EXPLORING THE ROLE OF L-ASPARAGINASE AND HTPX AS POTENTIAL TARGETS AGAINST *Neisseria gonorrhoeae*

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by

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DEDICATION

To my grandmother and my parents….

सर्वतीर्थमयी माता सर्वदेवमयः पिता।
मातरं पितरं तस्मात् सर्वयत्नेन पूजयेत।।

Mother is (the embodiment) of all pilgrimages,
father is (the embodiment) of all deities.
Hence, mother and father are to be revered with all efforts.

CERTIFICATE

This is to certify that the thesis titled "**Exploring the role of L-asparaginase and HtpX as potential targets against *Neisseria gonorrhoeae***" being submitted by **Mr. Shubham Vashishtha** to the Kusuma School of Biological Science, Indian Institute of Technology Delhi New Delhi for the award of the degree of '**Doctor of Philosophy**' is a record of the bonafide research work carried out by him, prepared under my supervision in conformity with the rules and regulations of 'Indian Institute of Technology Delhi'. The research report and the results presented 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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the entrepreneur Mayank (fufu), the crazy Vinayk (Vinny) dev, the rough one Ashish S, the love Gaurav Patial, the street-smart Namit, the half-head Deep, with them I have spent the most mind-blowing days. These guys were always there in all ups and down. I cherish all the memories that we have created and will create for the years ahead. Cheers to our friendship!

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At last, I would like to remember the almighty, who provides us with the strength to do good, be humble, stay curious, and move on to exploring the unknown.

Shubham Vashishtha

ABSTRACT

Neisseria gonorrhoeae is a gram-negative diplococci bacterium and a causative agent of gonorrhoeae, the second most prevalent sexually transmitted disease. The emergence of multiple drug-resistant 'super gonorrhoea' complicates the management and treatment of *Neisseria gonorrhoeae* infections. The antimicrobial-resistant (AMR) traits acquired by the pathogen are attributed to the cumulative mutations in the bacterial proteome, either in the direct targets of antibiotics or in the additional factors involved in AMR. A continuous evaluation of bacterial proteome is necessary to understand the global mutational landscape and identify conserved molecular targets and their respective inhibitors for therapeutic applications.

In this study, a comparative genomic analysis of the *N. gonorrhoeae* genes (*penA, ponA, 23s rRNA, rpoB, gyrA, parC, mtrR and porB*) proposed to confer resistance to drugs like ceftriaxone, cefixime, azithromycin and ciprofloxacin, was done. The Shannon entropy data of this analysis disclosed highly uncertain amino acid positions in the antibiotic target genes. When a similar analysis was performed on the whole proteome of *N. gonorrhoeae* MDR and XDR isolates, two proteins, the L-asparaginase and the HtpX, emerged as highly conserved targets. Interestingly, in the drug-susceptible isolates, these two proteins also showed complete conservation, emphasising their importance in the growth and survival of the pathogen. L-asparaginase is an amino hydrolase previously indicated to provide survival benefits to intracellular pathogens. HtpX is a putative metalloprotease, which is suggested to be involved in proteolytic control of membrane protein quality during stress. Thus, when functionally compromised using specific inhibitors, both NgA and NgHtpX are likely to act as duel targets.

Therefore, both NgA and NgHtpX were cloned in E. coli, expressed and purified to homogeneity and their biophysical and biochemical properties were evaluated. NgA showed

high stereospecificity and affinity for L -Asp, its natural substrate. Based on modelled structure, high-throughput screening and MD simulations, three FDA-approved drugs, pemirolast, thalidomide, and decitabine, displayed strong binding to NgA. The binding energies of the drugs were -20.14, -19.67, and -16.47 kcal/mol, respectively, compared to -6.82 kcal/mol for L-Asn. Subsequently, fluorescence quenching confirmed the high binding affinity of pemirolast thalidomide and decitabine to NgA with dissociation constants (K_d) 1.19 µM, 1.61 µM and 0.90 µM, respectively.

