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MOLECULAR DYNAMIC SIMULATIONS OF MlaC INHIBITION BY ANTIBIOTIC IN *Escherichia coli*

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Abstract

Antimicrobial resistance has emerged as a global public health concern. Gram-negative bacteria such as *Escherichia coli* (*E. coli*) pose a significant threat to human health due to their increasing antibiotic resistance. For instance, Shiga toxin-producing *E. coli* (STEC) is a strain that produces toxins that cause damage to the lining of the intestines and kidneys. Antibiotic exposures to STEC would induce the hemolytic uraemic syndrome and bloody diarrhea, a potentially fatal-condition to the patient. The outer membrane architecture in Gram-negatives, specifically the OmpC–Mla complex, maintains the outer membrane lipid asymmetry. The MlaC protein transfers phospholipids from outer membranes to inner membranes and ensures the integrity of the membrane. Inactivation of MlaC protein increases the penetrability of OM and increases the antibiotic's sensitivity. Therefore, screening for inhibitor compounds that can bind and inhibit the function of MlaC is a viable strategy for antibiotic development. This study aims to understand the interactions of four types of inhibitors in MlaC protein from *E. coli* via docking and molecular dynamic (MD) simulation. The four types of inhibitors namely albacarcin V, clorobiocin, 1-N,4-N-bis(3-phenylphenyl)piperazine-1,4-dicarboxamide (piperazine dicarboxamide) and -2-[2-[(6-oxobenzo[c]chromen-2-yl)carbamoyl]phenyl]benzoic acid (salicylanilide benzoate). The docking showed that the inhibitors fit into the lipid pocket of MlaC. MD for each system run at 100 ns showed that the system has stable Root Mean Square Deviation (RMSD), Root Mean Square Fluctuation (RMSF), and reasonable Radius of Gyration (Rg) value. The RMSD, RMSF and Rg were comparable to the native phospholipid binding in the crystal structure, which suggests the potential use of these four types of inhibitors. Salicylanilide benzoate was revealed to be the most stable in complex with MlaC, with the least deviation, least fluctuation, and most compact throughout the simulation.

INTRODUCTION

Gram-negative bacteria possess a three-layered envelope which includes the outer membrane (OM), followed by peptidoglycan and the inner membrane (IM). The membrane's first layer, OM consists of lipopolysaccharides (LPS) which are found at the outer leaflet of OM. The OM of Gram-negative bacteria is a layer which differentiates Gram-negative bacteria from Gram-positive bacteria [1]. The LPS layer allows small molecules such as amino acids

and small saccharides to pass through the membrane. The second layer, peptidoglycan, is a rigid exoskeleton composed of repetitive units. Gram-negative bacteria possess thinner peptidoglycan layers compared to Gram-positive bacteria [2]. The third layer, the IM layer, is a phospholipid bilayer responsible for multifunctional processes such as structure, transport, and protein biosynthesis [3].

The OM of Gram-negative bacteria is the primary cause of antibiotic resistance, such as β-lactams, quinolones, colistins, and other antibiotics. Most antibiotics must pass through the OM to reach their putative targets. For example, hydrophobic antibiotics would pass through a diffusion pathway, whereas hydrophilic antibiotics like β-lactams must pass through porins [3]. Gram-negative bacteria develop resistance by modifying their target site, modifying the antibiotic itself, or destruction of the antibiotic, antibiotic efflux via efflux transporters and reducing antibiotic influx by changing their outer membrane's features and porins. For example, work by Tsai et al., (2011) showed that mutations in the outer membrane porins OmpK35 and OmpK36 have increased resistance of *Klebsiella pneumoniae* to cefazolin and ceftazidime [4]. In Gram-negative multidrug resistance is primarily linked to extended-spectrum beta-lactamase production. However, new resistance mechanisms are increasingly evolving. Thus, developing new antibiotics or screening for inhibitors is necessary to overcome antimicrobial resistance.

