightened discussions about work and life. I am also thankful to all my Kusuma School friends, specially* ***Dibyakanti, Shivani, Kiruthika, Akansha, Late Ms. Devanshi, Medha, Yamini,*** *who made this school a lively place to do extra-curricular activities apart from research work. Individually naming every one of them is very tough for me as there are plenty of students who have helped me in enjoying the beauty of research at Kusuma School.*

I would also like to acknowledge the contributions of ***Sahil*** *from CBME who helped me with using their lab's facility when I required DLS/Zeta sizer or Fluorimeter.*

I thank ***IIT Delhi*** *and* ***Kusuma Trust*** *for providing financial assistance and infrastructure, respectively to carry out the research work.* ***IIT Delhi*** *also supported me financially to attend international conferences at Germany and Ireland. I would also like to thank* ***Department of Science and Technology, Govt. of India*** *for funding the project that I have worked on.*

This acknowledgement is incomplete without recognizing the contribution of my parents in my life. I am indebted for the love and support showered by them. It is because of them that I have

crossed every hurdle of my life and will continue to outpace them in future. No words can express how grateful I am to be their child. My father always supported me in every decision I took and gave me wings to fly higher and higher. My sister stood beside me through every thick and thin. My grandparents never missed out a day to call me when I was in a hostel. I am truly blessed to have such a wonderful, supporting and loving family.

Last but not the least, I thank Almighty God to stand with me at every step and giving me such wonderful people in my life.

Harsha Rohira

ABSTRACT

Blindness and vision impairment remains a major health concern in developing countries with more than 2 billion people visually compromised globally. In India, corneal opacities (8.2%) are the second leading cause of blindness among people of age 50 years and above. Major risk factors of corneal opacities include infectious and non-infectious keratitis, xerophthalmia, keratoconus, trauma, and few inherited and inflammatory disorders. Out of total visually compromised population, atleast 1 billion have preventable blindness owing to accurate diagnosis and timely management of ocular disorders. Present study aims at developing alternate strategies for efficacious management of fungal keratitis and keratoconus than the existing therapeutic regimes and also reduction in treatment duration as compared to the standard treatment protocols.

One of the approaches to increase therapeutic efficiency of any treatment protocol is to enhance the bioavailability of active drug molecules that will subsequently lead to reduced treatment duration and increased patient compliance. In past few decades, several nanocarrier mediated drug delivery strategies have been investigated. Cell penetrating peptides (CPPs), with an ability to translocate themselves across the cell membranes, have emerged as promising drug delivery vehicles for the management of various diseases including ocular disorders. Since the path breaking discovery of TAT, numerous CPPs have been designed and developed for their potential therapeutic applications *in vitro* and *in vivo*. The present study aims at assessing the potential of an established cell penetrating peptide, Tat_2, for efficacious management of fungal keratitis and keratoconus.

First part of the study investigates tissue penetration ability as well as antifungal efficacy of a CPP (Tat_2) conjugated standard drug (Natamycin) used for the treatment of fungal keratitis. Natamycin is the only FDA approved drug that is used as a first line of treatment for fungal keratitis caused by filamentous fungi, however natamycin is known for poor corneal penetration. To address this drawback, natamycin has been successfully conjugated with a cell penetrating peptide, Tat_2. Recently, we have shown the enhanced cellular uptake as well as antifungal efficacy of Tat_2 conjugated natamycin (Tat_2natamycin) *in vitro*. In the present study, tissue penetration ability and antifungal efficacy of Tat_2natamycin have been investigated and compared with natamycin alone *in vivo*. Results have shown that Tat_2natamycin exhibited five-

fold higher ocular penetration than natamycin alone when given topically. Complete resolution of fungal keratitis in 44% of the animals in Tat_2natamycin treated group as compared to only 13% of the animals in natamycin treated group further highlighted its increased antifungal efficacy. Hence, this conjugate is a promising antifungal strategy with enhanced ocular penetration as well as efficacy against selected fungal species.

In the second part of the study, cell penetrating ability of Tat_2 conjugated riboflavin has been assessed *in vitro*. Dresden protocol, involving riboflavin as a photosensitizer, is used as a standard therapy for the management of keratoconus. However, less ocular permeability of riboflavin across corneal epithelial layer necessitates the removal of corneal epithelial layer prior to the initiation of protocol. As a result of this, severe post-op complications including pain, infections, sterile infiltrates, corneal haze etc., have been reported. In the current study, we have conjugated a cell penetrating peptide, Tat_2, with riboflavin to enhance its cellular penetration across human corneal epithelial cells *in vitro*. Significantly enhanced cellular uptake of Tat_2riboflavin, in addition to negligible cytotoxicity, was observed in human corneal epithelial cell line. Upon quantification, Tat_2riboflavin was found to enter more than 80 % of the cell population as compared to approximately 0.1 % of the cells treated with riboflavin alone. The study demonstrates the potential of Tat_2riboflavin in modifying the standard Dresden protocol with respect to the epithelial debridement step, paving way for the *in vivo* studies.

