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Docking and Molecular Dynamic Simulations of *Legionella pneumophila* MurB Reductase for Potential Inhibitor Design

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Abstract

Legionella pneumophila is the causative organism for Legionnaires' disease, pneumonia and life-threatening prosthetic valve endocarditis. MurB reductase, one of the important enzymes for biosynthesis of peptidoglycan, a component of cell wall, was identified as common drug target against bacterial pathogens causing infective endocarditis including *Legionella pneumophila*. MurB reductase with FAD acts as a cofactor and catalyzes the NADPH-dependent reduction of UDP-N-acetylenolpyruvylglucosamine (UDP-GlcNAcEP) to UDP-N-acetylmuramic acid. In the present study, 360 structural analogs of FAD were docked to MurB reductase of *Legionella pneumophila* using sequential protocol of Glide v5.7 implemented in virtual screening workflow of Maestro v9.2. Among seven leads were obtained through docking analysis, only lead1 (XPGscore -13.27Kcal/mol) was observed to have better binding affinity towards MurB reductase as compared to cofactor FAD (XPGscore -13.25Kcal/mol). Molecular level interactions of the MurB reductase- lead1 docking complex showed good correlation with MurB reductase- FAD complex. Further, molecular dynamic simulations for MurB reductase - lead1 docking complex were performed using Desmond v3.0 to shed light on natural dynamic of the docking complex in solution on different timescales. Molecular dynamic simulations of MurB reductase - lead1 complex showed stable nature of the docking interactions.

Keywords: Legionnaire's disease; Infective endocarditis; MurB reductase; Virtual screening; Molecular dynamics

Introduction

Legionella pneumophila is significant cause of sporadic and epidemic community-acquired pneumonia (CAP) and nosocomial acquired pneumonia in both healthy and immune-suppressed hosts [1-3]. In patients with community-acquired pneumonia, the incidence ranges from 2 to 15% [4]. The mortality rate is the highest for bacteremic pneumococcal pneumonia and Legionnaires disease in patients with community-acquired pneumonia [4]. Legionnaires' disease primarily occurs in elderly male patients underlying diseases (cardiac, pulmonary or renal) with a habit of smoking [4]. Organ transplant recipients are generally susceptible to Legionnaires disease [4]. *Legionella* infection usually occurs through inhalation of contaminated aerosols produced by water systems such as cooling towers, showers, hot water distribution systems and faucets [5-9]. Other modes of transmission of *Legionella* are aspiration and direct instillation into the lung during respiratory tract manipulation. Prominent symptoms of the disease include headache, diarrhea, arthralgias or myalgias, neurologic symptoms including confusion, fever to 39°C, purulent sputum, hyponatremia, hepatic dysfunction, creatine phosphokinase [CPK] elevation, hypophosphatemia, proteinuria, and hematuria [1]. Bayer *et al.* findings suggested that the infection from *Legionella* led to prosthetic valve endocarditis [10]. Prosthetic valve endocarditis is a life-threatening microbial infection of the endocardial surface lining of the heart chambers and heart valves [11]. Cure for this patient needs prolonged parenteral antimicrobial therapy with either doxycycline or erythromycin, followed by prolonged oral therapy with these agents with duration of 6 to 17 months [10]. Subsequently, levofloxacin (or other fluoroquinolone) or azithromycin were reported as current treatment of choice for Legionnaires' disease [1]. Azithromycin and fluoroquinolones have been found to be superior to older macrolides in inhibiting the intracellular growth of *L. pneumophila* both in invitro and in animal models [1]. The existing treatments cure Legionnaires' disease efficiently within 5-10 days, however, in cases, where *L. pneumophila* infection led to prosthetic valve endocarditis, prolonged medication (6-17 months) is needed for treatment of the diseases. The present study was mainly designed to implement rational drug design method for development of new generation drug molecules for effective infective endocarditis therapy. Peptidoglycan is an important component of cell wall and provides structural integrity. Properly constructed peptidoglycan provides rigidity, flexibility and strength that are necessary for bacterial cells to grow and divide, while withstanding high internal osmotic pressure [12-15]. Peptidoglycan is composed of a β-1, 4-linked glycans of alternating N-acetyl-glucosamine and N-acetyl-muramic acid sugar [12-15]. The biosynthesis of peptidoglycan is a complex process involving several steps. UDP-N-acetylmuramate (UNAM) is the sugar building block for peptidoglycan biosynthesis. It is synthesized in a two-step process by the cytoplasmic enzymes MurA and MurB reductase. MurA transfers an enolpyruvyl group from phosphoenolpyruvate to UDP-N-acetylglucosamine to form UDP-N-acetylglucosamine enolpyruvate (UNAGEP). MurB reductase then reduces UNAGEP using NADPH to form UNAM. Subsequently, the ATPdependent Mur ligases MurC, MurD, MurE, and MurF successively add L-Ala, D-Glu, meso- A2pm or L-Lys and D-Ala-D-Ala to the nucleotide precursor, UNAM.