Gram-negative bacteria possess numerous protein systems, which functions to transport phospholipids, including PbgA/YejM, Tol–Pal complex, OmpC–Mla system, and other putative transporters. The OmpC–Mla system in Gram-negative bacteria plays a vital role in maintaining the OM of lipid symmetry [5]. The OM of the MlaA-OmpC/F complex, the IM of ATPase MlaFEDB complex, and a soluble periplasmic protein named MlaC are the three components of the pathway [6]. The Mla pathway in a Gram-negative bacterium has recently been identified as a crucial participant in the transportation of phospholipids through the bacterial envelope. Mla is required to keep the outer membrane barrier intact by transporting phospholipids between the OM and the IM. In this Gram-negatives, one of the most significant components in the Mla pathway is the MlaC protein. It is a protein that transfers phospholipids from OMs to IMs to ensure the integrity of the membrane [7]. In the OmpC–Mla pathway in *E. coli*, removal of any components of the system causes abnormal phospholipid accumulation in the outer leaflet of the OM, interrupting the lipid asymmetry. This system is responsible for removing mislocated phospholipids from the OM and transporting back to the inner membrane [5].

Our study shows the docking and simulation of four active antibiotic compounds to MlaC. The four active compounds selected in this study was based on the previous literature which showed binding activity to pockets of MlaC including 1-N,4-N-bis(3-phenylphenyl)piperazine-1,4-dicarboxamide (piperazine dicarboxamide), 2-[2-[(6-Ooxobenzo[c]chromen-2-yl)carbamoyl]phenyl]benzoic acid (salicylanilide benzoate), clorobiocin and albacarcin V. Piperazine dicarboximide consists of saturated aliphatic six-member heterocyclic compounds. It contains a sizeable polar surface area, hydrogen-bond acceptors and donors and provides a rigid structure [8]. Salicylanilide benzoate consists of a benzene ring with at least one carboxyl group and is also known as a nitrogen binding agent [9]. Clorobiocin and albacarcin V are active compounds produced by different *Streptomyces* spp. and are part of the aminocoumarins antibiotic. These compounds consist of an aromatic acyl backbone and the second moiety of 3-amino-4,7-dihydroxycoumarin and L-noviosyl sugar [10]. This structural component of the compound should enable binding at MlaC binding pockets [12-15]. All four compounds have no known activity for MlaC in *E. coli*. This is the first work to simulate the binding of these four compounds to MlaC in *E. coli* via docking and molecular dynamics simulation.

MATERIALS AND METHODS

Docking Simulation

The MlaC protein structure was obtained from the Protein Data Bank (PDB) (PDB ID: 5UWA). The crystal structure exists as a dimer, and each monomeric structure contains a lipid pocket for the phospholipid transfer between the inner membrane and the other membranes of *E. coli*. The docking analysis was generated using UCSF Chimera [11]. There were a few parameters that had been used for docking. In the receptor and ligand options, the MlaC protein was chosen as the receptor, and the selected inhibitor compounds, which are clorobiocin, albacarcin V, salicylanilide benzoate, and piperazine dicarboxamide as the ligand. The box size that delimitts the binding site would change according to the volume and geometry of the binding site of the system. In Chimera, the hydrogens were added to the structure. The charges were merged, and the non-polar hydrogens and lone pairs were removed. The waters and chains of non-standard residues were ignored. The charges were not merged for the ligand parameters, and the non-polar hydrogens and lone pairs were not removed. In the output parameter, three options were involved: the number of binding mods, the exhaustiveness of search, and the maximum energy difference (3 kcal/mol). All the options were set at the maximum.

Molecular Dynamic Simulation

Five docking systems were set up for molecular dynamic (MD) simulation, including native MlaC with the presence of phospholipid and four systems from the docking results. The starting model of MlaC was neutralized with 150 mM NaCl solution and solvated in the water cube model by the

CHARMM-GUI solution builder [12]. The box size was 10Å apart from the edge of the protein structure. Topology for ligand parameter was generated using CHARMM General Force Field (CgenFF), and all MD systems were run using CHARMM36m force field using Gromacs packages. Each system then undergoes an energy minimalization stage to avoid the steric clashes by using the steep decent step as it is robust and easy to implement. Each system was heated at 303 K for 100 ps during the constant Number of particles, Volume, and Temperature (NVT) ensemble to equilibrate the system at the desired temperature. This NVT equilibration was done based on the Verlet cutoff scheme. The constant number of particles, Pressure, and Temperature (NPT) were assembled in isothermal-isobaric using Parrinello-Rahman barostat for 1 ns, followed by the removal of all constrain and further NPT equilibration for another 1 ns. LINCS algorithm was used as a constraint algorithm to achieve 2 fs in timestep for simulation. Simulations were analyzed and visualized in Visual Molecular Dynamic Tools (VMD) [13], and graphs for RMSD, RMSF and gyration were generated using GRACE software.