Similarly, in the case of NgHtpX, the zinc-binding potential was established using fluorescence quenching (K_d =0.4 µM), and E141 was identified as a crucial residue required for zinc binding. A composite high throughput screening followed by MD identified pemirolast (-15.18 kcal/mol) and thalidomide (-19.13 kcal/mol) as highly efficient ligands. The binding was verified through fluorescence quenching, where pemirolast and thalidomide displayed binding affinities with dissociation constants (K_d) of 3.47 µM and 1.04 µM, respectively. Together, the pemirolast and thalidomide appeared to have a high binding affinity towards both NgA and NgHtpX targets. Conclusive evidence came from the cell viability assays where these drugs were found to impede the growth of *N. gonorrhoeae* culture effectively. Overall, our results provide high entropy positions in the targets of presently used antibiotics, which can be further explored to understand the AMR mechanism. Additionally, NgA, NgHtpX and their specific inhibitors identified can be explored further in combination therapy or dual therapy for managing gonococcal infections.

सार

नेइसेरिया गोनोरहोई एक ग्राम-नकारात्मक डिप्लोकोकी जीवाणु है और गोनोरिया का एक प्रेरक एजेंट है, जो दूसरा सबसे प्रचलित यौन संचारित रोग है। एकाधिक दवा प्रतिरोधी 'सुपर गोनोरिया' के उद्भव नेइसेरिया गोनोरिया संक्रमण के प्रबंधन और उपचार को जटिल बनाता है। रोगज़नक़ द्वारा अधिग्रहीत रोगाणुरोधी-प्रतिरोधी (एएमआर) लक्षणों को बैक्टीरिया प्रोटिओम में संचयी उत्परिवर्तन के लिए जिम्मेदार ठहराया जाता है, या तो एंटीबायोटिक दवाओं के प्रत्यक्ष लक्ष्य में या एएमआर में शामिल अतिरिक्त कारकों में। वैश्विक पारस्परिक परिदृश्य को समझने और चिकित्सीय अनुप्रयोगों के लिए संरक्षित आणविक लक्ष्यों और उनके संबंधित अवरोधकों की पहचान करने के लिए बैक्टीरियल प्रोटिओम का निरंतर मूल्यांकन आवश्यक है।

इस अध्ययन में, एन. गोनोरिया जीन (पेनए, पोनए, 23एस आरआरएनए, आरपीओबी, जीआईआरए, पार्ससी, एमटीआरआर और पोरबी) का एक तुलनात्मक जीनोमिक विश्लेषण किया गया था, जिसमें सीफ्रीएक्सोन, सेफिक्साइम, एज़िथ्रोमाइसिन और सिप्रोफ्लोक्सासिन जैसी दवाओं के लिए प्रतिरोध प्रदान करने का प्रस्ताव किया गया था। इस विश्लेषण के शैनन एन्ट्रापी डेटा ने एंटीबायोटिक लक्ष्य जीनों में अत्यधिक अनिश्चित अमीनो एसिड की स्थिति का खुलासा किया। जब नेइसेरिया गोनोरहोई एमडीआर और एक्सडीआर आइसोलेट्स के पूरे प्रोटिओम पर एक समान विश्लेषण किया गया, तो दो प्रोटीन, एल-ऐस्पैरजाइनेस और एचटीपीएक्स, अत्यधिक संरक्षित लक्ष्यों के रूप में उभरे। दिलचस्प बात यह है कि ड्रग-अतिसंवेदनशील आइसोलेट्स में, इन दो प्रोटीनों ने रोगज़नक़ के विकास और उत्तरजीविता में उनके महत्व पर जोर देते हुए पूर्ण संरक्षण भी दिखाया। एल-एस्पैरजाइनेज एक एमिनो हाइड्रॉलेज़ है जिसे पहले इंट्रासेल्युलर रोगजनकों को उत्तरजीविता लाभ प्रदान करने के लिए संकेत दिया गया था। एचटीपीएक्स एक पुटेटिव मेटालोप्रोटीज है, जिसे तनाव दौरान झिल्ली प्रोटीन गुणवत्ता के प्रोटियोलिटिक नियंत्रण में शामिल होने का सुझाव दिया गया है। इस प्रकार, जब विशिष्ट अवरोधकों का उपयोग करके

कार्यात्मक रूप से समझौता किया जाता है, तो एनजीए और एनजीएचटीपीएक्स दोनों द्वंद्व लक्ष्य के रूप में कार्य करने की संभावना रखते हैं।