सार:

नेत्रहीनता और दृष्टि हानि विकासशील देशों में एक प्रमुख स्वास्थ्य चिंता बनी हुई है, जिसमें विश्व स्तर पर 2 बिलियन से अधिक लोग दृष्टिबाधित हैं। भारत में, कॉर्नियल अस्पष्टता (8.2%) 50 वर्ष और उससे अधिक आयु के लोगों में अंधेपन का दूसरा प्रमुख कारण है। कॉर्नियल अपारदर्शिता के प्रमुख जोखिम कारकों में संक्रामक और गैर-संक्रामक केराटाइटिस, ज़ेरोफथाल्मिया, केराटोकोनस, आघात और कुछ विरासत में मिली और सूजन संबंधी विकार शामिल हैं। कुल दृष्टिबाधित आबादी में से, कम से कम 1 बिलियन लोगों को सही निदान और नेत्र संबंधी विकारों के समय पर प्रबंधन के कारण रोके जाने योग्य अंधापन है। वर्तमान अध्ययन का उद्देश्य मौजूदा चिकित्सीय व्यवस्थाओं की तुलना में फंगल केराटाइटिस और केराटोकोनस के प्रभावी प्रबंधन के लिए वैकल्पिक रणनीति विकसित करना और मानक उपचार प्रोटोकॉल की तुलना में उपचार की अवधि में कमी करना है।

किसी भी उपचार प्रोटोकॉल की चिकित्सीय दक्षता बढ़ाने के दृष्टिकोणों में से एक सक्रिय दवा अणुओं की जैव उपलब्धता को बढ़ाना है जो बाद में उपचार की अवधि को कम करेगा और रोगी के अनुपालन में वृद्धि करेगा। पिछले कुछ दशकों में, कई नैनोकैरियर मध्यस्थता वाली दवा वितरण रणनीतियों की जांच की गई है। सेल मर्मज्ञ पेप्टाइड्स (सीपीपी), सेल झिल्ली में खुद को स्थानांतरित करने की क्षमता के साथ, ओकुलर विकारों सहित विभिन्न बीमारियों के प्रबंधन के लिए आशाजनक दवा वितरण वाहनों के रूप में उभरे हैं। टैट की नई खोज के बाद से, कई सीपीपी को इन विट्रो और विवो में उनके संभावित चिकित्सीय अनुप्रयोगों के लिए डिजाइन और विकसित किया गया है। वर्तमान अध्ययन का उद्देश्य फंगल केराटाइटिस और केराटोकोनस के प्रभावी प्रबंधन के लिए एक स्थापित सेल मर्मज्ञ पेप्टाइड, टैट$_2$ की क्षमता का आकलन करना है।

अध्ययन का पहला भाग ऊतक प्रवेश क्षमता के साथ-साथ कवक केराटाइटिस के उपचार के लिए उपयोग की जाने वाली सीपीपी (टैट$_2$) संयुग्मित मानक दवा (नैटामाइसिन) की एंटिफंगल प्रभावकारिता की जांच करता है। नैटामाइसिन एकमात्र एफडीए द्वारा अनुमोदित दवा है जिसका उपयोग फिलामेंटस कवक के कारण होने वाले फंगल केराटाइटिस के उपचार की पहली पंक्ति के रूप में किया जाता है, हालांकि नैटामाइसिन खराब कॉर्नियल पैठ के लिए जाना जाता है। इस कमी को दूर करने के लिए, नैटामाइसिन को सेल मर्मज्ञ पेप्टाइड, टैट$_2$ के साथ सफलतापूर्वक संयुग्मित किया गया है। हाल ही में, हमने इन विट्रो में वर्धित कोशिकीय उत्थान के साथ-साथ टैट$_2$ संयुग्मित नैटामाइसिन (टैट$_2$नैटामाइसिन) की एंटिफंगल प्रभावकारिता को दिखाया है। वर्तमान अध्ययन में, ऊतक प्रवेश क्षमता और टैट$_2$नैटामाइसिन की

