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CaNCR63: A Nodule-Specific Cysteine Rich Peptide from *Cicer arietinum* L. Locks the Lateral Gate of the *E. coli* BAM Complex

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A B S T R A C T

The emergence of multidrug-resistant Gram-negative bacteria underscores the need for antibiotics with novel mechanisms of action. The essential *β*-barrel assembly machinery (BAM) complex, which is crucial for the biogenesis of outer membrane proteins (OMPs), represents as a promising therapeutic target. In this study, we investigate the potential role of CaNCR63, a nodule-specific cysteine-rich (NCR) peptide derived from chickpea (*Cicer arietinum*), as a novel inhibitor of BamA complex. Using a multi-step computational approach, we confirmed CaNCR63's antimicrobial peptide-like physicochemical characteristics and the presence of a conserved *γ*-core motif, justifying its selection. Comparative molecular docking between CaNCR63 and darobactin against the *E. coli* BAM complex showed a high-affinity binding configuration for CaNCR63 at the functionally significant lateral gate of BamA, a site also targeted by the known antibiotic darobactin. To validate this static model, a 100 ns all-atom molecular dynamics simulation of the CaNCR63-BamA complex within a model bacterial membrane was conducted. The simulation verified the formation of a dynamically stable complex, marked by a persistent network of intermolecular contacts and hydrogen bonds. Binding free energy calculations (MM/PBSA) suggested a strong, sub-nanomolar binding affinity (ΔG_{bind} = -12.75 kcal/mol), predominantly driven by electrostatic interactions. Additionally, analysis of system's dynamics showed that CaNCR63 binding allosterically modulates the flexibility of the entire BAM complex, limiting the conformational plasticity of the lateral gate. Collectively, these findings provide a cohesive, multi-scale model suggesting that CaNCR63 functions as a darobactinlike inhibitor by physically locking the lateral gate, thereby impeding the OMP assembly process. This research supports the potential of plant-derived NCR peptides as a promising chemical framework for developing new antibiotics targeting Gram-negative pathogens.

Introduction

The rise of antibiotic resistance has become one of the most critical public health issues of the 21st century. The World Health Organization (WHO) has continuously warned that without immediate and coordinated actions, common infections and even minor injuries could once again prove deadly (Sabtu, Enoch, and Brown 2015). Resistant pathogens are already responsible for millions of infections each year, leading to longer hospital stays, increased healthcare costs and higher mortality rates (Thorpe, Joski, and Johnston 2018). Worryingly, the development of new antibiotics has not kept pace with the rapid development of resistance, resulting in a dangerous gap in treatment options. In this context, multidrug-resistant (MDR) and extensively drug-resistant (XDR) organisms, often referred to as "superbugs," pose a particularly severe threat to global health security (Fatima *et al.,* 2023).

Among the various groups of resistant bacteria, Gram-negative organisms such as *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii Salmonella enterica*, etc. are particularly concerning (Chan-Tompkins 2011; Trigg 2023). These bacteria are significant contributors to hospital-acquired infections, such as ventilator-associated pneumonia, bloodstream infections, urinary tract infections and wound or surgical site infections, where treatment often fails, resulting in unfavorable clinical outcomes (Abban *et al.,* 2023).

This resistance is primarily due to their distinctive envelope structures. Unlike Gram-positive bacteria, which have a thick yet permeable peptidoglycan wall, gram-negative bacteria have developed a complex dual-membrane system. Their system includes an inner cytoplasmic membrane, a thin peptidoglycan layer and an asymmetric outer membrane rich in lipopolysaccharides (LPS). This configuration confers both structural strength and inherent resistance to numerous antibiotics (Chan-Tompkins 2011).

The outer membrane of Gram-negative bacteria acts as an effective permeability barrier, blocking the entry of many hydrophobic and hydrophilic molecules, along with the majority of standard antibiotics (Masi, R´efregiers, Pos, *et al.,* 2017). In addition to passive defense, the bacteria developed several adaptive mechanisms to counter the effects of therapeutic substances. Notably, these include multidrug efflux pumps that actively pump toxic substances out of the cell (Blair, Richmond, and Piddock 2014), periplasmic enzymes like *beta*-lactamases, which degrade antibiotics before they have reached vital targets (Schaenzer and Wright 2020) and the ability to create biofilms, which create a sheltered microenvironment for bacterial populations (Dincer, Uslu, and Delik 2020). These mechanisms significantly reduce the efficacy of available drugs, leaving healthcare providers with only last-resort treatments, such as polymyxins or colistin agents, which are associated with considerable toxicity and emerging resistance (Vardakas and Falagas 2017).

As the outer membrane is crucial for the survival of Gram-negative bacteria, the molecular machinery involved in its formation acts as an promising target for therapeutic intervention (Urfer *et al.,* 2016; Srinivas *et al.,* 2010). A key player in this process is the β-barrel assembly machinery (BAM) complex, which facilitates the folding and integration of outer membrane proteins (OMPs) (Xu, Guo, and Yu 2023). At the heart of this complex is BamA, a highly conserved insertase that is vital for the biogenesis of OMPs. These proteins are essential for nutrient uptake, cell communication and maintaining the structural integrity of the bacterial envelope. Owing to its critical role and structural accessibility, BamA is considered a prime target for the development of antibacterial drugs (Vij *et al.,* 2018; Di Somma *et al.,* 2022).

Along with this, recent progress in structural biology has offered remarkable insights into the structure and conformational dynamics of BamA. High-resolution crystal and cryo-EM structures of the *E. coli* BAM complex (e.g., PDB ID: 5D0O) demonstrate that BamA can shift between various functional states, such as the inward and lateralopen conformations (Seyfert *et al.,* 2023).