इसलिए, एल-ऐस्पैरजाइनेस और एचटीपीएक्स दोनों को ई. कोलाई में क्लोन किया गया, समरूपता के लिए अभिव्यक्त और शुद्ध किया गया और उनके जैवभौतिक और जैव रासायनिक गुणों का मूल्यांकन किया गया। एनजीए ने अपने प्राकृतिक सब्सट्रेट एल-एएसएन के लिए उच्च रूढ़िवादिता और आत्मीयता दिखाई। प्रतिरूपित संरचना, उच्च-थ्रूपुट स्क्रीनिंग और एमडी सिमुलेशन के आधार पर, तीन एफडीए-अनुमोदित दवाएं, पेमिरोलास्ट, थैलिडोमाइड और डिकिटाबाइन, ने एनजीए के लिए मजबूत बंधन प्रदर्शित किया। एल-एएसएन के लिए -6.82 किलो कैलोरी/मोल की तुलना में दवाओं की बाध्यकारी ऊर्जा क्रमशः -20.14, -19.67, और -16.47 किलो कैलोरी/मोल थी। इसके बाद, फ्लोरेसेंस क्वेंचिंग ने एनजीए को पृथक्करण स्थिरांक (केडी) 1.19 माइक्रोन, 1.61 माइक्रोन और 0.90 माइक्रोन के साथ पेमिरोलास्ट थैलिडोमाइड और डिकिटाबाइन के उच्च बाध्यकारी संबंध की पुष्टि की।

इसी तरह, एनजीएचटीपीएक्स के मामले में, प्रतिदीप्ति शमन (केडी = 0.4 माइक्रोएम) का उपयोग करके जस्ता-बाध्यकारी क्षमता स्थापित की गई थी, और इ141 को जस्ता बंधन के लिए आवश्यक एक महत्वपूर्ण अवशेष के रूप में पहचाना गया था। एक समग्र उच्च प्रवाह क्षमता स्क्रीनिंग के बाद एमडी ने पेमिरोलास्ट (-15.18 किलो कैलोरी/मोल) और थैलिडोमाइड (-19.13 किलो कैलोरी/मोल) को अत्यधिक कुशल लिगेंड के रूप में पहचाना। बाइंडिंग को प्रतिदीप्ति शमन के माध्यम से सत्यापित किया गया था, जहां पेमिरोलॉस्ट और थैलिडोमाइड ने क्रमशः 3.47 माइक्रोएम और 1.04 माइक्रोएम के पृथक्करण स्थिरांक (केडी) के साथ बाध्यकारी समानताएं प्रदर्शित कीं। साथ में, पेमिरोलास्ट और थैलिडोमाइड एल-ऐस्पैरजाइनेस और एचटीपीएक्स दोनों लक्ष्यों के प्रति एक उच्च बाध्यकारी संबंध रखते हैं। सेल व्यवहार्यता परख से निर्णायक सबूत मिले जहां इन दवाओं को एन गोनोरिया संस्कृति के विकास को प्रभावी ढंग से बाधित करने के लिए पाया गया। कुल मिलाकर, हमारे परिणाम वर्तमान में उपयोग किए जाने वाले एंटीबायोटिक दवाओं के

लक्ष्यों में उच्च एन्ट्रापी स्थिति प्रदान करते हैं, जिन्हें एएमआर तंत्र को समझने के लिए और खोजा जा सकता है। इसके अतिरिक्त, एल-ऐस्पैरजाइनेस, एचटीपीएक्स और उनके विशिष्ट अवरोधकों की पहचान गोनोकोकल संक्रमणों के प्रबंधन के लिए संयोजन चिकित्सा या दोहरी चिकित्सा में की जा सकती है।

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ABBREVIATIONS & SYMBOLS

mm	Millimetre
º C	Degree Celsius
mins	Minutes
µm	Micrometre
CO_2	Carbon Dioxide
%	Percentage
PID	Pelvic inflammatory disease
DGI	Disseminated gonococcal infection
NAATs	Nucleic acid amplification tests
WHO	World health organisation
CDC	Centres for disease control and prevention
AMR	Antimicrobial resistance
DHPS	Dihydropteroate synthase
UK	United Kingdom
COVID-19	Corona virus disease 2019
Bla_{-TEM}	Beta lactamase containing plasmid
DNA	Deoxyribonucleic acid
DUS	DNA uptake sequences
bp	Base pairs
PBP	Penicillin binding proteins
rRNA	Ribosomal Ribonucleic acid
MIC	Minimum inhibitory concentration
Mtr	Multiple transferable resistance