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Finally UDP-Nacetylmuramate intermediate is transported across the bacterial cell membrane and incorporated into the bacterial cell wall [12-15]. Priyadarshini et al., (2011) identified MurB reductase of *L. pneumophila* as a common drug target among the pathogens causing infective endocarditis [16]. The importance of MurB reductase as valid targets is supported by the fact that it is essential for survival of bacterial pathogens causing infective endocarditis including *L. pneumophila* and it is absent in the host. MurB reductase is also one of the well known targets for antibacterial therapy from the machinery for peptidoglycan biosynthesis [16]. Therefore, designing potential inhibitors targeting MurB reductase which is involved in cell wall biosynthesis would lead to osmotic lysis of bacterial pathogen. Tertiary structure of MurB reductase in complex with FAD and the detailed information on FAD binding site (allosteric site) presented by Priyadarshini *et al.* [16] would be vital for rational drug designing against organisms causing infective endocarditis including *L. pneumophila*. Therefore, FAD binding site of MurB reductase was explored with docking and molecular dynamic simulations to propose a potential inhibitor, its mechanism of binding in the MurB reductase allosteric site and to check the stability of the enzyme-inhibitor complex.

Material and Methods

Retrieval of MurB reductase

MurB reductase in complex with FAD of *L. pneumophila* retrieved from the protein model database (PMDB) [16,17] and allosteric site residues were defined.

High throughput virtual screening

Ligand based high throughput virtual screening was performed for FAD using interactive java applet based Ligand.Info Meta-database tool [18]. The tool retrieves structural analogues for the queried small molecule by implementing 2D geometry search techniques from eight renowned small molecule databases such as Havard's ChemBank, ChemPDB, KEGG Ligand, Druglikeliness National Cancer Institute (NCI), Anti-HIV NCI, Unannotated NCI, AkoS GmhB, Asinex Ltd etc. Maximum of 50 structural analogues of FAD were retrieved from each of eight structural databases of Ligand. Info [18-22]. Consequently, an in-house library of FAD structural analogues was compiled.

Molecular docking

Tertiary structure of MurB reductase and compiled ligand dataset were imported to Maestro v9.2 environment [23] for molecular docking to investigate binding affinity of the ligand dataset towards MurB reductase. Each atom of protein and ligands must be fixed for any potential aberrations before molecular docking for accurate prediction of binding affinity and interactions. The MurB reductase structure was preprocessed with the protein preparation workflow in the Maestro v9.2. All hydrogens were added to MurB reductase which subsequently minimized with the OPLS 2005 force field and the impact molecular mechanics engine setting the maximum root mean square deviation (RMSD) of 0.30 Å. Minimization was performed constraining the heavy atoms with the hydrogen torsion parameters turned off, to allow free rotation of the hydrogen atoms.

The ligands were prepared to expand protonation and tautomeric states at 7.0±2.0 pH units using LigPrep with Epik [24]. High-energy ionization / tautomer states were removed during post LigPrep evaluations. The post LigPrep parameters were restrained to report at four stereo isomers for each compound. Pharmacological properties of the prepared ligands were assessed through Lipinski's rule of five. The compounds with poor pharmacological properties were discarded. The compounds with reactive functional groups were eliminated by applying reactive filter parameters. A three phased subsequent docking protocol was implemented to the prepared protein and ligand dataset to rank the ligands based on their binding affinities towards MurB reductase and to study interaction of the best lead [19-22]. MurB reductase allosteric site was selected to generate a grid using Glide v5.7. Glide virtual high-throughput screening (VHTS), standard precision (SP) and extra precision (XP) methods were applied [25,26]. The XP docking method is highly accurate and generates 10000 poses for each ligand during docking and reports the best pose based on the energy term Emodel. The best poses of each ligand were further ranked based on XPGscore.

