DECIPHERING ALLOSTERIC REGULATION OF THE SARS-COV-2 MAIN PROTEASE AND ITS MODULATION BY SMALL MOLECULES

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by

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In reverence to Mahakaal and Lord Hanuman, symbols of strength, loyalty, and devotion,

and in deep gratitude to my parents, whose love and sacrifices have shaped my path.

CERTIFICATE

This is to certify that the thesis titled "Deciphering allosteric regulation of the SARS-CoV-2 main protease and its modulation by small molecules", submitted by Mr. Mohit Bhardwaj to the 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 him, which has been prepared under my supervision and guidance in conformity with the rules and regulations of the Indian Institute of Technology Delhi, India. The results prescribed in it have not been submitted in part or full to any other University for the award of any degree or diploma.

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ABSTRACT

The COVID-19 pandemic has highlighted the need for antiviral strategies that remain effective against viral evolution. The 3-chymotrypsin-like protease ($3CL^{pro}$) of severe acute respiratory syndrome coronavirus 2 is essential for viral replication and a major drug target. Most current inhibitors target the catalytic site, which is susceptible to resistance due to mutations. This study investigates both catalytic and allosteric mechanisms that regulate $3CL^{pro}$ activity, intending to identify alternative strategies for inhibition. We performed a comparative analysis of key catalytic residues (F140 and C145) and allosteric residues (N28 and R298) that contribute to enzyme activity and dimerization. The allosteric site affects not only enzymatic activity but also the oligomeric form of the protein. Mutational and computational analyses showed that changes in these residues disrupt critical interactions required for enzymatic function and oligomer stability. Both in-silico and in-vitro results confirmed that perturbation of these sites reduces proteolytic activity and alters the structural integrity of the enzyme. We further explored an allosteric pocket near residue Asn28 as a potential druggable site. Structure-based virtual screening identified novobiocin as a ligand for this region. Biophysical studies showed direct binding with sub-micromolar affinity ($K_d \sim 3 \times 10^{-7}$ M). Ligand binding reduced thermal stability and affected dimer formation. Enzymatic assays showed a strong decrease in catalytic turnover with minimal impact on substrate binding, indicating an allosteric mode of inhibition. Molecular docking and simulation analyses supported stable binding and revealed localized changes in protein dynamics. These findings establish that both catalytic and allosteric sites play essential roles in regulating $3CL^{pro}$ function. Targeting allosteric regions such as the Asn28-associated pocket offers a promising approach for antiviral development. This strategy may provide improved specificity and reduce the likelihood of resistance compared to active-site inhibitors.

सार

COVID-19 महामारी ने ऐसी एंटीवायरल रणनीतियों की ज़रूरत को उजागर किया है जो वायरस के बदलते स्वरूप (इवोल्यूशन) के खिलाफ भी असरदार बनी रहें। 'सीवियर एक्यूट रेस्पिरेटरी सिंड्रोम कोरोनावायरस 2' (SARS-CoV-2) का 3-काइमोट्रिप्सिन-जैसा प्रोटीएज़ (3CLpro) वायरस के रेप्लिकेशन (प्रतिकृति बनाने) के लिए ज़रूरी है और दवा के लिए एक मुख्य टारगेट है। अभी इस्तेमाल हो रहे ज़्यादातर इनहिबिटर (रोकने वाले) कैटेलिटिक साइट को टारगेट करते हैं, जो म्यूटेशन के कारण रेजिस्टेंस (प्रतिरोध) विकसित होने के प्रति संवेदनशील होती है। यह स्टडी 3CLpro की गतिविधि को नियंत्रित करने वाले कैटेलिटिक और एलोस्टेरिक दोनों तरह के मैकेनिज़्म की जांच करती है, ताकि इसे रोकने के लिए वैकल्पिक तरीके खोजे जा सकें। हमने मुख्य कैटेलिटिक रेसिड्यू (F140 और C145) और एलोस्टेरिक रेसिड्यू (N28 और R298) का तुलनात्मक विश्लेषण किया, जो एंजाइम की गतिविधि और डाइमेराइज़ेशन में योगदान करते हैं। एलोस्टेरिक साइट न केवल एंजाइम की गतिविधि को प्रभावित करती है, बल्कि प्रोटीन के ओलिगोमेरिक रूप को भी प्रभावित करती है। म्यूटेशनल और कंप्यूटेशनल विश्लेषणों से पता चला कि इन रेसिड्यू में बदलाव एंजाइम के काम करने और ओलिगोमर की स्थिरता के लिए ज़रूरी अहम इंटरैक्शन को बाधित करते हैं। इन-सिलिको और इन-विट्रो दोनों परिणामों ने पुष्टि की कि इन साइट्स में गड़बड़ी से प्रोटीओलिटिक गतिविधि कम हो जाती है और एंजाइम की संरचनात्मक अखंडता बदल जाती है। हमने रेसिड्यू Asn28 के पास एक एलोस्टेरिक पॉकेट को दवा के लिए संभावित साइट के तौर पर और खोजा। स्ट्रक्चर-आधारित वर्चुअल स्क्रीनिंग से इस क्षेत्र के लिए नोवोबायोसिन (novobiocin) की पहचान लिगैंड के रूप में हुई। बायोफिजिकल स्टडीज़ ने सब-माइक्रोमोलर एफिनिटी ($K_d \sim 3 \times 10^{-7}$ M) के साथ सीधे बाइंडिंग दिखाई। लिगैंड बाइंडिंग ने थर्मल स्थिरता को कम किया और डाइमर बनने की प्रक्रिया को प्रभावित किया। एंजाइमेटिक परीक्षणों से पता चला कि सबस्ट्रेट बाइंडिंग