BC	Before Christ
Opa	Opacity protein
LOS	Lipoligosaccharide
PorB	Major outer membrane protein
ASGPR1	Asialoglycoprotein receptor 1
CEACAM	Carcino-embryonic antigen-related cell adhesion molecule
AV	Antigenic variations
PMNL	Polymorphonuclear lymphocytes
WBC	White blood cells
PRR	Pathogen recognition receptors
IgG	Immunoglobulin G
NADPH	Nicotinamide adenine dinucleotide phosphate
ROS	Reactive oxygen species
O_2^-	Superoxide anion
H_2O_2	Hydrogen peroxide
$HO^\bullet$	Hydroxyl free radicle
MPO	Myeloperoxidase enzyme
ILs	Interleukins
H^+	Hydrogen ion
L-asnase	L-asparaginase
HSPs	Heat shock proteins
MDR	Multiple drug resistance
XDR	Extreme drug resistance
NgA	*Neisseria gonorrhoeae* L-asparaginase

ALL	Acute lymphoblastic leukaemia
Ntn	N-terminal nucleophile
TCM	Traditional Chinese medicine
FDA	Food and Drug administration
MD	Molecular dynamics
PDB	Protein data bank
BLAST	Basic Local Alignment Search Tool
FASTA	Fast alignment
MSA	Multiple sequence alignment
ID	Identity document
PubMLST	Public databases for molecular typing and microbial genome diversity
CSV	Comma-separated values
BIGSdb	Bacterial isolate genome sequence database
MEGA	Molecular evolutionary genetics Analysis
MAFFT	Multiple sequence alignment using the Fourier transformation program
UPGMA	Unweighted Pair-Group Method with Arithmetic Averaging
SE	Shannon Entropy
Log	Logarithm
Å	Angstrom
PHYRE	Protein Homology/analogy recognition engine
I-TASSER	Iterative Threading Assembly Refinement
ADMET	Chemical absorption, distribution, metabolism, excretion, and toxicity

GROMACS	Groningen Molecular Simulation
SPC	Simple point charge
PME	Particle Mesh Ewald
NPT	Constant number, pressure, temperature
NVT	constant number, volume, temperature
K	Kelvin
ps	picosecond
ns	nanosecond
MA	Massachusetts
USA	United States of America
MM-PBSA	Molecular mechanics-based Poisson Boltzmann surface area
SASA	Solvent accessibility surface area
PCR	Polymerase chain reaction
LB	Luria broth
IPTG	Isopropyl-ß-D-thiogalactopyranoside
rpm	Rotations per minute
mM	Micromolar
PMSF	Phenylmethylsulfonyl fluoride
Ni-NTA	Nickle Nitriloacetic acid
mg	Milligram
ml	Millilitres
GFC	Gel filtration chromatography
FPLC	Fast protein liquid chromatography
SDS	Sodium dodecyl sulfate

PAGE	Polyacrylamide gel electrophoresis
GE	General electronics
TCA	Trichloroacetic acid
nm	Nanometre
min	Minutes
MM	Michaelis Menten
LB	Lineweaver Burk
UV	Ultraviolet
CD	Circular dichroism
mdeg	Millidegrees
λ_{ex}	Fluorescence excitation maxima (nm)
λ_{em}	Fluorescence emission maxima (nm)
DSC	Differential scanning calorimetry
FTS	Fluorescence-based thermal shift assay
T_m	Thermal transition midpoint
DMSO	Dimethyl sulfoxide
Asn	Asparagine
Gln	Glutamine
SEC	Size exclusion chromatography
kDa	Kilodaltons
λ_{222}	Ellipticity at 222 nm
λ_{340}	Fluorescence excitation maxima at 340 nm
hERG	Human ether-a-go-go-related gene
kCal	Kilocalories

HIV	Human immunodeficiency virus
RMSD	Root means square deviations
EC	Enzyme commission
NgHtpX	*Neisseria gonorrhoeae* HtpX
Zn^{2+}	Zinc ion
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
REMA	Resazurin microtiter assay
IC_{50}	Half of maximal inhibitory concentration