Lower XPGscore for a ligand indicates better binding affinity towards protein. The cutoff XPGscore parameter for XP docking was set to 0.0Kcal/mol, a constraint set to discard ligands with positive XPGscore from the final docking output.

Molecular dynamic (MD) simulations

MD simulations were performed to docking complex of MurB reductase-lead1 to evaluate the stability, conformational changes and getting insights into the natural dynamic on different timescales in solution. Simulations were carried out by using Desmond v3.0 [27-29] implemented in Maestro v9.2 graphical user interface. The system was embedded with simple point charge (SPC) water model and neutralized by replacing solvent molecules with counter ions. The final system with approximately 40,914 atoms was simulated through a multistep protocols devised in Maestro v9.2. In brief, the full system was minimized with maximum 2000 iterations of a hybrid of the steepest descent and the limited memory Broyden–Fletcher– Goldfarb–Shanno (LBFGS) algorithms, with a convergence threshold of 50.0kcal/mol/Å2 followed by a similar unrestrained minimization with a convergence threshold of 5.0kcal/mol/Å2. The minimized system was relaxed with three subsequent short span simulations with NVT ensemble (constant number of atoms N, volume V and temperature T) for a simulation time of 12 picoseconds (ps) restraining all non-hydrogen solute atoms, NPT (constant number of atoms N, pressure P and temperature T) ensemble for a simulation time of 24 ps restraining all non-hydrogen solute atoms and NPT ensemble, without restraints, for a simulation time of 24 ps. The three simulations were implemented in Berendsen thermostat with default parameters for time steps, temperature and velocity resampling devised in Maestro v9.2 [27-29]. These initial minimization and simulations were performed to relax the model before implementing a longer simulation time. The relaxed system was simulated for a simulation time of 5000 ps with a time step of 2 femtosecond (fs), NPT ensemble using a Berendsen thermostat at 310 K and velocity resampling for every 1 ps. The simulated system was analyzed for stability of the docking complex. Energy fluctuations and RMSD of the complex in each trajectory were analyzed with respect to simulation time. The root mean square fluctuations (RMSF) of overall atoms, backbone and side chains of MurB reductase were analyzed for each residue. The docking complex was analyzed and monitored for consistency in hydrogen bonding interactions.

Results and Discussion

The MurB reductase-FAD structural complex as a platform for rational drug designing

MurB reductase has a decisive role in formation of N-acetyl-muramic acid subunit of peptidoglycan layer. It is involved in maintaining structural integrity, flexibility and rigidity of *L. pneumophila* cell wall and saves the pathogen from osmotic lysis in host immune system. MurB reductase of *L. pneumophila* was identified as a common drug target among bacterial pathogens causing infective endocarditis [16], hence, it is worth mentioning to implement rational drug designing procedure to the MurB reductase for identifying novel lead molecules. Homology models were successful in rational drug designing against many microbial pathogens [19-22]. Therefore, MurB reductase in complex with FAD was retrieved from PMDB (PM0077283) (Figure 1). The allosteric site residues (Trp63, Leu64, Gly65, Leu66, Gly67, Ser68, Asn69, Thr84, Ala103, Ile128, Pro129, Gly130, Thr131, Gly133, Gly134, Ala135, Arg137, Met138, Ala140, Gly141, Cys142, Arg176, Glu184, Trp185, Phe186, Arg212, Asn222, Gly224 and Phe261) were localized. Trp63, Gly65, Leu66, Gly67, Asn69, Pro129, Thr131, Arg137, Gly141, Phe186 and Arg212 were considered as important residues of allosteric site as they were involved in intermolecular hydrogen bonds with FAD [16]. Therefore, discovery of lead molecules that would block these residues by interacting with better binding affinity compared to FAD would be ideal step towards designing competitive inhibitor against MurB reductase.

Lead identification

The FAD based 2D-structural analog search led to compilation of a ligand dataset of 360 compounds. 10730 protonation and tautomeric states were generated from the ligand dataset. 1628 compounds were screened through post LigPrep evaluations, 312 of them have passed Lipinski's filter. Subsequently, 303 conformations were found to have drug like properties without any reactive functional group. Therefore, these 303 conformations were selected for three levels of docking. Through subsequent screening during HTVS, SP and XP docking seven molecules were reported with good binding affinity with MurB reductase. Comparison of XPGscore of seven leads with FAD deciphered that only lead1 was having a better binding affinity with XPGscore of -13.27Kcal/mol compared to that of FAD with XPGscore of -13.25Kcal/mol (Supplementary Figure 1). Therefore, lead1 would competitively occupy MurB reductase allosteric site instead of FAD (Figure 2A, Figure 2B). Blocking of MurB reductase allosteric site with lead1 would stop reduction of UDP-N-acetylglucosamine enolpyruvate to UDP-N-acetylmuramic acid.