RESULTS AND DISCUSSION

Molecular Docking

The docking simulation between the MlaC protein with the inhibitors, which are piperazine dicarboxamide, salicylanilide benzoate, clorobiocin and albacarcin V, showed that all these inhibitors successfully bind to the pocket of the MlaC protein (Figure 1). However, docking simulation is insufficient to determine whether the ligand has docked within the binding site in a stable because it is not simulated in a real condition in which proteins are found involving the time or temperature [14].

MD analysis was used and estimated the ligands' binding affinity toward the MlaC protein's target site. In our analyses, we compared the binding energy, which energy is the primary parameter in a molecular study to generate the strength and affinity between the protein and the ligand. The binding energy of these compounds was ranked from the lowest numbers to the highest. In docking analysis, the lowest figures binding energy is depicted as a stronger binding [15]. It is also affected by the number of binding resitudes of both molecules.

Our analyses showed that the strongest binding is by, piperazine dicarboxamide complex, which is -12.5 kcal/mol. The second lowest binding energy value was occupied by salicylanilide benzoate and clorobiocin, with binding energy of -11.4 and -10.1 kcal/mol, respectively (Table 1). Finally, albacarcin V had the highest value for binding energy, which was -9 kcal/mol, showing that albacarcin V had the lowest binding affinity towards MlaC protein. This result suggested that piperazine dicarboxamide to be the best compound for the MlaC protein and is the best potential inhibitor for the MlaC comparaed to the the three other compounds. Interestingly, although 1-N,4-N-bis(3-phenylphenyl) piperazine-1,4-dicarboxamide showed the strongest binding energy, Chlorobiocin had the highest number of binding residues.

Several interactions occur between the ligand and amino acid. The residues of each ligand show several amino acids essential in establishing intermolecular interactions with the compound (Figure 2). In piperazine dicarboxamide, Ala108, Arg143, Ile137, and Leu64 residues formed tight binding with MlaC. Salicylanilide benzoate formed tight binding with residues Leu109, Leu148, Phe39, Met166, and Val68. Clorobiocin interacted with Ala35, Arg143, Asp139, Ile137, Leu64, Met111, and Phe39. Albacarcin V showed interaction with Val67 and Val162.

MD Simulation

Root-Mean-Square Deviation (RMSD)

The conformational stability of each of the systems was evaluated through root-mean-square deviation (RMSD) (Figure 3). RMSD calculates the average displacement of the atoms throughout the simulation relative to a reference structure [16]. In this study, the deviation of the C-alpha with respect to the C-alpha of the whole system was calculated with lower RMSD values between $\approx 0.2 – 0.3$ nm, indicating a system with higher structural stability [17]. In general, all four inhibitors showed stable RMSD values with an average of 0.1 to 0.2 nm across the 100 ns simulation time and receiving plateau after 50 ns. In comparison to the native inhibitor, which deviates between the value of 0.15 nm, clorobiocin, dicarboxamide, and benzoic acid showed the overall value of 0.2 nm with fluctuation in the beginning and stabilized towards the end.

Interestingly, even though salicylanilide benzoate showed a higher RMSD value compared to the native inhibitor, the RMSD was revealed to be the most stable compared to other tested inhibitors in the study. As reflected in Figure 3, the MlaC-salicylanilide benzoate complex does not deviate much from the initial towards the end of the simulation. Meanwhile, albacarcin V showed the most fluctuating value in the first 20 ns, which eventually stabilized after 40 ns, similar to the RMSD of the native inhibitor.