A key structural characteristic is the lateral gate, composed of β-strands 1 and 16, which opens and closes to aid in the insertion of OMPs into the outer membrane (Ritzmann *et al.,* 2022). These conformational transitions create temporary but targetable pockets that can be explored for inhibition using small molecules or peptides.

Several studies have shown that BamA can be inhibited by both peptides and small molecules (Kaur *et al.,* 2021; Peterson, Doyle, and Bernstein 2022; Yang *et al.,* 2023; Morgan E. Walker *et al.,* 2025b; Peterson, Doyle, and Bernstein 2022). For example, the ribosomal peptide

darobactin attaches to BamA, stabilizing a laterally closed conformation and thus obstructing OMP biogenesis. Similarly, macrocyclic peptides discovered through mRNA display can embed themselves within the BamA β-barrel lumen, hindering the initiation of the assembly process. Both computational and experimental data indicate that small molecules can bind at the lateral gate, disrupting β-augmentation and gating dynamics (Haysom *et al.,* 2023). Together, these studies reveal at least two separate inhibitory sites on BamA, the lateral gate seam and the intra-barrel lumen, both of which are accessible to peptides.

Although there have been encouraging developments, the exploration of naturally occurring peptide frameworks as inhibitors of BamA is still in its early stages. A particularly interesting group of candidates is legume nodule-specific cysteine-rich (NCR) peptides.

These NCR peptides are small, positively charged antimicrobial peptides stabilized by disulfide bonds and are known for their broad-spectrum activity and ability to influence bacterial physiology. In chickpeas (*Cicer arietinum*), a variety of NCRs have been discovered, including CaNCR63, which has shown strong antimicrobial effects (Lima *et al.,* 2022). Given the known vulnerability of BamA to inhibition by peptides, CaNCR63 stands out as a promising candidate for targeting this crucial bacterial protein.

This study explores the potential of CaNCR63 as a BamA inhibitor through a comprehensive *in silico* approach. We began by examining its physicochemical properties and similarity to antimicrobial peptides (AMPs), followed by the development of precise three-dimensional structural models. Subsequently, molecular docking was employed to predict the potential binding configurations of CaNCR63 at both the lateral gate and the intra-barrel lumen of BamA.

The top-ranking complexes were subjected to explicitsolvent molecular dynamics (MD) simulations within a membrane environment to assess binding stability, interaction networks and free energy profiles. Our findings identify specific interaction patterns, such as backbone β-augmentation at the lateral gate and electrostatic anchoring within the lumen, which mirror previously known BamA–peptide interactions. Collectively, these computational insights provide a foundation for the future experimental validation of CaNCR63 as a potential antibacterial agent.

Materials and Methods

Peptide sequence, AMP-likeness and physicochemical profiling

The primary sequence ”KMICKTRVDCKKYRCPRSK IKDCVKGYCRCVRKK” of CaNCR63, a six-cysteine NCR peptide from *Cicer arietinum*, was curated from the chickpea NCR catalog (Montiel *et al.,* 2017). To establish its antimicrobial peptide (AMP) potential and guide downstream modeling, physicochemical and AMP-like descriptors were systematically computed. Parameters, including sequence length, net charge at physiological pH 7, hydrophobic ratio, Boman index and instability index, were derived. Probabilistic AMP-likeness scores were obtained using two complementary approaches: the AMPir R package (default classifiers) and the CAMP-R3 server (support vector machine, random forest and discriminant function models; default thresholds).

Peptide 3D Modeling and Receptor Structure Retrieval

Initial three-dimensional conformers of CaNCR63 were modeled using the SWISSMODEL server, which generated structures based on homologous templates. Each conformer was then evaluated for stereochemical accuracy, with a specific focus on the geometry of its disulfide bonds. For the receptor, the *E. coli* BAM complex was retrieved from the Protein Data Bank (PDB ID: 5D0O) (Gu *et al.,* 2016).

The structure was then prepared for docking by removing non-essential heteroatoms and crystallographic water molecules by using UCSF Chimera, while any missing side chains were rebuilt using standard refinement tools (Gu *et al.,* 2016; Haysom *et al.,* 2023; Morgan E. Walker *et al.,* 2025b).

Molecular Docking of CaNCR63 to the BamA Complex

Molecular docking was performed to predict the binding pose and affinity of CaNCR63 by using the Dockit pipeline. The minimized, low-energy conformers of CaNCR63 were treated as ligands, while the prepared BamA receptor structure (PDB ID: 5D0O) was kept rigid to maintain the integrity of the binding pocket. The docking strategy was designed to thoroughly probe the

mechanistically significant lateral gate region, a site known to be the binding target for the antibiotic darobactin.

Membrane embedding and system building

The most promising BamA–CaNCR63 complexes identified from docking were embedded into explicit lipid bilayers using the CHARMM-GUI Membrane Builder. Symmetric bilayers were constructed to approximate the native *E. coli* inner membrane composition, consisting of phosphatidylethanolamine (POPE), phosphatidylglycerol (POPG) and diphosphatidylglycerol (DPPG). Peptide disulfide connectivity was preserved throughout the embedding and patching processes. Each system was solvated with TIP3P water molecules and neutralized with 150mM NaCl to mimic the physiological ionic strength. The following force fields were assigned: CHARMM36m for proteins and peptides and CHARMM36 for lipid components. The final periodic simulation boxes were approximately 150 A˚ in the lateral dimensions, with exact sizes determined from CHARMM-GUI outputs.