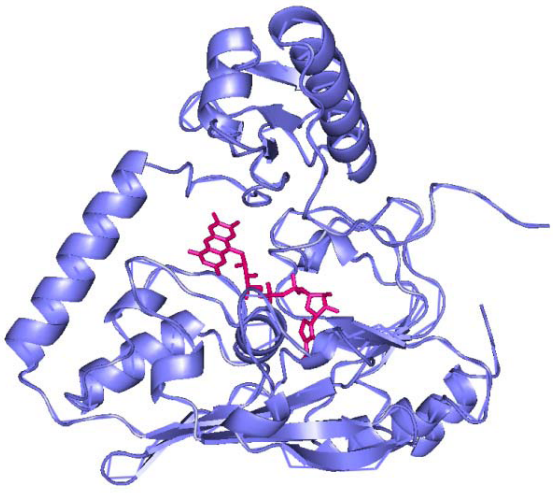

Figure 1: MurB reductase (violet) of *L. pneumophila* with FAD (pink).

The molecular interactions of docking complex of MurB reductase-lead1 showed that the residues such as Ser68, Asn69, Thr131 and Glu184 were involved in intermolecular hydrogen bonding; Leu62, Trp63, Leu64, Gly65, Leu66, Gly67, Ser68, Asn69, Ser70, Thr84, Ala103, Cys107, Trp125, Ile128, Pro129, Gly130, Thr131, Gly133, Gly134, Ala135, Arg137, Met138, Ala140, Ala179, Glu184, Trp185 and Phe186 were involved in good van der Waal contacts (Figure 2A, Figure 2B). The binding modes were correlated well with binding complex of MurB reductase-FAD hence justified the aspect of proposing lead1 as potential inhibitor of MurB reductase (Figure 2A, Figure 2B).

MD simulation studies

The conformations obtained after simulations are more stable and credible than the docked conformations. This can be explained by the fact that docking methods have some inherent drawbacks. However, MD simulations are carried out closer to the physiological environment condition. Therefore, the binding orientations of lead molecules predicted through MD simulations show better correlation to their biologically active states [30-34]. The influences of lead1 and MurB

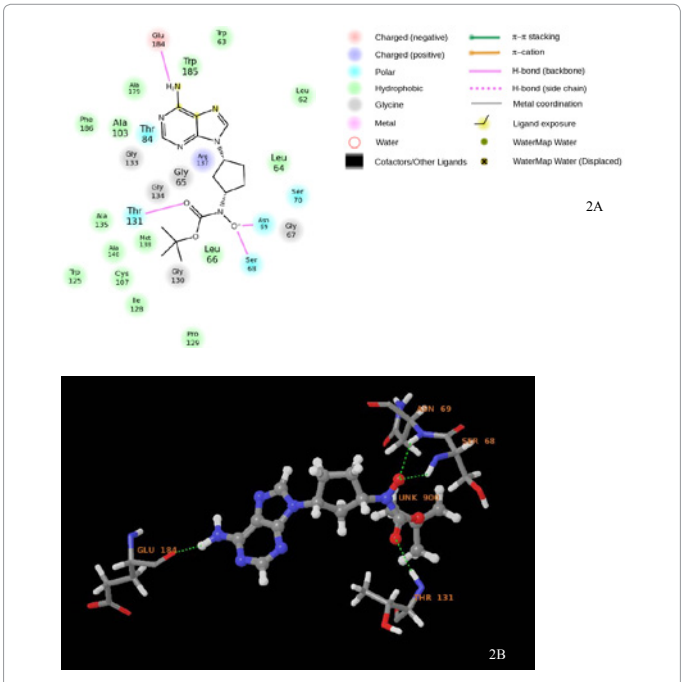

Figure 2: A) Docking interactions of lead1 with allosteric site of MurB reductase. B) Allosteric site residues of MurB reductase forming hydrogen bonds with lead1.