पर बहुत कम असर के साथ कैटेलिटिक टर्नओवर में भारी कमी आई, जो एलोस्टेरिक तरीके से रोकने का संकेत देता है। मॉलिक्यूलर डॉकिंग और सिमुलेशन विश्लेषणों ने स्थिर बाइंडिंग का समर्थन किया और प्रोटीन डायनामिक्स में स्थानीय बदलावों का खुलासा किया। ये निष्कर्ष बताते हैं कि कैटेलिटिक और एलोस्टेरिक दोनों साइट्स 3CLpro के काम को नियंत्रित करने में अहम भूमिका निभाती हैं। Asn28-से जुड़ी पॉकेट जैसे एलोस्टेरिक क्षेत्रों को टारगेट करना एंटीवायरल विकास के लिए एक आशाजनक तरीका है। यह रणनीति एक्टिव-साइट इनहिबिटर की तुलना में बेहतर विशिष्टता प्रदान कर सकती है और रेजिस्टेंस की संभावना को कम कर सकती है।

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ABBREVIATIONS AND SYMBOLS

A_{280}	Absorbance of the protein-dye conjugate at 280 nm
bp	Base pair
cm	Centimeter
CD	Circular dichroism
°C	Degree Celsius
Da	Dalton
dH2O	Distilled water
ddH2O	Double distilled water (sterilised distilled water)
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylene diamine tetra-acetic acid (disodium salt)
ε	Weight extinction coefficient of the solute
$3CL^{pro}$	3-chymotrypsin-like protease
g	Gram
HCl	Hydrochloric acid
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid

IPTG	Isopropyl-1-thio-β-D-galactopyranoside
kb	Kilo base
k_{cat}	Turnover number
KCl	Potassium chloride
kDa	Kilodalton
K_d	Equilibrium dissociation coefficient
K_m	Michaelis coefficient
l	Sample pathlength
LB	Luria-Bertani
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate
mg	Milligram
min	Minute
ml/l	Milliliter per liter
µM	Micromolar
µmol	Micromole
µg	Microgram
mM	Millimolar
M	Molar
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2

COVID-19	Coronavirus Disease of 2019
MW	Molecular weight
NaCl	Sodium chloride
NSPs	Non-structural proteins
NaOH	Sodium hydroxide
nm	Nanometer
FDA	Food and Drug Administration
RMSD	Root mean square deviation
RMSF	Root mean square fluctuation
RoG	Radius of gyration
MERS	Middle East respiratory syndrome
MD Simulation	Molecular Dynamics Simulation
OD_{600}	Optical density at 600 nm
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PCA	Principal component analysis
GFC	Gel Filtration Chromatography
pI	Isoelectric point
DSC	Differential scanning calorimetry
HTS	High-throughput screening

SPR	Surface Plasmon Resonance
Psi	Pounds per square inch
rpm	Revolutions per minute
[S]	Substrate concentration
$S_{0.5}$	Substrate concentration at 1/2 V_{max} (Hill model)
F-C reagent	Folin–Ciocâlteu's reagent
SDS	Sodium dodecyl sulphate
SOC	Super Optimal broth with Catabolite repression
Tm	Melting temperature
k_d	Dissociation rate constant
k_a	Association rate constant
ITC	Isothermal Titration Calorimetry
UV	Ultra violet
TCA	Trichloroacetic acid
V_{max}	Maximum reaction velocity
CBB	Coomassie brilliant blue
v/v	Volume to volume
TBE	Tris-borate-EDTA
w/v	Weight to volume