Molecular dynamics simulations

All molecular dynamics (MD) simulations were performed with GROMACS (versions 2023/2024) using a leap-frog integrator and a 2 fs time step. Neighbor lists were generated using the Verlet cutoff-scheme with a 1.2 nm cutoff for the short-range interactions. Long-range electrostatic interactions were treated using the Particle Mesh Ewald (PME) method with a real-space cutoff of 1.2 nm. Van der Waals interactions were truncated at 1.2 nm using a force-switch modifier between 1.0 and 1.2 nm. The bond lengths involving hydrogen were constrained using the LINCS algorithm. Temperature was maintained at 303.15 K using the Velocity-rescale (v-rescale) thermostat with a 1.0 ps time constant. Pressure was controlled semi-isotropically at 1 bar using the C-rescale barostat, with a time constant of 5.0 ps and a compressibility of 4.5e-5 bar^{-1}.

Prior to production runs, each system underwent the standard CHARMM-GUI equilibration protocol, consisting of steepest-descent energy minimization followed by staged NVT and NPT equilibration phases with gradually released positional restraints on proteins, peptides and lipids. For a 100 ns production run, the total number of steps should be set to 50,000,000 (nsteps = 50000000). To save trajectory snapshots every 10 ps as stated, the output frequency should be set to 5,000 steps (nstxout-compressed = 5000). These protocols closely follow previously established MD studies of ligandbound BamA in membrane environments (Kuo *et al.,* 2023; Haysom *et al.,* 2023; Morgan E. Walker *et al.,* 2025b).

Trajectory Analysis and Binding Energetics

Simulation trajectories were post-processed to remove periodicity and center the protein–peptide complexes within the simulation box. Structural stability was assessed by calculating the backbone root-mean-square deviation (RMSD) over time, while flexibility profiles were quantified using per-residue root-mean-square fluctuations (RMSF), and overall compactness was evaluated using the radius of gyration. Interfacial stability was characterized by monitoring the formation and persistence of hydrogen bonds at the peptide–BamA and peptide–lipid interfaces. In addition, contact map and hydrogenbond occupancy analyses were performed to identify recurring interaction motifs that contributed to complex stabilization. To quantify the thermodynamic favorability of CaNCR63 binding to BamA, the binding free energy (ΔG_{bind}) was estimated from these trajectories using the MM-PBSA approach. Calculations were performed on equilibrated snapshots extracted from the production windows (typically 40–100ns).

Results and Discussion

Physicochemical Profiling and In Silico Analysis Confirm the Antimicrobial Potential of CaNCR63

Initial characterization of the CaNCR63 peptide confirmed its strong antimicrobial peptide (AMP) activity. The three-dimensional model (Figure 1) reveals a compact, stable fold maintained by three disulfide bonds, which is critical for its function. Physicochemical characterization (Table 1) revealed a strongly cationic nature, with a net charge of +11.6 and an isoelectric point (pI) of 10.6, properties known to facilitate electrostatic attraction to the negatively charged surfaces of bacterial membranes. Furthermore, a low instability index (11.00) and a negative GRAVY score (-0.947) indicate that CaNCR63 is a stable, hydrophilic peptide well-suited to function in the aqueous periplasm where the BAM complex is located. The presence of a conserved gamma-core motif (GYCRC at residues 25-29), a known feature

in many AMPs, provided a strong rationale for investigating its interaction with a key bacterial target (Yount and Yeaman 2004).

To further validate its potential, the peptide sequence was analyzed by eight different *in silico* prediction tools (Table 2). Consistent with previous experimental reports of its broad-spectrum activity against pathogens like *A. baumannii* and *C. albicans* (Lima *et al.,* 2022), six out of the eight predictors (75%) classified CaNCR63 as a putative AMP. This strong computational consensus reinforced its selection as a candidate for targeting the essential BamA protein.

Molecular Docking Predicts a High-Affinity Binding Pose at the BamA Lateral Gate

Given its characteristic AMP properties, we hypothesized that CaNCR63 could directly inhibit the essential BamA complex. Molecular docking was performed using the *E. coli* BamA structure (PDB: 5D0O) and the results were compared to those of the known inhibitor, darobactin. The docking revealed a distinct "funnel-shaped" energy landscape for both ligands, where the most energetically favorable poses were also the most structurally consistent (Figure 2c), indicating a specific binding event. CaNCR63 achieved a top-scoring affinity of -8.1 kcal/mol. While less than darobactin's -10.5 kcal/mol, this value still reflects a strong and potentially biologically relevant interaction.

Crucially, the top-ranked poses for both CaNCR63 and darobactin were located in the same functional pocket: the periplasmic region of BamA, adjacent to the lateral gate formed by β-strands 1 and 16 (Figure 2a, b). Analysis of the static docked model identified a robust network of interactions stabilizing the CaNCR63 pose, including numerous hydrogen bonds and two key salt bridges between ASP 512/ARG 526 of BamA and ARG 31/ASP 21 of the peptide, respectively (Table 3, Figure 2d). This generated a precise, testable hypothesis: CaNCR63 functions as a competitive inhibitor by occupying the lateral gate in a manner analogous to darobactin.

Molecular Dynamics Simulations Validate a Stably Bound Complex

To test the stability of the docked pose, the top-ranked BamA-CaNCR63 complex was subjected to a 100 ns all-atom MD simulation in a model bacterial membrane. The simulation confirmed the formation of a stable and persistent complex. The backbone RMSD of the entire complex equilibrated after approximately 40 ns and remained stable (Figure 4A). Similarly, the radius of gyration (Rg) of the complex indicated a consistent, compact structure (Figure 4B).