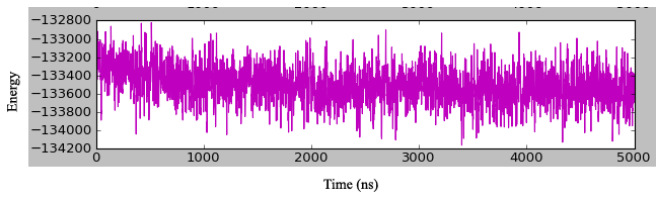

Figure 3: Energy plot of MD simulations for MurB reductase-lead1 docking complex as a function of time.

reductase on the structural and dynamical properties of the allosteric site region have been clarified by analyzing the trajectory data obtained from the MD simulations [35]. The trajectory data of MurB reductase-lead1 docking complex were plotted for energy (Figure 3), RMSD (Figure 4) and RMSF (Figure 5).

The energy plot showed that the energy of the system was stable throughout the simulations (Figure 3). The analysis of the RMSD plot for backbone and heavy atoms (Figure 4) showed that after a small rearrangement from the initial conformation, the complex was relatively stable during entire MD simulation period [29]. The RMSD for atoms after 300 ps remained within the limit of 2 Å (Figure 4). The average RMSD of backbone and heavy atoms, for the MurB reductase-lead1 docking complex during 5000 ps simulation time were 2.34 Å and 2.76 Å, respectively. The RMSD range for backbone atoms varied from 0.0 Å to 3.28 Å while that of heavy atoms varied from 0.0 Å to 3.67 Å. The lower RMSD during MD simulations for MurB reductase-lead1 docking complex showed consistent nature of docking conformations. The root mean square fluctuations (RMSF) of a given residue in the MD trajectories were calculated by averaging over all the atoms of the given residue (Figure 5) [35]. RSMF of most of the residues were within the limit of 2.5 Å. Fluctuations for a few residues were exceeded 3 Å. The lower atomic fluctuations of the allosteric site and the backbone atoms indicated small conformational changes [33,35]. The RSMF values of N-terminal regions (residues 1-5), residue segment 50-55 and C-terminal region (residue 301) revealed that MurB reductase experienced larger side chain rearrangements in these regions (Figure 5). The energy plot, RMSD plot and RSMF plot analysis revealed that the MurB reductase-lead1 docking complex was stable during 5000 ps MD simulation time. Lead1-MurB reductase molecular interactions were monitored to assess the structural flexibility of the docking complex. During MD simulations the hydrogen bonding patterns of lead1 - MurB reductase complex were reproduced. Interactions of lead1 and allosteric site residues of MurB reductase involved in hydrogen bonds (Figure 2A, Figure 2B and Figure 6) were monitored. In the docking complex, (Figure 2A, Figure 2B) atom 4669 (O) of lead1 was observed to be involved in two hydrogen bonds with atom 2870 (H) of Asn69 and atom 2865 (H) of Ser68. The first trajectory of MD simulations (Figure 6) showed existence of an additional hydrogen bond between atom 4699 (O) of lead1 and atom 2880 (H) HG of Ser70 along with the above two hydrogen bonds. The hydrogen bond distances of Ser68, Asn69 and Ser70 with oxygen of lead1 (atom 4669) were monitored in all trajectories (Figure 7). The results showed that hydrogen bond formed by Ser68 (atomic distance ≤ 2.4 Å) and Asn69 (atomic distance ≤ 2.3 Å) were 100% conserved during MD simulations. The hydrogen bond between Ser70 and lead1 (atomic distance 2.5 Å in ~ 95% trajectories) was conserved in ~95% of MD simulation time. As the atom 4699 (O) of lead1 involved in three hydrogen bonds with MurB reductase allosteric site residues such as Ser68, Asn69 and Ser70, the docking complex is deemed to be highly stable. Leu66 (Supplementary Figure 2) and Arg137 (Supplementary Figure 3) were involved in hydrogen