एंटिफंगल प्रभावकारिता की जांच की गई है और इन विवो में अकेले नैटामाइसिन के साथ तुलना की गई है। परिणामों से पता चला है कि टैट$_2$नैटामाइसिन ने शीर्ष रूप से दिए जाने पर अकेले नैटामाइसिन की तुलना में पांच गुना अधिक ओकुलर पैठ का प्रदर्शन किया। टैट$_2$नैटामाइसिन उपचारित समूह में 44% जानवरों में कवक केराटाइटिस का पूर्ण समाधान, जबकि नैटामाइसिन उपचारित समूह में केवल 13% जानवरों ने इसकी बढ़ी हुई एंटिफंगल प्रभावकारिता पर प्रकाश डाला। इसलिए, यह संयुग्म एक आशाजनक ऐंटिफंगल रणनीति है जिसमें बढ़ी हुई ओकुलर पैठ के साथ-साथ चयनित कवक प्रजातियों के खिलाफ प्रभावकारिता भी है।

अध्ययन के दूसरे भाग में, इन विट्रो में टैट$_2$ संयुग्मित राइबोफ्लेविन की कोशिका भेदन क्षमता का मूल्यांकन किया गया है। ड्रेसडेन प्रोटोकॉल, जिसमें राइबोफ्लेविन को फोटोसेंसिटाइज़र के रूप में शामिल किया जाता है, केराटोकोनस के प्रबंधन के लिए एक मानक चिकित्सा के रूप में उपयोग किया जाता है। हालांकि, कॉर्नियल एपिथेलियल परत में राइबोफ्लेविन की कम ओकुलर पारगम्यता प्रोटोकॉल की शुरुआत से पहले कॉर्नियल एपिथेलियल परत को हटाने की आवश्यकता होती है। इसके परिणामस्वरूप, दर्द, संक्रमण, बाँझ घुसपैठ, कॉर्नियल धुंध आदि सहित गंभीर पोस्ट-ऑप जटिलताओं की सूचना मिली है। वर्तमान अध्ययन में, हमने इन विट्रो में मानव कॉर्नियल उपकला कोशिकाओं में अपनी सेलुलर पैठ बढ़ाने के लिए राइबोफ्लेविन के साथ एक सेल मर्मज्ञ पेप्टाइड, टैट$_2$ को संयुग्मित किया है। मानव कॉर्नियल एपिथेलियल सेल लाइन में नगण्य साइटोटोक्सिसिटी के अलावा, टैट$_2$राइबोफ्लेविन के महत्वपूर्ण रूप से बढ़े हुए सेलुलर उत्थान को देखा गया था। परिमाणीकरण पर, टैट$_2$राइबोफ्लेविन अकेले राइबोफ्लेविन के साथ इलाज किए गए लगभग 0.1% कोशिकाओं की तुलना में 80% से अधिक सेल आबादी में प्रवेश करने के लिए पाया गया था। अध्ययन एपिथेलियल डिब्रिडमेंट चरण के संबंध में मानक ड्रेसडेन प्रोटोकॉल को संशोधित करने में टैट$_2$राइबोफ्लेविन की क्षमता को प्रदर्शित करता है, इन विवो अध्ययनों के लिए मार्ग प्रशस्त करता है।

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ABBREVIATIONS AND SYMBOLS

%	Percent
®	Registered
™	Trade Mark
°C	Degree Celsius
α	Alpha
β	Beta
γ	Gamma
µ	Micro
κ	Kappa
µg	Microgram
µL	Microlitre
AmB	Amphotericin B
AMD	Age-related Macular Degeneration
APTES	3-Aminopropyl Triethoxysilane
ARVO	The Association for Research in Vision and Ophthalmology
ASO	Antisense Oligonucleotide
ATP	Adenosine Triphosphate
BAB	Blood Aqueous Barrier
BAK/BAC	Benzalkonium Chloride
BL	Bowman's Layer
BN	Brown Norway
BRB	Blood Retinal Barrier
CAP	Cellulose Acetate Phthalate
CBA	Cytokine Bead Assay
CFU	Colony Forming Unit
CLR	C-Type Lectin Receptor
CLSI	The Clinical & Laboratory Standards Institute
CLSM	Confocal Laser Scanning Microscopy
CNV	Choroidal Neovascularization
CO_2	Carbon dioxide