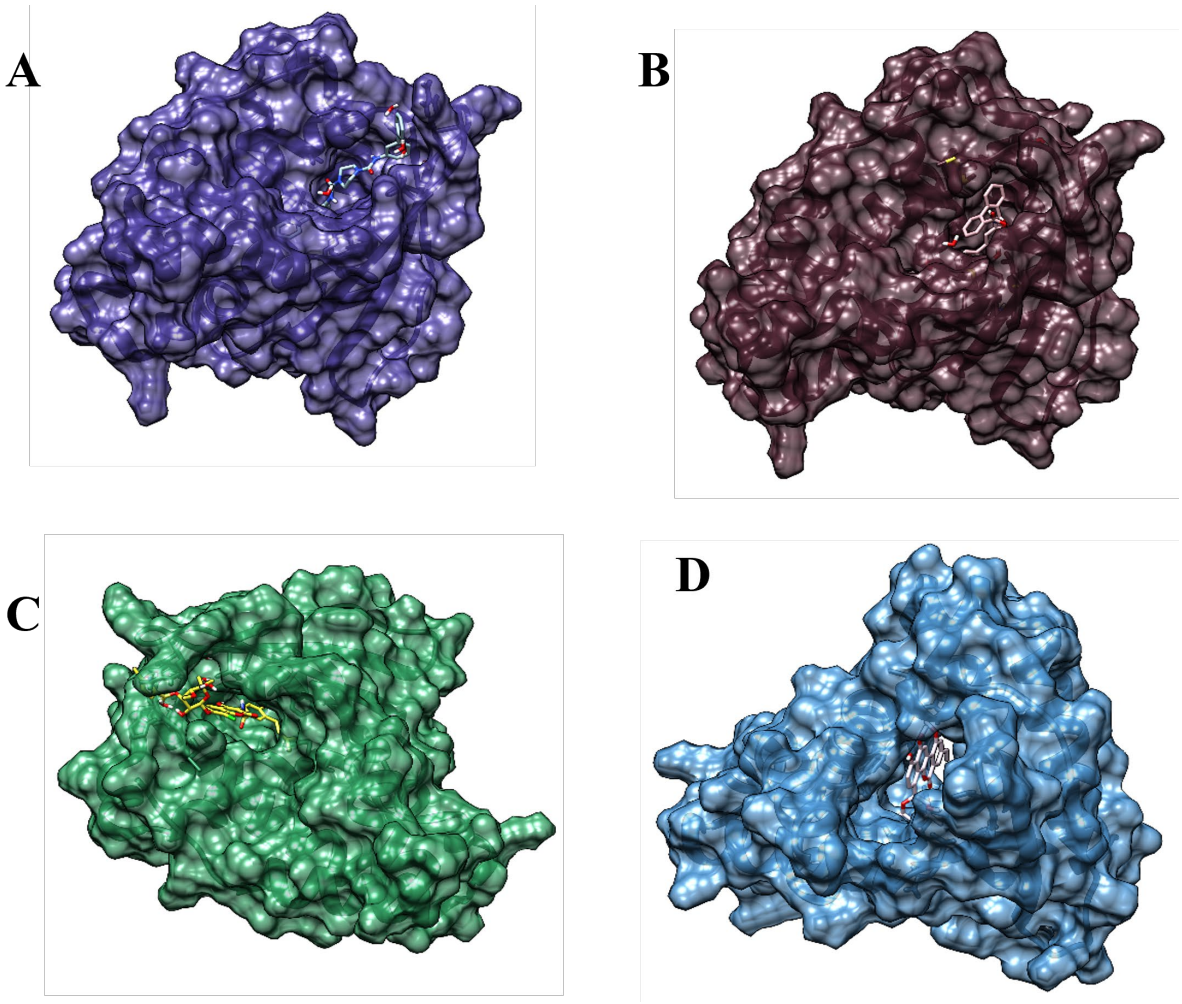

Figure 1. Results for docking simulation with A) piperazine dicarboxamide, B) salicylanilide benzoate C) clorobiocin and D) albacarcin V.

Table 1. The binding energy and residues of the MlaC protein with the selected compounds

Bioactive Compounds	Binding energy (kcal/mol)	Binding Residues
1-N,4-N-bis(3-phenylphenyl)piperazine-1,4-dicarboxamide	-12.5	Ala 108, Arg 143, Ile 137, Leu 64
2-[2-[(6-Oxobenzo[c]chromen-2-yl)carbamoyl]phenyl]benzoic acid	-11.4	Leu 109, Leu 148, Phe 39, Met 166, Val 68
Chlorobiocin	-10.1	Ala 35, Arg 143, Asp 139, Ile 137, Leu 64, Met 111, Phe 39
Albacarcin V	-9.0	Val 67, Val 162