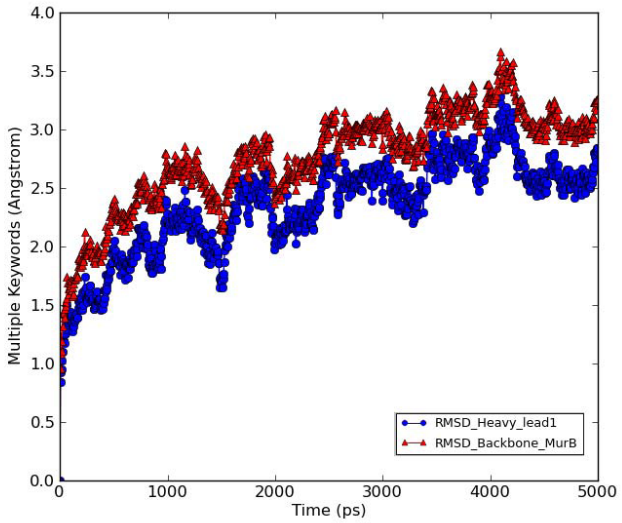

Figure 4: RMSD of protein backbone atom and heavy atoms with respect to time over the course of the 5000 ps MD simulation run.

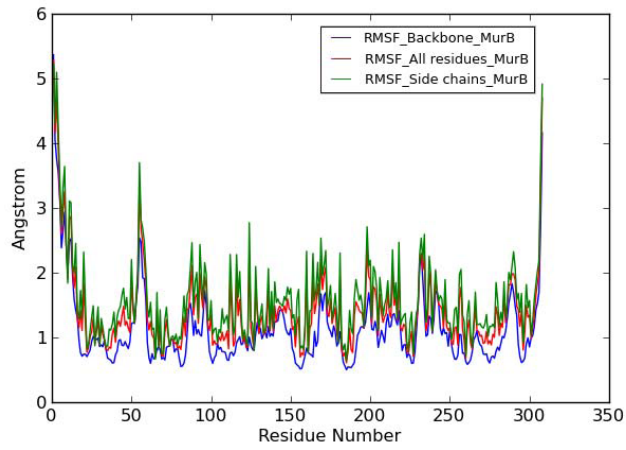

Figure 5: RMSF for the all atoms, backbone and side chains of MurB reductase - lead1 docking complex during the course of MD simulation time (5000 ps).

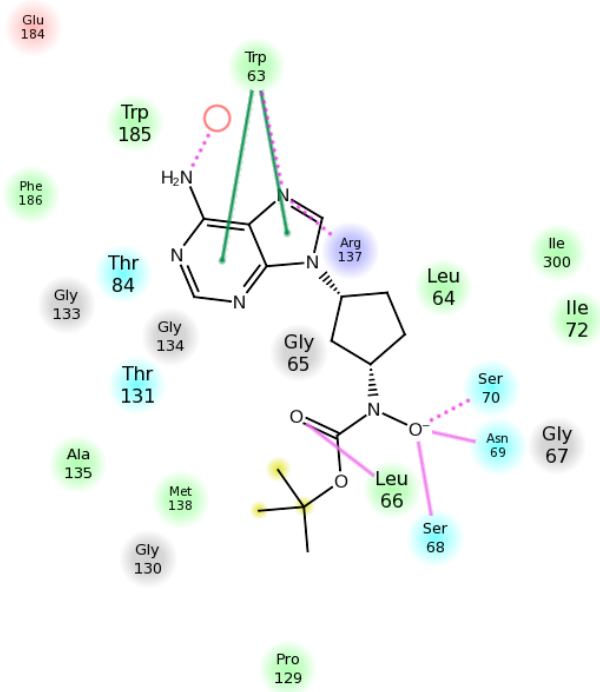

Figure 6: Interactions of MurB reductase - lead1 complex in the first trajectory of molecular dynamic simulations. Legends for the color representations were shown in figure 2.