The stability of the binding interface was assessed by monitoring intermolecular contacts. The number of noncovalent contacts and hydrogen bonds maintained a stable plateau after initial equilibration, demonstrating a persistent interaction network (Figure 3A, C). The CaNCR63 peptide itself remained stably folded within the binding pocket, as shown by its own backbone RMSD (Figure 6A).

The per-residue fluctuations (RMSF) of the peptide and each BAM subunit were also analyzed, revealing localized changes in dynamics upon binding (Figure 5 and 6B). Together, these results provide strong evidence that the docked configuration represents a true, dynamically stable bound state.

Binding Free Energy Calculations Reveal a Sub-Nanomolar Affinity

To quantify the strength of the stable CaNCR63–BamA interaction, the binding free energy was calculated using the MM/PBSA method over the equilibrated segment of the trajectory (last 60 ns).

The analysis revealed an exceptionally favorable average binding free energy (ΔG_{bind}) of -12.75 kcal/mol (Table 4). This value corresponds to a predicted dissociation constant (Kd) in the sub-nanomolar range (≈0.5 nM), which is characteristic of high-affinity, drug-like biological complexes.

The decomposition of the energy components confirmed that the binding is overwhelmingly driven by favorable electrostatic forces (ΔE_{EEL} = −239.52 kcal/mol), which powerfully overcome the energetic penalty of desolvating the charged residues upon binding (ΔG_{solv} = +224.53 kcal/mol).

This confirms that the network of salt bridges and hydrogen bonds identified in the docked configuration served as the predominant stabilizing force underpinning the formation of this potent inhibitory complex.

Table.1 Physicochemical Properties of the Peptide

Property	Value
Molecular Weight (Da)	4125.140
Isoelectric Point (pI)	10.600
Net Charge (at pH 7)	11.620
Aliphatic Index	48.530
Instability Index	11.000
GRAVY Index	-0.947

Table.2 *In Silico* Antimicrobial Peptide (AMP) Prediction for CaNCR63

Prediction Tool	Value / Class	Classification	Reference
ampir	0.990	AMP	**(Fingerhut *et al.*, 2020)**
AmpGram	0.686	AMP	**(Oliveira Maciel, Pires, and Franco 2017)**
CAMPR4 (RF)	0.520	AMP	**(Waghu *et al.*, 2016)**
CAMPR4 (SVM)	0.510	AMP	**(Waghu *et al.*, 2016)**
CAMPR4 (DA)	0.510	AMP	**(Waghu *et al.*, 2016)**
CAMPR4 (ANN)	0.510	AMP	**(Waghu *et al.*, 2016)**
APD3	48.96	Non-AMP	**(G. Wang, Li, and Z. Wang 2016)**
DBAASP	**Non-AMP**	**Non-AMP**	**(Pirtskhalava *et al.*, 2021)**

Table.3 Interaction analysis between BamA (Chain A) and CaNCR63 (Chain F) from the docked complex.

BamA Residue	CaNCR63 Residue	Interaction Type
GLU 470	CYS 27	Hydrogen Bond
PRO 476	ARG 16	Hydrogen Bond
GLY 482	ARG 16	Hydrogen Bond
GLY 487	ARG 28	Hydrogen Bond
ARG 488	ARG 31	Hydrogen Bond
SER 508	TYR 26	Hydrogen Bond
ASP 512	ARG 31	Hydrogen Bond
THR 514	VAL 30	Hydrogen Bond
ARG 526	ASP 21	Hydrogen Bond
GLY 584	ASP 8	Hydrogen Bond
GLY 584	VAL 7	Hydrogen Bond
GLY 584	LYS 11	Hydrogen Bond
ASN 594	ASP 21	Hydrogen Bond
TYR 618	ASP 8	Hydrogen Bond
SER 657	LYS 4	Hydrogen Bond
SER 658	LYS 4	Hydrogen Bond
SER 658	ILE 2	Hydrogen Bond
GLN 775	ASP 8	Hydrogen Bond
THR 809	MET 1	Hydrogen Bond
LEU 581	VAL 7	Hydrophobic
ASP 512	ARG 31	Salt Bridge
ARG 526	**ASP 21**	Salt Bridge

Table.4 Binding free energy components for the BamA-CaNCR63 complex. All values are in kcal/mol.

Energy Component	Value (kcal/mol)
Van der Waals Energy (ΔE_{VDW})	-0.22
Electrostatic Energy (ΔE_{EEL})	-239.52
Polar Solvation Energy (ΔG_{solv})	+224.53
Total Binding Energy (ΔG_{bind})	-12.75

Figure.2 3-D structure of the CaNCR63

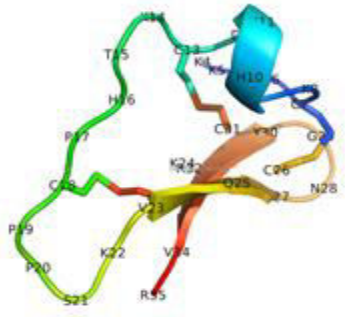


Figure.2 Comparative analysis of ligand docking to the BamA protein. The left panel shows the best docking poses for (a) the BamA-Darobactin complex and (b) the BamA- CaNCR63 complex. The right panel displays (c) the binding affinity funnel plot for both ligands and (d) a detailed view of the key interacting residues within the BamA-CaNCR63 binding site.