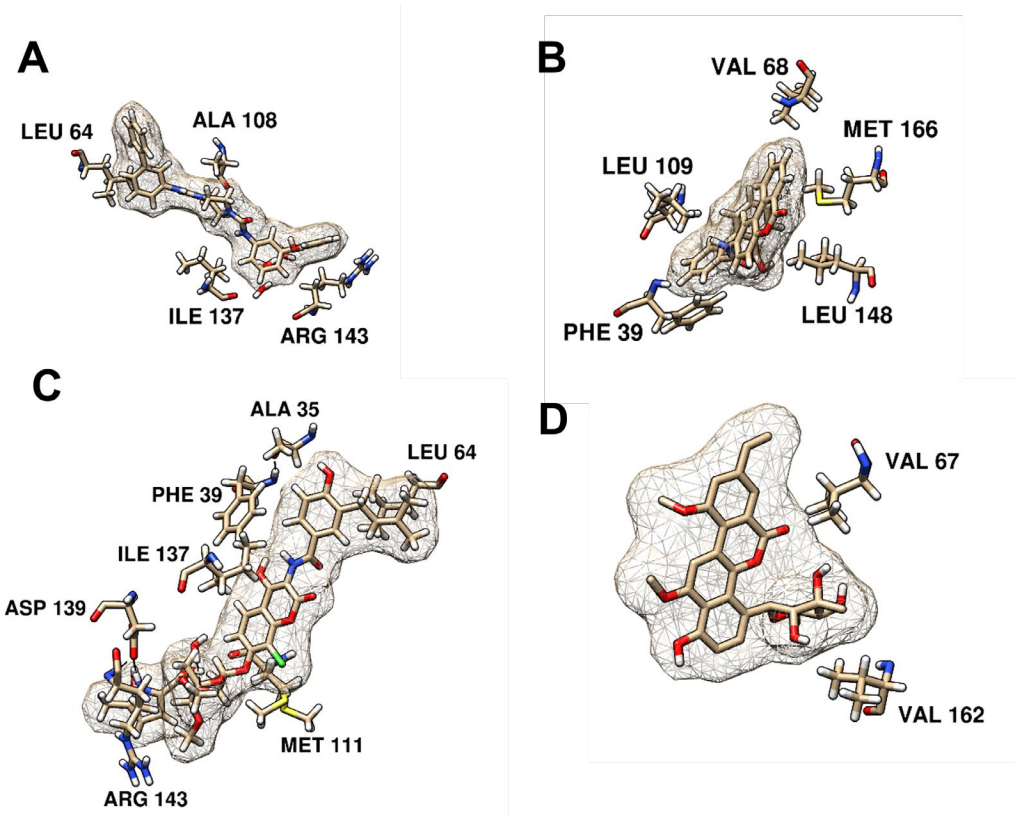

Figure 2. Amino acid interaction with A) piperazine dicarboxamide B) salicylanilide benzoate C) clorobiocin and D) albacarcin V.