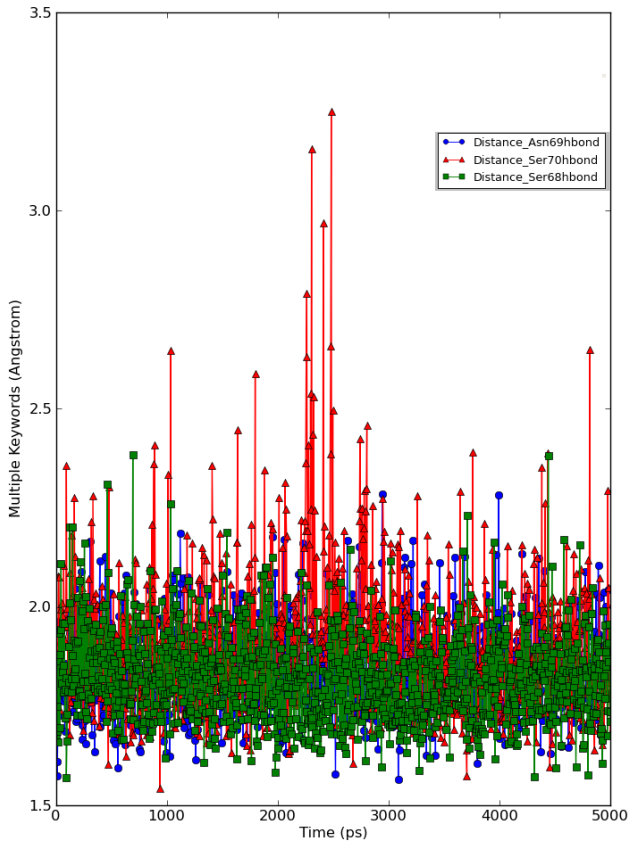

Figure 7: Distances of three hydrogen bonds of Ser68, Asn69 and Ser70 with 4669 (O) of lead1 during total course of 5000 ps MD simulation run.

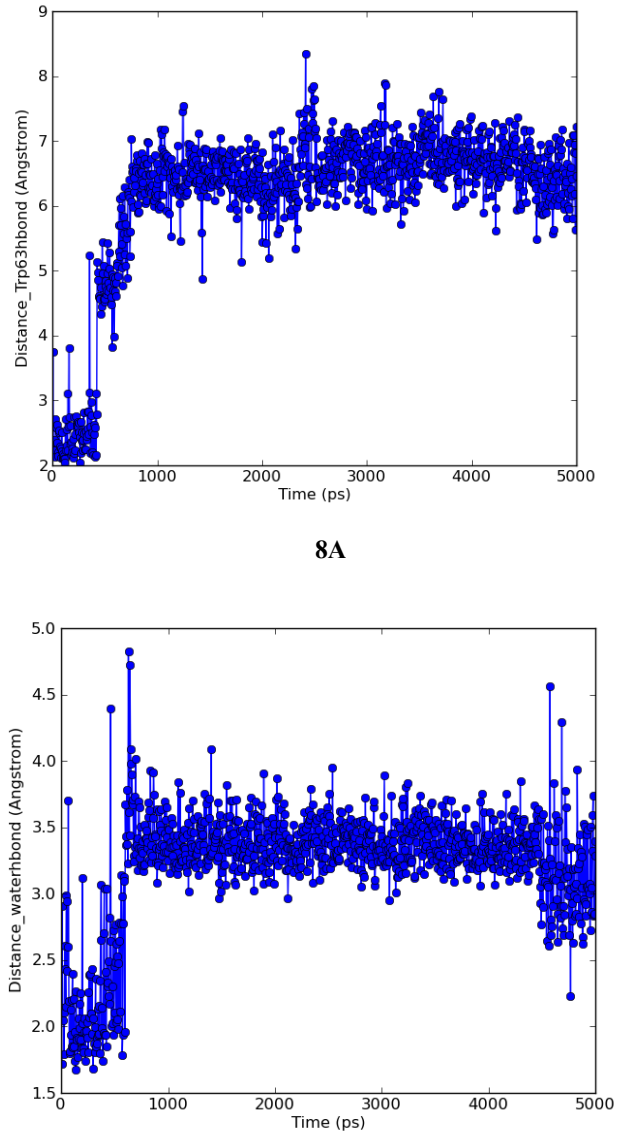

Figure 8: A) Distances of Trp63 atom 2832 (H) HE1 and atom 4707 (N) of lead1 during total course of 5000 ps MD simulation run. B) Distances of SPC397 atom 5866 (O) and atom 4334 (H) of lead1 during total course of 5000 ps MD simulation run.