CPCSEA	Committee for the Purpose of Control and Supervision of Experiments on Animals
CPP	Cell Penetrating Peptide
CS	Chitosan
CXL	Corneal Collagen Crosslinking
DALK	Deep Anterior Lamellar Keratoplasty
Da	Dalton
DM	Descemet’s Membrane
DME	Diabetic Macular Edema
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DOC	Sodium Deoxycholate
DPX	Dibutyl phthalate Polystyrene Xylene
EB	Entry Blocker
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
ELP	Elastin-Like Polypeptide
EMA	European Medicines Agency
EVA	Ethylene Vinyl Acetate
ex/em	Excitation/emission
FA	Flucinolone Acetonide
FBS	Fetal Bovine Serum
FC	Flucytosine
FGF	Fibroblast Growth Factor
FIC	Fractional Inhibitory Concentration
FICI	Fractional Inhibitory Concentration Index
FITC	Fluorescein Isothiocyanate
FK	Fungal Keratitis
FU	Fluorouracil
gm	Gram
GAP	Global Action Plan

GBD	Global Burden of Disease Study
HA	Hyaluronic Acid
HCE	Human Corneal Epithelial
HCl	Hydrochloric Acid
HDL	High Density Lipoprotein
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
HPMC	Hydroxypropyl Methylcellulose
HSV	Herpes Simplex Virus
IAEC	Institutional Animal Ethics Committee
ICAMB	Intra-Cameral Amphotericin B
ICRS	Intracorneal Ring Segment
IFN	Interferon
IL	Interleukin
JNK	c-Jun N-terminal Kinase
Kg	Kilogram
LK	Lamellar Keratoplasty
LOX	Lysyl Oxidase
MALDI-ESI	Matrix-Assisted Laser Desorption/Ionization- Electrospray Ionization
MAP	Model Amphipathic Peptide
MDR	Multi Drug Resistant
ME	Micro Emulsion
MEND	Multifunctional Enveloped Nano Device
mg	Milligrams
MIC	Minimum Inhibitory Concentration
MIC_{90}	Minimum Inhibitory Concentration inhibiting more than 90% of the isolates
min	Minute
ml	Millilitre
mM	Millimolar
MRP	Multi-drug Resistance Protein
MSVI	Moderate And Severe Vision Impairment

$MTat_2$	Mutated TAT Dimer
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
MUTT	Mycotic Ulcer Treatment Trial
NBP	Nucleolin Binding Peptide
NCSS	National Council for the Social Studies
NE	Nano Emulsion
NLRP	Nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin domain containing Proteins
nm	Nanometre
NOD	Nucleotide-Binding Oligomerization Domain
NP	Nanoparticle
NPCBVI	National Programme for Control of Blindness & Visual Impairment
OH	Hydroxyl group
ORA	Ocular Response Analyzer
PAA	Poly (Acrylic Acid)
PACK-CXL	Photoactivated Chromophore for Keratitis-Corneal Collagen Crosslinking
PAMAM	Poly(Amidoamine)
PBS	Phosphate Buffered Saline
PCF	Poly Sulfone Capillary Fibre
PCL	Polycaprolactones
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
PDT	Photodynamic Therapy
PEG	Polyethylene Glycol
PFA	Paraformaldehyde
PGA	Polyglycolic Acid
PIC	Protease Inhibitory Cocktail
PK	Penetrating Keratoplasty
PLA	Poly Lactic Acid
PLGA	Polylactic-co-Glycolic Acid
PMMA	Polymethyl Methacrylate

POD	Peptide for Ocular Delivery
POE	Poly Ortho Esters
PRR	Pattern-Recognition Receptors
PTD	Protein Transduction Domains
PVA	Poly Vinyl Alcohol
RGP	Rigid Gas Permeable
RIPA	Radioimmunoprecipitation Assay
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
rpm	Revolutions per minute
RVO	Retinal Vein Occlusion
SCL	Soft Toric Contact Lens
SD	Standard Deviation
SEM	Scanning Electron Microscopy
SLN	Solid Lipid Nanoparticle
SO	Singlet Oxygen
SODI	Soluble Ocular Drug Insert
SPF	Specific Pathogen Free
TAT	Transactivator of Transcription
Tat_2	TAT Dimer
TLR	Toll-like Receptor
TNF	Tumor Necrosis Factor
TPK	Therapeutic Penetrating Keratoplasty
USFDA/FDA	U.S. Food and Drug Administration
UV	Ultraviolet
UVA	Ultraviolet-A
VE-TPGS	Vitamin E- D-α-Tocopheryl Polyethylene Glycol 1000 Succinate
VI	Visual Impairment
WHA	World Health Assembly
WHO	World Health Organization
ZNF	Zinc Finger