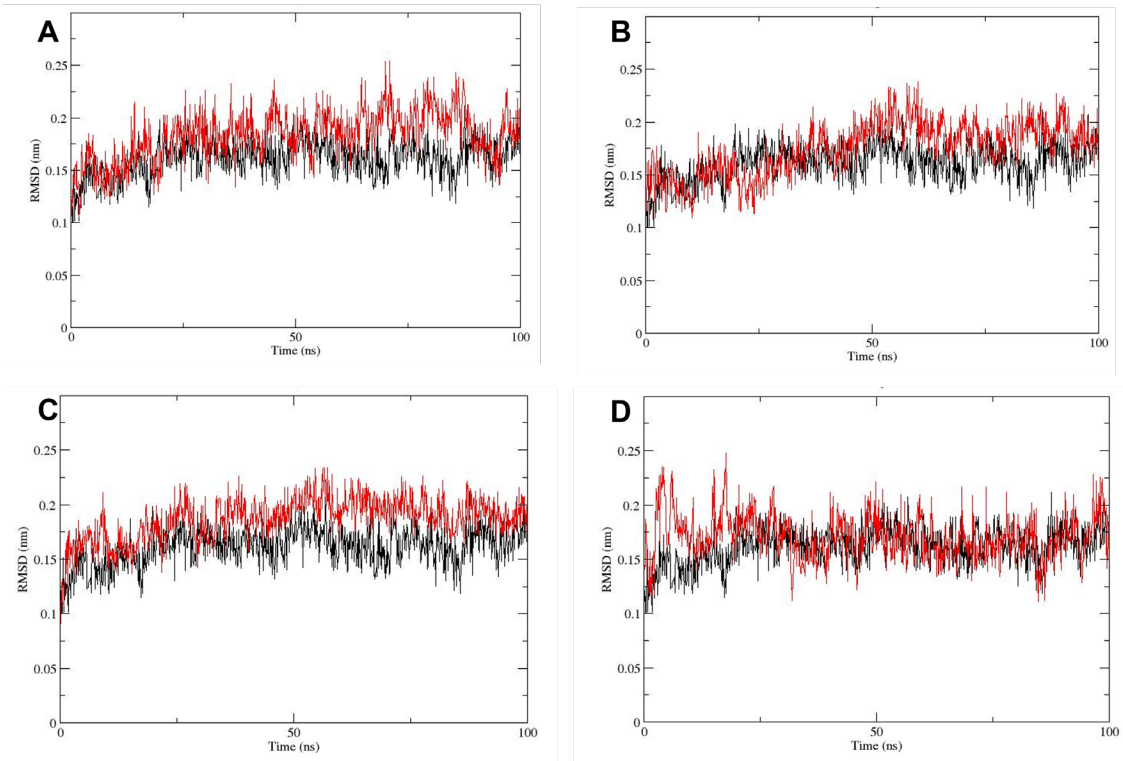

Figure 3. Root Mean Square Deviation (RMSD) comparison between native lipid binding (black line) with RMDS value of inhibitor (red line). RMSD value comparison between native lipid with: (A). (B) piperazine dicarboxamide. (C) salicylanilide benzoate. (D) albacarcin V.

Root-Mean-Square Fluctuation (RMSF)

It was also important to study the fluctuation of each targeted amino acid or residues, which could provide additional information on the structural stability and protein interactions during the simulation relative to the average structure [16]. This can be achieved through the root-mean-square fluctuation of each residue (Figure 4). Based on the result, the protein residues of each residue numbering from 25 to 220 were subjected to RMSF compared to the native inhibitor. However, unlike the rest of the inhibitors, albacarcin V revealed more fluctuating and least stabilized residual interactions with the highest RMSF of 0.45 nm contributed by the residues between 110 to 140. This is followed by dicarboxamide, which reached 0.4 nm RMSF and clorobiocin. Meanwhile, salicylanilide benzoate revealed a more stabilized RMSF value similar to that of the native inhibitor, suggesting possible similar residues might be involved in both proteins, which could be crucial for the inhibitory actions – as demonstrated in another study by Kakhar et al., [18]. Intriguingly, all inhibitors also showed similar residual fluctuations, mainly between the 125 to 200 residues, which supported the potential binding residues identified in the molecular docking of MlaC-inhibitors.