bond formation with lead1 in ~50% times. More than 50% of the trajectories didn't show existence of hydrogen bonds between Glu184 and Lead1 (Supplementary Figure 4) while that of Thr131 didn't show hydrogen bonds with lead1 in any of the trajectories during total course of simulation time (Supplementary Figure 5). In the first trajectory of MD simulations, one additional hydrogen bond and two pi-pi staking were observed between lead1 and Trp63 (Figure 6). To evaluate stability of these interactions the atomic distances of Trp63 atom 2832 (H) HE1 and atom 4707 (N) were monitored. Existence of hydrogen bond and pi-pi stackings were studied till 500 ps simulation time (Figure 8A). A receptor-ligand water bridge (one water molecule) was also observed during trajectory analysis (Figure 6) between SPC397 atom 5866 (O) and atom 4334 (H) of lead1. The water bridge was observed during first 750 ps and 4500 ps onwards in most of the trajectories of MurB reductaselead1 docking complex (Figure 8B) [32]. The molecular docking and MD simulation results such as energy analysis, RMSD and RMSF proved that the binding affinity of lead1 towards MurB reductase was highly stable with the proposed binding orientations. It showed novel insight into the natural dynamic by revealing presence of Water Bridge, pi-pi stacking and additional hydrogen bonds on different timescales of MurB reductase-lead1 docking complex in solution. Therefore, lead1 can act as potential competitive inhibitor of MurB reductase to stop its activity. Peptidoglycan biosynthesis pathway is absent in human. Comparative analysis of FAD binding site in MurB reductase of *Legionella* with thiredoxine reductase of human (PDB ID: 2CFY) showed that FAD binding site residues (allosteric site) were not conserved (Figure 9). Dym and Eisenberg (2001) also reported the existence of variable sequence motifs in FAD binding site in different organisms [34]. Therefore, Lead1 proposed in the present study would not act as competitive inhibitor for human enzymes with FAD as a cofactor. Lead1 is having good pharmacological properties [35-39], hence, could be considered for designing inhibitor against MurB reductase which in turn would be useful for developing new therapeutic insights on disease such as Legionnaires' disease, pneumonia and life-threatening prosthetic valve endocarditis.

Conclusion

MurB reductase is absent in human, therefore, compounds inhibiting the enzyme would have significant contribution towards rational drug design against bacterial pathogens of public health importance such as Legionnaires' disease, pneumonia and life-threatening prosthetic valve endocarditis etc. The allosteric site of

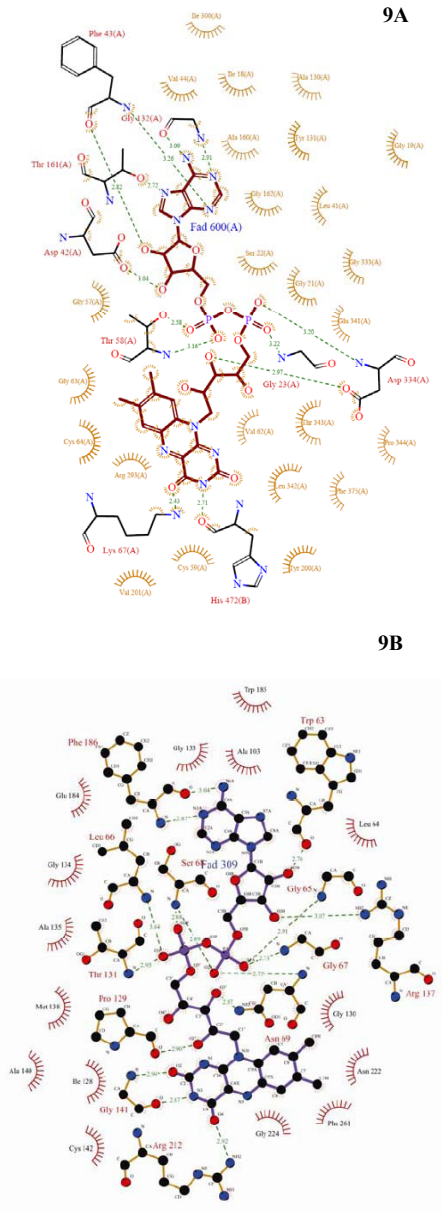

Figure 9: A) Interaction of human thioredoxin reductase with FAD. B) Interaction of MurB reductase with FAD complex.

MurB reductase from *Legionella pneumophila* was selected to propose a novel potential inhibitor through virtual screening. The binding affinity of the proposed lead was accessed and analyzed through molecular docking and molecular dynamic simulation studies. Energy, RMSD and RMSF analysis of the MurB reductase-lead1 docking complex showed that the complex was highly stable in all trajectories. The molecular interactions such as hydrogen bonds in the complex were stable during MD simulations. The physiological environment system created during simulations showed existence of additional one water bridge and two pi-pi stackings in the docking complex. The binding orientations of lead1 compared favorably with FAD in the allosteric site of MurB reductase. The suggested lead would be useful for designing and developing novel therapeutics against *L. pneumophila.*

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