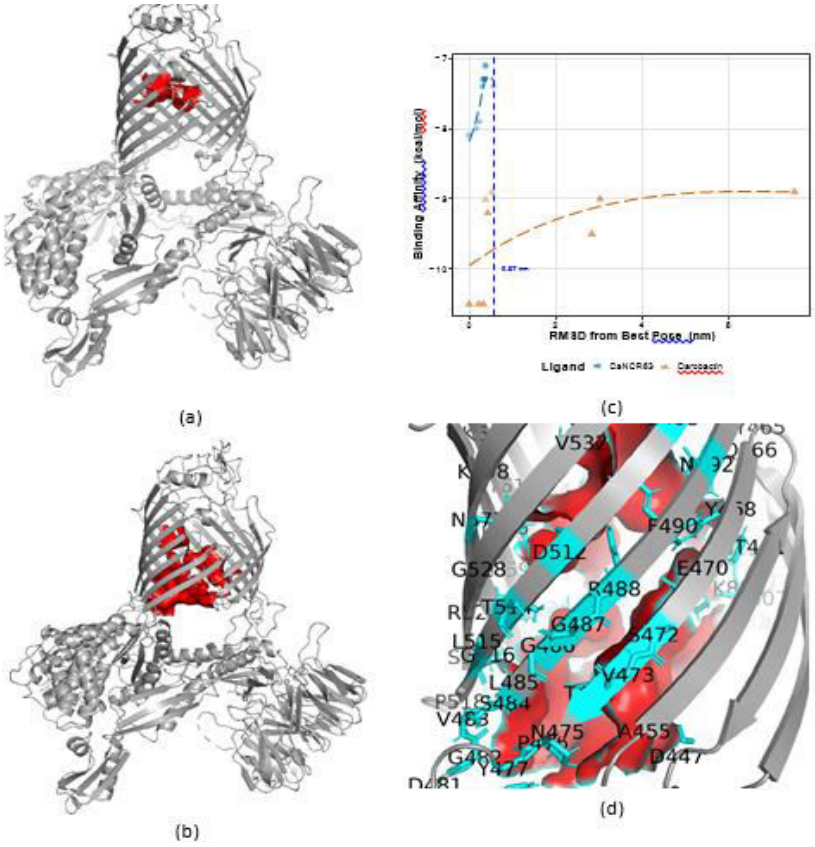


Figure.3 Analysis of CaNCR63-BamA intermolecular interactions over the 100 ns MD simulation. (A) Total noncovalent contacts. (B) Minimum distance. (C) Number of intermolecular hydrogen bonds. (D) H-bond distance distribution. (E) H-bond angle distribution. The stable plateaus in (A-C) demonstrate a persistent binding interface.

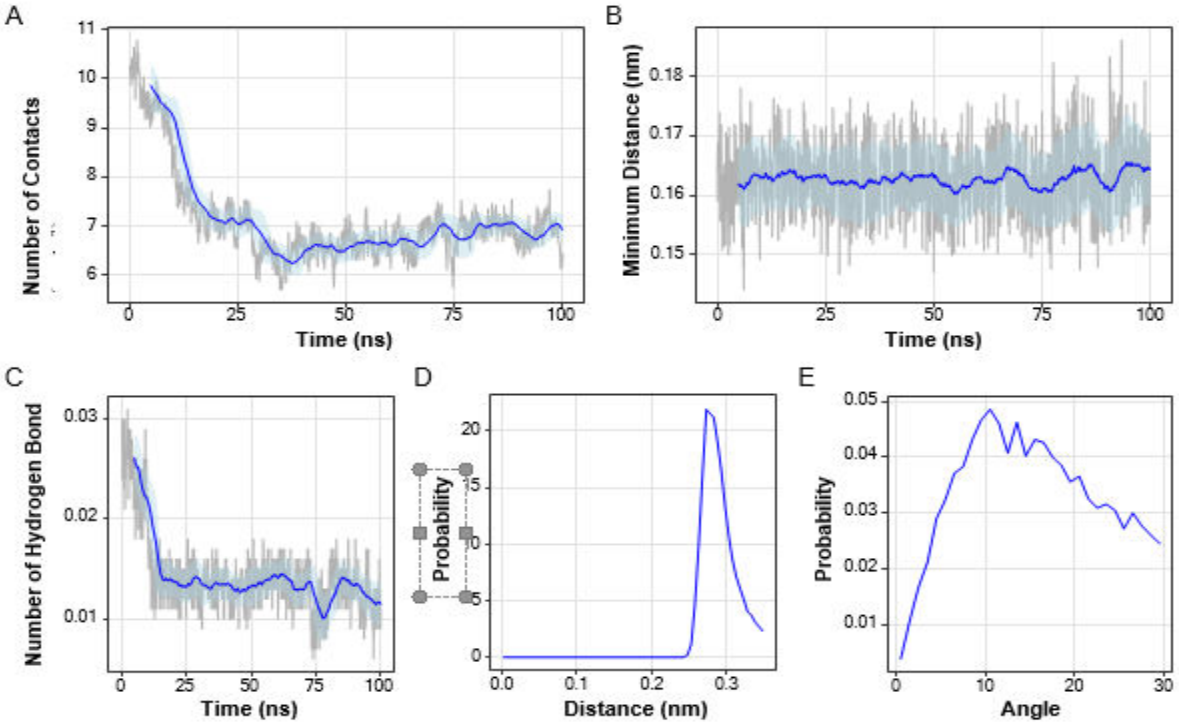


Figure.4 Global stability of the BamABCDE-CaNCR63 complex. (A) Backbone RMSD of the apo-BamA (red) and the complex (blue). (B) Radius of Gyration (Rg) of the apo-BAM complex and the CaNCR63-bound complex. The stable RMSD and Rg trajectories confirm the structural integrity of the complex.