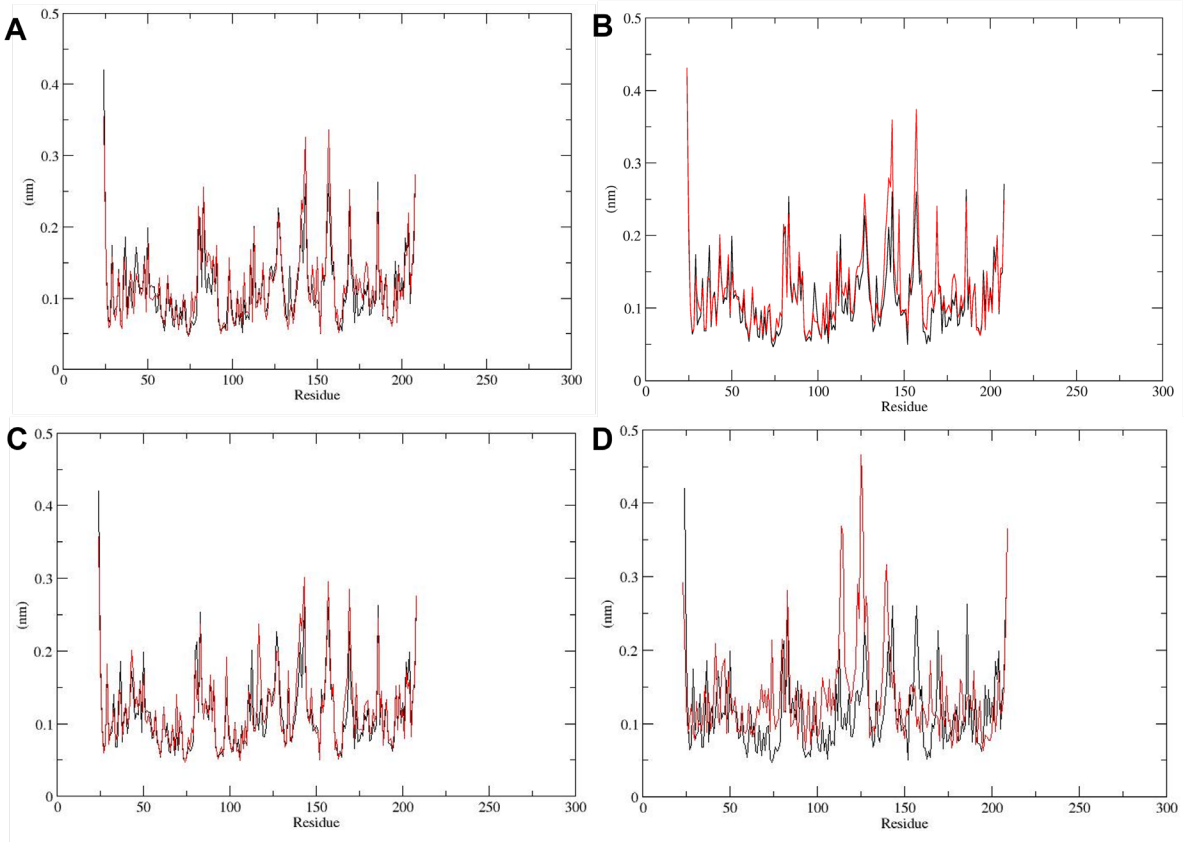

Figure 4. Root Mean Square Fluctuation (RMSF) comparison between native lipid binding (black line) with RMSF value of inhibitor (red line). RMSF value comparison between native lipid with: (A) clorobiocin. (B) piperazine dicaboxamide. (C) salicylanilide benzoate. (D) albacarcin V.

Radius of Gyration (Rg)

In order to evaluate the overall compaction of the MlaC-inhibitors complexes and prove the stability of the whole system throughout the simulation period, the radius of gyration (Rg) analysis was performed (Figure 5). According to [19], the Rg can be defined by the mass-weighted root mean square distance of a collection of atoms from a common centre of mass where a constant value can indicate a more stably folded structure when subjected to force fields which could interfere and disrupt the protein folding [17]. Hence, a higher Rg plot could also suggest more loosely packed amino acid residues and compactness and vice versa [17]. As shown in Figure 4, dicarboxamide and albacarcin V revealed the most fluctuating Rg values over the simulation course. Even though albacarcin V showed a lower Rg value in comparison to the native inhibitor, the whole system was unstable and showed a more dispersed and least stabilized Rg with increasing simulation time, especially towards the last 10 ns period. This can also be seen in the Rg value of dicarboxamide, indicating significant changes in the compactness of the complex conformation. Furthermore,

salicylanilide benzoate revealed the most stable value of 1.75 nm throughout the simulation time, reflecting the Rg of the native inhibitor. From the Rg plots, it is evident that unlike the rest of the complexes, the Mlac-salicylanilide benzoate complex showed the most stably folded and unaffected Rg, suggestive of stronger amino acid interactions and bonds of the complex.

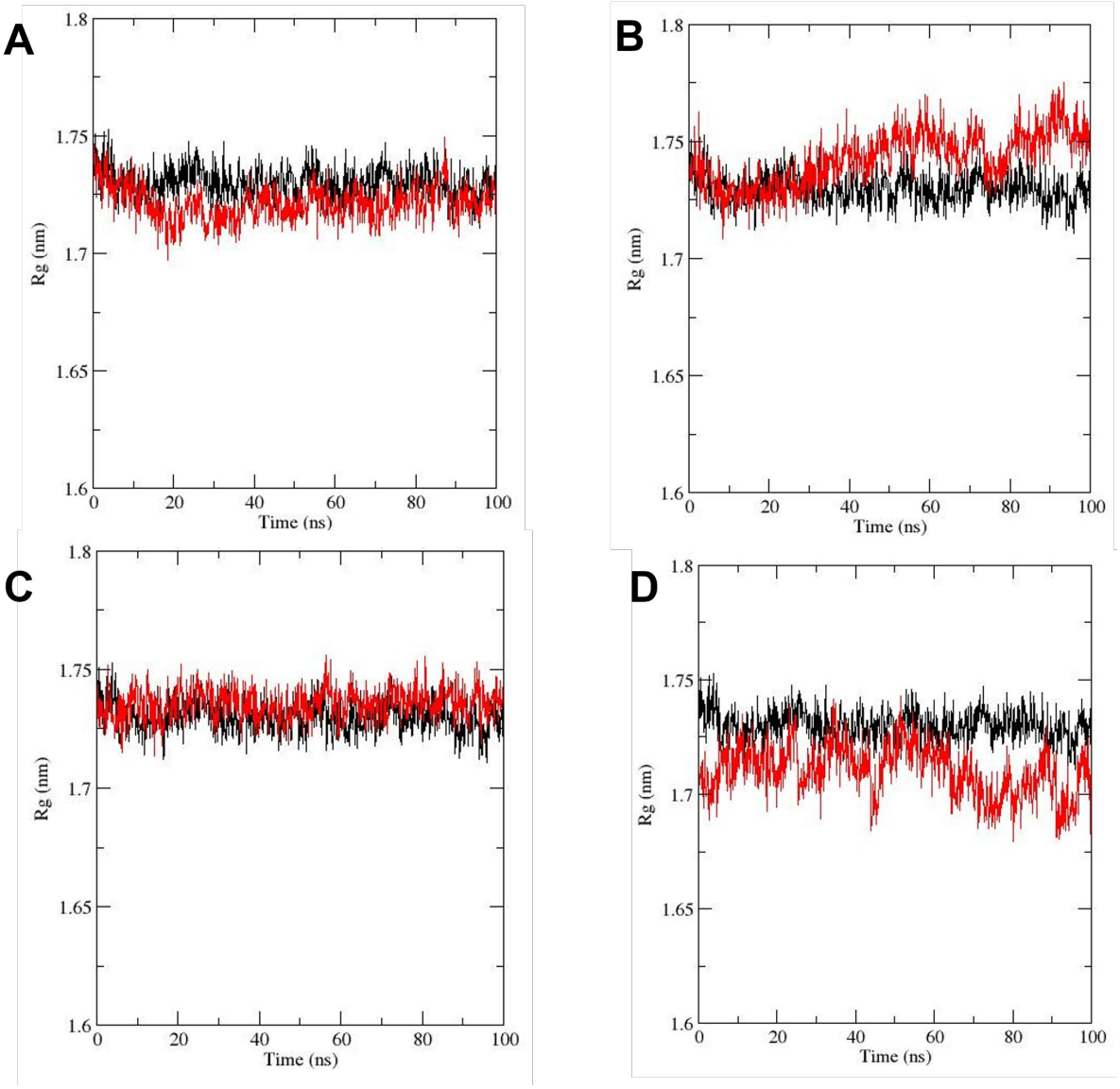

Figure 5. Gyration comparison between native lipid binding (black line) with gyration value of inhibitor (red line). Gyration value comparison between native lipid with: (A) clorobiocin. (B) piperazinedicaboxamide. (C) salicylanilide benzoate. (D) albacarcin.

Conclusively, all three analyses of RMSD, RMSF and Rg demonstrated structural stabilities and compactness of the inhibitory proteins relative to the native inhibitor even when subjected to a force field for 100 ns. In comparison to the other inhibitors (chlorobiocin, piperazine dicarboxamide and albacarcin V), salicylanilide benzoate was revealed to be the most stable in complex with MlaC, with the least deviation, least fluctuation and most compact throughout the simulation [20]. Further, the RMSF analysis also revealed the highest interaction between the residues of 125 to 200, reflecting the molecular docking analysis, while constantly stable Rg value indicates tighter protein folding of the complex. Hence, the MlaC-salicylanilide benzoate complex is more ideal than the other inhibitors.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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