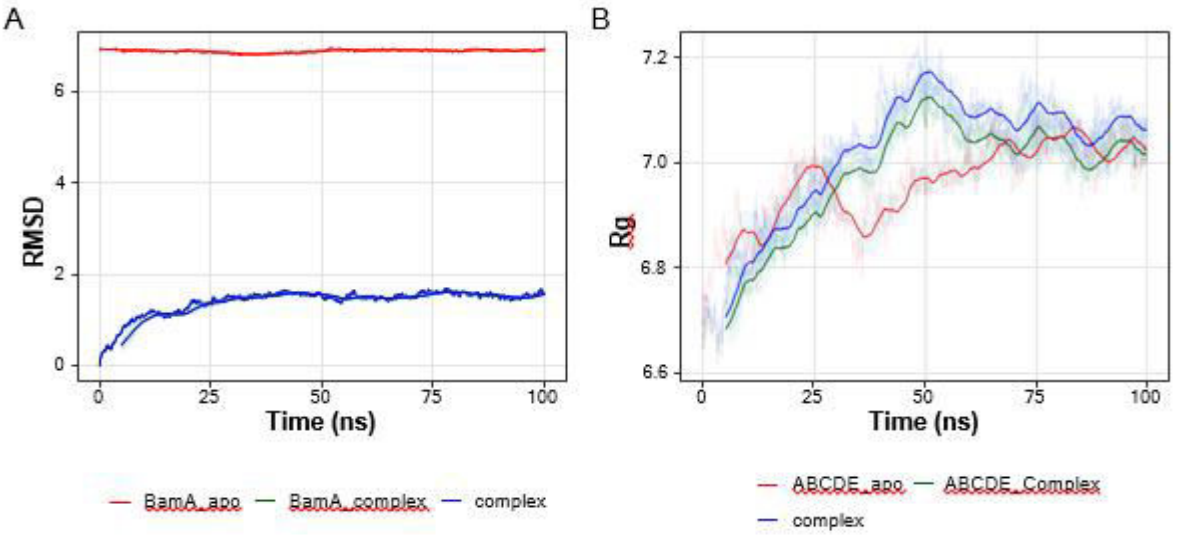


Figure.5 Subunit-specific stability and flexibility. Backbone RMSD (left panels) and RMSF (right panels) are shown for each subunit of the BAM complex in both the peptide- bound (complex) and unbound (apo) states. (A, B) BamA. (C, D) BamB. (E, F) BamC. (G, H) BamD. (I, J) BamE.

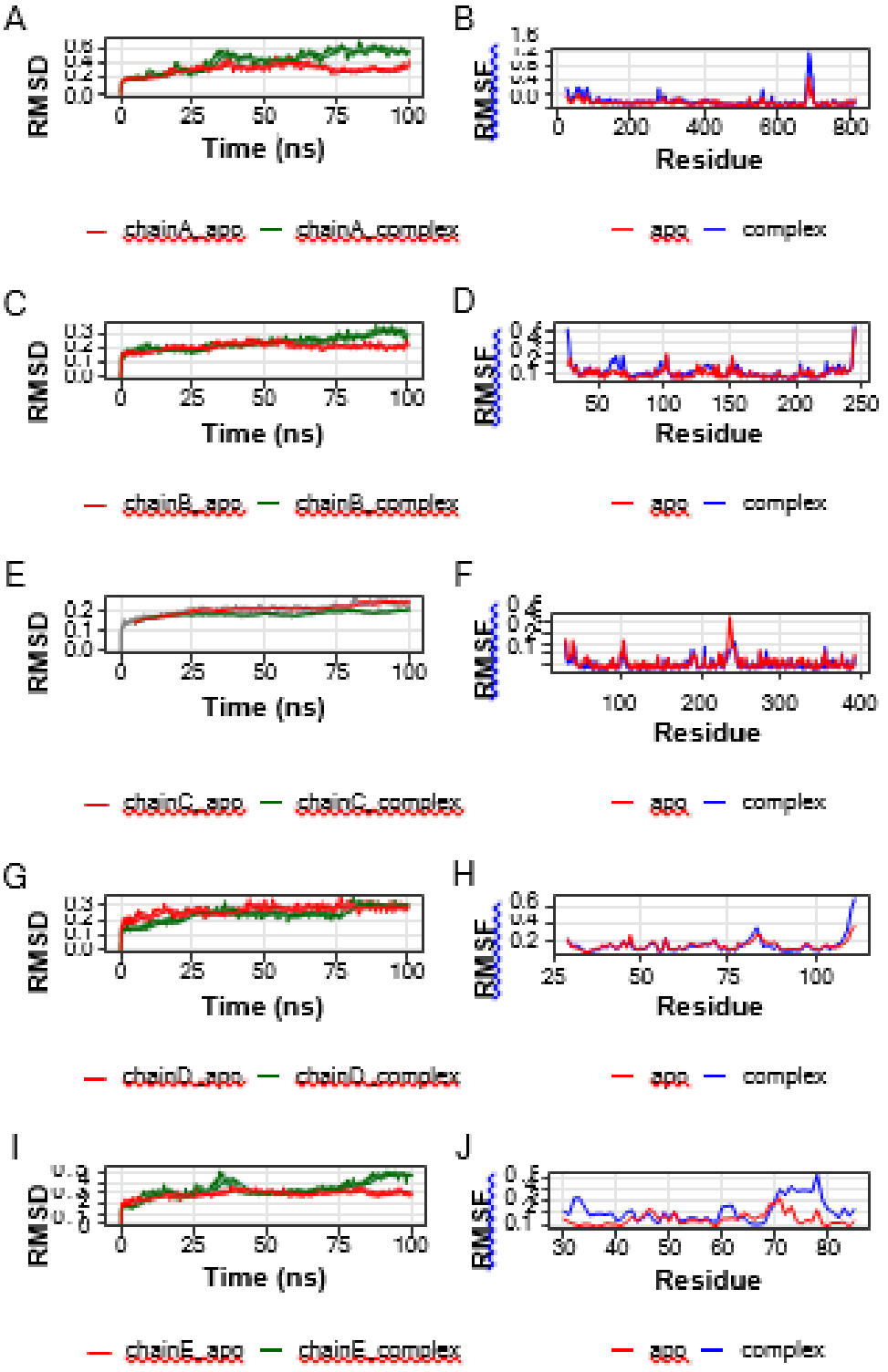
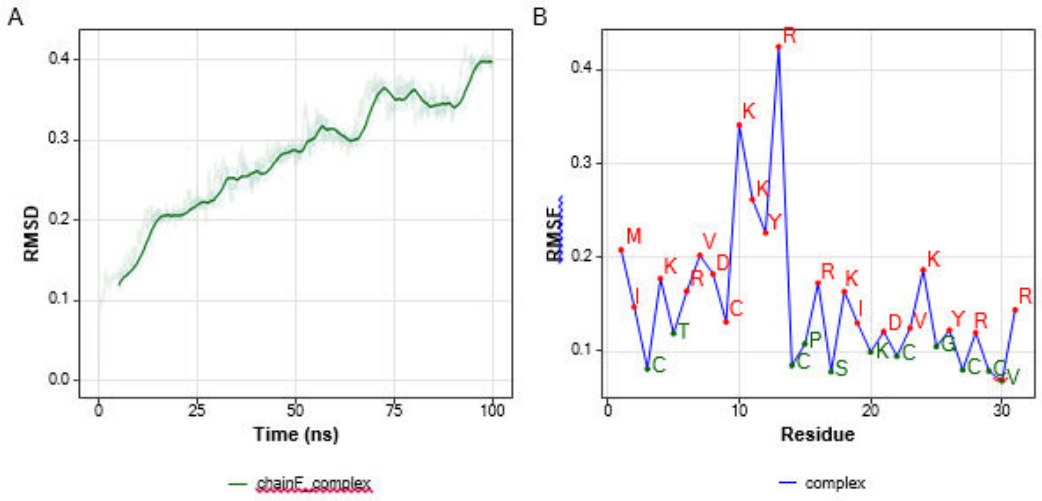


Figure.6 Conformational stability of the bound CaNCR63 peptide. (A) Backbone RMSD of the peptide during the 100 ns simulation. (B) Per-residue RMSF of the peptide. The stable RMSD indicates the peptide maintains a well-defined conformation within the binding pocket.



The rapid emergence of multidrug-resistant Gram-negative bacteria constitutes a global health emergency, making the discovery of novel antibiotics with new mechanisms of action a priority (Sabtu, Enoch, and Brown 2015; Fatima *et al.,* 2023). The *β*-barrel assembly machinery (BAM) complex has emerged as a promising target (Xu, Guo, and Yu 2023; Vij *et al.,* 2018). It is essential for bacterial viability, highly conserved across pathogenic Gram-negative species, and located in the outer membrane, making it accessible to external agents (Noinaj, Gumbart, and Buchanan 2017). Importantly, it lacks a human homolog, which significantly reduces the potential for host toxicity and is not essential for growth and hence lacks resistance if not (Gu *et al.,* 2016). Our findings reveal that the plant-derived peptide CaNCR63 acts as a potent inhibitor of the central component of the BAM complex, BamA, employing a sophisticated mechanism of action.

A Stable, High-Affinity Complex at the BamA Lateral Gate

The multi-stage computational analysis paints a clear and cohesive understanding of how CaNCR63 interacts with the essential BamA component of the BAM complex. The initial docking prediction of a favorable binding pose at the BamA lateral gate (Figure 2, Table 3) was validated by 100 ns of all-atom molecular dynamics simulation. Throughout the simulation, the peptide remained stably bound, as reflected by the equilibrated RMSD of the complex and peptide (Figure 4A and Figure 6A). This stability differentiates the interaction from transient nonspecific associations and establishes CaNCR63 as a true molecular binder. The observed stability is underpinned by a robust network of noncovalent interactions, including a significant number of hydrogen bonds and salt bridges, which stabilize after an initial equilibration phase (Figure 3).

The calculated binding free energy of -12.75 kcal/mol corresponds to a predicted sub-nanomolar dissociation constant (Kd ≈ 0.5 nM), indicative of a high-affinity drug–target interaction. This predicted affinity is comparable to, and potentially stronger than, that reported for the clinically significant antibiotic darobactin (estimated Kd in the nanomolar to low micromolar range) (Imai *et al.,* 2019; Haysom *et al.,* 2023; Kaur *et al.,* 2021). Although MM/PBSA calculations may overestimate the absolute energies, the magnitude of this value strongly supports a highly stable complex dominated by electrostatic interactions. Taken together, these findings suggest that CaNCR63 bhomologyinds to BamA in a manner consistent with potent inhibitory peptides, reinforcing its potential as a novel scaffold for next-generation antibiotics targeting gram-negative pathogens.

A Darobactin-like Inhibitory Mechanism via Lateral Gate Sequestration

The mechanism of binding appears to be dominated by strong electrostatic interactions, as evidenced by the

MM/PBSA energy decomposition (Table 4) and the specific salt bridges identified in the initial docked pose between charged residues on BamA (ASP 512, ARG 526) and CaNCR63 (ARG 31, ASP 21) (Table 3). This mode of action is highly consistent with the known biology of NCR peptides, which are often highly cationic and have evolved to interact potently with microbial targets (Lima *et al.,* 2022; Tiricz *et al.,* 2013). The fact that CaNCR63, a peptide involved in symbiotic plant-microbe relationships, exhibits such strong binding to an essential bacterial protein is a fascinating example of evolutionary co-option, where molecules developed for one biological purpose have potent applications in another (Roy *et al.,* 2020).

Crucially, the binding pose of CaNCR63 adjacent to the lateral gate—formed by β-strands 1 and 16—positions it to be an effective inhibitor in a manner analogous to darobactin (Gu *et al.,* 2016). Darobactin functions by mimicking a β-strand and binding to the lateral gate, stabilizing a closed or partially closed conformation and thereby preventing the initial engagement of OMP substrates (Kaur *et al.,* 2021; Haysom *et al.,* 2023; Ritzmann *et al.,* 2022). Our RMSF analysis provides compelling evidence of a similar functional outcome.

The altered flexibility profiles for BamA subunits upon CaNCR63 binding, particularly in the periplasmic loops that constitute the lateral gate (Figure 5), suggest that the stable interaction of CaNCR63—as demonstrated in our work—restricts the conformational dynamics, which is essential for the OMP folding cycle, as discussed by (Gu *et al.,* 2016; Noinaj, Gumbart, and Buchanan 2017).

By locking the gate, CaNCR63 leads to the same functional outcome as darobactin: inhibition of outer membrane biogenesis and subsequent bacterial death. This inhibitory strategy is distinct from other recently discovered macrocyclic peptide inhibitors, which bind within the BamA lumen and appear to trap BamA in a conformation that blocks the initiation of OMP assembly via a non-competitive, allosteric mechanism (Morgan E Walker *et al.,* 2025a).

Broader Implications: Allosteric Modulation and the Role of the Membrane Environment

Recent studies have highlighted the intricate relationship between BamA's conformational state, its local membrane environment and its function. The native asymmetric bilayer enhances the conformational heterogeneity of BamA, allowing it to sample a wider range of states, including inward-open and lateral-open states, than in detergent micelles (Gopinath and Joseph 2022). Furthermore, the fluidity of the outer membrane is a critical determinant of BAM efficiency; a highly fluid membrane, such as that in LPS-deficient strains, can compromise BamA folding activity and sensitize the machinery to inhibitors (Storek *et al.,* 2018). Our RMSF data (Figure 5), showing peptide-induced changes in the dynamics of all five Bam subunits, suggest that CaNCR63 binding does not merely act as a static "plug." Instead, it actively modulates the dynamics of the entire complex. This allosteric effect, propagated from the lateral gate, could disrupt the coordinated motions between BamA and its accessory lipoproteins (BamB-E), which are necessary for efficient OMP assembly. For instance, the presence of a peptide may alter the mechanical properties of the β-barrel. Darobactin has been shown to increase the energetic stability of BamA while decreasing its mechanical stiffness, effectively "softening" the barrel (Ritzmann *et al.,* 2022). By stabilizing a specific conformational state, CaNCR63 likely disrupts the delicate balance of flexibility and rigidity required for BamA to cycle through the conformations necessary for substrate recognition, folding, and release.

In conclusion, the comprehensive *in silico* investigation strongly supports the hypothesis that the chickpea-derived peptide CaNCR63, is a potent, high-affinity inhibitor of the *E. coli* BAM complex. Through a combination of molecular docking and extensive all-atom molecular dynamics simulations, we have demonstrated that CaNCR63 forms a dynamically stable complex by anchoring to the functionally critical lateral gate of the BamA subunit. The calculated sub-nanomolar binding affinity, driven predominantly by electrostatic interactions, is indicative of a highly specific and potent drug-target interaction, reinforcing the potential of plant-derived NCR peptides as a novel source of antibacterial agents.

The mechanism of action elucidated by the study is particularly significant, as it is functionally analogous to that of the known antibiotic darobactin. By physically occupying and stabilizing the lateral gate, CaNCR63 effectively sequesters the machinery, preventing the conformational changes necessary for outer membrane protein biogenesis. Furthermore, our findings suggest that this binding event initiates an allosteric cascade,

disrupting the dynamics of the entire multi-protein complex. This sophisticated mechanism, which targets the function of a whole molecular machine, represents a promising strategy for combating Gram-negative pathogens and may present a higher barrier to the development of resistance.

While these computational findings provide a robust foundation, the clear path forward requires rigorous experimental validation. The immediate next steps should involve biochemical assays to confirm the predicted binding affinity, followed by *in vitro* functional assays to demonstrate the inhibition of OMP assembly. Ultimately, solving the high-resolution structure of the BamA-CaNCR63 complex would provide definitive proof of our model and serve as an invaluable blueprint for the future rational design and optimization of this promising class of peptide-based antibiotics in the urgent fight against antimicrobial resistance.

Author Contributions

Rajendra Choure: Conceived the original idea and designed the model the computational framework and wrote the manuscript; Sarang Lobhi: Formal analysis, writing review and editing; Mitesh Disale: Validation, methodology; Rupendra Jadhav: writing—reviewing

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

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